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Using extracellular vesicles as a template for autoimmune diagnostic screening

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FA01

Ral GTPases promote metastasis by controlling biogenesis and organotropism of extracellular vesicles

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Introduction: Primary tumours secrete large amounts of extracellular vesicles (EVs), which play critical roles in preparing distant sites for a pre-metastatic niche formation, thereby promoting metastasis and even determining metastatic organotropism. Whether biogenesis, secretion rates and organotropism of EVs are linked remains unknown. We have recently shown that Ral GTPases control EVs secretion in nematodes as well as in mouse mammary tumour cells (Hyenne et al. JCB 2015). Since both RalA and RalB are overexpressed or over-activated in various human cancers, we aimed to investigate the mechanisms by which these two GTPases control EVs secretion and to determine how this affects metastatic progression, with a focus on breast cancer.

Methods: We used 4T1 mouse mammary carcinoma cells knocked down for either RalA or RalB and determined their ability to induce orthotopic tumours and metastasis in a syngeneic mouse model. In vitro, we investigated EV secretion mechanisms using confocal and electron microscopy (EM). EVs were isolated either by UC or SEC and characterized by NTA, EM, RNA sequencing and mass spectrometry. The function of EVs was assessed using a transwell assay. Finally, we tracked the organotropism of fluorescently labelled EVs and their capacity to induce pre-metastatic niches in mice.

Results: We show that RalA and RalB promote lung metastasis of breast cancer cells in mice without affecting their invasive behaviours. We found that RalA and RalB control the biogenesis of exosomes, by acting on the formation of multi-vesicular bodies though the phospholipase PLD1. As a consequence, knock down of RalA or RalB reduces the levels of secreted EVs and modifies their RNA and protein contents. These differences alter the pro-tumoural function of EVs, as demonstrated with an in vitro permeability test. Importantly, we show in vivo that EVs from RalA or RalB depleted cells have a decreased lung organotropism and, as a consequence, are less efficient in priming lung metastasis. Finally, we show that high expression of RalA or RalB is associated with a bad prognosis in human breast cancer patients.

Summary/Conclusion: Altogether, our study identifies Ral GTPases as central molecules linking the mechanisms of EVs secretion, their dissemination and their capacity to promote metastasis.

FA02

Nuclear proteins are recruited into tumour-derived extracellular vesicles upon expression of tetraspanin Tspan8

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Introduction: Tetraspanin Tspan8 is a transmembrane protein that exhibits a unique expression pattern, being overexpressed in many cancer types, but undetectable in most healthy tissues. Although there is increasing evidence of an effect of Tspan8 in invasion, metastasis, and regulation of extracellular vesicle cargo, the molecular mechanisms of Tspan8 are yet not fully understood.

Methods: To study the function of Tspan8, we have established a fibrosarcoma model consisting of the parental cell line (HT1080) and its derivatives expressing Tspan8 (HT1080-Tspan8) either fused with

different fluorescent tags or tag-free. Life imaging, STED and STORM microscopy were used to determine the intracellular localization of Tspan8. Co-immunoprecipitation from nuclei lysates was performed to detect direct and indirect interacting partners of Tspan8. Small EVs were purified from cell-conditioned media using SEC and subjected to mass spectroscopy and NGS for a comprehensive comparative analysis of the proteome and transcriptome of the EVs.

Results: The results of the proteome analysis showed a strong effect in the protein cargo of EVs upon Tspan8 expression. Remarkably, among 20 of the most regulated targets, several histones and ribosomal proteins were enriched in the EVs derived from HT1080-Tspan8 cells. In line with this finding, life imaging and super-resolution microscopy revealed that, while a majority of the intracellular Tspan8 is located on the cell membrane or intracellular membranes, -as it is known for other tetraspanins-, a portion of Tspan8 is located on the nuclear envelope. In fact, several histones co-immunoprecipitated with Tspan8, indicating their interaction.

Summary/Conclusion: Our data show that the expression of Tspan8 in the tumour cells greatly impacts EV cargo. Moreover, localization of Tspan8 on the nuclear envelope, together with the enhanced recruitment of nuclear and ribosomal proteins to the EVs, suggests a new mechanism of action of Tspan8.

Funding: European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No 722148.

FA03

Genetically encoded probes provide insight into extracellular vesicle cargo release in cells

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Introduction: Extracellular vesicles (EVs) modulate tissue development, regeneration and disease through the transfer of proteins, nucleic acids and lipids between cells. Currently, the mechanism of cytosolic delivery of EV cargo is largely unknown. Here, we unravel how EVs release their cargo in recipient cells.

Methods: EVs were isolated from GFP-CD63 and CD63-RFP expressing HEK293 T cells by ultracentrifugation. GFP-CD63 and CD63-RFP EVs were added to HEK293 T cells stably expressing anti-GFP fluobody and fluorescently tagged galectin-3, respectively. CLEM and fluorescence microscopy were employed to visualize fluorescent markers in recipient cells. Bafilomycin A1 and U18666A were used to inhibit endosomal acidification and cholesterol export from lysosomes, respectively.

Results: Fluorescent galectin-3 which binds to beta-galactosides present at the luminal side of endosomes was used to detect endosomal permeabilization. The absence of galectin-3 recruitment to endosomes in presence of CD63-RFP EVs showed that endosome permeabilization is not the mechanism behind EV cargo release. GFP-CD63 EV addition to cells expressing anti-GFP fluobodies resulted in the formation of fluobody punctae, reflecting cytosolic exposure of EV cargo. Subsequent CLEM of the fluobody punctae revealed endosomes as the underlying cellular compartments from where cargo release takes place. Neutralization of endosomal pH and accumulation of endosomal cholesterol blocked cargo release, showing that EV cargo release is dependent on endosomal pH and cholesterol level.

Summary/Conclusion: We show that genetically encoded cytosolic probes and CLEM offer an excellent approach to study both the mechanism and efficiency of EV cargo release in cells. We provide experimental evidence that EV cargo release occurs from endosomes.

Funding: The research was supported by Dutch technology foundation TTW and Netherlands organization for scientific research NWO, de Cock-Hadders Stichting, and Erasmus Mundus NAMASTE scholarship.

FA04

Towards reference intervals of extracellular vesicles in human plasma by flow cytometry

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Introduction: Flow cytometers with submicrometer sensitivity can characterize single extracellular vesicles (EVs) in clinical samples. Hitherto, there is no consensus about the concentrations of EVs in plasma from

healthy humans. To determine cut-off values for diagnoses, reference intervals of EVs in plasma are needed. To establish such reference intervals, (1) a significant number of healthy donors should be included, (2) the presence of non-EV particles, residual platelets, lipoproteins, and haemolysis should be quantified, (3) flow cytometry signals should be in SI units. The long-term aim of this study is to determine reference intervals of EV concentrations in human plasma within known dynamic ranges of the detectors.

Methods: (1) To establish a clinical reference, we collected blood from 224 healthy volunteers and prepared platelet-free plasma. (2) We performed quality control measurements including residual platelet count, serum index, and lipid spectrum. (3) We measured all samples by flow cytometry (Apogee A60-Micro) and used custom software (MATLAB R2018b) to automate calibration of all signals and data processing. Scatter signals were calibrated in comparable units of scattering cross-section (nm2) and diameter (nm). Fluorescence signals were calibrated in units of molecules of equivalent soluble fluorophores (MESF).

Results: The quality controls showed that most residual platelet concentrations ranged from 10^5 to 10^6 per mL except for one outlier, while the serum index and lipid spectrum were normally distributed. Preliminary results of the first 21 donors analysed, show that within the EV size range of 162–1,000 nm, the median concentration of CD61+ EVs is 3.9·10^8 per mL (APC>150 MESF), CD62p+ EVs is 1.1·10^7 per mL (PE>83 MESF), CD235a+ EVs is 5.2·10^7 per mL (PE>123 MESF), and CD45+ EVs is 1.8·10^7 per mL (APC>91 MESF).

Summary/Conclusion: We have developed reliable procedures for establishing reference intervals of EV concentrations, within a well-defined size and fluorescence intensity range, in human plasma by flow cytometry. We are currently applying these procedures to 224 samples to obtain, for the first time, EV reference intervals for human plasma.

Funding: Pol, E. van der is supported by the Netherlands Organisation for Scientific Research – Domain Applied and Engineering Sciences (NWO-TTW), research programmes VENI 15924.

OT01

Symposium Session 01: Advances in Separation and Concentration I

Chair: Juan Manuel Falcon-Perez – Exosomes laboratory and Metabolomics Platform, CIC bioGUNE, CIBERehd, Bizkaia, Spain. / IKERBASQUE, Basque Foundation for Science, Bizkaia, Spain.

Chair: Navneet Dogra – Icahn Shool of Medicine at Mount Sinai

OT01.1

Beyond size-exclusion: dual-mode chromatography improves purity of extracellular vesicles from plasma

Jan Van Deun, Ala Jo, Huiyan Li, Hsing-Ying Lin, Ralph Weissleder, Hyungsoon Im and Hakho Lee

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Introduction: Purifying extracellular vesicles (EVs) from complex biological fluids is a critical step for reliable EV analysis. Plasma lipoprotein particles (LPPs) are a significant confounding factor as they outnumber EVs >10,000-fold. Given their size overlap, LPPs cannot be completely removed using standard size-exclusion chromatography (SEC). We noticed the contrast in surface charge properties between EVs (-) and ApoB100-containing LPPs (+). Exploiting these charge discrepancies, we combined ion exchange in tandem with size-exclusion chromatography to obtain an LPP-depleted EV population.

Methods: The dual-mode chromatography (DMC) column was constructed as a layered combination of size-exclusion and ion exchange resins. DMC performance for EV enrichment from plasma samples was compared to standard SEC by Western blot (CD63 and ApoA1), ELISA (ApoB100), nanoparticle tracking analysis and transmission electron microscopy (TEM). Additional analytical methods included single vesicle imaging and integrated magnetoelectrochemical exosome (iMEX) assay.

Results: The DMC strategy removed the majority of plasma LPPs: >97% of high-density lipoprotein/HDL (similar to SEC) and >99% of (very) low-density lipoproteins/(V)LDL (>60-fold more efficient than SEC). Additionally, applying DMC resulted in a relatively high EV isolation yield. TEM provided qualitative confirmation of LPP removal after DMC operation. Furthermore, DMC-prepared samples led to better analytical outcomes in single vesicle imaging and iMEX. DMC operation was simple, fast (15 min/sample) and equipment-free (i.e. gravity-driven).

Summary/Conclusion: DMC is a novel, single-step chromatography approach for EV enrichment. It produced enriched EV populations and improved outcome of EV immunoassays by lowering biological background. We envision further investigations with different biofluids and analytical modalities (e.g., nucleic acid detection) to broaden its applicability. Such efforts would further confirm DMC as a powerful EV preparation strategy that can seamlessly replace the current SEC-based EV isolation.

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OT01.2

Tangential flow for analyte capture of extracellular vesicles

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Introduction: The use of extracellular vesicles for diagnostic and therapeutic applications has seen a major interest increase in recent years because of their capacity to exchange components such as nucleic acids, lipids and proteins between cells. Isolation of a pure population of EVs is the first step in studying their

physiological functions since contamination of EV preparations with non-EV proteins can lead to incorrect conclusions about their biological activities. We have developed a new method termed tangential flow for analyte capture (TFAC) using ultrathin nanomembranes to purify extracellular vesicles from pure, highly complex biological fluids such as blood plasma, resulting in a new method for extracellular vesicle purification.

Methods: The TFF microfluidic devices are assembled through a layer stack process using patterned Polydimethylsiloxane (PDMS) sheets with the membranes sandwiched between top and bottom channels. Undiluted plasma was tested in both normal flow filtration (NFF) and tangential flow filtration (TFF) modes on ultrathin nanomembranes. We have utilized a pore patterning technique called nanosphere lithography (NSL) that uses close-packing of nanoscale beads to pattern pores in an ultrathin membrane.

Results: NFF of undiluted plasma resulted in a protein cake of ~8 μm on the membrane, which prevented further transport across the membrane and EVs were buried in the formed cake that were impossible to identify. However, TFAC as a modified version of TFF, led to capturing CD63 positive EVs on the pores of the membrane with little evidence of protein fouling.

NSL allows us to fabricate nanopockets (bowls with a single pore at the base) with various diameter, depth and pore diameter. Using NSL, we further utilize nanopocket membranes to purify EV samples in TFAC devices. This nanomanufacturing technology will allow us to pattern nanopockets with various diameter, depth and pore diameter which increases the efficiency of capturing of EVs. Furthermore, nanopockets can be modified and coated by specific EV markers to capture different subpopulation of EVs based on size and affinity and further allows identifying the phenotypic subsets of EVs by combining both size and affinity-based techniques.

Summary/Conclusion: We have developed a method for the capture and release of nanoparticles such as EVs called TFAC using ultrathin nanomembranes. NSL technology can be applied to fabricate nanopockets with different physical and biochemical properties. Utilizing nanopocket membranes in TFAC system will allow us to separate different subpopulations of EVs based on size and affinity.

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OT01.3

The addition of a size exclusion chromatography step to various urinary extracellular vesicle concentrating methods reveals differences in the small RNA profile

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Introduction: Urinary extracellular vesicles (EVs) and their RNA cargo are a novel source of biomarkers for various diseases, however non-vesicular RNA (e.g. associated with proteins) is also present within urine. This study aimed to identify the optimal method for isolating and enriching EVs from human urine prior to small RNA analysis.

Methods: Three EV concentration methods, ultracentrifugation (UC); a precipitation-based kit (PK); and ultrafiltration (UF), were compared using 50 mL aliquots of pooled healthy volunteer urine. EVs were then separated from protein contaminants by size-exclusion chromatography (SEC). Presence of EVs was confirmed by transmission electron microscopy and Western blotting, and EVs were quantified using nanoparticle tracking analysis (NTA). Small RNA content of concentrated urine and fractions obtained by SEC (EVs and proteins) were evaluated with the Agilent Bioanalyzer small RNA chip.

Results: EV recovery following SEC of concentrated samples was 35–78%, however particle: protein ratio (indicating EV purity) was approximately 10x greater after SEC, regardless of the concentrating method used. UF+SEC yielded the highest number of EVs (per mL of urine) compared with PK+SEC and UC+SEC. Small RNA analysis from UF-concentrated urine (prior to SEC treatment) identified peaks at 20 nucleotides (nt) and 60 nt. Following SEC, RNA analysis indicated that EV fractions contained mostly small RNA of ~60 nt, whereas the protein factions contained small RNA of ~20 nt in size (consistent with miRNAs).

Summary/Conclusion: UF+SEC provided the best balance between EV recovery (per mL urine) and particle: protein ratio. These data indicate that most of the 20 nt sized RNAs, presumably miRNAs, are not within EVs in urine. EV preparations obtained after UC, PK and SEC (regardless of concentrating method) contain predominantly ~60 nt sized small RNA. These data outline the importance of removing non-vesicular proteins and RNA from urine EV preparations prior to small RNA analysis.

Funding: This research has been funded by PetPlan Charitable Trust.

OT01.4

The use of rEV for the optimization of EV separation and characterization by AF4

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Introduction: The reproducibility of extracellular vesicle (EV) research has been hampered by the infinite number of separation and measurement techniques and the lack of appropriate reference materials (Van Deun et al., Nat Methods, 2017). Recombinant extracellular vesicles (rEV) were developed as a biological reference material to overcome these limitations (Geeurickx et al. Nat Comm 2019). Since rEV have EV-like physical an biochemical characteristics and as they are trackable and distinguishable from sample EV they can be used as a spike-in material for data normalization and method development, and as a quality control. We used rEV to optimize EV separation by asymmetrical flow field-flow fractionation (AF4).
Methods: An AF4 long channel column with a frit inlet driven by the eclipse system (Wyatt) was coupled to a UV detector (Shimadzu), MALS Dawn Helios-II (Wyatt) and fluorescent detector (Agilent). A spacer of 350 μm and a regenerated cellulose membrane of 10 kDa were used. PBS supplemented with 0.02% NaN3 was used as a running buffer. Light scatter profiles and UV profiles were analysed as well as the fluorescent emission spectrum as the rEV are GFP positive. Fractions were collected and analysed by nanoparticle tracking analysis (NTA) and western blot. We also estimated the repeatability and reproducibility of the AF4 technique by light scatter and fluorescence profiles as well as the recovery efficiency by NTA.
Results: In a first step 4*10^10 rEV isolated from conditioned medium by a velocity gradient were injected in the AF4 system to optimize the EV characterization protocol. Later concentrated conditioned medium was spiked with 1*10^11 rEV and injected in the AF4 column to optimize EV separation from non-EV contaminants. The most optimal separation protocol was obtained by varying detector and cross-flow settings. This protocol shows elution of monodisperse particles at each time point and size distribution estimations by AF4 correspond to size determination by NTA and electron microscopy.
Summary/Conclusion: We were able to optimize the AF4 protocol for characterization of EV by AF4 as well as for separation of EV from crude conditioned medium samples by using rEV. We demonstrate that rEV are suitable for method development and that AF4 has high potential as an EV separation technique.

OT01.5

Comparative evaluation of EV isolation methods for EV subpopulation analysis in human urine, plasma and cell culture media

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Introduction: Extracellular vesicles (EVs) are membrane-enclosed particles of variable sizes that are released by any cell types to the extracellular space and are identified in all body fluids. A shortcoming in EV research is the lack of standardized isolation protocol for various sample types, resulting in heterogeneous outcomes in downstream analyses. In this study, we compared the EV isolation purity and efficiency among ultracentrifugation (UC), precipitation, size-exclusion chromatography (SEC) and a microfluidic tangential flow filtration device (Exodisc) in human plasma, urine and cell culture media (CCM).
Methods: All EVs were isolated by different isolation methods and characterized per MISEV2018 guidelines. Single-particle interferometric reflectance imaging senor (SP-IRIS) with optional fluorescence and nano-flow (nFCM) were used for single particle analysis.
Results: In CCM, total particle yield of Exodisc was about 5 times higher than those of the rest three methods. Size distribution differed per sample, but the ranges were comparable between the different isolation methods. The total protein amount of SEC, precipitation and Exodisc were similar which were 6–10 times higher than that of UC. UC had the highest particle-to-protein ratio followed by Exodisc. Precipitation and SEC had low ratios. When loading 9ug of total protein for Western blot, CD9, CD81, CD63 and Flot1 could only be detected in UC and Exodisc samples, but not precipitation or SEC. SP-IRIS and nFCM demonstrated consistent purity findings. In urine, total particle yields of Exodisc and SEC were about 4 times higher than

those of the rest two methods. The total protein amount of precipitation was 3 times higher than Exodisc and SEC, 10 times higher than UC. SEC had the highest particle-to-protein ratio followed by UC and Exodisc. Precipitation had low ratios. In plasma, total particle yields of Exodisc and precipitation were 100 times higher than those of the rest two methods. And so were the total protein amount. SEC had the highest particle-to-protein ratio followed by UC. Exodisc and precipitation had low ratios. Western blot, SP-IRIS and nFCM demonstrated consistent purity findings in urine and plasma. To evaluate particle capture efficiency, we spiked a known number of density-gradient UC purified EVs to each method and the recovery rate of UC, precipitation, Exodisc and SEC was 8.6%, 31%, 50.1% and 42%, respectively.

Summary/Conclusion: The order of EV isolation purity in CCM is UC, Exodisc, SEC and precipitation. In urine it's SEC, Exodisc, UC and precipitation. And in plasma, this order is SEC, UC, Exodisc and precipitation. Exodisc and SEC have similar high isolation efficiency followed by precipitation. UC has low efficiency for EV capture.

OT01.6

A capillary-channelled polymer (C-CP) fibre spin-down tip approach for the isolation and biomarker characterization of extracellular vesicles of ovarian cancer origin

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Introduction: Extracellular vesicle (EVs) profiling has shown promise for disease detection through less invasive sampling (liquid biopsies). Current diagnostic tools for ovarian cancer are invasive or only semi-informative. Thus, use of EVs could prove useful in early disease detection. Demonstrated is a hydrophobic interaction chromatography (HIC)-based capillary-channelled polymer (C-CP) fibre tip spin-down process for the isolation of ovarian cancer EVs for use in diagnostics.

Methods: Polyester C-CP fibre micropipette tips are employed in the isolation of EVs from biological matrices including cell culture media, urine, and blood plasma in a spin-down solid-phase extraction (SPE) approach. EVs were isolated from standards of healthy urine origin and from SKOV3 cells (human ovary adenocarcinoma). The C-CP fibre isolation method (taking less than 5 mins and 10 μL sample volumes) preserves the morphology and functionality of EVs as confirmed by SEM, TEM, and confocal fluorescence microscopy.

Results: The dynamic binding capacity of EV standards on a 1 cm PET C-CP fibre tip was found to be ~7E11 particles (50%). The release of EVs was confirmed using dot blot analysis for CD9, CD81, and CD63 tetraspanin proteins. Immobilized EVs were subjected to immunolabeling to allow the positive identification of a profile of ovarian cancer biomarker proteins (HER2, CD24, EGFR, EPCAM, CA125).

Summary/Conclusion: This new EV isolation method introduces a simple capture mode, allowing for direct immuno-characterization and imaging on the fibre surface. This offers a unique and cost-effective opportunity for clinical analyses related to early detection and diagnosis of ovarian cancers (and others). The long-term goal is the creation of a rapid EV isolation and biomarker detection platform.

Funding: Support from the National Science Foundation, Eppley Foundation for Scientific Research, Gibson Foundation, Prisma Health System and ITOR Biorepository are gratefully acknowledged.

OT01.7

Development and optimization of purification method of exosomes by tangential flow filtration and ion-exchange chromatography approach

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Introduction: Extracellular vesicles (EVs) such as exosomes have significant therapeutic potential, however, translation of EV-based therapies has been slowed down because of the biomanufacturing challenges. The isolation of EVs, especially exosomes, is inherently challenging due to their small size, and heterogeneity in the mixture. The current isolation methods either have low recovery rate, aggregation, damaging the structure, time consuming or co-precipitation of contaminants. Specially, it is difficult to process larger sized samples by centrifugation-based or immunoaffinity based methods because of the time and cost associated with these methods.

Methods: To overcome these roadblocks, we developed and optimized alternative purification techniques to isolate EVs with higher purity and yield by using tangential flow filtration (TFF) coupled with ion-exchange chromatography. We used bioreactor platform to produce EVs from serum-free medium using BM-MSC and HEK293 s cells. BM-MSCs were cultured on

stirred tank bioreactors using microcarriers which provide a high surface area to volume ratio for the optimal cell growth and EVs production. Impellers were used to enhance mixing and maintain homogeneous culture conditions that can be easily monitored and controlled.
Results: Depth filtration was applied for clarification of conditioned medium. We screened different types of filters during depth filtration for the best recovery of EVs. TFF membranes with different pore sizes were used to optimize the purity and yield of EVs. Because of the negatively charged nature of EVs, anion exchange chromatography was chosen to capture and separate TFF purified vesicles by their surface charge characteristics. We compared monolith based and membrane-based anion exchange columns to remove contaminants and purify exosomal fractions. The purity, size and presence of exosomal markers in isolated EVs at each step of purification was evaluated by F-NTA, nano-FCM and tetraspanins based ELISA kits.
Summary/Conclusion: In summary, our optimized methods improved the speed of isolation and purity of EVs to the clinical grade. The production and isolation methods of exosomes that we developed here will be easily expandable to support large-scale and cGMP compatible bio-manufacturing in the future.

OT01.8

Use of an alternating current electrokinetic microelectrode chip to positively identify oncology, neurology, and infectious disease samples through plasma extracellular vesicle analysis

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Introduction: Cancer, neurological, and infectious diseases are leading causes of death, with early detection needed to improve outcomes. Extracellular vesicles (EVs) in the blood contain disease biomarkers, but current methods do not allow rapid analysis, and are often limited to one biomarker type.
Methods: We developed methods using alternating current electrokinetics (ACE) to isolate EVs from blood-based samples and analyse the EVs in situ with downstream assays for protein and nucleic acid biomarkers. We investigated if we could identify tuberculosis (TB) donor samples, protein and nucleic acid biomarkers in EVs derived from cancer cell lines, and Alzheimer's disease (AD) protein biomarker levels.
Results: EV isolation was confirmed by positive identification of the proteins CD9, CD63, and CD81 and measurement of EV mRNAs using a direct RT-ddPCR assay. Different disease models were analysed following method development.

TB was used as a model for infectious disease, with 20 TB positive and 20 TB negative samples isolated on ACE chips and analysed for levels of lipoarabinomannan and Ag85. Using a cut-off above the negatives, the AUC of ROC curves were 0.9975 and 1.0, respectively.

For oncology, cancer cell lines were cultured and EVs isolated from supernatants were spiked into human plasma for analysis. Levels of PD-L1 or Glypican-1 on EVs were able to be measured following ACE capture. Additionally, DNA and RNA mutations known to be present in the cell lines were able to be detected using NGS and qRT-PCR, respectively.

Using AD samples as a neurological disease model, Tau and phospho-tau T181 (p-tau T181) in human donor plasma were detected. In 8 AD and 5 healthy donor samples, p-tau T181 signal increased 134% in diseased versus healthy donors.
Summary/Conclusion: ACE chips are an innovative EV isolation and analysis platform that allow rapid disease sample detection in a wide range of studies with high sensitivity and specificity.

OT02
Symposium Session 02: Cancer Progression

Chair: Hector Peinado – Microenvironment and Metastasis Group, Molecular Oncology Program, Spanish National Cancer Research Center (CNIO)

Chair: Hidetoshi Tahara – Department of Cellular and Molecular Biology, Graduate School of Biomedical & Health Sciences, Hiroshima University

OT02.1

Identification of critical modifying factors in EV-based communication between colorectal cancer and stromal cells using the organoid model

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Introduction: Colorectal cancer (CRC) is one of the most frequent causes of cancer-related death. In the majority of CRC patients, mutation in the Apc gene is among the first genetic events. It leads to uncontrolled activation of the Wnt pathway, and thus, to adenoma formation. Some of these adenomas may then further progress to CRC with the accumulation of other mutations. The 3D organoids maintain the cellular and genetic heterogeneity of in vivo tissues and haves proved to be so far the best ex vivo model of human cancers. Here we analysed the EV-based communication between cancer cells and fibroblasts by i) identifying factors that substantially increase EV release from intestinal cancer cells and ii) by determining cargo components of EVs that enhance tumour cell proliferation.
Methods: We used commercially available and patient-derived fibroblasts and CRC organoids. The Medical Research Council of Hungary approved all experiments with human samples and informed consent was obtained from patients. EVs were studied by using antibody-coated beads, TRPS, NTA, TEM and Western-blotting. We introduced Apc mutation into wild type murine small intestinal organoids by CRISPR-Cas9.
Results: We found that in CRC patient-derived organoid cultures, small EVs were preferentially secreted. We observed that Apc mutation and the accumulation of the extracellular matrix component collagen critically enhanced EV secretion in intestinal organoids. Furthermore, we showed that amphiregulin, present on fibroblast-derived EV, contributed to the maintenance of the intestinal stem cell pool and to cell proliferation in epidermal growth factor-dependent CRC organoids.
Summary/Conclusion: By proving the key role of mutations, collagen deposition and EV-bound amphiregulin in the release intensity and functions of the EVs, we identified novel mechanisms in the progression if CRC.
Funding: This work was funded by OTKA-NN 118018, by the National Competitiveness and Excellence program NVKP_16-0007 (National Research, Development and Innovation Office, Hungary) and by the National Excellence Program in Higher Education (Ministry of Human Resources, Hungary).

OT02.2

Prostate cancer-derived EVs induce a pro-inflammatory phenotype in the stroma

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Introduction: Since 90% of patients with metastatic prostate cancer (PC) develop bone metastasis, identifying the mechanism that drives this process is essential. Most EV research has been focused on the role of exosomes in mediating the pre-metastatic niche formation. However, most of these studies do not separate exosomes from large EVs. Our preliminary studies have demonstrated that a subclass of EVs known as Large Oncosomes (LO) can reprogram prostate

fibroblasts, at the primary tumour site, promoting angiogenesis and enhancing the migration and invasion of PC cells in vitro and tumour growth in vivo. The bone marrow is the initial site of entry into the bone microenvironment for disseminating tumour cells (DTCs) and is a rich source of nutrients that houses various cells types including bone marrow derived mesenchymal stem cells (BM-MSC) and immune cells such as neutrophils, which have been implicated in metastasis. Here we investigate the role of LO in reprogramming BM-MSCs and driving bone metastasis in PC.

Methods: Differential centrifugation, density gradient centrifugation, TRPS, RNA sequencing, qPCR, migration assay, invasion assay, chemotaxis assay.

Results: We report that PC-derived EVs induce distinct gene expressions changes in BM-MSCs. RNA-Seq analysis identified inflammatory and immune regulating cytokines as top differentially expressed genes (DEG) in BM–MSC. Moreover, LO induced a more potent response in BM-MSC in comparison to Exo and to non-treated controls. The genes enriched in LO treated BM-MSC were associated with tumour cell motility. In agreement with the gene expression data, LO-treated BM-MSC attracted migration and invasion of significantly more PC cells than Exo -treated BM-MSCs. In addition, the top DEG expressed in EV treated BM-MSC were identified as potent neutrophil chemoattractant proteins. In line with the RNAseq findings, the LO-treated BM-MSC demonstrated enhanced chemotaxis of neutrophils towards them in comparison with Exo or vehicle-treated BM-MSC. Finally, we show that the observed differences in BM-MSC's response to LO and Exo may be mediated by distinct molecular pathways.

Summary/Conclusion: The results from this study provide novel insight into how tumour derived EVs alter the bone marrow microenvironment and how they may drive bone metastasis in prostate cancer.

OT02.3

The αvβ6 integrin in cancer cell-derived small extracellular vesicles enhances angiogenesis

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Introduction: Prostate cancer (PrCa) cells crosstalk with the tumour microenvironment by releasing small extracellular vesicles (sEVs). sEVs isolated from PrCa cell media, express the epithelial-specific αvβ6 integrin, a surface receptor for fibronectin and vitronectin. The αvβ6 integrin is not detectable in healthy prostate tissues but is highly expressed in PrCa. In this study, we hypothesized that αvβ6 in cancer sEVs plays a crucial role in angiogenesis.

Methods: The sEVs isolated from PrCa cell media were characterized by nanoparticle tracking analysis, iodixanol density gradients and expression of sEV markers. The αvβ6-negative endothelial cells (HMEC1) were incubated with αvβ6-positive sEVs from PrCa cells to evaluate the transfer of αvβ6 by immunoblotting (IB) and FACS. The effect of αvβ6-positive sEVs on motility, tube formation and angiogenic signalling were assessed by Boyden chamber, angiogenesis assays and IB in HMEC1.

Results: We demonstrate for the first time that the αvβ6 is de novo expressed on endothelial cell surface by sEV-mediated protein transfer. PrCa cell-derived αvβ6-positive sEVs, significantly promote the motility and the formation of nodes, junctions and tubules by HMEC1. Mechanistically, we demonstrate that HMEC1 treatment with sEVs from PC3 cells that endogenously express αvβ6, decreases pSTAT1(Y701), a negative regulator of angiogenesis, while upregulating survivin, an inducer of angiogenesis. HMEC1 treatment with sEVs isolated from PC3 cells harbouring CRISPR/Cas9-mediated downregulation of β6, or shRNA-mediated downregulation of β6, results in increased levels of pSTAT1(Y701). This sEV treatment also results in a decrease of survivin in sEVs and HMEC1.

Summary/Conclusion: Overall, our findings show that αvβ6 in prostate cancer sEVs regulates a novel pro-angiogenic signalling pathway.

Funding: This study was supported by NCI R01-224769 (LRL); P01-140043 (LRL and DCA).

OT02.4

Prostate cancer exosomes promote bone metastasis in a cholesterol-dependent manner

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Introduction: Advanced prostate cancer (PCa) is associated with elevated cholesterol levels; however, the mechanisms underlying this association are not well understood. Further, the development of bone metastases is one of the most lethal processes in PCa, and is also associated with elevated cholesterol. Interestingly, recent work has demonstrated that exosome-mediated intercellular communication is dependent upon cholesterol levels in the target cell population. Here, we investigated whether PCa exosomes promote pre-metastatic niche formation and bone metastasis via intercellular communication with bone marrow-resident myeloid cells in a cholesterol-dependent fashion.
Methods: Exosomes from enzalutamide resistant (EnzR) PCa cells (CWR-R1) and normal prostate epithelial cells (PNT2) were isolated via ultracentrifugation, while exosomes from patient serum samples were isolated using ExoQuick Ultra (System Biosciences). Intracardiac injection of luciferase-expressing EnzR CWR-R1 cells into C.B.-17 SCID mice was used as a bone metastatic PCa mouse model, and tumour burden was monitored by BLI. Alterations in extracellular matrix (ECM) composition were determined via immunohistochemistry and RNA sequencing. Sequencing of small RNAs was performed on exosomal RNA samples obtained from conditioned media and patient serum. A nanoparticle mimic of high-density lipoprotein was used for targeted reduction of myeloid cell cholesterol.
Results: Data revealed that educating mice with PCa exosomes altered the composition of the bone marrow ECM in a pro-tumorigenic manner. Specifically, EnzR CWR-R1 exosomes reduced thrombospondin-1 (TSP1) expression and enhanced versican (VCAN) expression. Both changes have been reported to occur in advanced PCa. Furthermore, educating mice with EnzR CWR-R1 exosomes enhanced metastatic tumour burden in a PCa mouse model. Exosome-mediated pre-metastatic niche formation and enhanced tumour burden were both significantly diminished by targeted reduction of myeloid cellular cholesterol. Moreover, we found that exosome-induced downregulation of TSP1 was specifically mediated by exosomal miR-4443. This miRNA was also over-represented in the serum of late stage PCa patients compared to patients with early stage disease and healthy controls. Finally, reduced TSP1 and increased VCAN expression were also found in bone tissues obtained from patients with bone metastatic PCa.
Summary/Conclusion: These results offer insights into mechanisms of PCa bone metastasis, and specifically suggest an exosome-mediated mechanism for the link between elevated cholesterol and advanced, bone metastatic PCa. Finally, these results suggest that exosomal miR-4443 may be a viable diagnostic and therapeutic target for PCa.
Funding: This work was funded in part by a grant from the Prostate Cancer Foundation.

OT02.5

Low aggressive HNSCC-derived EVs regulates metastatic potential of highly metastasized HNSCC.

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Introduction: Extracellular vesicles (EVs) are secreted from cells, and carry bioactive proteins and RNA cargoes. Increasing numbers of studies have identified key roles for exosomes in driving aggressive tumour behaviours, including metastasis. However, the detailed mechanisms and responsible factors in the EV cargo are still unclear. Recently, immune system has been considered as an important factor in establishing and maintaining metastasis. Our goal is to identify the role of head and neck squamous cell carcinoma (HNSCC) derived small EVs (SEVs) in tumour metastasis from the study analysing the effects of SEVs on metastasis and tumour immunity.
Methods: SEVs were collected from the conditioned media of HNSCCs and purified through cushioned density gradient ultracentrifugation. An orthotopic mouse model was used for the assessment of tumour angiogenesis and metastasis. MOC1 (inflammation-inducing rarely metastasizing murine HNSCC line) and MOC2 (highly metastasizing murine HNSCC line) were used for this study. MOC1 and MOC2 cells were transplanted into mice tongues orthotopically, and MOC1/MOC2 derived SEVs or PBS were injected into the tumour twice in a week. Two weeks after tumour transplantation, mice were sacrificed and tumours were sectioned for pathological analysis and FACS analysis. In FACS analysis, the number and species of tumour-infiltrated immune cells were measured.
Results: Injection of SEVs from MOC1 into MOC2 tumours suppressed frequency of lymph node metastasis. On the other hand, injection of SEVs from MOC2 into MOC1 tumours didn't promote metastasis. CD4 positive T-cell distribution in MOC2 tumour was significantly changed by MOC1 SEV injection. T-cell deprivation treatment using anti-CD3 antibody

increased the frequency of metastasis in MOC1-SEV treated MOC2 tumours. From the result of proteomics analysis on MOC1 and MOC2 SEVs, immune-regulated proteins and metastasis-suppressing proteins were observed in MOC1 SEVs.

Summary/Conclusion: We find that low aggressive HNSCC SEVs affect metastasis of highly metastasized HNSCC, and also find that changing immune cell distribution may be related to the result. This mechanism and finding contributes to understanding the possible role of HNSCC SEVs on metastasis as well as on the tumour immune microenvironment.

Funding: This work was supported by the NIH under award numbers R01CA163592 and R01CA206458 to AW.

OT02.6

Desmoglein 2 enhances squamous cell carcinoma tumour development through extracellular vesicles in an IL-8/miR-146a-dependent mechanism

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Introduction: The cadherin Dsg2 is a stem cell marker that is upregulated in many different cancers, including SCCs, and its expression correlates with poor prognosis. Dsg2 activates mitogenic signalling and plays a key role in cell proliferation, migration, and survival. We recently showed that Dsg2 enhances EV release, but the mechanism by which these EVs modulate tumorigenesis is not fully understood.

Methods: We established SCC cell lines stably expressing wildtype Dsg2 or a palmitoylation deficient mutant, Dsg2cacs. EVs were isolated by sequential ultracentrifugation, iodixanol gradient separation, or qEV Izon column, and analysed by NTA and BCA. Tumour xenografts were established by subcutaneous injection of 106 cells in SCID mice and monitored up to 4 weeks. Cytokine profiling was determined by antibody array. miRNA expression was analysed by RNAseq and confirmed by qPCR.

Results: Dsg2 enhanced EV release by 40% and promoted a ~ fivefold increase in tumour size in xenograft models. Tumour growth was increased when control cells were treated with a single 20 μg dose of EVs. Loss of palmitoylation, which altered membrane trafficking of Dsg2, reduced EV release (~55%) as well as tumour development. Plasma EVs from xenograft mice reflected in vitro particle counts from SCC cell lines. A cytokine array analysis was performed revealing that Dsg2-EVs were enriched with pro-inflammatory cytokines including IL-8, a potent chemotactic and angiogenic factor. Most importantly, IL-8 was surface-bound on EVs. Furthermore, RNAseq revealed miR-146a, a negative regulator of IL-8, to be significantly downregulated in response to Dsg2. Treatment with miR-146a mimic or miR-146a inhibitor decreased or increased, IL-8 expression in SCC cells, respectively.

Summary/Conclusion: In summary, Dsg2 plays a key role in SCC tumour development by increasing EV biogenesis and downregulating miR-146a, which in turn upregulates IL-8 synthesis and release which can promote invasion, angiogenesis and metastasis.

Funding: NIH R01

OT03

Symposium Session 03: Cardiovascular Disease

Chair: Elena Aikawa, MD PhD – Center for Interdisciplinary Cardiovascular Sciences, Division of Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School

Chair: Edit Buzás – Semmelweis University, Department of Genetics, Cell and Immunobiology, MTA-SE Immune-Proteogenomics Extracellular Vesicle Research Group, Budapest, Hungary and HCEMM_SE Extracellular Vesicle Research Group

OT03.1

Towards identification of protein components mediating cardiac repair by cardiac progenitor cell-derived extracellular vesicles

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Introduction: Stem- and progenitor cell transplantation therapy holds great promise for regenerating damaged heart tissue. Several lines of evidence suggest that its efficacy is mainly caused by secreted extracellular vesicles (EVs). Indeed, cardiac progenitor cell (CPC)-derived EVs have been shown to protect the myocardium against ischaemia/reperfusion injury in several preclinical models. However, the underlying mechanisms for CPC-EV-mediated cardioprotection remain elusive. Here, we utilized the proteomic composition of CPC-EVs released during different culture conditions, to unravel protein-mediated effects of CPC-EVs on the endothelium.
Methods: CPCs were stimulated with calcium ionophore (ca ion-EVs) or vehicle (control-EVs) for 24 hours and EVs were isolated from serum-free conditioned medium using size exclusion chromatography. EV concentration and size was assessed using NTA. EVs were functionally characterized based on endothelial cell activation by western blotting and an endothelial cell scratch assay. The proteomic composition of both EV conditions was profiled using mass spectrometry. CPC-EV knockouts for specific proteins were generated using CRISPR/Cas9 technology.
Results: We found enhanced phosphorylation of ERK1/2 and AKT in endothelial cells and increased wound closure after stimulation with control-EVs, but not after stimulation with ca ion-EVs. Proteomic analysis identified a total of 1411 EV-associated proteins, with 79 proteins uniquely expressed in control-EVs. Another 68 proteins were revealed as candidate proteins, based on their relative enrichment in control-EVs compared with ca ion-EVs. GO analysis demonstrated that differentially expressed proteins were involved in vascular endothelial growth factor signalling, extracellular matrix organization and angiogenesis. To investigate the involvement of the individual candidate proteins on endothelial cell activation, knockout EVs of multiple proteins were generated and functionally characterized.
Summary/Conclusion: A specific set of EV proteins is identified that may be functionally responsible for the activation of endothelial cells upon exposure to CPC-EVs. Generating knockout EVs for each of these proteins will help to investigate their individual roles. This may lead to a better mechanistic understanding of the use of CPC-EVs as therapeutics for cardiac repair.
Funding: ERC-2016-COG-725229 EVICARE grant.

OT03.2

Hypoxia enhances the therapeutic potential of human CD34+ stem cell exosomes in ischaemic hindlimb repair

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Introduction: Human CD34+ stem cell therapy has emerged as a promising approach for the treatment of

ischaemic cardiovascular disease. We have previously shown that human CD34+ cell-derived exosomes (CD34Exo) improve perfusion and function of the ischaemic tissues. Hypoxia is shown to modulate the secretion and content of exosomes in both cardiovascular and cancer research. Therefore, we hypothesized that hypoxia can modulate the content and regenerative efficacy of human CD34Exo.

Methods: CD34Exo were isolated from primary human CD34+ stem cells cultured under hypoxia (1.5% O2, H-CD34Exo) or normoxia (20% O2, N-CD34Exo) using density gradient ultracentrifugation. CD34Exo size was measured using TRPS, NTA, and DLS and surface protein expression was determined using imaging flow cytometry. Function of CD34Exo was assessed using cell viability, migration and Matrigel tube formation assays in vitro and a mouse hind limb ischaemia model (HLI) in vivo. Protein content of hypoxic or normoxic CD34Exo was evaluated via LC-MS/MS and 2-D -DIGE followed by LC-MS/MS.

Results: We did not observe any significant differences in size or in quantity of exosomes secreted from H- or N-CD34 cells. Both H- and N-CD34Exo expressed CD9, CD81 and CD63 surface markers. Interestingly, H-CD34Exo significantly improved cell viability, migration and tube formation of HUVECs in vitro compared to N-CD34Exo. In the same line, H-CD34Exo also significantly improved perfusion (ratio: 0.93 ± 0.05 v 0.77 ± 0.02) and prevented ischaemic limb amputation (0% v 37.5%) as compared to N-Exo ($p < 0.05$; $n = 7–8$) in a murine (BalbC nude) model of hind limb ischaemia. Flow cytometry and confocal microscopy indicated that H-Exo was uptaken by endothelial cells in the ischaemic limb. Remarkably, we detected several proteins (including a fragment of hemopexin) and miRNAs (mir-210) that could be responsible for the proangiogenic and beneficial function of H-CD34Exo. We have also demonstrated that removal of surface proteins diminished the pro-angiogenic function of CD34Exo.

Summary/Conclusion: Hypoxia enhanced the proangiogenic and regenerative potential of CD34Exo, and thus, may represent a more efficient clinical strategy for CD34Exo therapy. Our research is clinically important to improve therapeutic angiogenesis in diabetic and cardiovascular patients with compromised stem cell populations.

OT03.3

MiRNA-192 and −432 depleted c-kit+ progenitor cell exosomes promotes the proliferation, migration of mesenchymal stem cells and regulates inflammation

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Introduction: Exosomes, a subset of membrane nanovesicles, transfer cellular information by passing proteins and nucleic acids between cells. Exosomes have been implicated as the mechanistic unit in stem cell therapy, as inhibition of exosome synthesis abrogates the effects of cell therapy following cardiac injury. More importantly, increasing evidence indicates that miRNAs (miRs) within exosomes serve as important signalling molecules to regulate inflammation, recruit stem cells, and repair diseased tissue. Among exosomal miRs, miR-192 and −432 are known to decrease angiogenesis, cell migration, and increase inflammation in various types of cells. Here, we investigated the inhibition of these negative miRs as a means to improve the reparative capacity of c-kit+ progenitor cell (CPCs) exosomes.

Methods: CPCs were isolated from three paediatric patients using magnetic-bead sorting. 2′-O-methylated RNA duplexes inhibited miR-192 and −432 expressions in CPCs. Exosomes (inhExos) were isolated from miR-inhibited CPC conditioned medium. MiR expression in exosomes and CPC was quantified by qRT-PCR. Migration and proliferation of mesenchymal stem cells (MSCs) were assessed two days post-exosome treatment. For inflammation analysis, THP1 cells with/without TNFα exposure were treated with exosomes and the expression of IL-6, −8, and −10 was quantified by qRT-PCR. Finally, the angiogenic potential of inhExos was tested by tube formation of cardiac endothelial cells.

Results: Inhibitor treatment of CPCs decreased exosomal miR-192 and −432 expression. Treatment with inhExos enhanced MSC migration and proliferation compared with normal CPC exosome (norExo). Moreover, inhExos showed promising results for immune regulation, as TNFα-induced inflammation was decreased in THP1 exposed to inhExos for 4 h. However, tube formation capacity is slightly decreased (~20%) by inhExo compared to norExo.

Summary/Conclusion: Exosomes from miR-192 and −432-depleted CPCs may be a promising strategy for

the treatment of various cardiac diseases, as they enhanced stem cell recruitment and proliferation, and regulated inflammation and angiogenesis. While other studies focus on boosting the reparative potential of exosomes by increasing positive miR and mRNA cargo, the inhibition of negative miR in exosomes could be an overlooked strategy for the treatment of cardiac disease.

OT04

Symposium Session 04: Cellular Uptake and Fusion

Chair: David R F. Carter – Oxford Brookes University

Chair: Neta Regev-rudzi – Weizmann Institute of Science

OT04.1

Endo-lysosomes as an alternative intracellular location for EV cargo delivery with disease relevance

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Introduction: Extracellular vesicles (EV) are lipid-bilayer nanovesicles that carry macromolecules and act as paracrine vectors for cell-to-cell communication. The processes regulating EV biogenesis are largely known, whereas how EV cargo is delivered to recipient cells remains poorly understood. A simple mechanism proposed is direct EV fusion with the cell membrane that liberate cargo into the cytosol. In this study, we observed that cargo release occurs also at an alternative intracellular location and that this acquires a disease relevance.
Methods: EV were isolated by serial centrifugation and characterized. For uptake studies, EV were traced by labelling donor cells with a lipophilic dye or by over-expressing GFP-CD63. Uptake was assessed by cyto-fluorimetry or by live confocal imaging. Co-localization studies were performed with ectopic marker expression or by immune staining. Protein-protein interaction was analysed by bi-molecular fluorescence complementation (BiFC). Prion-like transmission was studied using a pro-fibrillogenic Tau fragment in donor cells and full-length Tau in recipient cells. For quantification of subcellular localization, an automated algorithm based on machine learning was developed. Lysosomal stress was monitored by nuclear translocation of TFE3 and lysotracker staining. Antibodies directed against pathogenic epitopes of Tau were employed to assess prion-like transmission.
Results: EV were taken up by recipient cells through an endocytic process and accumulated in endo-lysosomes (EL). When cells were exposed to EV carrying a pro-fibrillogenic Tau, recipient cells accumulated Tau within EL by an autophagic process. Direct interaction of EV-Tau and cellular Tau in EL favoured the appearance of pathological epitopes. Cells displaying this condition showed an increased EL stress and cytotoxicity.
Summary/Conclusion: In this study, for the first time we report that EL represent a critical subcellular location where transcellular prion-like transmission mediated by EV of a neurodegeneration-associated protein occurs. Thus, the degradative pathway most likely involved in the recycle of EV and endogenous proteins is highjacked in disease. These findings represent a novel mechanism for EV acting as vector for transcellular propagation of Tau, which opens up new therapeutic interventions trying to halt the disease.
Funding: Supported by Gelu foundation.

OT04.2

Anti-human Fab fragment of CD9 antibody prevents the endocytosis of melanoma and colon cancer-derived extracellular membrane vesicles and nuclear transfer of their cargos

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Introduction: Interfering with the mechanisms regulating intercellular communication mediated by extracellular membrane vesicles (EVs) may find relevance especially in oncology where cancer cell-derived EVs have an implication in the malignant transformation of tumour microenvironment. Our laboratories recently demonstrated a novel intracellular pathway in which a fraction of endocytosed EV-associated proteins is transported into the nucleoplasm of the host cell via a subpopulation of Rab7+ late endosomes entering into the nucleoplasmic reticulum. Here, we have investigated the effect of a monovalent Fab antibody against the tetraspanin CD9 (referred hereafter as CD9 Fab), on the internalization of EVs and nuclear transfer of their cargo proteins.

Methods: To monitor the intracellular transport of EV-associated proteins, we used bioengineered fluorescent EVs containing CD9-GFP fusion protein from FEMX-I melanoma, SW480 colorectal cancer and bone marrow-derived mesenchymal stromal cells (MSC) as donors and the same cell types as recipients. EVs were enriched by differential centrifugation from 72 h serum-free conditioned media and characterized by ZetaView nanoparticle tracking analysis, zeta-potential and immunoblotting. CD9 Fab was prepared from 5H9 hybridoma cells using the Pierce Fab purification kit.
Results: We previously demonstrated that silencing CD9 both in EVs and recipient cells strongly decreased the endocytosis of EVs and abolished the nuclear transfer of their cargos. Here we show that CD9 Fab significantly reduced the cellular uptake of CD9-GFP+ EVs and the nuclear transfer of their proteins in melanoma, colorectal cancer and MSC used as receptor cells in a dose-dependent manner. The effect on the nuclear transfer is probably a direct consequence of the endocytosis inhibition of EVs. In contrast, the divalent, intact CD9 antibody stimulated both events.
Summary/Conclusion: The effect of CD9 Fab appears independent of the used EV-donor cell types or receptor cells, probably due to the widespread expression of CD9 both at plasma membrane and EV surface. In conclusion, by impeding intercellular communication in the tumour microenvironment, CD9 Fab-mediated inhibition of EV uptake, combined with direct targeting of cancerous cells could lead to the development of novel anti-cancer therapeutic strategies.

OT04.3

A bright, versatile reporter for multivesicular body trafficking and exosome secretion and uptake

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Introduction: Live imaging of exosomes is one of the required tools to understand the function of exosomes. Our previous live-cell reporter, pHluorin-CD63 allows dynamic subcellular monitoring of exosome secretion in migrating and spreading cells. However, there were some caveats to its use, including dim fluorescence and the inability to make cell lines that stably express the protein.
Methods: A stabilizing mutation, M153 R is incorporated in the pHluorin moiety and now exhibits stable expression in cells and superior monitoring of exosome secretion. A dual-tag reporter was created by incorporating a further pH-insensitive red fluorescent protein, mScarlet to the C-terminus of pHluo_M153 R-CD63. Cancer cells stably expressing the constructs were imaged using a variety of microscopy techniques in vitro as well as in vivo. Purified small EVs labelled with pHuo_M153 R-CD63 were imaged using immunogold transmission electron microscopy (TEM) and quantitated for the half-life in the blood circulation using flow cytometry.
Results: pHluo_M153 R-CD63 and pHluo_M153 R-CD63-mScarlet are exclusively detected in exosome-enrich small EV preparations. Immunogold TEM visualizes the pHluo_M153 R tag is located on the surface of small EVs. Live cell imaging reveals pHluo_M153 R-CD63–positive puncta left behind migrating cells suggesting the deposition consists of exosomes. Those puncta and trails are not only positive for exosome markers such as CD63, Alix, and TSG101 but also correspond to small EVs observed by a scanning electron microscopy. The dual-tag reporter allows visualization of the exosome lifecycle, including multivesicular body (MVB) trafficking, MVB fusion, exosome uptake and endosome acidification.
Summary/Conclusion: Using pHluo_M153 R-CD63 construct, we demonstrate superior visualization of exosome secretion in multiple contexts and a role of exosomes in promoting leader-follower behaviour in collective migration by observing that exosomes are secreted at the front of migrating cells and left behind in exosome trails. The dual-tag reporter allows visualization of the entire exosome lifecycle. We anticipate that these reporters will be broadly useful to investigate regulation and functions of exosome secretion and uptake in diverse physiological conditions.
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OT04.4

Uncovering novel genes regulating EV-mediated functional RNA transfer using a CRISPR/Cas9-based reporter system

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Introduction: Extracellular vesicles (EVs) play a pivotal role in intercellular communication through functional transfer of bioactive cargo, including RNA molecules. Despite increasing interest in EV-mediated RNA transfer, our understanding of the pathways and mechanisms regulating EV-mediated RNA delivery and processing is limited due to a lack of suitable readout systems. We recently developed a novel CRISPR/Cas9-based reporter system that allows study of EV-mediated RNA transfer at single-cell resolution. Here, we further validate this system by studying the role of known targets involved in EV uptake and intracellular membrane trafficking, and subsequently employ this system to uncover various novel genes that play a regulatory role in functional RNA transfer.
Methods: We employed a novel CRISPR/Cas9-based stoplight reporter system, in which eGFP expression is activated upon functional delivery of targeting single guide RNAs (sgRNAs) stably expressed by donor cells. Intercellular functional RNA transfer was assessed by measuring eGFP expression in acceptor cells using fluorescence microscopy and flow cytometry after direct co-culture, transwell co-culture, and upon addition of isolated EVs. Potential roles of various genes in intercellular RNA transfer were assessed by RNAi-mediated target knockdown in acceptor cells, prior to co-culture experiments. RNAi knockdown was confirmed by qPCR analysis.
Results: A significant activation of eGFP expression was observed in acceptor cells after direct co-culture and transwell co-culture with donor cells expressing sgRNAs, as well as after addition of EVs from cells expressing sgRNAs. Reporter activation was substantially decreased after knockdown of multiple targets involved in EV uptake through endocytosis and/or intracellular membrane trafficking. Based on these results, a potential role of various novel genes in intercellular RNA transfer was studied in acceptor cells. These experiments uncovered various novel targets involved in ECM binding, endocytosis, intracellular membrane trafficking, as well as various Rho GTPase interactors.
Summary/Conclusion: We previously demonstrated a CRISPR/Cas9-based reporter system that allows the study of functional delivery of small non-coding RNAs with single-cell resolution. Here, we show that this novel approach allows the study of specific genetic targets and pathways in EV-mediated functional RNA delivery, and unravel the regulatory pathways that dictate the underlying processes.
Funding: This work was supported by the Biotechnology and Biological Sciences Research Council (BB/M024393/1), the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 721058, and the Dutch Research Council (NWO) VI.Veni.192.174.

OT04.5

Quantitative characterization of extracellular vesicle uptake and content delivery within mammalians cells

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Introduction: Extracellular Vesicles (EVs), including exosomes, are thought to mediate intercellular communication through the transfer of biomolecules from donor to acceptor cells. Occurrence of EV-content delivery within acceptor cells has not been unambiguously demonstrated, let alone quantified, and remains debated.
Methods: We developed a cell-based assay in which EVs containing luciferase-tagged cytosolic cargo are loaded on unlabelled acceptor cells. Measurement of luciferase activity associated with acceptor cells revealed EV uptake efficacy. Additional cell fractionation procedure that separates membranes from cytosol revealed the occurrence of EV-content release within the cytosol of acceptor cells.
Results: Results from dose-responses, kinetics, and temperature-block experiments suggest that EV-uptake is limited (1% spontaneous rate at 1h), does not depend on bona-fide EV-receptor, at least for the tested acceptor HeLa cells. Yet, further characterization of this limited EV-uptake, through cell fractionation that separates membranes from cytosol, revealed the occurrence of EV-content release within the cytosol of acceptor cells. Cytosolic release is inhibited by Bafilomycin-A and overexpression of IFITM proteins, which prevent virus content delivery.
Summary/Conclusion: Our results show that EV-content release requires endosomal acidification and suggest the involvement of membrane fusion.
Funding: ANR18-CE15-0008-01 and ARC PJA 20171206453 and PGA1 RF20180206962.

OT05

Symposium Session 05: Single EV Analysis

Chair: Shannon Stott - Department of Medicine, Harvard Medical School

Chair: Joanne Lannigan – Flow Cytometry Core, University of Virginia School of Medicine

OT05.1

Droplet-based Single Extracellular Vesicle Sequencing for Rare Immune Subtype Discovery

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Introduction: Glioblastoma is a highly malignant brain tumour with a poor prognosis. Its ability to develop therapeutic resistance result in devastating clinical outcomes. To solve the intractable problem, we need highly sensitive diagnostics that can detect the molecular changes during treatments. Extracellular vesicles (EVs) can be a potential biomarker to monitor treatments and the host cell EV mapping can better reflect molecular changes in the tumour immune microenvironment. We have developed a droplet-based single EV protein sequencing platform that overcomes limitations of current bulk measurement technologies, which make it difficult to discover a rare EV population in the presence of high background.

Methods: We multiplex protein measurements to profile hundreds of proteins at a time by using an antibody-DNA conjugate and sequencing. We barcode each EV in droplets and make amplicons that are comprised of both EV barcodes and antibody barcodes for sequencing. Barcoded antibodies are made using TCO-tetrazine click reaction and EVs are labelled with these barcoded antibodies. The labelled EVs are encapsulated into droplets with barcoded beads that serve as a template for EV barcodes. We then perform extension to make amplicons that contain both EV barcodes and antibody barcodes for sequencing.

Results: We successfully fabricated barcoded beads using a split-pool approach and validated by observing a fluorescence decrease of the SYBR Green after DNA strand denaturation. We used a 3-channel droplet maker to encapsulate barcoded beads, single EV, and master mix into droplets. Close packing of barcoded beads allowed >95% encapsulation into droplets. Both droplet and tube-based methods achieved a similar high amplification efficiency (Ct < 30 for 300 EVs). We confirmed the amplicon size by running a gel, which showed the right amplicon size (~150 bp) from the droplet and tube prepared samples and no signal from the negative control.

Summary/Conclusion: The droplet-based single EV profiling platform has the ability to identify rare immune EV subtypes in the peripheral blood, which would otherwise be impossible to detect due to the co-presence of abundant normal EVs. This cutting-edge technique has the potential to revolutionize treatment monitoring of high-cost immunotherapies, avoid unnecessary toxicities, and enhance personalized medicine capabilities.

Funding: Schmidt Science Fellows, in Partnership with the Rhodes Trust

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QuantBio graduate student award at Harvard University.

OT05.2

Advancing extracellular vesicle characterization with quantitative single-molecule localization microscopy

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Introduction: Single-molecule localization microscopy (SMLM) can detect individual biomolecules with nanoscale precision. Although the technique has been routinely used to quantify biomolecules in cells, several technical challenges have impeded a similarly robust quantification in extracellular vesicles (EVs) secreted by cells. To address this, we developed optimized analytical protocol followed

by quantitative SMLM to robustly assess EVs derived from either cultured cells or patient biofluids.

Methods: Size exclusion chromatography was used to isolate EVs. EV membranes were labelled with various fluorescent reagents; core beads or size exclusion columns efficiently separated excess fluorescent molecules from labelled EVs. While imaging conditions needed to be significantly optimized when EVs were detected with fluorescent lipophilic membrane dyes, EVs were easily detected with lectins conjugated to fluorescent dyes typically used for SMLM. To ensure that the SMLM imaging data was robustly quantified, we optimized our analysis algorithms.

Results: We used our methodology to assess EVs isolated from plasma of patients with pancreatic cancer. Informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board. We determined the number of isolated EVs, their size, and the abundance of several biomarkers. According to our results, patients with pancreatic cancer exhibited a unique population of larger EVs containing epidermal growth factor receptor and carbohydrate antigen 19–9.

Summary/Conclusion: The methodological advancement in analytical preparation, SMLM imaging, and quantitative analysis can help identify sub-populations of EVs. As quantitative SMLM of EVs may represent a new diagnostic paradigm, our ultimate goal is to advance single molecule characterization of EVs for precision medicine.

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OT05.3

An evaluation of four orthogonal single-particle analysis platforms

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Introduction: In this study, we compared four orthogonal technologies for sizing, counting, and phenotyping of EVs. The platforms were: single-particle interferometric reflectance imaging senor (SP-IRIS) with optional fluorescence, nanoFCM nanoflow (NF), nanoparticle tracking analysis (NTA) with fluorescence, and microfluidic resistive pulse sensing (MRPS). Results from these platforms were compared with results from standard EV characterization techniques such as transmission electron microscopy (TEM) and western blot (WB).

Methods: Human T lymphocyte H9 (high CD81, low CD63) and promonocytic U937 (low CD81, high CD63) cells were chosen for their distinct tetraspanin profiles without abnormalities that might result from genetic manipulation. EVs were isolated from culture conditioned medium (CCM) by differential ultracentrifugation (dUC) and size exclusion chromatography (SEC) and characterized per MISEV2018 guidelines. Synthetic particles (silica and polystyrene spheres) with known concentrations and mixed size distributions were also tested.

Results: Particle counts from NF and MRPS were consistent, while NTA detected approximately one order of magnitude lower for CCM derived EVs, but not for synthetic particles. SP-IRIS events could not be used to estimate particle concentrations. For sizing, NF, MRPS, and SP-IRIS returned similar size profiles, with smaller sizes predominating (per power law distribution), but with sensitivity typically dropping off below diameters of 60 nm. NTA detected a population of particles with a mode diameter above 100 nm. Additionally, SP-IRIS, NF, and MRPS were able to identify at least three of four distinct size populations in a mixture of silica or polystyrene nanoparticles. Finally, for tetraspanin phenotyping, the SP-IRIS platform in fluorescence mode and NF were able to detect at least two markers on the same particle.

Summary/Conclusion: Based on the results of the study, we can draw conclusions about existing single-particle analysis capabilities that may be useful for EV biomarker development and mechanistic studies.

Funding: This project is funded by MH118164 and UG3CA241694.

OT05.4

Analysis of tetraspanin expression and spatial arrangement for EV subpopulations using single particle interferometric reflectance imaging and immunofluorescence.

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Introduction: Protein expression on single extracellular vesicles (EVs) is of great interest due to its

importance to EV organotropism. Yet, most techniques rely on bulk characterization, or are severely restricted by the diffraction limit. The ExoView R100 (NanoView Biosciences) combines interferometry, immunocapture, and immunofluorescence, introduced as an alternative technique to multiplex protein detection on single EVs below the limit of diffraction. Here, we use this technique to characterize tetraspanin multiplexing on EVs and to identify spatial patterning of tetraspanins using steric hindrance of antibodies (Abs).

Methods: EVs were isolated from conditioned media from SKOV-3 cell culture or human serum. EVs were incubated overnight on chips to allow immunocapture by anti-CD9, anti-CD63, or anti-CD81. Chips were then incubated with three fluorescent Abs against the same epitopes and imaged on the ExoView R100. Following concentration optimization, EVs were tested after pre-incubating with carboxy-fluorescein diacetate succinimidyl ester (CFSE) or fluorescent Abs against tetraspanins.

Results: Using different concentrations of EVs, binding curves could be fit to characterize binding kinetics of Abs. Maximum concentration of EVs could be identified that minimized fluorescent overlap. Bright-field interferometry (detection limit ~ 50 nm) distinguished 10x fewer bound EVs than fluorescent detection, while pre-labelling EVs with CFSE produced 10x more detectable EVs than immunofluorescence. Interestingly, EVs captured by one tetraspanin did not necessarily show high fluorescent detection of the same tetraspanin. Upon pre-incubating EVs with a single Ab, vastly different expression profiles were identified, indicating significant steric hindrance between Abs. Furthermore, pre-incubating EVs with anti-CD9 Ab significantly decreased detection of CD81 with less impact on CD63. This discrepancy indicated possible spatial patterning of tetraspanins with CD9 and CD81 closely colocalizing on the EV surface.

Summary/Conclusion: This combination of interferometry, immunocapture, and immunofluorescence produces unique information about size distribution of EVs and single EV protein profile. This data corroborates that EVs have distinct subpopulations of tetraspanins and indicates that tetraspanins may be spatially patterned.

OT06

Symposium Session 06: MSC EV Therapeutics

Chair: Andrew Hoffman, DVM, DVSc – University of Pennsylvania School of Veterinary Medicine

Chair: Michael Davis – Emory University

OT06.1

Regulation of liver homoeostasis, regeneration and diseases by mesenchymal stem cell-derived apoptotic extracellular vesicles

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Introduction: Billions of cells undergo apoptosis and produce apoptotic extracellular vesicles (ApopEVs) each day, whereas the roles of ApopEVs in regulating the organismal health and disease remain poorly understood. Mesenchymal stem cells (MSCs) emerge as critical contributors to tissue homoeostasis, while MSCs suffer from apoptosis in regenerative transplantation. In this study, we investigated the function and mechanisms of MSC-derived ApopEVs in regulating the organismal homoeostasis.
Methods: Fas mutant (Fasmut) and Caspase 3 knockout (Casp3-/-) mice were applied for apoptotic and ApopEV deficiency. Mouse bone marrow MSCs were cultured and apoptosis was induced by Staurosporine (STS). MSC-derived ApopEVs were collected by serial centrifuges and were infused into mouse circulation via caudal vein. Tracing of ApopEVs were performed by radioisotope or fluorescent labelling. Liver homoeostasis was evaluated at the histological and functional aspects. Liver regeneration was induced by partial hepatectomy (PHx). Acetaminophen (APAP) was used to establish acute liver drug injury. High-fat diet (HFD) was used to establish type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD).
Results: After systemic injection, MSC-derived ApopEVs migrate to liver and can be uptaken by liver macrophages and hepatocytes. Fasmut and Casp3-/- mice develop hepatomegaly with structural disorders, which particularly reveals hepatocyte polyploidization. Furthermore, Fasmut and Casp3-/- mice demonstrate liver glucose and lipid metabolic disorders. Importantly, MSC-derived ApopEV infusion significantly rescues structural and metabolic dysfunction in Fasmut and Casp3-/- mice. Mechanistically, ApopEVs use the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) protein for interactions with recipient organelles thus transferring signalling molecules. Moreover, MSC-derived ApopEV infusion promotes liver regeneration after PHx, prevents APAP-induced liver injury, and ameliorates NAFLD in T2D.
Summary/Conclusion: MSC-derived ApopEVs serve as crucial regulators of liver homoeostasis, regeneration and diseases. These findings indicate potential significant roles of ApopEVs in maintaining the organismal health and in developing therapeutics for diseases.
Funding: The Postdoctoral Innovative Talents Support Program of China (BX20190380) and the General Program of China Postdoctoral Science Foundation (2019M663986).

OT06.2

MSC-sEVs restore structural and mechanical properties in a rabbit model of cartilage injury

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Introduction: We previously reported that small extracellular vesicles derived from mesenchymal stem cells

(MSC-sEVs) mediate osteochondral regeneration in rats. However, the therapeutic effects of these MSC-sEVs/exosomes in restoring the mechanical competence of the repaired cartilage for joint function in a clinically relevant animal model remain to be addressed. To investigate this, we compared the structural and mechanical properties of the repaired cartilage in a rabbit model after intraarticular administration of MSC-sEVs and hyaluronic acid (HA) with that of HA alone, which is widely used as visco-supplementation.

Methods: Bilateral osteochondral defects were surgically created on 17 rabbits. Immediately after surgery and at days 7 and 14 post-surgery, 9 rabbits received 1-ml injections of 200 μg MSC-sEVs and HA in both knees, and 8 rabbits received 1-ml injections of HA in both knees. At 6 and 12 weeks, macroscopic evaluation, histological scoring and compressive testing at different points on the repaired cartilage were performed.

Results: Defects treated with MSC-sEV/HA showed improvements with time in macroscopic and histological scores and mechanical properties than defects treated with HA alone. In contrast, HA treated defects showed some repair at 6 weeks, but this was not sustained, as evidenced by significant deterioration of histological scores and a plateau in mechanical properties from 6 to 12 weeks. By 12 weeks, the MSC-sEV/HA repaired tissues demonstrated significantly better macroscopic score (10.33 vs 7.5; $P < 0.001$) and histological score (21.87 vs 11.11; $P < 0.001$). Mechanical strength as measured by the Young's modulus was significantly higher in the MSC-sEV/HA repaired cartilage than that in HA repaired tissues [defect centre (13.41 vs 3.95MPa; $P = 0.001$) and overall periphery (12.22 vs 3.37MPa; $P = 0.012$], and approximated that of the adjacent native cartilage.

Summary/Conclusion: Our findings demonstrated that MSC-sEVs and HA not only improved tissue morphology of the repaired cartilage but also promoted functional mechanical competence. This study establishes a clinically translatable protocol for use of MSC-sEVs for cartilage repair.

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OT06.3

Mesenchymal stromal cell exosomes educate myeloid cell populations to alleviate neonatal hyperoxia-induced lung injury

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Introduction: Mesenchymal stromal/stem cell (MSC)-exosome (MEx) treatment has shown considerable promise in experimental models of bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH). Mechanisms by which MEx afford their beneficial effects remain incompletely understood and here, we embark into investigating them through assessment of MEx biodistribution and impact on immune cell heterogeneity.

Methods: Newborn FVB mice were exposed to hyperoxia (HYRX, 75% O2) at birth and returned to room air at postnatal day (PN) 14. Mice received a bolus MEx dose at PN4. Adoptive transfer studies were used to determine the role of MEx-educated myeloid cells in vivo. Mice were harvested at PN4, 7, 14, or 28 to characterize MEx biodistribution and for assessment of pulmonary parameters.

Results: MEx therapy effectively ameliorated core features of HYRX-induced neonatal lung injury, improving alveolar simplification, pulmonary fibrosis, vascular remodelling and blood vessel loss. Exercise capacity testing and assessment of PH showed functional improvements following MEx therapy. Biodistribution studies demonstrated that MEx localize in the lung, where they interact with lung monocytes/macrophages. Whole lung mass cytometry (CyTOF) revealed that MEx treatment promotes a pro-homoeostatic shift in lung immune cell apportion, replenishing the early HYRX-induced depletion in pulmonary CD45+ immune cells, restoring alveolar monocyte and macrophage populations and suppressing cellular inflammation. Ex vivo and in vivo analysis showed that MEx promotes a "pro-resolving" CCR2- monocyte phenotype. Notably, adoptive transfer of MEx-educated bone marrow-derived myeloid cells (BMDMy), but not naïve BMDMy, restored alveolar architecture, blunted fibrosis, improved vascular remodelling and pulmonary blood vessel loss.

Summary/Conclusion: MEx treatment ameliorates core features of experimental BPD, restoring lung architecture, decreasing pulmonary fibrosis and vascular muscularization, ameliorating PH and improving exercise capacity. The beneficial actions of MEx are associated with modulation of immune cell phenotypes, arising from MEx-monocyte interaction. Furthermore, adoptive transfer of MEx-educated BMDMy rescued, at least in part, alveolar architecture, reduce fibrosis, improve vascular remodelling and pulmonary blood vessel loss.

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OT06.4

Immunomodulatory small extracellular vesicles derived from mesenchymal stem cells: A potential cell-free therapy for acute and chronic pulmonary vascular diseases

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Introduction: Vascular inflammation plays a critical role in acute respiratory distress syndrome (ARDS) and pulmonary arterial hypertension (PAH). Despite decades of research, there is no curative therapy for either condition. Mesenchymal stem cells (MSCs) have shown preclinical efficacy, mediated by release of extracellular vesicles. Hence, MSC-derived small extracellular vesicles (sEVs) can harness the benefits of MSCs with advantages in cost and safety. This study aims to evaluate the immunomodulatory effects of sEVs in preclinical ARDS and PAH.
Methods: MSC-sEVs were characterized by nanoparticle tracking analysis, electron microscopy and western blot. Live fluorescence imaging measured in vitro and in vivo distribution of sEVs. Using a lipopolysaccharide (LPS)-induced mouse model of acute lung injury (ALI), a time course study of inflammatory response guided endpoint analyses. Cell count and cytokines were measured in bronchoalveolar lavage fluid (BALF) and histological lung injury was assessed. In ALI mice, saline, MSCs, MSC conditioned media or sEVs were administered 0.5 h post-LPS. Using a monocrotaline (MCT)-induced rat model of PAH, animals received saline or sEVs at day 3. Haemodynamic changes and right ventricular hypertrophy were evaluated at 3 weeks.
Results: MSC-sEVs were 72 nm in size with CD63/81 expression. PKH26-labelled sEVs were taken up by endothelial cells. In the ALI time course study, cell count and IL1b in BALF peaked at 24h post-LPS, whereas IL6 peaked at 10h. Histology showed significant intra-alveolar cell infiltrate at 72h. MSC conditioned media attenuated IL1b in BALF, whereas a trend towards reductions in IL1b and cell count were seen from delivery of MSCs and sEVs. Using fluorescence imaging, lung accumulation of DiR-labelled sEVs was highest when administered 1 h post-LPS as compared to 5 h, 10 h or 24h. For PAH rats, sEVs reduced right ventricular systolic pressure (51.4 ± 8.3 mmHg) as compared to control (68.1 ± 0.4 mmHg; P = 0.012), whereas no changes were observed for right ventricular remodelling.
Summary/Conclusion: These findings demonstrate the potential of MSC-sEVs to be used as a cell-free immunomodulatory therapy for acute and chronic lung vascular diseases. Additional live and ex-vivo biodistribution studies will determine optimal timing of sEV administration, tissue distribution and clearance in both ALI and PAH.

OT06.5

Changes in extracellular vesicle protein cargo after pro-inflammatory priming of umbilical cord mesenchymal stem cells

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Introduction: Human Umbilical Cord Mesenchymal Stem Cells (UCMSCs) have been shown to suppress inflammatory responses in studies of autoimmune diseases. These therapeutic effects can be attributed to paracrine signalling, by which extracellular vesicles (EVs) are one of the essential components. This study looks at how the culture conditions of UCMSCs affects the type of EVs they secrete. It also aims to identify an EV population with an anti-inflammatory potential for the treatment of autoimmune diseases.
Methods: UCMSCs were isolated and culture expanded in a Quantum® cell expansion system, then grown at 21%O2, 5%O2 and primed with a pro-inflammatory cocktail. EVs were isolated from UCMSC conditioned media by differential ultracentrifugation using a sucrose cushion and characterised by Transmission Electron Microscopy and Nanoparticle Tracking Analysis. EV markers were analysed using a europium-based immunoassay, Macsplex Exosome Detection kit and immunoblotting. A proximity-based extension assay was used to identify 92 inflammatory proteins in the EVs.
Results: There was no difference in EVs cultured at 21%O2, 5%O2 or with pro-inflammatory conditions when analysed for size and morphology. All EVs displayed the tetraspanin markers (CD9/63/81) and

internal proteins (Alix, Hsp70). EVs from primed cells showed a > twofold increase of 5 CC chemokines and a > sixfold increase in CXCL5 and CSF-1. Protein cargo did not differ in EVs from 21%O2 and 5%O2.

Summary/Conclusion: This study showed that pro-inflammatory culture conditions alter EV protein cargo, evidenced by the increased production of chemotactic and angiogenesis associated proteins. Upcoming RNAseq analysis will show if UCMSC culture conditions also affect miRNA expression in EVs. Ongoing functional studies will determine how changes in EV cargo correlates with changes in T-cell proliferation and polarisation.

Funding: This work is fund by the Orthopaedic Institute Ltd, Keele University and the RJAH Orthopaedic Hospital Charity.

OT07

Symposium Session 07: Neurodegenerative Diseases

Chair: Eva-Maria Albers Kramer – Institut für Entwicklungs- und Neurobiologie Zelluläre Neurobiologie AG Extrazelluläre Vesikel

Chair: Xandra Breakefield – MGH

OT07.1

Alzheimer's disease biomarkers in plasma extracellular vesicles of neuronal origin correlate with brain pathology in mice

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Introduction: Multiple studies have shown that neuronal-derived extracellular vesicles (NDEs) in blood contain Alzheimer's disease (AD) biomarkers, especially Tau. However, the convergent validity of Tau in blood NDEs in relation to brain pathology is yet to be determined. To address this, we measured total and phosphorylated Tau levels in matched NDE and brain tissue samples from AD mouse models.
Methods: We collected the cortex, hippocampus and plasma of 4 2xTg-AD, 15 3xTg-AD, and 9 Wild Type (total of 28 mice; 14 female, 14 male; age: mean = 8.17, SD = 1.61, 5–11 months). Plasma samples were collected retro-orbitally for 5 weeks and at euthanasia via heart puncture. NDEs from the pooled serial blood collections (NDE1) and the single endpoint (NDE2) were immunocaptured by targeting the neuronal marker L1CAM. We measured human total Tau and pThr181-Tau (p-Tau) in NDEs and cortex and hippocampus homogenates using a Luminex multiarray.
Results: Overall, there were strong positive correlations for both total Tau and p-Tau between NDEs and brain tissues across mice types. Total tau in NDEs showed positive correlations with levels in the cortex and hippocampus ($r = 0.6885$ and 0.7026, $p < 0.0001$, cortex vs NDE1 and NDE2; $r = 0.4958$, $p = 0.0073$, hippocampus vs NDE1; $r = 0.6058$, $p = 0.0006$, hippocampus vs NDE2). Levels of p-Tau in NDE1 showed positive correlations with levels in the cortex ($r = 0.4640$, $p = 0.0155$) and hippocampus ($r = 0.6086$, $p = 0.0008$); however correlations were not observed for NDE2 ($r = 0.1152$, $p = 0.6273$ vs cortex; $r = 0.0531$, $p = 0.7926$ vs hippocampus).
Summary/Conclusion: Tau levels in circulating NDEs reflect levels in cortex and hippocampus across AD model mice, supporting their convergent validity as "liquid biopsy" biomarkers for AD.
Funding: This research was supported in part by the Intramural Research Program of the National Institute on Ageing, National Institutes of Health.

OT07.2

Exosomal ceramide mediates neurotoxicity of Amyloid beta (Aβ) in Alzheimer's disease.

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Introduction: Amyloid beta is a pathologic hallmark of Alzheimer's disease (AD), however, the mechanism of Aβ neurotoxicity is not fully understood. It has been reported that exosomes associate with Aβ, but it is not clear how this association affects Aβ neurotoxicity.
Methods: Here we utilized several techniques to isolate exosomes from the sera of wild type (WT) and AD transgenic mouse model.

(5xFAD) as well as Alzheimer's patients and healthy controls. We used Exoquick, Exoeasy, sequential ultracentrifugation, and size exclusion chromatography. Particles' size and number were characterized by nanoparticle tracking analysis (ZetaView).
Results: We report that the sphingolipid ceramide mediates neurotoxicity of Aβ. We show that sera from AD transgenic mouse model (5xFAD) and AD patients, but not the WT or healthy controls, contain a subpopulation of astrocyte-derived exosomes that are enriched with ceramide and are prone to aggregation (termed astrosomes) as confirmed by nanoparticle tracking and cluster analyses. When taken up by

Neuro2A cells and human iPS cell-derived neurons, these astrosomes are shuttled to mitochondria where they induce mitochondria clustering, evident by elevation of expression of the fission protein dynamin-related protein1 (Drp1). Using proximity ligation assays, we show that Aβ forms a complex with voltage dependent anion channel 1 (VDA1), a protein that functions as a gatekeeper for the entry and exit of mitochondrial metabolites and is a key player in mitochondria-mediated apoptosis. Complex formation colocalized with ceramide cotransported with Aβ by astrosomes. The interaction between Aβ and VDAC1 leads to caspase3 activation and subsequently apoptosis. Aβ-associated exosomes, not Aβ alone, induced neurite fragmentation and neuronal death, suggesting that association with astrosomes substantially enhances Aβ neurotoxicity in AD. Interestingly, the novel ceramide analogue N-oleoyl serinol (S18) prevented the aggregation of exosomes, and Aβ association with astrosomes, and reduced Aβ interaction with VDAC1, suggesting that exosomal ceramide mediated binding of Aβ to astrosomes and mitochondrial damage.

Summary/Conclusion: Our data suggest that the association of Aβ with ceramide in astrosomes enhances Aβ interaction with VDAC1 and mediates Aβ neurotoxicity in AD, which can be prevented by novel ceramide analogues.

Funding: This work is supported by NIH R01AG034389 and R01NS095215, and VA 1 I01 BX003643.

OT07.3

Blood-derived extracellular vesicles in Multiple Sclerosis: the effects of Particulate Matter exposure

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Introduction: Multiple Sclerosis is the most common chronic inflammatory demyelinating disease of the central nervous system, affecting more than 2 million people worldwide. MS is a multifactorial, immune-mediated disease caused by complex genetic and environmental interactions. In recent years, extracellular vesicles (EVs) have been described as powerful mediators of the modulation of biological processes (e.g. inflammatory and immune response) following environmental exposures such as Particulate Matter (PM), and have been described altered in MS. We characterized EVs in 48 patients with MS and 20 healthy subjects matched for age and gender and evaluated the effects of PM exposure on EV release patients with MS compared with controls.

Methods: EVs isolated from blood samples were characterized by nanotracking analysis and by flow cytometry after labelling with the following markers: CD14 + (monocyte), CD61+ (platelet), CD66+ (neutrophil), CD25+ (T-reg), and CD105+ (endothelium). PM10 and PM2.5 concentrations at the residency of each subject were obtained from the regional air quality monitoring network.

Results: We observed decreased concentrations of CD14+ ($p < 0.05$), CD61+ ($p < 0.003$), CD66+ ($p < 0.009$), CD25+ ($p < 0.003$), and CD105+ ($p < 0.003$) in patients compared with controls. In cases, PM was inversely associated with CD14+ EVs (PM2.5, $\beta = -0.03$; $p < 0.05$), CD66+ EVs (PM2.5 $\beta = -0.03$; $p < 0.03$), and CD25+ EVs (PM10 $\beta = -0.02$; $p < 0.04$; PM2.5, $\beta = -0.03$; $p < 0.02$). On the contrary, in controls PM was positively associated with CD14+ EVs (PM10 $\beta = 0.02$; $p < 0.03$; PM2.5, $\beta = 0.03$; $p < 0.02$).

Summary/Conclusion: Our findings showed a different composition of blood-derived EV subpopulations in patients compared with controls. Moreover, we observed that patients and controls react differently to PM exposure in terms of blood-derived EV release, suggesting the involvement of this mechanism in the modulation of both inflammatory and immune responses, and thus in MS pathogenesis.

OT07.4

Plasma neuronal and astrocyte-derived exosomes serve as biomarkers of neurodegeneration and systemic bioenergetic effects in male Cynomolgus monkeys self-administrating oxycodone

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Introduction: Opioid use disorder (OUD) is currently a health emergency in the USA affecting millions of people. OUD is a complex issue requiring a multi-pronged strategy. At the biological level, there is an urgent need to understand the dynamic molecular changes and adverse effects associated with opioid addiction. Here, we aimed at identifying the

biosignature of brain cells-derived exosomes associated with opioid addiction in a non-human primate (NHP) model of OUD in which Cynomolgus monkeys perform cognitive tasks and self-administer (SA) intravenous oxycodone daily. We also characterized the systemic adverse effects of the brain cells-derived exosomes from drug-naïve and oxycodone SA monkeys.

Methods: We isolated total exosomes (TE) by ultracentrifugation and ExoQuick methods from the plasma of male monkeys self-administrating oxycodone for 3 years and naive monkeys. Subsequently, from the TE population, we isolated neuron-derived exosomes (NDE) and astrocytes-derived exosomes (ADE) using surface biomarkers L1CAM (L1 Cell Adhesion Molecule) and GLAST (Glutamate Aspartate Transporter), respectively. This novel method involved streptavidin coated magnetic beads and photo-cleavable (PC) biotin, providing us biologically intact exosomes useful for co-culture studies. We characterized the exosomes by nanoparticle tracking analyses (NTA), Western blotting, flow cytometry, immunogold labelling, transmission electron microscopy (TEM), ELISAs and mass spectrometry. Respirometric profiling in cardiac myoblasts and monocytes following exosomes treatment was performed by Seahorse XF.

Results: The quality of isolated exosomes (TE, NDE, and ADE) was confirmed by NTA (size distribution and concentration), Western blotting (e.g. CD9) and TEM (size and shape). NTA did not show any significant difference in exosomes size and concentration (number per ml) between control and oxycodone SA groups. Flow cytometry (e.g. L1CAM and GLAST) and immunogold labelling (CD9, CD63 and L1CAM) confirmed the purity of NDE and ADE isolated from TE. Proteomics analyses of TE, NDE and ADE identified several unique proteins present in exosomes from the oxycodone SA group. Interestingly, we observed significantly higher expression of neurodegeneration markers neurofilament light protein (NFL) and alpha-synuclein in NDE and ADE of oxycodone SA group compared to controls. Furthermore, TE treatment of H9C2 cardiac myoblasts and RAW264.7 monocytes significantly compromised their mitochondrial metabolism (basal and maximum respiratory capacity).

Summary/Conclusion: These results suggest the utility of plasma exosomes as biomarkers for better understanding of the neurodegenerative and systemic effects of oxycodone addiction.

Funding: DA049267, DA017763.

OT08

Symposium Session 08: Bacterial and Fungal Evs

Chair: Amy Buck – The University of Edinburgh

Chair: Cherie Blenkiron – The University of Auckland

OT08.1

Vesicles released during Mycobacterium tuberculosis infection: immunomodulatory (glyco)lipids and role in host-pathogen interactions

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Introduction: The tuberculosis disease remains one of the top 10 causes of death worldwide. Mycobacterium tuberculosis (Mtb) has evolved strategies to evade immune responses and to persist within the hostile intracellular environment of alveolar macrophages. The current lack of efficient anti-tuberculosis strategies is largely due to our incomplete understanding of the host-pathogen interactions of Mtb infection. Vesicles released by the bacillus itself (bacterial membrane vesicles, BMV) and by infected cells (host extracellular vesicles, HEV) have immunomodulatory properties in vitro and when administered to animals. If vesicles likely play key role in host-pathogen interactions of the Tuberculosis infection, their content in bacterial factors, their uptake, trafficking and interaction with host cells receptors remain incompletely deciphered.
Methods: BMV and HEV have been purified by combining differential centrifugation, density gradient and exclusion chromatography. After characterization by microscopy, NanoSight and western blot, their content in bacterial (glyco)lipids has been characterized by the use of high sensitivity mass spectrometry-based lipidomic approach. BMV have been tested for their capacity to activate reporter cell lines of pattern recognition receptors. In addition, fluorescent-labelled BMV have been used to study their uptake by host cells thank to super-resolution microscopy.
Results: We have undertaken to characterize the content, the trafficking and interaction with pattern recognition receptors of BMV and HEV released during infection by mycobacteria of variable virulence. We have importantly optimized the purification of BMV showing that lipoproteins aggregates are co-purified with vesicles on density gradient. SFC-MS lipidomic analyses allowed the characterization of the repertoire of immunomodulatory bacterial lipids released by BMV and HEV, which excluded a continuum between these two release pathways. Preliminary, assays have shown that these vesicles are capable to interact with different pattern recognition receptors including TLR and lectins. Finally, we have been able to visualize fluorescent-labelled vesicles uptake by macrophages using superresolution microscopy.
Summary/Conclusion: During M. tuberculosis infection, the bacillus as well as infected cells release vesicles that harbour different content in immunomodulatory bacterial (glyco)lipids, including strain-specific lipids. These vesicles likely play important role in host-pathogen interactions by modulating immune response beyond the infected cells, in part through their interaction with different pattern recognition receptors.
Funding: Fondation pour la Recherche Medicale, Fondation FonRoga.

OT08.2

Blood-based detection of tuberculosis using outer membrane vesicles

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Introduction: Conventional diagnoses of Mycobacterium tuberculosis (Mtb) rely on quantifying bacteria in sputum samples, which make it incapable of measuring the body's total bacterial load and diagnosing patients that have difficulty producing sputum – such as children and those that are HIV-positive. Nanoscale (30–200 nm) outer membrane vesicles (OMVs), which are shed from their bacterial cells of origin and circulate in the bloodstream, have been found to contain rich molecular information from their mother cells. Despite the diagnostic potential, their nanoscale size in the presence of high background has complicated the use of these promising biomarkers for clinical diagnosis of tuberculosis.

Methods: Here we report two complementary approaches to systematically discover and clinically detect Mtb-derived OMVs using protein and RNA biomarkers. First, we employ a digital droplet ELISA on whole, unprocessed samples to detect and quantify the presence of these OMVs using surface protein markers. Second, we have developed a platform to specifically enrich for Mtb-derived OMVs using our previously developed magnetic nanopore platform, wherein millions of nanofluidic devices are operated in parallel, increasing throughput relative to a single nanofluidic device by a million-fold. Using this approach, we identify RNAs that are specifically enriched in Mtb-derived OMVs and can be used to identify TB strain, infectious activity, and total body burden.

Results: Using these platforms, we enriched for Mtb-derived OMVs from plasma and profiled their cargo, both proteins and RNA. We first determined a panel of protein biomarkers for multiplexed detection of OMVs through a digital droplet sandwich ELISA. We then tested our protein markers on spiked plasma samples as models for clinical TB samples. Simultaneously, we performed RNA sequencing and discovered a panel of RNA biomarkers that are preferentially enriched in OMVs. We picked ten of the most highly-expressed RNA biomarkers and also tested for them on spiked plasma samples using our magnetic nanopore platform.

Summary/Conclusion: These results demonstrate the capability of OMV biomarkers in the development of novel liquid biopsy based Mtb diagnostics. Building on this work, we are working with clinical collaborators to test our assays on clinical samples from Philadelphia and west Africa.

Funding: NIH

OT08.3

Mechanisms of innate immune regulation by exosomes released during infection with pathogenic gram-negative bacteria

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Introduction: A dearth of knowledge exists regarding the molecular mechanisms by which host exosomes regulate immune response to infections caused by gram-negative pathogens. To address this gap in knowledge, our laboratory has been using two well-established model organisms; Yersinia pestis (Yp), and Burkholderia pseudomallei (Bp). Yp and Bp cause the emerging human diseases plague and melioidosis respectively. Currently, no licenced vaccines or highly effective therapeutics are available for either disease.

Methods: EX were purified from naïve U937 monocytes (EXu) and infected U937 (EXi) by serial centrifugation followed by sucrose density gradient purification, and characterized by TEM, ZetaView nanoparticle tracking, and exosome markers (CD63, TSG101, and Flotillin-1). Immune responses of naïve U937 cells and response mechanisms were analysed following treatment with equivalent amounts of EXi or EXu (as control). These included macrophage differentiation assays, multiplex measurements of inflammatory cytokines, bacterial clearance assays, quantitative protein microarray analysis of 173 host signalling proteins, siRNA knockdown of EXi-induced cytokines in recipient cells, and mass spectrometry (MS) analysis of EXi contents. For all assays, at least four biological replicates were performed.

Results: EXi induce monocyte differentiation to macrophages and dramatic release of IL-6, IL-8 and IL-10 cytokines, effects that are also seen when monocytes are infected with the bacteria. The EXi also induce a substantial increase in the capacity of the recipient monocytes to clear bacteria in an IL-6-dependent manner. Specific host signalling molecules are strongly modulated by the EXi, including p38, Jak2 and ALK for EXi-Yp, influencing the observed phenotypes. MS analysis showed lack of LPS in EXi-Yp and demonstrated the presence of specific bacterial proteins that have antigenic properties.

Summary/Conclusion: We have identified some of the molecular mechanisms by which EXi assist the host in clearing infection. EXi prime distant naïve monocytes through modulation of distinct pathways such as p38 to mount immune responses similar to when they become infected. These include differentiation to macrophages and migration to infection site for increased IL-6-dependent bacterial clearance.

Funding: NIH grant (1R15AI137981-01) and U.S. MRMC grant (W81WH-15-T0003)

OT08.4

Human pathogenic fungus Candida albicans induces immunomodulatory TGF-β1-transporting vesicles from human immune cells

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Introduction: Recruitment of monocytes to sites of infection is important in restricting growth and invasion of various microorganisms such as pathogenic fungi C. albicans. Beside complement supported phagocytosis and extracellular trap formation, human monocytes secrete extracellular vesicles which are crucial in cellular communication in physiology and pathophysiology as they transfer proteins, lipid, and nucleic acids. The current study attempts to shed light on immune evasion mechanisms by C. albicans via extracellular vesicles.

Methods: Human monocytes were isolated by magnetic beads technique and extracellular vesicles were isolated using polymer precipitation or ultracentrifugation or size exclusion chromatography. Vesicles were characterized by ELISA, LC/MS-based proteomics, confocal laser scanning microscopy (CLSM) as well as electron- and dynamic light scattering microscopy, etc. CRISPR-CAS9 based genome editing was performed to knockout CD11b in human monocytic THP-1 cells. Effect of isolated vesicles were determined by using proximity ligation assay (PLA), ELISA, Western Blot, next generation RNA sequencing, QPCR, Immunohistochemistry, etc.

Results: Here we show for the first time that human blood derived monocytes alone and in a whole blood model strongly induced and released extracellular vesicles in response to the pathogenic fungus C. albicans. One induced population carried the anti-inflammatory cytokine TGFβ-1. Release of these vesicles is triggered by binding of soluble β-glucan from C. albicans to the CR3 receptor on monocytes as demonstrated by CRISPR-CAS9 based CR3 genome editing in THP-1 cells, and by using CR3 knock out mice. Isolated TGF-β1-transporting vesicles reduced the inflammatory response in human M1 macrophages and in a whole blood model. The anti-inflammatory effect by TGF-β1-transporting vesicles is investigated in detail and results in inhibition of IL-1β gene transcription.

Summary/Conclusion: Showing that human apoptotic bodies similarly induced TGF-β1-transporting vesicles from human monocytes we hypothesize that C. albicans hijacks this new CR3-dependent anti-inflammatory vesicle pathway for immune escape.

Funding: This work was supported by the "Deutsche Forschungsgemeinschaft" TransRegio Funginet 124 projects C4, C5, C6 and Z2.

OT09

Symposium Session 09: EV Biogenesis I

Chair: Guillaume van Niel – Institute of Psychiatry and Neurosciences of Paris U-1266 INSERM

Chair: Maureen Barr – Rutgers University

OT09.1

Fragmentation of extracellular ribosomes and tRNAs shapes the extracellular RNAome

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Introduction: To date, most research involving extracellular RNAs has focused in RNAs encapsulated inside extracellular vesicles (EVs) or in total unfractionated biofluids. It is known that exRNAs also exist outside vesicles or in lipoprotein particles. However, nonvesicular exRNAs remain widely uncharacterized despite being a feasible source of contaminants in EV preparations. Our interest in nonvesicular exRNAs arises from the observation that some small RNAs, such as specific tRNA-derived fragments, have much higher relative representation in this extracellular fraction. At least in part, this enrichment seems to be a consequence of their differential extracellular stability.

Methods: To get a representative picture of the whole set of RNAs released to the extracellular nonvesicular space by cultured human cells, we inhibited extracellular degradation by adding recombinant ribonuclease inhibitor to the cell-conditioned media and studied the kinetics of RNA release and degradation. High-resolution iodixanol gradients were used to separate EVs from extracellular RNPs or vesicle-free RNA. The conversion rate between parental ncRNAs and their fragments was studied by high-throughput sequencing and Northern blot.

Results: The inhibition of extracellular RNase activity revealed the presence of full-length tRNAs and ribosomes in the extracellular space of a variety of malignant and non-malignant cell lines. Extracellular ribosomes co-isolate with EVs purified by ultracentrifugation or size-exclusion chromatography, but not with EVs purified by density gradients.These ncRNAs are substrates of extracellular RNases, demonstrating an extracellular biogenesis route for the formation of ncRNA-derived fragments, some of which achieve remarkable stability and can be detected in biofluids. We also highlight the immunoregulatory potential of purified RNA-containing extracellular complexes.

Summary/Conclusion: In conclusion, ribonuclease inhibition dramatically shapes extracellular RNA profiles and uncovers a population of extracellular ribosomes, tRNAs and other coding and noncoding RNAs which exists outside EVs. Although these RNAs are prone to degradation, some of their fragments can accumulate in cell culture media and in biofluids. This dynamic view of exRNAs impacts our understanding of RNA secretion mechanisms and may offer a window to new molecules with biomarker potential. In contrast, EVs confer an RNase-protected environment and contain more full-length ncRNAs (tRNAs, YRNAs, 7SL RNAs, rRNAs depending on vesicle size) than their fragments.

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OT09.2

CD47 interactions with exportin-1 regulate targeting of m7 G-capped RNAs to extracellular vesicles

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Introduction: CD47 is a ubiquitously expressed membrane protein that functions as a receptor for thrombospondin-1 and the counter receptor for signal regulatory protein-α in phagocytes. High expression of CD47 is associated with a poor prognosis for some cancers. Conversely, CD47 blocking agents are in clinical trials for enhancing innate and adaptive antitumor immunity in cancer patients. These studies suggest utility of CD47 as a diagnostic and prognostic biomarker and as a therapeutic target. CD47 is also expressed on extracellular vesicles (EVs), and we reported that CD47 expression identifies a distinct population of EVs from those that express the traditional EV markers CD63 or MHC1. CD47-, CD63- and MHC1-enriched vesicles contain distinct small RNA populations (PMID: 29416092), and these differ in RNA content from EVs that lack any of these markers. The mechanisms by which CD47 directly or indirectly regulates which RNAs are packaged into EV remain unknown.
Methods: To elucidate the mechanism by which CD47 regulates EV RNA composition and function, we performed global miRNA microarray analysis between EVs produced by wild type and CD47-deficient T cells. Results were further validated using real-time PCR and RNA-immunoprecipitation. Interactions between CD47 and exportin-1/Ran complex was identified by mass spectrometry and confirmed by using co-immunoprecipitation, subcellular localization, flow cytometry, and confocal and electron microscopy.
Results: EV released from CD47-deficient human T cells and in cd47-/- mouse plasma were enriched in 5′-7-methylguanosine-capped microRNAs and mRNAs that depend on the exportin-1/RanGTP pathway. Knockdown of CD47 in wild type cells or thrombospondin-1 treatment correspondingly enhanced levels of capped-RNAs released in EV and re-expressing CD47 in null cells decreased their levels. Mass spectrometry and co-immunoprecipitation identified specific interactions of CD47 with components of the exportin-1/Ran nuclear export complex and its known cargos and between the CD47 cytoplasmic adapter ubiquilin-1 and the exportin-1/Ran complex. Interaction of CD47 with exportin-1 was inhibited by leptomycin B, which inactivates exportin-1 and increased levels of cap-dependent RNAs in EV released from wild type but not CD47-deficient cells.
Summary/Conclusion: These findings indicate that CD47-dependent thrombospondin-1 signalling regulates cytoplasmic levels of cap-dependent RNAs in T cells at least in part through ubiquilin-1- and GTP-dependent physical interactions with the exportin-1/Ran transport complex, which regulate levels of specific pre-miRNAs and mRNAs available for sorting into EVs.
Funding: This work was supported by the Intramural Research Program of the NIH/National Cancer Institute (ZIA SC 009172).

OT09.3

Role of membrane protein palmitoylation in extracellular vesicle biogenesis in squamous cell carcinoma

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Introduction: Desmoglein 2 (Dsg2), is a palmitoylated cadherin that is involved in cell-cell adhesion. Interestingly, Dsg2 promotes mitogenic cell signalling and is upregulated in many cancers, including SCC, contributing to poor prognosis and survivability. We recently demonstrated that Dsg2 promotes EV release, but the mechanism by which Dsg2 enhances EV biogenesis and role of palmitoylation is poorly understood.
Methods: Pharmacological drug inhibitors 2-bromopalmitate, GW4869, and Bafilomycin A1 were used. Stable SCC cell lines were established by retrovirus infection expressing GFP, wild type Dsg2/GFP, or palmitoylation deficient Dsg2cacs/GFP. EVs were isolated by sequential ultracentrifugation and iodixanol gradient separation and analysed by NTA. Proteins associated with the endocytic pathway were analysed by immunofluorescence and imaged by confocal microscopy or immunoblotting and signals were quantitated using ImageStudio.

Results: Here we demonstrate that the effect of Dsg2 on EV release was reduced by the palmitoylation inhibitor 2-bromopalmitate. Furthermore, mutations that prevented palmitoylation (Dsg2cacs) dramatically abrogated EV release by targeting of un-palmitoylated Dsg2 to the lysosomes for degradation. Dsg2 increased expression and subcellular localization of FLOT1, a membrane lipid raft protein critical for membrane invagination. Dsg2 also altered membrane localization of several early (EPS15 and EEA1), but not late (Rab7, Rab11, and HRS), endocytic pathway proteins. Loss of palmitoylation in the Dsg2cacs mutants abrogated these effects. Finally, Dsg2-induced EV release was abrogated by the sphingomyelinase inhibitor GW4869 or augmented by the V-ATPase inhibitor Bafilomycin A1.

Summary/Conclusion: The combined results of the drug treatments and functional mutations of Dsg2 suggest that Dsg2 plays a critical role in EV biogenesis by modulating proteins involved in early endosome sorting and is dependent on post-translational palmitoylation.

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OT09.4

Translation initiation factor eIF4E reprograms the vesiculation of cancer cells

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Introduction: The translation initiation factor eIF4E (4E) is an oncogenic protein that is upregulated in 30% of cancers including a subgroup of acute myeloid leukaemia (AML) patients. EIF4E regulates post-transcriptional RNA processing including the nuclear export and/or translation of mRNA transcripts. In particular, it selectively increases the expression of genes that have a prominent role in cancer progression such as MYC, cyclin D1, and MCL1. Furthermore, our lab pioneered studies demonstrating that a subset of 4E-high AML patients is clinically responsive to treatment with a 4E inhibitor (Ribavirin) indicating the importance of 4E in AML progression and its relevance as a therapeutic target. We investigated an as yet unexplored perspective of 4E- whether the oncogenic role of 4E is in part mediated by its function as a master regulator of vesiculation.

Methods: To assess mRNA export and identify 4E-bound mRNA targets that correspond to vesiculation-related genes and associated cargo, we used cellular fractionation and RNA immunoprecipitation techniques. To determine whether 4E regulates the number of extracellular vesicles (EVs) released as well as their protein and RNA cargo we used Nanoparticle Tracking Analysis (NTA) as well as mass spectrometry, antibody microarrays, and RNA sequencing technologies.

Results: EIF4E upregulates cellular protein levels of the vesiculation marker CD63 by increasing its nuclear export. In addition to increased cellular expression, CD63, CD81, CD9, and flotillin-1 proteins are elevated in EVs released from 4E-high cells. This is also associated with an increased release of vesicles that are 50–200 nm in size. Currently, we have validated the upregulation of several receptors and cytosolic proteins in EVs isolated from 4E-overexpressing cells that function in cell growth, migration, invasion, and stemness. The most abundant RNAs in our EV preparations are microRNAs (miRs) and we have confirmed downregulation of several of these.

Summary/Conclusion: Our work shows that 4E reprograms the vesiculation of cancer cells changing the release and cargo of EVs. This may impact cellular communication and tumour biology, which we are currently addressing in functional studies. We hope that these studies will highlight novel therapeutic strategies for AML patients.

Funding: Leukemia and Lymphoma Society, Cole Foundation

OT10

Symposium Session 10: EV-based Therapeutics I

Chair: Dolores Di Vizio – Cedars-Sinai Medical Center

OT10.1

Intranasal administration of neural stem cells-derived extracellular vesicles promotes neurogenesis and reduces neuroinflammation and amyloid plaques in a mouse model of Alzheimer's disease

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Introduction: Cognitive and memory impairments worsen with time in Alzheimer's disease (AD), likely due to a progressive loss of hippocampal neurogenesis, and escalation of neuroinflammation. These changes are also accompanied by increased deposition of amyloid plaques in the brain.

Methods: In this study, using the 5XFAD mouse model, we examined the efficacy of extracellular vesicles (EVs) shed from the rat subventricular zone neural stem cells (SVZ-NSCs) for disease modification. We first purified EVs from the rat SVZ-NSC cultures through ion-exchange chromatography and then administered intranasally to 3-months old 5XFAD mice (~75 billion/week for two weeks). Two months later, the functional effects of EV treatment were quantified through a series of behavioural tests, and animals were euthanized for quantification of hippocampal neurogenesis, oxidative stress, neuroinflammation, and amyloid plaque deposition.

Results: In comparison to AD mice receiving vehicle, AD mice receiving NSC-EVs displayed improved cognitive function to discern minor changes in the environment in an object location test, better spatial recognition memory in an object-in-place test, and improved pattern separation ability in a pattern separation test. Besides, EV-treated AD mice displayed no anhedonia in a sucrose preference test. Analyses of neurogenesis using the birth-dating marker 5′-bromodeoxyuridine and the newly born neuron marker doublecortin revealed maintenance of a higher level of hippocampal neurogenesis in AD mice receiving EVs, in comparison to vehicle-treated AD mice. Moreover, analyses of brain tissues from EV-treated AD mice revealed decreased concentrations of oxidative stress markers malondialdehyde and protein carbonyls and elevated levels of antioxidants catalase and superoxide dismutase. Also, the concentration of proinflammatory cytokines tumour necrosis factor-alpha and interleukin-1 beta and the extent of amyloid plaques were significantly reduced in EV treated AD mice. Immunohistochemical analysis showed reduced hypertrophy of astrocytes.

Summary/Conclusion: Intranasal administration of NSC-derived EVs restrains the deterioration of cognitive and mood dysfunction of AD by maintaining higher levels of neurogenesis and curtailing the progression of neuroinflammation.

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OT10.2

Protective effects of extracellular vesicles derived from induced pluripotent stem cells in acute kidney injury model

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Introduction: Induced pluripotent stem cells (iPSC) present a wide range of applications, including cellular therapy. However, the tumorigenic potential is a limitation in the iPSC use. The extracellular vesicles (EV) have been shown to mimic the beneficial effects of cells, as in

the case of mesenchymal stromal cells, opening new perspectives in the use of iPSC in regenerative medicine. The aim of this study is to evaluate the potential of iPSC-EV in the treatment of kidney disease.

Methods: The iPSC were generated from skin fibroblast after informed consent of healthy donors (CytoTune®-iPS 2.0 Sendai Reprogramming Kit – Protocol: Clementino Fraga Filho UH 38583914.7.0000.5257/933.018). The EV were isolated from iPSC supernatants (cultured 24 h in mTeSR-1 medium) by ultracentrifugation (100,000 g for 2 h at 4°C). Characterization of iPSC-EV was performed using ZetaView, TEM, ExoView™ Tetraspanin Kit and MACSPlex Exosome Kit. For in vitro injury, renal epithelial cells were cultured under hypoxia (1% O2). For in vivo injury, male Wistar rats were submitted to bilateral renal arterial clamping (45 min) followed by reperfusion without or with injection of iPSC-EV (Protocol approval: Federal University of Rio de Janeiro – 043/19). Kidney damage was assessed by histological and immunohistochemistry analyses (PCNA, TUNEL and ED-1). Modulation of RNA levels was assessed by RT2 Profiler PCR array.

Results: The results show that iPSC-EV reduce renal cell death, tissue damage, macrophage infiltration, promote mitochondria protection and ameliorate renal function. The iPSC-EV mechanism of action is related to the regulation of key genes known to prevent damage caused by oxidative stress like GSTK1, SOD1, SOD3, TXN1 and TXNRD2. Characterization of iPSC-EV showed that iPSC-EV can carry important molecules that can support renal recovery as EpCAM and Prominin-1.

Summary/Conclusion: iPSC-EV presents renoprotective properties, acting on different aspects of AKI. This presents a new relevant application of iPSC as a source of EV for therapeutic purpose in kidney diseases.

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OT10.3

Epigenetic regulation of foetal hypoplastic lungs by amniotic fluid stem cell-derived extracellular vesicles

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Introduction: Incomplete lung development, also known as pulmonary hypoplasia (PH), is a recognized cause of neonatal death. We have previously shown that experimental PH can be rescued by the administration of extracellular vesicles derived from amniotic fluid stem cells (AFSC-EVs) through an RNA-mediated mechanism. This effect was not observed with EVs derived from mesenchymal stromal cells (MSC-EVs). The aim of this study was to 1) evaluate which RNA species were responsible for PH rescue, and 2) to define the mechanism behind this effect.

Methods: EVs were isolated and characterized from conditioned medium of rat AFSCs and rat MSCs (control group) using ultracentrifugation. EVs were assessed for size (nanoparticle tracking analysis), morphology (TEM), and expression of CD63, Hsp70, Flo-1, and TSG101 (Western).

To identify the mediators of AFSC-EVs, we used DESeq (FDR<0.01) to differentially analyse RNA from:

A) ASFC-EV and MSC-EV cargo, isolated with SeraMir and sequenced with NextSeq.
B) lung epithelial cells from rat PH lungs treated with vehicle or AFSC-EVs. Epithelial cell RNA was isolated with miRvana and sequenced with NextSeq.

We correlated AFSC-EV cargo miRNA with validated mRNA targets that were downregulated after EV conditioning in lung epithelial cells.

Results: Of the RNA species contained in ASFC-EV and MSC-EV cargo, miRNAs were the most proportionally different between the two EV populations. AFSC-EVs were enriched for miRNAs that are critical for lung development, such as miR17 ~ 92 and their paralogues that control lung branching morphogenesis. AFSC-EV administration to PH lung cells significantly downregulated 35 genes, which formed 415 miRNA-mRNA reported interactions.

Summary/Conclusion: AFSC-EVs contain many RNA species in their cargo, but miRNAs are the main effectors of their ability to rescue underdeveloped foetal lungs. We have identified for the first time that AFSC-EV biological effect on underdeveloped foetal lungs is in part due to the release of miR17 ~ 92 cluster.

Funding: CIHR-SickKids Foundation grant.

OT10.4

Bottom-up assembly of fully-synthetic extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are considered as key elements for future therapeutic and diagnostic procedures. However, despite enormous research efforts to understand their physiological relevance and several greatly successful clinical trials, EVs are currently not authorized for clinical routines by American or European regulation and approval agencies. This is especially because therapeutic EVs are produced or isolated from cell cultures or biofluids, both of which are subjected to batch-to-batch variations and ill-defined contaminations. Therefore, complementary technologies that produce EVs as reproducible and defined as state of the art nanotherapeutics, would revolutionize the application of EVs in clinical settings and provide the scientific community with a holistic understanding of EV-mediated signalling processes. In our study, we achieve de novo bottom-up assembly of fully synthetic EVs (fsEVs) that comprise identical physiological and therapeutic functionalities to natural EVs.
Methods: We applied droplet-based microfluidic synthesis to sequentially amalgamate synthetic lipids, proteins and nucleic acids into defined vesicles that display analogous therapeutic capabilities to natural EVs. FsEVs were characterized by electron and confocal microscopy, dynamic light scattering and mass spectrometry and tested on organotypic models or in vivo.
Results: Using previously described EVs as "nature-given" blueprints, we assembled several fsEVs in their exact molecular composition. In particular, we produced wound-healing promoting EVs composed of several exosomal proteins, lipids and microRNAs and showed that their therapeutic performance on human skin wounds is equivalent to that of natural EVs. Besides their high molecular complexity, being composed of dozens of different molecular building-blocks, the presented fsEVs are completely defined on a quantitative level. Based on this, we achieved a stoichiometric understanding of cell-vesicle interactions.
Summary/Conclusion: By applying bottom-up synthesis of fsEVs for quantitative studies on EV signalling, we not only provide innovative and safe compounds for EV-therapeutics but also a vastly new perspective on the application spectrum of extracellular vesicles in fundamental research.

OT10.5

lncRNA EPHA6-1 in IFNβ-induced small extracellular vesicles promotes cytotoxicity of NK cells by acting as a ceRNA for hsa-miR-4485 to up-regulate NKp46 expression

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Introduction: Small extracellular vesicles (sEVs) contain functional molecules from their cell of origin and can enter recipient cells for intercellular communication. IFNβ has been shown to induce some lncRNAs to regulate host immune response and play a major role in the positive regulation of the activity of natural killer (NK) cells. Here, we aim to clarify whether IFNβ induced sEVs can regulate the cytotoxicity of NK cells by transferring specific lncRNAs into NK cells.
Methods: EVs were purified from A549 with/without IFNβ treatment by serial centrifugation followed by sucrose density gradient purification. ELISA assay were performed to demonstrate the cytotoxicity of NK cells. qPCR and Western Blot were used to verify the expression of NKp46.
Results: Surprisingly, IFNβ induced sEVs can strengthen the cytotoxicity of NK cells. Through human transcriptome array (HTA) we found the expression levels of 69 lncRNAs were significantly changed within sEVs isolated from A549 cells following IFNβ treatment. Additionally we found a specific sEV cargo, linc-EPHA6-1, acted as a competing endogenous RNA (ceRNA) for hsa-miR-4485 which subsequently up-regulate the natural cytotoxicity receptor (NKp46) expression. Furthermore, we verified over-expression of linc-EPHA6-1 significantly enhance the cytotoxicity of NK cells against Zika virus-infected A549 cells.
Summary/Conclusion: Our results demonstrated that IFNβ-induced linc-EPHA6-1 wrapped in sEVs can regulate the cytotoxicity of NK cells. Our study provides a novel link between type I IFN and NK cells, which are two major players for the host innate immunity against pathogen infections.
Funding: This work was financially supported by the Chinese Academy of Medical Sciences Innovation Fund for Medical Sciences (2016–12 M-3-025 to LC); the National Key Research and Development Program from Ministry of Science and Technology of China (2018YFE0107500 to LC) and the National Natural Science Foundation of China (NSFC 81702017 to S. Li).

OT11

Symposium Session 11: Viruses

Chair: Shilpa Buch – University of Nebraska Medical Center

Chair: Malene M. Møller – Department of Clinical Immunology, Aalborg University Hospital

OT11.1

Unbiased quantitative proteomic analysis identifies novel cellular components specifically incorporated into endogenous EV subtypes, HIV-1 particles and virus-modified EVs

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Introduction: HIV-infected T cells release simultaneously viral particles and small extracellular vesicles (sEVs) including MVB-derived exosomes and plasma membrane-derived EVs. sEVs and HIV share many physical and chemical characteristics, which makes their separation difficult. Although several approaches have been used to obtain sEVs free of virus they leave a majority of sEVs within HIV preparations. For this reason, the function of sEVs during HIV infection remains unclear.
Methods: We have developed a novel un-biased proteomic profiling approach to identify specific markers of the virus or sEV subtypes released by a human T lymphoma cell line. Our approach was to combine differential centrifugation of medium/small EVs contained in the CCM with quantitative mass spectrometry to generate protein abundance profiles across the different sub-fractions. We generated an interactive database to define groups of proteins with similar profiles, suggesting their release in the same EVs.
Results: We thus identified different categories of EVs, which bear different surface proteins, e.g. different combinations of T cell surface markers, integrins or tetraspanins. In EVs released by infected cells, we identified cellular proteins behaving like HIV proteins, and several that changed behaviour after infection, either moving towards or away from the HIV cluster. We identified two cell-derived proteins that are included in the viral particles and one that is specific of non-viral sEVs that are modified by infection, and analysed their respective roles in controlling EV composition or virus infectivity.
Summary/Conclusion: Our approach presents a powerful tool for identification of common cargoes of given EV subtypes, and could be now used to identify modifications of EV composition in any given physiological or pathological situation.
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OT11.2

The encephalomyocarditis virus Leader modulates autophagic pathways to promote the release of virions inside extracellular vesicles

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Introduction: Recent data indicate that naked viruses belonging to the picornaviridae family can be released from host cells via enclosure in extracellular vesicles (EV). EV cloak virus particles in a host-derived "envelope" and can thereby affect antiviral immune responses and disease severity. A better understanding of the formation and function of EV-enclosed viruses is therefore required. Previously, we showed the presence of the autophagosome marker LC3 in EV isolates from encephalomyocarditis virus (EMCV) infected cells, suggesting the involvement of a secretory autophagy

pathway in EV-mediated virus release. However, little is known about the viral and host factors that regulate this process. Here, we have assessed the role of the EMCV Leader, a viral protein that is dispensable for replication but is required for symptomatic disease.

Methods: Cells were infected with wildtype virus or a mutant carrying an inactive Leader. EV produced during the infection were isolated using differential ultracentrifugation and density gradient purification. EV were characterized by high resolution flow cytometry and their infectivity determined using end-point dilution assay. In addition, the fate of autophagosomes in infected cells was monitored using a reporter assay for autophagosome-lysosome fusion and analysis of the secretion of autophagosomal proteins.

Results: Inactivation of the EMCV Leader strongly reduced the release of EV-enclosed virus. Whereas autophagosomes are typically degraded, we show this is blocked by the Leader. Instead, autophagosomes fuse with the plasma membrane, as indicated by the secretion of autophagy marker LC3 during infection with wildtype but not the mutant virus. Pharmacological reactivation of degradative autophagy in infected cells resulted in a strong reduction in the release of EV and EV-enclosed virus. Similarly, the reduction in EV-enclosed virus release in the absence of the Leader could be partially reversed by drugs that promote the secretion of autophagosomes.

Summary/Conclusion: Our data supports a role for secretory autophagy in the release of viruses in EV, a pathway that is regulated by the EMCV Leader. These findings highlight an unconventional route for EV formation that intersects with autophagosomal compartments and contributes to viral pathogenesis.

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OT11.3

ZIKV induced DEFA1B blocks ZIKV adsorption and retard cell cycle by regulating ORC1 expression

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Introduction: Zika virus (ZIKV) causes a public health emergency of international concern because of its correlation with microcephaly. During viral infection, the innate immune response quickly to produce some endogenous functional molecules which can prevent viral invasion or replication. Extracellular vesicles (EVs) contain molecules from their cell of origin under virus infection and can enter recipient cells for intercellular communication. Here, we aim to clarify whether ZIKV induced EVs can regulate viral pathogenicity by transferring specific RNA.

Methods: EVs were purified from A549 with/without ZIKV infection by serial centrifugation followed by sucrose density gradient purification. Human transcriptome array (HTA) was used to found RNA expression within EVs. Flow Cytometry was used to determine cell cycles. ZIKV replication was assayed by qPCR and Western Blot. Flow Cytometry was used to determine cell cycles.

Results: Through HTA we found the defensin alpha 1B (DEFA1B) expression was significantly increased within EVs isolated from ZIKV infected A549 cells. Additionally, we found that the extracellular DEFA1B but not the intracellular DEFA1B exerts anti-ZIKV effect mainly before entry step. Surprisingly, up-regulate DEFA1B can retard cell cycles of host cell. We verified DEFA1B could bind with the origin recognition complex 1 (ORC1) which is required to start DNA replication during the cell cycle. Furthermore, up-regulate DEFA1B decreased the ORC1 level in nuclear. Interestingly, EVs with DEFA1B can internalize into recipient cells and inhibit their cell cycles.

Summary/Conclusion: Together, our results demonstrated that ZIKV infection can induce DEFA1B wrapped in EVs, and DEFA1B not only exerts anti-ZIKV effect but also regulate cell cycles which may affect neurodevelopment. Our study provides a novel viewpoint that DEFA1B act as first-line anti-viral molecules during ZIKV infection also correlate with neurodevelopment by retarding cell cycles.

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OT11.4

Extracellular vesicles mediate bacterial-immune cell interactions during respiratory viral-bacterial co-infections

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Introduction: Respiratory infections are a major cause of morbidity and mortality worldwide and host-derived extracellular vesicles (EVs) play important roles in

mediating these infections. During respiratory infection, EVs are shown to have a modulatory effect: promoting or suppressing infection dependent on the pathogen and cell type. In the age of next-generation sequencing, we now appreciate that many respiratory infections are polymicrobial in nature, with viral-bacterial co-infections correlating with worse disease outcomes. Epidemiological studies correlate acute viral infections with the increased likelihood and severity of both acute and chronic secondary bacterial infections; however, the exact mechanisms of these interactions remain poorly understood. EVs have been understudied in the context of respiratory viral-bacterial co-infections; thus, their role in mediating these infections is relatively unknown. Unpublished data from the lab shows that in airway epithelial cells (AECs), viral infection induces the release of EVs that associate with Pseudomonas aeruginosa (Pa) and promote biofilm growth. Here, we aim to expand upon these findings and determine how AEC EVs mediate Pa-immune cell interactions during respiratory viral-bacterial co-infection.

Methods: To determine how exposure to EVs impacts Pa-immune cell interactions, EVs were isolated from the apical secretions of AECs and co-cultured with Pa. EV-treated Pa was then co-cultured with macrophages to evaluate EV impact on Pa uptake and survival.

Results: In preliminary experiments using control EVs, we observed that EVs associate with Pa. Interestingly, during co-culture with macrophages, EV-treated Pa are more susceptible to phagocytosis in comparison to non-treated Pa. However, after 4 hours of co-culture with macrophages, EV-treated Pa are able to survive and replicate, while non-treated Pa are effectively controlled by the macrophages.

Summary/Conclusion: These findings suggest that while Pa-EV association promotes Pa uptake, it may ultimately enhance Pa immune evasion and survival. Ongoing experiments in the lab are evaluating the mechanism of Pa-EV association and how EVs from virus-infected AECs affect the phenotypes observed with control EVs. Notably, this is one of few reports of a mammalian EV influencing the pathogenesis of a bacterium; thus, results from these experiments will define the function of AEC EVs in regulating bacterial-immune cell interactions during respiratory co-infections.

OT11.5

Using machine learning with neuronal EV target proteins and clinical data to predict cognitive impairment in HIV infection

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Introduction: Objective biomarkers are needed to assess and predict neuronal function and cognitive impairment. In people ageing with chronic infections, such as HIV, determining the mechanism of impairment will be important when therapies are available.

Methods: Sixty plasma samples from HIV-infected people were obtained from NIH-sponsored AIDS banks. Clinical and epidemiological data were collected. All underwent neuropsychological testing and 40 were considered impaired. Neuronal extracellular vesicles (nEVs) were isolated from plasma and assayed for high-mobility group box 1 (HMGB1), neurofilament (NF-L) and phosphorylated tau-181 (p-tau) proteins.

Results: Using 3 different algorithms, Support Vector Machines (SVM) performed the best with an area under the curve (AUC) value of 0.82 ± 0.16. Using 4 different combinations of clinical data and the 3 nEV protein targets, selected clinical data and HMGB1 best predicted cognitive impairment (AUC = 0.82). The most important features included CD4 count, HMGB1, NF-L and education.

Summary/Conclusion: Specific clinical features plus nEV HMGB1, an inflammatory marker, were the best predictors of cognitive impairment. Previous published data showed nEV p-tau-181 elevated in Alzheimer's disease and in this study p-tau had no importance in assessing HIV-associated cognitive impairment. nEV target discovery can be improved to better identify neuronal damage, possibly to differentiate other neurodegenerative diseases and hopefully recovery after therapies are identified.

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OT11.6

Who comes out first: Extracellular Vesicles (EV) or Viruses?

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Introduction: In recent years, we have been able to separate and characterize Extracellular Vesicles (EVs) from several different viruses including HIV-1, HTLV-1, Rift Valley Fever Virus and Ebolavirus. However, to date it is not clear whether there is a timing difference between EV and virus release from infected cells.

Methods: EV isolation by Nanoparticle capture and differential centrifugation, EV quantification by Nanoparticle

tracking analysis, Western Blot, RT-qPCR, virus rescue assay.

Results: We have attempted to address the kinetics of EV and virus release from multiple-infected cells using serum starvation experiments from infected (100%) cells. These infected cells were initially put in G0 quiescent stage using serum starvation. Both supernatants and cell pellets were collected post-induction release (20% FBS + PMA/PHA) at 0, 3, 6, 12, and 24 hours and examined for the presence of EV, autophagy and viral proteins as well as viral RNA expression. Results from supernatants of uninfected cells showed a peak of tetraspanin proteins (CD63, CD81, and CD9) at 6 hours and a gradual decrease of all EV associated proteins by 24 hours. However, the EV from HIV-1 infected cells showed all three tetraspanins present at 3 hours and expression gradually increased up to 24 hours. When compared to HTLV-1 infected cells, the three tetraspanin proteins peaked at 6 hours and expression continued to decrease up to 24 hours. HTLV-1 infected cells also showed a unique pattern of CD81 expression. Autophagy associated proteins (LC3A, LC3B and p62) from uninfected cells and HTLV-1 infected cells plateaued at 6 hours, whereas in HIV-1 infected cells their expression continued to increase and peaked at 24 hours. HIV-1 viral proteins (p24, gp120, Nef) expression was present at 6 hours and continued to increase and peaked at 24 hours. HTLV-1 proteins (p19 and gp46/61) peaked at 6 hours and gradually decreased overtime. HIV-1 and HTLV-1 RNA gene expression analysis was performed, and data correlated with viral protein expression. Additionally, EVs release was quantified and showed significant increase of EV concentration overtime in both uninfected and infected samples. Finally, experiments of infectivity from 6- and 24-hour supernatants were performed on three naive cells. HIV-1 supernatant 6- hour sample was found not to be infectious. However, HIV-1 was successfully rescued from 24-hour sample.

Summary/Conclusion: Collectively, our data indicates that EV release may occur prior to viral release in infected cells, thereby implicating a potentially significant effect of EVs on uninfected recipient cells prior to subsequent viral infection.

Funding: This work was supported by National Institutes of Health (NIH) grant NS099029.

OP1 = PT15

Oral with Poster Session 1: Lumgs

Chair: Uta Erdbrügger – University of Virginia

Chair: Peter Kurre, MD – Comprehensive Bone Marrow Failure Center, Children's Hospital of Philadelphia; Perelman School of Medicine, University of Pennsylvania

OP1.01 = PT15.01

Human urinary extracellular vesicles carry surface markers that are indicative of haematopoietic origin

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Introduction: Urinary extracellular vesicles (uEVs) are important intercellular communicators. By systems biology integration, uEVs prove to be relevant in genitourinary disease detection. However, it has recently been shown that labelled EVs administered to the circulation can be detected in the urinary system, as well. Thus, this pilot study aimed at phenotyping haematopoietic surface markers on uEVs to create enough plausibility for future non-invasive biomarker studies of circulation and immune disorders that may translate into urine but are not yet timely recognized.
Methods: Urine was obtained from healthy men signing a written informed consent (n = 31). Sampling was approved by the local ethics committee and in compliance with the Declaration of Helsinki. Cell-free urine was obtained by serial centrifugation and 10 ml, each, were utilized for the MACSPlex Exosome Kit, human (Miltenyi Biotec). The manufacturer's recommendations were followed to examine 37 distinct uEV surface markers of CD9+/CD63+/CD81+ vesicles in a multiplexed bead-based manner including respective controls. The Accuri C6 (BD) was utilized for data acquisition.

For further MISEV2018-compliant characterization, CD9+/CD63+/CD81+ uEVs were isolated by immunoaffinity and analysed by fluorescence nanoparticle tracking (f-NTA), transmission electron microscopy (TEM) and western blotting (WB).

Urinary creatinine (Ucrea) was determined to control for variances in urinary dilutions and used for data normalization.

Results: Except CD209, all other 36 surface markers could be identified. The most abundant markers were CD9 and CD63, which were detected in 93% of samples, followed by CD133/1 (84%), CD326 (81%), CD81 and CD24 (77%, each). CD3 (42%), CD9, CD45 (39%), CD49e (32%) and CD81 showed similar relative median fluorescent intensities (rMFI), while CD63 yielded significantly higher ($p = 0.009$) and all other markers significantly lower rMFI ($p < 0.011$).

TEM and f-NTA revealed cup-shaped vesicles (137 ± 22 nm) with 8.8 ± 7.0 E + 10 particles/g Ucrea. WB indicated uEV isolates that were positive for Alix, Syntenin, TSG101, CD9, CD63 and CD81 without any uromodulin or calnexin contamination.
Summary/Conclusion: Our results imply that considerable quantities of circulatory EVs are, indeed, filtered into urine and could serve as valuable non-invasive biomarkers for systemic dysfunctions.

OP1.02 = PT15.02

Cardiovascular risk markers are strongly related to numbers of circulating extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are small plasma membrane-derived vesicles released from various cells, which potentially affect many physiological and pathophysiological processes, and are emerging as a potential novel biomarker in cardiovascular diseases (CVDs). However, there is little information about the association of circulating EV levels with traditional cardiovascular risk markers and CVD risk score.
Methods: • Subjects (n = 40) aged 40–70 yrs with moderate risk of CVDs were recruited and assessed for body mass index (BMI), blood pressure (BP) and plasma lipid profile (triacylglycerol, total cholesterol and high-density lipoprotein).

• EVs were isolated from platelet-free plasma by size exclusion chromatography and analysed by both Nanoparticle Tracking Analysis (NTA) and flow cytometry (FCM). NTA was used to measure the concentration and size distribution of EVs population, and EVs were phenotyped by FCM via a 3-colour panel, which included Annexin V (for the majority of circulating EVs), CD41 (for platelet-derived EVs) and CD105 (for endothelial-derived EVs).

• The association between risk markers and EV numbers was examined by Pearson's correlation coefficient and stepwise multivariate regression model. Analysis of covariance (ANCOVA) was performed after adjustment for various variables to determine the correlation between the quartile range of EV numbers and 10-yr CVD risk detected by QRISK2.

Results: EV numbers, as determined by NTA, were strongly associated with BMI ($r = 0.602$, $p < 0.001$), blood pressure (systolic BP: $r = 0.359$, $p = 0.023$; diastolic BP: $r = 0.550$, $p < 0.001$) and plasma triacylglycerol levels ($r = 0.703$, $p < 0.001$). Plasma total cholesterol level was positively associated with platelet-derived EVs, determined by FCM ($r = 0.330$, $p = 0.038$). A multivariate regression model demonstrated that plasma triacylglycerol and diastolic BP independently predicted total EV numbers, with plasma triacylglycerol concentrations explaining 49.4% of the variance for total EV numbers. An additional 9.3% of the variance in total EV numbers was predicted by diastolic BP. ANCOVA of the 10-yr CVD risk score in the quartile range of total EV numbers were positively and independently associated.

Summary/Conclusion: BMI, blood pressure, plasma triacylglycerol and total cholesterol levels are strongly associated with EV numbers. Plasma triacylglycerol and diastolic BP independently predict circulating EV numbers. Elevated numbers of EVs are independently associated with 10-yr CVD risk.

Funding: This project is supported by Biotechnology and Biological Sciences Research Council (BBSRC)-Diet and Health Research Industry Club (DRINC) in the UK.

OP1.03 = PT15.03

Extracellular vesicles from cardiosphere-derived cells potentiate regulatory T cells

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Introduction: Extracellular vesicles from cardiosphere-derived cells (CDC-EVs) are known to be anti-inflammatory in various disease models. To further dissect the mechanism, we examined the effects of CDC-EVs on T lymphocytes.

Methods: Naïve CD4 + T cells were isolated from secondary lymphoid organs of Foxp3-RFP reporter mice, using magnetic-activated and fluorescence-activated cell sorting. Cells were subsequently polarized into effector subtypes (Th1, Th2, and Th17), as well as regulatory T cells (Tregs), and the effects of exposure to human-derived CDC-EVs on proliferation and cytokine production were assessed. CDC-EVs were isolated from serum-free, 15-day conditioned medium, using ultrafiltration by centrifugation.

Results: After polarization and culture for 5 days, CDC-EVs resulted in dose-dependent and cell-specific proliferative responses. Effector T cells (Th1, Th2, Th17) showed either no change in proliferation (Th1) or decrease in proliferation (Th2, Th17), compared to the vehicle control. In contrast, Tregs proliferated much more than control ($P < 0.0001$). Next, we sought to characterize the changes in cytokine production by each effector T cell and Tregs. Compared to the vehicle control, exposure of polarized effector T cells to CDC-EVs had little effect on the expression of characteristic cytokine genes, including Ifnγ and Tnfα (Th1), Il4 and Il13 (Th2), or Il17a and Il17 f (Th17). In contrast, exposure of Tregs to CDC-EVs resulted in ~1000-fold increase in expression of Il10, a key paracrine agent utilized by Tregs in suppression of inflammation. This response was specific to CDC-EVs insofar as it was not recapitulated with dermal fibroblast exosomes. Concentrations of IL-10 in the culture media of CDC-EV-conditioned Tregs mirrored the increases in gene expression.

Summary/Conclusion: CDC-EVs potentiate Tregs by increasing their proliferation and enhancing production of IL-10. This offers an attractive therapeutic approach to inflammatory diseases that relies on harnessing an endogenous mechanism of immunosuppression.

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OP1.04 = PT15.04

Prostanoids impair platelet reactivity, thrombus formation and platelet extracellular vesicle release in patients with pulmonary arterial hypertension

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Introduction: Prostanoids (epoprostenol, treprostinil and iloprost) induce vasodilation in advanced pulmonary arterial hypertension (PAH) but also inhibit platelet activation, thereby increasing the risk of bleeding. Therefore, the platelet function and extracellular vesicle (EV) concentrations were measured in PAH patients treated with prostanoids and compared to patients with PAH not receiving prostanoids.

Methods: Venous blood was collected from 42 patients treated with prostanoids (study group; n = 42, 50 ± 16 years, 70% female) and 38 patients not treated with prostanoids (control group; n = 38, 55 ± 19 years, 65% female). Platelet reactivity was analysed in whole blood by impedance aggregometry using arachidonic acid (AA; 0.5 mM), adenosine diphosphate (ADP; 6.5 μM) and thrombin receptor-activating peptide (TRAP; 32 μM) as agonists. In a subset of patients, concentrations of EVs from platelets (CD61+ and CD62p+; PEVs), leukocytes (CD45+, LEVs) and endothelial cells (CD146+, EEVs) were measured in platelet-depleted plasma by flow cytometry (A60-Micro). Platelet-rich thrombus formation was measured using a whole blood perfusion system.

Results: Compared to the control group, patients treated with prostanoids had lower platelet reactivity in response to AA and ADP ($p = 0.01$) and lower concentrations of PEVs and LEVs ($p \leq 0.05$). Furthermore, thrombus formation was delayed ($p \leq 0.003$) and thrombus size was decreased ($p = 0.008$) on prostanoids. Epoprostenol did not affect platelet reactivity in vitro, but decreased the concentrations of CD61+ PEVs ($p = 0.04$). In contrast, treprostinil and iloprost decreased both platelet reactivity in response to AA and ADP ($p \leq 0.05$) and the concentrations of PEVs ($p \leq 0.08$). All prostanoids delayed thrombus formation and decreased thrombus size ($p \leq 0.04$).

Summary/Conclusion: Patients with PAH treated with prostanoids have increased risk of bleeding both due to impaired platelet aggregation, EV release and thrombus formation, compared to patients not treated with prostanoids. Antiplatelet effect of prostanoids varies: whereas epoprostenol decreases the release of PEVs, treprostinil and iloprost impair platelet aggregation.

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OP1.05 = PT15.05

Nanoflow cytometry identifies an imbalance of epithelium- and neutrophil-derived extracellular vesicles in the airway environment of paediatric cystic fibrosis patients

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Introduction: Progressive lung disease is the leading cause of mortality in cystic fibrosis (CF), a chronic condition characterized by recruitment of polymorphonuclear neutrophils (PMNs) into the airways. Newly arrived PMNs are exposed to extracellular vesicles (EVs) from the airway epithelium and PMNs recruited before them. In controlled experiments, these EVs were necessary and sufficient to induce pathological changes including reduced bacterial killing and immunosuppressive activities towards macrophages and T-cells. However, children with CF do not always show a high PMN presence in their airways, which suggests that the balance between PMN recruitment and the activity of other cells is still in flux in early stage disease.

Methods: We utilized spectral nanoflow cytometry to profile the single EV content of the bronchoalveolar lavage fluid (BALF) from 17 CF children (<6 years of age). For nanoflow cytometry, EVs were stained with Di-8-ANEPPs, and with EpCAM, CD66b and CD115 (to ascertain epithelial, PMN, and macrophage origins, respectively). Violet side scatter and/or fluorescence threshold triggering were used for EV detection.

Results: The ratio of neutrophil- to epithelial-derived EVs in CF BALF correlated positively with the percentage of PMNs that are present in the airways ($p = 0.003$, Spearman's rho = 0.689). This ratio also

correlated with the PRAGMA disease score, which quantifies airway damage by chest computed tomography (p = 0.001, rho = 0.857).

Summary/Conclusion: Using a method to quantify EVs from specific cell types in vivo, we demonstrated that the ratio of PMN- and epithelial cell-derived EVs tracks with airway damage and neutrophil influx, suggesting a critical interplay between these cells in early CF disease. This EV-focused method can be applied to other diseases in which sampling cells is difficult. Future experiments will use CF BALF biobanks to strengthen data presented here.

Funding: CF Foundation (TIROUV15A0), Emory Pediatrics Flow Core.

OP1.06 = PT15.06

The potential of crude extracellular vesicle microRNAs for the diagnosis of community-acquired pneumonia and for the detection of pneumonia-related sepsis as a severe secondary complication

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Introduction: Circulating cell-free microRNAs (miRNAs), often associated to extracellular vesicles (EVs), are essential for cell-cell communication in the pathogenesis of infectious pulmonary disorders. As early pneumonia diagnosis is often clinically challenging, advances in disease detection could improve outcomes. We characterized crude EV miRNAs as potential biomarkers for community-acquired pneumonia and sepsis as a severe secondary complication.

Methods: 142 individuals were enrolled into our study, subdivided into a training (volunteer n = 27, pneumonia n = 12, sepsis n = 28) and testing cohort (volunteer n = 20, pneumonia n = 18, sepsis n = 37). After precipitating crude EVs from sera (miRCURY Exosome Isolation Kit-Serum and Plasma) and extracting total RNA, small RNA sequencing was performed. miRNAs were selected as biomarker candidates by differential gene expression analysis (DESeq2) and sparse partial-least-squares discriminant analysis (mixOmics). Technical and biological validation was performed by reverse transcription quantitative real-time PCR. Group classification was predicted by partial-least-squares discriminant analysis. Gene targets and causal networks were identified by ingenuity pathway analysis.

Results: Differential gene expression analysis revealed 29 significantly regulated miRNAs in pneumonia compared to volunteers, and 25 miRNAs in pneumonia related to sepsis. Based on sparse-partial least discriminant analysis, group separation was achieved by 12 miRNAs as discriminators with high sensitivity and specificity (area under the curve of the receiver operated curve: volunteer: 0.982, pneumonia: 0.965, sepsis: 0.992). miR-193a-5p (log2FC = 1.86, padj = 1.49E-6) and miR-542-3p (log2FC = 1.67, padj = 3.29E-5) differentiated between pneumonia and volunteers and miR-1246 (log2FC = −2.41, padj = 1.78E-04) between pneumonia and sepsis. Expression levels of miR-193a-5p and miR-1246 were related to disease severity. miR-542-3p was higher expressed in pneumonia compared to volunteers and had equal expression in patient groups. Prediction of group classification in the testing cohort was 73.33%. Signalling networks were constructed for "cellular and humoral immune response", "antimicrobial response" and "pathogen influenced signaling" involving the significantly regulated miRNAs.

Summary/Conclusion: Crude EV miRNAs are potentially novel biomarkers for community-acquired pneumonia and may help to identify patients at risk for progress to sepsis allowing early intervention and treatment.

Funding: This work was supported by a grant from the German Federal Ministry for Economic Affairs and Energy (protocol number ZF4247001MD6).

OP1.07 = PT15.07

microRNA exosome cargo from induced sputum: new tool for approaching asthma research

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Introduction: It remains unclear the specific mechanisms that lead to airways inflammation in asthma and the subsequent remodelling of the airways. Exosomes, small extracellular vesicles, has become in an important mechanism of cell-to-cell communication and participate in diverse biological processes including inflammation. In this study, we hypothesize that exosomes and

their miRNA cargo play an important role in the proinflammatory status of the upper airway of asthma patients, especially in those patients with severe asthma.
Methods: In a pilot study,3healthy subjects had induced sputum using standard methods. After several centrifugation steps, we were able to isolate exosomes from sputum supernatant by both precipitation and Size Exclusion Cromatography (SEC). Exosome size was observed with Transmission Electron Microscopy (TEM) and the protein markers CD63 and CD81 were analysed by Western Blot (WB). Then, total RNAs were isolated from sputum exosomes and 9 miRNAs (miR-103a-p, miR-191-5p, miR-320a, miR-200b-3p, miR-185-5p, miR-223-3p, miR-21-5p, miR-155-5p, let-7 g-5p), were evaluated by RT-qPCR. After the optimization of the methodology, 10 healthy adults subjects and 10 patients with persistent moderate-severe asthma, matched by age and sex were selected and induced sputum was collected.
Results: Exosomes isolated with both methodologies (precipitation and SEC) were observe under the TEM with a correct range of size. Furthermore, WB assay displayed a coherent protein profile for the exosome markers CD63 and CD81. However, SEC displayed low signal and the variability of between subjects was to higher. Using the optimized method of precipitation, we observed that after normalization, miRNA-320a showed a significant increased ($p = 0.02$) in asthma patients compared to control. This miRNA has been linked with an active proinflammatory status.
Summary/Conclusion: Our results confirm the presence of exosomes in induced sputum with promising applications in the field of asthma. The upregulation of exosomal miR-320a, which is related with inflammation, suggest that exosomes could play a crucial role in the chronic inflammation of airway described in asthma patients.

OP1.08 = PT15.08

Human nrf2-active multipotent stromal cell exosomes reverse pathologic delay in the healing of cutaneous diabetic wounds

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Introduction: Multipotent stromal cells (MSCs) have attracted much attention for their capacity to accelerate wound healing. Exosomes, nanosized extracellular vesicles, may be key to translating MSC therapy. We previously found that nuclear factor erythroid 2-related factor 2 (Nrf2) regulates MSC promotion of diabetic tissue repair. Here, we explore a novel role of Nrf2 in exosome biogenesis and investigate whether exosome treatment recapitulates the effects MSCs have on healing.
Methods: Exosomes were harvested by differential ultracentrifugation of conditioned bone marrow derived MSC media. For Nrf2-active exosomes, MSCs were incubated with potent Nrf2 activator, CDDO-Im. Exosomes and MSCs were vigorously characterized. Full-thickness humanized-stented wounds were created on adult Leprdb/db diabetic mice (db/db). Exosomes were injected intradermally and circumferentially to the wound margin.
Results: MSCs adopt an adherent fibroblast morphology, demonstrate robust osteogenic, chondrogenic, and adipogenic differentiation, express >95% positive MSC markers (CD44, CD73, CD90, and CD105) and <5% express negative markers (CD45, CD31, CD14, CD19, or HLA-DR). Immunoblotting of MSC exosomes shows enrichment for positive exosomal markers CD81, CD9 and TSG101. Nanoparticle tracking analysis (NTA) shows a nanoparticle population with mean diameter of 168.0 ± 6.5nm. Transmission electron microscopy of exosomes reveals flattened cup-like spheres. NTA demonstrates that Nrf2-active human MSCs increase exosome secretion by 54%, compared to Nrf2-baseline MSCs ($p < 0.05$). Both Nrf2-baseline and Nrf2-active exosome treatment significantly reduced closure time to 15.5 and 14 days respectively, compared to 29.8 days for vehicle-treated wounds ($p < 0.05$). This reduction eliminated the delay in closure time compared to wounds of C57/B6 mice. Nrf2-active exosome treatment of db/db wounds reduced closure time by a further 2.6 days compared to untreated C57/B6 wounds. At day 10, exosome-treated db/db wounds have significant decreases in epithelial gap, expanded granulation tissue, and greater density of CD31+ vessels compared to vehicle-treated wounds.
Summary/Conclusion: Enhancing Nrf2 function in MSCs multiplies exosome yield. Our results demonstrate exosome-based therapies hold tremendous promise and warrant further investigation for rapid translation.
Funding: PSF Pilot translational grant, WHS 3 M Fellowship, NYU CTSI Translational Pilot Project

OP1.09 = PT15.09

Extracellular vesicles from adipose tissue end endothelial cells of obese humans share miRNA cargos and increase prostate cancer aggressiveness in conjunction with Twist1

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Introduction: Obesity increases prostate cancer aggressiveness and adipose tissue (AT) is a rich source of extracellular vesicles (EV) that have been shown to contribute to pro-oncogenic effects in various malignancies. Twist1 is a key mediator of tumour cell metastasis.. The goal of this study was to determine molecular and phenotypic changes of prostate cancer cells in response to EVs from obese human AT and the role of different levels of endogenous Twist1.

Methods: EV were harvested from human AT (ATEV) obtained from bariatric subjects or from AT endothelial cells treated with proinflammatory cytokines (PIC-EV) to mimic the obese AT environment. EVs were isolated by ultracentrifugation and characterized by electron microscopy, NTA and protein markers. We determined the effect of ATEV and PIC-EV on PC3-ML prostate cancer cells proliferation and invasion. EV miRNA cargo and transcriptome of PC3-ML cells treated with ATEV or PIC-EV were assessed using NanoString. To establish the contribution of Twist to the EV-related phenotypic and molecular changes in recipient cells, we used PC3-ML lines stably overexpressing or deficient in Twist1.

Results: ATEV from obese subjects and EV-PIC from AT endothelial cells both reduced invasion and increased proliferation in wild-type PC3-ML cells. A molecular signature showing decreased expression of genes mediating invasion, adhesion and metabolism supported these functional effects. Also ATEV and EV-PIC shared a subset of miRNA that target multiple MMPs, inhibit glycolytic genes and target cell cycle inhibitory genes. PC3-ML overexpressing Twist1 showed an increase in both proliferation and invasiveness and this phenotype was supported by the transcriptomic analysis following EV treatment.

Summary/Conclusion: EV produced by obese AT or by AT endothelial cells share a subset of miRNA that in conjunction with increased Twist1 expression contribute to tumorigenesis and metastasis of prostate cancer cells in vitro.

Funding: American Heart Association

OF12

Symposium Session 12: Biodistribution & Antiviral Defence

Chair: Lorraine O'Driscoll – School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin

Chair: Ana Claudia Trocoli Torrecilhas – Department of Biological Sciences, Universidade Federal de Sao Paulo

OF12.1

Development of non-invasive clinically applicable in vivo tracking of extracellular vesicles using magnetic resonance imaging (MRI)

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Introduction: As researchers continue to explore the therapeutic potentials of extracellular vesicles (EVs) for the treatment of many diseases, there is a growing unmet need for real-time in vivo monitoring of these therapeutic EVs after they are injected into a subject to understand their safety, targeting, and effectiveness. While current optical imaging solutions like bioluminescence and fluorescence are useful for EV tracking studies in animal models, there is limited utility in clinical applications. Here we present a novel EV tracking solution utilizing clinically applicable MRI technology.
Methods: To generate trackable EVs, cells were labelled with a clinically applicable novel magnetic agent. EVs secreted by the labelled neural stem cells and amniotic fluid stem cells (AFSCs) were isolated by differential ultracentrifugation. The viability and morphology of labelled-cells were evaluated, and the in vitro MR properties of their derived EVs were analysed by magnetometer. A proof of concept in vivo biodistribution study was conducted by injecting labelled EVs into WT and Alport mice (a model of chronic kidney disease) via retro-orbital and intra-cardiac routes and tracking them via MRI at 10 min and 3 hr post-injection.
Results: The magnetic label did not affect the physiological characteristics of the cells. The MR detectability of labelled-EVs was confirmed by in vitro/ex vivo MRI phantoms. MRI studies showed that homing of AFSC EVs to the kidney injected intra-cardiacally into Alport mice were more efficient versus the retro-orbital route, and Prussian blue staining of kidney sections confirmed the MR findings.
Summary/Conclusion: We have developed a clinically applicable novel magnetic nanoparticle agent that can be used to label and track the biodistribution of EVs in living subjects using non-invasive, safe, and effective MRI technology that's widely available. This technology is highly adaptable and can be deployed in both preclinical and clinical settings.
Funding: Children's Hospital Los Angeles Small Animal Imaging Core Pilot Grant

OF12.2

EV biodistribution studies with a novel non-invasive, dynamic in vivo imaging method reveal diverse organ reactions to circulating EV: No EV retention, EV retention, and active EV recruitment

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Introduction: A central question in EV biology is the fate of circulating EV. This can be evaluated by developing non-invasive EV bioimaging techniques in mice in order to benefit from transgenic and knock-out models. Recent reports described EV biodistribution in vivo using optical (fluorescence) and nuclear imaging. But the physicochemical properties of the probes impact EV integrity, labelling efficiency, background signals and observation timecourse.
Methods: We developed the radiolabeling of red blood cells (RBC) and EV with [18 F]fluorodeoxyglucose (18 F-FDG). We used RBC-derived EV in their native, intact form, without pre-experimental processing (no centrifugation or filtration). We tracked 18 F-FDG in vivo by PET-Scan, within seconds of EV, RBC or free

18 F-FDG injection, and during their dissemination in blood and recruitment by organs over one hour. EV and RBC biodistribution were confronted to the kinetics of free 18 F-FDG.

Results: We collected images of the biodistribution of RBC, and RBC-derived EV. Nuclear imaging was well suited for accurate studies of EV organotropism, with high sensitivity, excellent signal-to-noise ratio, very low signal absorption by tissues and an inherent quantitative tomographic nature. EV-specific signals were mostly accumulated within minutes of injection (tail vein), in the spleen and liver, with a small part in the bone marrow (femurs). Signals in other compartments were largely transient and linked to tissue perfusion and blood volume. We selected the most drastic control conditions to secure a correct interpretation of the data. This made kidneys, hearts and brains unavailable for analysis. Hence the new approach came with limitations, but we describe how "free" 18 F-FDG signals can be used to draw sound conclusions for EV.

Summary/Conclusion: We propose that three types of compartments coexist in control mice at rest: Active EV-capturing organs with high capacity and specificity including the spleen, and to a lesser degree the bone marrow; Passive EV-retaining organs with high capacity, including the liver; and EV-neutral organs where transient signals only mirror tissue perfusion. We also report how EV biodistribution patterns are altered in ageing animals, as an example. We hope that this novel, non-invasive, quantitative, dynamic whole-body imaging approach will help characterize native cell-derived EV and help set standards for the reproducibility of EV bioimaging in mice.

Funding: FRM grant "BiFace", Inserm COPOC, CNRS.

OF12.3

A novel in vivo Drosophila model of vesicle-mediated intercellular communication

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Introduction: Extracellular vesicles (EV) are important mediators of intercellular communication; however, basic principles of EV biogenesis and loading remain largely unknown. A limited repertoire of tools has thus far made these processes challenging to research. The development of an EV-transfer reporter in a genetically tractable organism such as Drosophila has allowed us to study mechanisms of cargo loading in vivo and has provided us with a platform to explore fundamental aspects of EV biology.

Methods: We have developed a bioinformatic pipeline to analyse the properties embedded in the 3′UTR of mRNAs enriched in EVs released by Drosophila cells. In parallel, we have adapted a Cre-LoxP system for use in fruit flies that appears to be proficient to reveal the exchange of bioactive molecules between secretory and recipient cells.

Results: Taking advantage of computational methods, we uncovered sequence motifs that preferentially appear in combinations along the 3′UTR. These sequence motifs occur within characteristic secondary structures, in a way that is more variable and motif dependent than previously reported. Identified motifs also show similarities to known binding sites for RNA binding proteins; a feature potentially important for EV-loading. In parallel, we developed a Drosophila in vivo system to detect cell communication in complex tissues and between different cell types. Using this system, we studied the biological significance of specific sequence motifs and identified their ability to modulate mRNA EV-transfer in a context dependent and evolutionarily conserved manner.

Summary/Conclusion: In summary, we have developed a novel tool to study cell communication in complex tissues, and shown its effectiveness to study principles of EV biogenesis and loading. Beyond improving our understanding of EV biology and providing a novel tool to the scientific community, we hope this knowledge will pave the way to harnessing EVs as a means of remotely manipulating cell communication in many biological contexts.

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OF12.4

Dietary cross-species communication: context-dependent role of bovine extracellular vesicles in cancer progression

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Introduction: The idea of cross-kingdom, species and inter-individual transfer of bioactive compounds via

extracellular vesicles (EVs) is a recent avenue. However, the bioactivity and bioavailability of these dietary compounds upon consumption is highly debated. It has been proposed that EVs from diet can absorbed by consuming organisms, be bioavailable in various organs and exert phenotypic changes. Milk is the most vastly consumed beverage and is an abundant source of EVs that may act as signalosomes. Whether these milk-derived EVs can serve as cross-species messengers and have a biological effect on host organism has been poorly understood.

Methods: Bovine milk-derived EVs were isolated by ultracentrifugation and OptiPrep density gradient centrifugation. The EVs were characterised by TEM, NTA, quantitative proteomics and RNA-Seq. EVs were orally administered to various mice models of colorectal, breast and pancreatic cancer. Primary tumour burden was monitored, and the rate of metastases was measured by imaging and qPCR. Immune cells were analysed by FACS. Mechanistic insights were obtained using quantitative proteomics, confocal microscopy and biochemical experiments.

Results: We demonstrated that upon oral administration, bovine milk-derived EVs were able to survive the harsh degrading conditions of the gut and be bioavailable in peripheral tissues. Interestingly, oral administration of milk-derived EVs reduced the primary tumour burden in various cancer models and attenuated cancer cachexia. Intriguingly, despite the reduction in primary tumour growth, milk-derived EVs accelerated metastasis in breast and pancreatic cancer mice models. Timing of EV administration was critical as oral administration after resection of the primary tumour reversed the pro-metastatic effects of milk-derived EVs in breast cancer. Biochemical and quantitative proteomics analysis highlighted the induction of epithelial-to-mesenchymal transition and senescence upon treatment with milk-derived EVs.

Summary/Conclusion: Taken together, we were able to demonstrate the capacity of bovine milk-derived EVs in mediating cross-species communication and regulating cancer progression in a context-dependent manner.

OF12.5

Bacterial membrane vesicles (MVs) – a bacterial innate defence system against viral infection

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Introduction: In order to survive the constant onslaught of phage, bacteria have evolved diverse defence mechanisms that act at every stage of the phage life cycle. It has been suggested that bacterial membrane vesicles (MVs) may play a key role in innate bacterial defence against phage infection by acting as a decoy to prevent phage adsorption. Nearly a decade has passed since MVs were first proposed as a decoy, but details of how bacteria utilize MVs to defend against phages remain poorly understood. Here we use the laboratory-built nano-flow cytometer (nFCM) to reveal details of the interaction between MVs and phages at the single-particle level, and to provide new insights into innate defence mechanisms of MVs.

Methods: S. Typhimurium was used as the model system. Differential ultracentrifugation and density gradient centrifugation were used to isolate and purify MVs and bacteriophage P22. Cryo-TEM was used to determine the morphologies of MVs and phage P22. The purity of MV isolates was validated by measuring the particle concentration before and after Triton X-100 treatment. Monodisperse silica nanoparticles were used as the size reference standards to measure the size distribution of MVs via single-particle light scattering detection. The purity of phage P22 was verified by concurrent detection of side scatter and fluorescence signals of single phages upon nucleic acid staining by SYTO 82.

Results: By incubating MVs and AF532-labelled P22, the number of phages adsorbed on single MVs were accurately quantified. We found that S. Typhimurium and MVs it secretes express different affinity for phage P22 attachment. The binding ability of P22 to MVs is greater than that of bacteria. We confirmed that P22 can inject their nucleic acids into MVs, and these nucleic acids can be degraded by non-specific nucleases inside MVs for the first time. Besides, by labelling the nucleic acids of MVs with SYTO 82, we were able to distinguish three different subpopulations of MVs.

Summary/Conclusion: Taking advantage of the superior sensitivity of nFCM in single-particle analysis, we developed a novel approach to the characterization of the interaction between MVs and phages. Our study revealed that bacteria produce MVs as bait to attract viral adsorption and nucleic acids injection.

Funding: This research was supported by the National Natural Science Foundation of China (Grants 21934004, 21627811 and 21475112).

OF12.6

In vivo tracking of extracellular vesicles: comparison across radioactive, fluorescence and bioluminescence approaches

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Introduction: The development of EVs for therapeutic applications requires an in-depth understanding of their in vivo biodistribution and pharmacokinetic profile. In this study, we have made a comprehensive comparison of nuclear, fluorescent, and bioluminescent imaging technologies to identify the most suitable in vivo EV tracking method.

Methods: EVs were purified from Expi293 F cell supernatant by differential centrifugation followed by iodixanol density gradient separation and further characterized following MISEV guidelines. Engineered Expi293 F cells were used to generate EVs carrying mCherry or NanoLuc (Nluc) proteins. The membrane of naïve EV was labelled with Indium111(In111)-DTPA or Xenolight DiR post-EV isolation. CT26 tumour-bearing BALB/c mice were intravenously dosed with 1 × 1011 EVs followed by imaging at 1 h, 4h and 24h using SPEC/CT and IVIS systems. Tissue distribution and blood circulation profile of EVs were analysed from ex vivo samples up to 24h post-injection.

Results: Xenolight DiR and (In111)-DTPA were the most suitable EV labels for live whole-body animal imaging, ex vivo organ imaging, and tissue lysate quantification. Nluc was appropriate for ex vivo imaging and tissue lysates quantification, but suboptimal for live imaging with limited sensitivity. mCherry EVs were found not suitable for in vivo tracking studies due to high background signal fluorescence. Ex vivo organ quantification of In111-DTPA and DiR showed that naïve EVs mainly accumulate in liver, followed by spleen, kidneys, and lungs at 24h post-dose, with less than 5% EV exposure to the tumours. Interestingly, Nluc-EVs accumulated mainly in the lungs, regardless of the small size of the particles injected and the absence of aggregation. Blood circulation profile of In111-DTPA and Nluc EVs showed rapid clearance of vesicles from circulation, with 15% of injected dose detected in blood after 30 min and less than 5% after 16 h.

Summary/Conclusion: Radionuclide imaging is an excellent technology to detect EVs in vivo and ex vivo with high resolution and sensitivity but requires advanced infrastructure for radiolabeling. The optical methods have limited tissue penetration and sensitivity but can be improved with the right selection of the dye. These results contribute to the understanding of the biodistribution and pharmacokinetics of EVs and are highly relevant to exploiting their potential for targeted delivery to diseased tissues in vivo.

OF13

Symposium Session 13: EV Characterization

Chair: Emanuele Cocucci – Comprehensive Cancer Center, Ohio State University, USA

OF13.1

Analysis of extracellular vesicles by size exclusion chromatography coupled with on-line fluorescence detection and microfluidic resistive pulse sensing

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Introduction: New methods for quantifying extracellular vesicles (EVs) in complex biofluids are critically needed. We report the development of a new technology combining size exclusion chromatography (SEC), a commonly used EV purification technique, with fluorescence detection of specifically labelled EVs (Flu-SEC).
Methods: Flu-SEC was validated using red blood cell derived EVs (REVs). Size and concentration measurements were performed by microfluidic resistive pulse sensing (MRPS) using the nCS1 instrument (Spectradyne LLC, USA). PE-CD235a (anti-Glycophorin A) and Alexa647-WGA (wheat germ agglutinin) were used to label REVs. Flu-SEC experiments were performed on a liquid chromatography system using a Tricorn 5/200 glass column filled with Sepharose CL-2B gel (GE Healthcare).
Results: A log-normal size distribution was obtained for REVs with a mean diameter of 163.5 ± 0.7 nm and standard deviation of 30.6 ± 0.6 nm. The concentration of REVs measured by MRPS was 3.14*10E11 particles/mL. The fluorescence chromatograms of the REV samples labelled with PE-CD235a and with Alexa647-WGA show the typical features of the separation of EVs from soluble proteins with SEC and enables the determination of the labelling efficiency of the markers. The linear range for quantification of EVs in our experiments spans over two orders of magnitude ranging from 10E9 particles/mL to 10E11 particles/mL. The LOD depends on the type of the label. In our experiments the lowest LOD was 10E9 particles/mL for Alexa647-WGA.
Summary/Conclusion: The results indicate that Flu-SEC is a quantitative technique with very good linearity over a wide range of concentrations, though the limit of detection depends largely on the employed label (Sci. Rep. 9, 19868, 2019). Moreover, the ratio of EV-bound and free-antibody molecules can be also determined by Flu-SEC, which can be used to calculate the labelling efficiency of the used marker.
Funding: This work was supported by the National Research, Development and Innovation Office (Hungary) under grant numbers PD 121326 and NVKP_16-1-2016-0007. ZV was supported by the Janos Bolyai Research Fellowship.

OF13.2

The CONAN assay: purity grade and concentration of EV microlitre formulations by colloidal nanoplasmonics.

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Introduction: We will share our latest advancements in the use of colloidal and nanoscience concepts and technologies to assign a purity grade to and quantify microlitre volume solutions of extracellular vesicles

(EVs). Control over such properties is constantly experienced by researchers to be critical for EV proper manipulation, engineering and translation. However, the need for characterization methods that strike the balance between robustness, working volume, cost and accessibility remains unmet.

Methods: The COlorimetric NANoplasmonic (CONAN) assay we developed consists of a solution of gold nanoparticles (AuNPs) into which 1–2 μL of the EV formulation is added. The solution turns blue if the formulation is pure, while stays red if soluble exogenous single and aggregated proteins (SAPs) are present. The colour shift is visible by the naked eye and can be quantified by conventional UV-Vis spectroscopy, providing a quantitative index of purity and an estimation the EV molar concentration (particle number).

Results: The assay specifically targets SAPs, and not the EV-related proteins, with a detection limit < 50 ng/μL (an order of magnitude higher resolution than the Bradford protein assay). For pure solutions, the assay also allows for determining the EV number, as the colour shift is linearly dependent to the AuNP/EV molar ratio. Instead, it automatically reports if the solution bears SAP contaminants, thus avoiding counting artefacts. Experiments, conducted on EV separated from Milk and Ascaris Suum culture medium, are repeatable, with an error below 20%.

Summary/Conclusion: CONAN proves to be robust and reliable, while displaying appealing performances in terms of cost (inexpensive reagents, run by standard microplate reader), working volumes (1–2 μL) and time (the procedure takes less than one hour). The ability to assign a quantitative purity grade is, up to date, a unique peculiarity of this assay. Finally, the assay is potentially extendable to all classes of natural and artificial lipid micro- and nanoparticles.

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OF13.3

Membrane-sensing peptides for extracellular vesicles analysis

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Introduction: Small extracellular vesicles (sEV) present fairly distinctive lipid membrane features in the extracellular environment. These include high curvature, lipid packing defects and a relative abundance in lipids such as phosphatidylserine and ceramide. sEV membrane could be then considered as a "universal" marker, alternative or complementary to traditional characteristic surface-associated proteins.

Here we introduce the use of membrane sensing peptides as new, highly efficient ligands to directly integrate sEV capturing and analysis on a microarray platform.

Methods: We designed and synthesized membrane-sensing peptide ligands as molecular baits for small EV and we demonstrate their use in a microarray platform as valuable alternative/complement to antibodies.

EVs from blood serum and plasma were isolated by ultracentrifugation, characterized by TEM, NTA, WB. Samples were analysed by label-free, single particle counting and sizing on peptide microarrays coupled to fluorescence co-localization immune staining with fluorescent anti-CD9/anti-CD63/anti-CD81antibodies.

Results: Peptide microarrays were realized using a click-chemistry strategy for optimal peptide surface orientation and used to analyse EVs from human blood. Membrane sensing peptides showed a capturing capacity higher than anti-tetraspanin antibodies. In addition to purified vesicles, peptide ligands were tested with pure serum showing capacity to capture EVs even from complex samples. In order to get insights into the EV-peptide binding mechanism and verify whether it is directly mediated by the lipid membrane, trypsin-treated EVs were captured on peptide microarrays demonstrating that binding is not directly mediated by surface associated proteins.

Summary/Conclusion: We introduced the use of membrane sensing peptides as a novel class of molecular ligands for integrated sEV isolation and analysis, reporting for the first time on peptide microarrays for extracellular vesicles. Given their affinity to the membrane of small EV, these molecules can serve as general baits, enabling vesicles capturing unbiased by differential surface protein expression. These new class of molecular probes may be integrated with the use of protein markers towards improved small EV isolation and characterization. Compared to proteins and antibodies, peptides are characterized by low cost of preparation, remarkable stability and ease of chemical manipulation, offering virtually unlimited possibilities for experimental design. We anticipate that this new class of ligands, may greatly enrich the molecular toolbox for EV analysis.

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OF13.4

High-resolution size-based profiling and morphological analysis of extracellular vesicles by scanning electron microscopy

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Introduction: Extracellular vesicles (EVs) have been found to mediate intercellular communication in physiological and pathological processes. Nevertheless, the understanding of EVs bio-functionality remains elusive, mainly because of their high heterogeneity in molecular content, but also in size (30–2000 nm). Therefore, accurate size measurements of EVs are highly desired, particularly for exploiting their full diagnostic/therapeutic potential.

Currently available techniques, such as Nanoparticle Tracking Analysis (NTA), cannot accurately measure EVs smaller than 70 nm and are not capable to distinguish them from protein aggregates. On the contrary, electron microscopy (EM) techniques allow high-resolution size-profiling and morphological analysis of EVs over their whole size range. However, their low throughput combined with several long preparatory steps have prevented EM from being routinely used for EV size profiling.

Methods: We shall present a method improvement in throughput and reproducibility of EV size-analysis by scanning EM (SEM). The technique is based on covalent EV capture onto a silicon wafer, using the protocol reported by Cavallaro et al. up to the Glutaraldehyde step. After immobilization, Critical Point Drying (CPD) is performed to dehydrate EVs before SEM, while preserving their shapes.

Results: SEM images, showing the comparison in densities of EVs prepared by covalent and non-covalent coupling to substrate, indicated a good capture efficiency of our covalent protocol. The size distribution analysis showed good agreement between NTA and SEM for EVs >80 nm. For smaller EVs, SEM is more sensitive than NTA, thus more suitable to check the purity of EV-isolation techniques. Last, atomic-force microscopy (AFM) measurements was also used to validate our measurements.

Summary/Conclusion: To conclude, SEM can be used to accurately estimate EV size distributions in a wide range, overcoming the well-known limitations of NTA.

Funding: Erling Persson Foundation

OF13.5

Detailed Characterization of how different extracellular vesicles isolation methods results in different amount of Periostin protein association.

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Introduction: Extracellular vesicles (EVs) are membrane vesicles secreted into extracellular space, by almost all cellular populations, playing a major role in cell-to-cell communication. It has been already demonstrated that changes in luminal or surface protein cargos of these vesicles, may reflect the status of producing cells. For this reason, EVs are considered as potential biomarkers in several types of diseases ranging from cancer diagnosis to heart rejection.

Periostin (POSTN) is a matricellular protein associated with EVs, and its level is considered a possible biomarker, which indicate malignancy and poor clinical outcome in different types of cancer.

Here we extensively characterize the presence of POSTN associated on EVs, showing how different isolation methods can drastically affect the amount of POSTN content in extracellular vesicles fraction.

Methods: Serial ultracentrifugation steps or size exclusion chromatography were used to isolate EVs from primary culture of cardiac progenitor cells. EVs were characterize, according to MISEV guidelines, by Western Blot, NTA, FACS and CryoTEM analysis methods. POSTN amount, associated with EVs, was analyses by Western Blot and ELISA. Furthermore, functional tests were performed on H9C2 cardiomyoblast cell line, treated with the same amount of EVs from different isolation methods; cells response were analysed by Western Blot.

Results: EVs, from both the isolation methods, showed TSG101, Syntenin1, CD63 positivity while GRP94 was absent. NTA showed no differences, in terms of amount and size of particles. By FACS analysis EVs resulted enriched with CD63, CD9 and CD81. CryoTEM showed a similar morphology in the two preparations with presence of protein contaminant in the ultracentrifuge pellet. In vitro, H9C2 treated with EVs showed activation of pFAK after 10′ of treatment,

this induction was 1.5 times higher in cells treated with EVs isolated with ultracentrifuge compared to EVs isolated with SEC, confirming a drastic effect of POSTN protein contamination. Furthermore, by Phospholipase-C treatment, we found that POSTN is bound to EVs surface through a GPI anchor.

Summary/Conclusion: These results suggest that selection of a proper isolation method is critically relevant in EVs studies, in particular when protein analysis is considered. Different isolation methods dramatically influence protein amount in extracellular vesicles and consequentially their function. Furthermore, in this study we show for the first time, that POSTN is actually bound to EVs surface and not carried in their lumen as previously believed.

Funding: This work has been supported by The Swiss National Science Foundation under grant N° 310030_169194.

OF14

Symposium Session 14: Extracellular RNA

Chair: Takahiro Ochiya – Tokyo Medical University

Chair: Michael Pfaffl – Division of Animal Physiology and Immunology, Technical University of Munich

OF14.1

Extracellular vesicles – a tattletale for rare gene editing events

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Introduction: Since its discovery, gene editing has provided the ability to meticulously change genes with a profound effect on both therapeutics and molecular research. Even with new tools constantly being developed to increase efficiency and precision of the technique, the repair mechanisms post-gene editing are still error prone making it critical to detect and/or select a desired gene-corrected cell clone. Since the contents of extracellular vesicles (EVs) reflect the cells that produced them, if a gene editing event occurs, the EV cargo should contain the gene corrected products, such as a protein or RNA species. The catch lays in the fact that EVs are by their nature very heterogeneous and only a small fraction of the population may harbour the gene edited products.
Methods: We designed a CD63 construct with a genomic DNA target sequence for detection of a desired gene editing event. The gene editing target harbours a premature stop codon. Only when the desired gene editing event occurs to correct stop codon truncations by genomic missense or frameshift mutations, is a bioluminescent signal detected, as it then allows the CD63 tethered luciferase reporter to be translated.
Results: This reporter detects gene corrections 2 days post-introduction of Cas9 and a sgRNA targeting the stop codon. Next-Generation Sequencing confirmed that the signal resulted from 1.12% gDNA changes. Our observations highlight the sensitivity of our system to detect even highly inefficient non-homologous end joining repair after a double-strand break within the target DNA. The CD63 construct also contains a membrane surface immune affinity tag to facilitate isolation of cells that encode the full length reporter, excluding the non-gene edited cells, without the need for single cell FACS. Moreover, the latter tag enables isolation of a pure EV population from these corrected cells to be isolated in a 1–2 hr procedure from the cell media. These EVs are detectable with luminescence if the reporter is fully expressed in the target cells. Our data shows that with this construct, EVs can be selected out of a heterogeneous pool of EVs that contain RNA solely expressed in the corrected cell. The latter observation allows the EVs derived from corrected cells to report on RNA derived from CRISPR/Cas9 events without the need for cell lysis and gene sequencing.
Summary/Conclusion: A CD63 transgenic reporter protein contained in the membrane of cells and EVs may be used to detect and select out correctly gene edited EV-donor cells early on, reducing effort in avoiding cells with off targets effects.

OF14.2

Y-RNA subtype ratios in plasma extracellular vesicles are cell type specific and are candidate biomarkers for inflammatory diseases

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Introduction: Major efforts are made to identify changes in the microRNA (miRNA) and messenger RNA content of patient plasma to discover novel disease-associated biomarkers. MiRNA in plasma can be associated to various macromolecular structures, including extracellular vesicles (EV), lipoprotein particles (LPP) and ribonucleoprotein particles (RNP). Besides miRNA, plasma contains various other non-coding RNA species, of which some are contained in EV. Members of the Y-RNA family have been detected in EV from various cell types and are among the most abundant non-coding RNA types in plasma. We previously showed that shuttling of full-length Y-RNA into EV is modulated by TLR-activation of EV-producing immune cells. This suggested that Y-RNAs may have potential as biomarker for immune-related diseases.
Methods: We separated RNA-containing structures in plasma based on differences in size, density, and resistance to protease/RNase treatment. Using RT-qPCR, we quantified full-length Y-RNA subtypes (Y1, Y3, Y4) in EV from various blood-related cell types cultured with or without LPS-stimulation. Inflammation-induced changes in Y-RNA were assessed in plasma samples from a human endotoxemia study.
Results: Full-length Y-RNA in plasma was mainly found in EV (early SEC-fractions, density 1.11–1.18 g/ml). In contrast, specific miRNAs were either enriched in LPP (e.g. miR-122), in both EV and LPP (e.g. miR-16 and miR-21), or in EV (e.g. miR-150). EV-enclosed full-length Y-RNA was resistant to enzymatic degradation, while LPP-bound miRNAs were degradation sensitive. We discovered that EV released by different blood cell types varied in Y-RNA subtype ratios. These ratios remained stable upon LPS-stimulation of the EV-producing cells. In endotoxemia plasma samples, the neutrophil-specific Y4/Y3 ratios and PBMC-specific Y3/Y1 ratios changed significantly during systemic inflammation. Importantly, the plasma Y-RNA ratios strongly correlated with the number and type of circulating immune cells during the inflammation process.
Summary/Conclusion: Cell type specific "Y-RNA signatures" in plasma EV can be determined without prior EV-enrichment, and may be further explored as biomarkers to diagnose inflammatory responses or other immune-related diseases.

OF14.3

Mining public EV Small RNA-seq data with miREV – Insights into potential reference transcripts and abundant miRNAs

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Introduction: Although recent endeavours such as EV-TRACK or the MISEV guidelines have made real headway in the continuing effort of improved characterization and standardization of extracellular vesicles (EV) methodologies, comparability between EV studies is still hampered by the multitude of experimental setups scientists can choose from. Different biological contexts or EV isolation methods will likely yield distinct miRNA profiles, which makes choosing suitable miRNAs as references or as potential biomarkers from literature difficult at best. At the same time, repositories for RNA-seq data harbour a wealth of EV-related data, which can be utilized in creating a comprehensive miRNA overview to detect influences of these different experimental procedures or disease contexts.
Methods: Recent literature as well as publicly available data bases (e.g. SRA, ENA, GEO) were searched for EV-related Small RNA-Seq data sets comprising a large variety of different species, tissues and diseases. Raw RNA-Seq data was processed to exclude poor quality data and reads mapping to other RNA species (e.g. rRNA, tRNA) before generating standardized miRNA read counts. To account for variable analysis setups, count lists were normalized by 6 commonly used normalization methods for abundance analyses and potential, stably expressed reference transcripts were evaluated by 3 different established algorithms (geNorm, BestKeeper, NormFinder).
Results: miRNA abundances and potential miRNA references are made available in an easy-to-use web tool (http://141.40.217.80:3838/miREV/). In a first big feasibility study, 654 data sets focusing on blood derived EVs were implemented. After strict filtering, 407 data sets (of 654) and 326 candidate miRNAs (of 2632) remained. Applying normalization methods and stability algorithms, 3 miRNAs turned out to be the most stable in blood derived EVs: miR-30d-5p, miR-140-5p, miR-23a-3p. In the near future, another 1,200 datasets from over 40 published EV studies will be implemented to further validate miREV applicability. Results can be refined by the user by filtering for, amongst others, specific disease/treatment context, tissues of origin or isolation methods to match individual experimental setups as close as possible. All findings (normalized read counts as well as potential reference transcripts) are visualized to easily facilitate

comparisons and can be downloaded as lists for further analysis.

Summary/Conclusion: miREV represents a new and comprehensive tool for scientists working in EV transcriptomics. By summarizing publicly available small RNA-seq data sets, it assists in finding suitable miRNAs as qPCR references or for biomarker research without the need to run prior RNA-seq yourself. In the future, the miREV framework can be easily expanded to include data from further species or experimental setups.

OF14.4

Presence of vault RNA and proteins indicates contamination of extracellular vesicle preparations with vault particles

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Introduction: Extracellular vesicles (EVs) contain a variety of molecular cargo such as proteins and nucleic acids. Vault particle components (vtRNA and proteins) have been repeatedly reported in the literature as part of the EV cargo. Here, we assessed the presence of vault particle components in EVs prepared by commonly used isolation methods to determine whether they were bona fide EV cargo.

Methods: EVs were isolated by differential centrifugation, size exclusion chromatography (SEC) and Dynabead immunocapture. Preparations were characterised by TEM, NTA, and western blotting for EV markers. Abundance of vtRNAs was determined by small RNA sequencing and qPCR. Major vault protein (MVP) abundance was determined by western blotting. RNase and proteinase K protection assays were used to investigate whether vault components were protected by EV membranes.

Results: EVs isolated by all methods were positive for EV markers, such as CD63, CD9 and TSG101. VtRNAs and MVP co-purified with EV markers by differential centrifugation and SEC. RNase and protease treatment of EV preparations demonstrated that vtRNA and MVP were not protected within the EV membrane. Immunocapture of EVs already pelleted by ultracentrifugation showed co-purification of MVP. Whereas, EVs captured directly from conditioned medium were not MVP-positive.

Summary/Conclusion: Commonly used isolation techniques, such as differential centrifugation and SEC, can lead to contamination of EVs with vault particle components. The current study highlights the importance of following the MISEV2018 guidelines when determining the topology of EV-associated components, or if indeed they are EV cargo or contaminants that have been co-purifed.

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OF14.5

Staphylococcus aureus secrete immunomodulatory RNA and DNA via extracellular vesicles

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Introduction: Bacterial-derived RNA and DNA can function as ligands for intracellular receptor activation and induce downstream signalling to modulate the host response to bacterial infection. The mechanisms underlying the secretion of immunomodulatory RNA and DNA by human pathogens, such as Staphylococcus aureus, and their delivery to intracellular host cell receptors is not well understood. Recently, extracellular membrane vesicle (MV) production has been proposed as a general secretion mechanism that could facilitate the delivery of functional bacterial nucleic acids into host cells. S. aureus produce membrane-bound, spherical, nano-sized, MVs packaged with a select array of bioactive macromolecules and they have been shown to play important roles in bacterial virulence and in immune modulation through the transmission of biologic signals to host cells. The present study sought to examine the nature of the association between nucleic acids and MVs produced by S. aureus. We also sought to analyse the immunostimulatory potential of MV-associated RNA and DNA, and to evaluate receptor-mediated recognition of MV-associated RNA and DNA molecules by innate immune cells.

Methods: By following a stringent purification protocol, we characterized the RNA and DNA content of MVs produced by actively growing S. aureus. Nuclease protection assays were performed to determine whether MV-associated nucleic acids are protected from degradation. We assessed the immunomodulatory potential of MV-associated RNA and DNA by treating cultured mouse macrophages with MVs and measuring the induction of Interferon-β mRNA using qPCR.

Results: We found that S. aureus secretes RNA and DNA molecules that are mostly protected from degradation by their association with MVs. We demonstrate that MVs can be delivered into cultured macrophage

cells through endocytosis and subsequently stimulate a potent IFN-β response in recipient cells via activation of endosomal Toll-like receptors.

Summary/Conclusion: Our findings show for the first time an MV-mediated pathway by which S. aureus-derived immunomodulatory nucleic acids are delivered to host cells and activate endosomal nucleic acid receptors. This study advances our understanding of the mechanisms by which bacterial nucleic acids are trafficked extracellularly to trigger the modulation of host immune responses.

OF14.6

Urinary extracellular vesicles transcriptome in diabetic kidney disease

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Introduction: Urinary extracellular vesicle (UEV) transcriptome could potentially reflect the kidney gene expression profile and serve as virtual/liquid biopsy. In order to explore this possibility, we performed mRNA sequencing of UEVs from individuals with type 1 diabetes to assess whether it can capture a "kidney enriched genes" expression signature that could lead to novel biomarker discovery for diabetic kidney disease.

Methods: The study included 75 type 1 diabetic individuals (41 normoalbuminuric, 15 microalbuminuric and 19 macroalbuminuric). Urine samples were collected either overnight (N = 38) or during 24-hours (N = 37) and UEVs were isolated from 20–30 ml of urine by differential centrifugation. The EVs quality was ensured by Electron microscopy (EM), western blotting and EV-RNAs – profiling with the Bioanalyzer. Isolated RNAs were subjected to RNA sequencing after cDNA library preparation (ultra-low amount protocols) using HiSeq 2000 (Illumina) pair-end protocol. The association between kidney specific gene expression levels (>4 fold higher compared to other tissues, N = 53) and degree of albuminuria or glomerular filtration rate was explored.

Results: Isolated EV quality appeared good by EM and Western blotting. RNA quantity and quality were sufficient for sequencing of all samples with >5 million pair end reads. We detected on average expression of 12,642 genes. Principal component analysis (PC) of the expression of all genes did not reveal any systematic batch differences between the overnight and 24-hour urine collections. Comprehensive look-up of 53 kidney-enriched genes revealed expression of >73% (total 39) of these genes in urine EVs with high expression of five kidney-specific genes (SLC12A3, SLC12A1, NPHS2, AQP2 and SLC22A12). PC analysis combining the impact of 39 kidney-enriched genes revealed that most macroalbuminuric patients clustered together along the PC1 axis, and the axis also correlated with the albumin-to-creatinine ratio ($p = 0.0004$) explaining 11% of the variance ($P = 0.005$) in the whole data set. The PC1 axis also showed correlation with HbA1c ($P = 0.003$), but not with diabetes duration, BMI, age and eGFR.

Summary/Conclusion: Our results show that urinary EV transcriptome can capture kidney specific gene expression signatures suggesting its potential as a virtual kidney biopsy.

Funding: BEAt-DKD, IMI-JU, GA115974 and Academy of Finland (317599)

OF15

Symposium Session 15: EV-based Therapeutics II

Chair: Chantal Boulanger – Université de Paris, Paris Cardiovascular Research Center, Inserm, Paris, France

OF15.1

Engineering extracellular vesicles for targeted delivery of Cas9 to T cells

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Introduction: Extracellular vesicles (EVs) are nanoscale lipid particles secreted by all cells that mediate intercellular communication by transferring encapsulated proteins and nucleic acids between cells. Intrinsic properties, such as non-toxicity and non-immunogenicity, and the ability to engineer EVs to incorporate desired cargo and targeting moieties make them an attractive delivery platform for a wide range of biomolecules. One such application is the use of EVs to deliver Cas9 ribonucleoprotein for targeted and translatable in vivo gene editing. Overcoming this delivery barrier could enable novel therapeutic strategies, such as the inactivation of HIV proviral DNA in T cells to eliminate the latent viral reservoir.
Methods: We developed a novel system for actively loading functional Cas9 ribonucleoprotein into EVs and for engineering these vesicles to target delivery of Cas9 to recipient T cells. To promote specific interactions with recipient cells, we developed a novel, high-affinity EV targeting platform by displaying single-chain variable fragments (scFvs) on the surface of the vesicles. To enhance vesicle uptake by T cells, we used viral glycoproteins to promote vesicle fusion at the cell surface.
Results: We demonstrated directed loading of Cas9 ribonucleoprotein into engineered vesicle populations. We enhanced receptor-dependent EV targeting to T cells ~100-fold as compared to a non-targeted EV control. Our approach, as well as display of fusion-enhancing proteins, was successfully used to increase EV uptake by recipient T cells, which exhibit low basal rates of endocytosis and are difficult to deliver cargo to by other means.
Summary/Conclusion: The ability to direct EV uptake to desired cell types and uptake pathways within those cells may ultimately enable functional delivery of biomolecular cargo for a wide range of targeted therapies.
Funding: This work was supported by the Third Coast Center for AIDS Research (CFAR), an NIH funded centre (P30 AI117943).

OF15.2

Exosomal AAVs in Myocardial Repair: Escaping Neutralizing Antibody and Enhancing Delivery

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Introduction: Due to their safety profile, tissue tropism and long-term transgene expression, adeno-associated viruses (AAVs) have become the vector of choice for human gene therapy. However, pre-existing neutralizing antibodies (NAbs) to many AAV serotypes pose a critical challenge for the translation of gene therapies to clinic. Here, we describe the use of exosomal AAVs (eAAV) as a robust cardiac gene delivery system that enhance transduction efficiency while shielding from pre-existing humoral immunity to the viral capsid.
Methods: We developed an ultracentrifugation-based purification strategy to obtain eAAV specimens from AAV-producing HEK-293 T cells, and used electron microscopy-based visualization, confocal microscopy-based colocalization studies, qPCR, immunoblotting, dynamic lights scattering, ExoView technology and protease assays to characterize eAAV morphology, contents and mechanism of action. We then evaluated efficiency of heart targeting for eAAV9 or eAAV6 and standard AAV9 or AAV6 encoding for EGFP, mCherry or firefly luciferase in different human cell lines in vitro, in black mouse and in passive immunity nude mouse model in vivo using flow cytometry, confocal microscopy, Langendorff perfusion system and

bioluminescence imaging. Last, to test therapeutic efficacy, eAAV9 or AAV9 vectors encoding for SERCA2a, a sarcoplasmic reticulum calcium adenosine triphosphatase, were injected intramyocardially in post-myocardial infarction mice preinjected with NAbs. Echocardiography and histochemistry were used to evaluate cardiac function and remodelling.

Results: We confirmed eAAV represent vesicular fractions that exhibit common exosome morphology and antigen expression, along with the presence of virus particles, and demonstrated that eAAV infectious entry potentially involves trafficking via endocytic compartments. Regardless of the presence or absence of NAbs, we showed in functional studies that eAAVs are more efficient in cell transduction in the same titre ranges as standard AAVs. Remarkably, eAAV9-SERCA2a outperformed standard AAVs significantly improving cardiac function even in the presence of NAbs (%EF 55.14 ± 3.50 compared to 27.31 ± 1.63 at 6 weeks, respectively).

Summary/Conclusion: Delivery of standard AAVs protected by carrier exosomes (i.e. eAAVs) is a promising approach to evade pre-existing NAbs while still efficiently transducing myocardium, which can be applied in the broader population of patients with myocardial infarction and may result in higher gene delivery efficacy.

OF15.3

Computational Modelling of Mesenchymal Stem Cell Exosomes Predicts Cardiac Improvements in Preclinical and Clinical Models of Hypoplastic Left Heart Syndrome

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Introduction: The palliation of hypoplastic left heart syndrome (HLHS), a complex congenital heart disease, places significant demand on the right ventricle (RV) to sustain systemic circulation, resulting in high RV dysfunction-associated mortality. Cell-based therapies may improve RV adaption to single ventricle physiology, specifically through the release of pro-reparative exosomes. The transplantation of bone marrow-derived mesenchymal stem cells (MSCs) in a pulmonary artery banding (PAB) swine model improves RV function and led to the ELPIS clinical trial: Allogeneic Human MSC Injection in Patients with HLHS. Here, we model circulating MSC exosome cargo to predict RV functional recovery in preclinical PAB model and validate its translational potential in ELPIS subjects.

Methods: HLHS patients (n = 3) after Glenn procedure and swine (n = 3) after PAB were given RV injections of allogeneic/xenogeneic MSCs. Donor-specific, HLA-I+, exosomes were isolated from plasma. In swine, exosomes were collected and RV fractional area change (FAC) was measured post-MSC-injection. In the ELPIS patients, exosomes were collected and outcome measurements (FAC, stroke volume (SV), RV mass) were recorded 6 and 12-months post-injection. Exosomal mRNA, microRNA (miRNA), and proteins were quantified and partial least squares regression (PLSR) reduced the dimensionality of the datasets to build a swine model, upon which ELPIS outcome predictions were made.

Results: Multiomics analysis of swine exosome cargo revealed miRNA to be the largest contributor to overall variance. In swine and ELPIS patients, miRNAs were similarly expressed (98%, fold-change<2). PLSR reduced the dimensionality of the swine miRNA dataset to 50 miRNAs with the highest weighted coefficients for changes in FAC. Pathway analysis of miRNA targets revealed links to smooth muscle cell proliferation and cardiac chamber development. Importantly, the swine miRNA PLSR model predicted ELPIS patient improvements in FAC, SV, and RV mass with strong correlation ($r > 0.9$).

Summary/Conclusion: These findings support the use of: (1) swine PAB model for RV failure in HLHS, (2) circulating donor-specific MSC-exosomal miRNA as a novel, non-invasive biomarker of patient outcomes, and (3) computational modelling to predict improvements and inform clinical trials.

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OF15.4

RNA nanoparticles as EV displaying ligands for specific cancer targeting and efficient RNAi delivery in vivo

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Introduction: EVs have been shown promising potential as a drug delivery vehicle, especially nucleic acid therapeutics. However, the overall short of specificity

to target cancer cells has led to low therapeutic efficacy and potential toxicity. RNA nanotechnology is the bottom-up self-assembly of nanometre-scale RNA architectures. We previously discovered a stable phi29 pRNA three-way junction (3WJ) motif and used it to construct multivalent RNA nanoparticles with high chemical and thermodynamic stability. The resulting arrow-shape RNA nanoparticles are homogenous, uniform in size and shape, and can harbour different functionalities while retaining their tertiary folding and independent functionalities both in vitro and in vivo. This flexible platform using RNA nanotechnology to achieve tumour-specific targeting has been demonstrated over the last decade. Here we introduce a strategy to take advantage of both EVs and RNA nanotechnology to develop a versatile platform for efficient target-specific delivery of siRNAs for cancer treatment.

Methods: We design membrane-anchoring arrowtail 3WJ RNA nanoparticles to display tumour targeting ligand (PSMA RNA aptamer or EGFR RNA aptamer or folate) on BIRC5 siRNAs loaded EVs (Fig.1). Nanoparticles were characterized by Nanoparticles Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM). EVs were produced by Hollowfiber bioreactor and purify by Tangential Flow Filtration (TFF) follow by ultracentrifugation. Cell binding were evaluated by flowcytometry and confocal microscopy and gene knockdown effect were assay by quantity reverse transcription-PCR (qRT-PCR). Formulated EVs were introduced to tumour (prostate, triple negative breast cancer, colon PDX) xenograft mice by tail-vein injection and evaluate in vivo tumour inhibition.

Results: 1) We found the orientation of arrow-shaped RNA can be used to control ligand display on EVs membranes for specific cell targeting. 2) By placing membrane-anchoring cholesterol at the tail of the arrow results in display of RNA aptamer or folate on the outer surface of the EVs and enhance cancer cell binding and uptake. 3) Taking advantage of the RNA ligand for specific targeting and EVs for efficient cytosolic delivery, the resulting ligand-displaying EVs or plant derived EVs-like nanovesicles were capable of specific delivery of siRNA to cells, and efficiently blocked tumour growth in three cancer models.

Summary/Conclusion: We developed an RNA-EVs based nanoparticles platform and shown the flexibility for different cancer type treatment.

Related publications:

Pi F et.al. Nature Nanotechnology. 2018, 13:82.
Li Z et.al. Sci Rep. 2018, 8:14644
Zheng Z & Li Z et.al. J Control Release. 2019, 311:43.

OF16

Symposium Session 16: Cancer Biomarker

Chair: Carolina Soekmadji – QIMR Berghofer

OF16.1

Multiplex nanoscale flow cytometry to study the circulating extracellular vesicle biome in patients with pancreatic cancer versus screen negative controls

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Introduction: Extracellular vesicles (EVs) contain plasma membrane surface markers that provide insights into their cell source. Until now, our understanding of the circulating EV-biome has been limited by the lack of cell- and size-specific EV quantitation methods. We have developed and validated a multiplex nanoscale flow cytometry approach to image cell- and size-specific EV populations using a novel human "EV-Lyoplate" with 3 differently coloured monoclonal antibodies per well in a 96 well plate format (n = 242 separate antibodies with isotype, stained PBS, unstained plasma, and quant-beads controls per plate). We hypothesized that platelet poor plasma samples from patients diagnosed with pancreatic cancer would have significantly different EV-biome profiles than screen negative study subjects.
Methods: Study subjects were enrolled and sampled before clinically scheduled endoscopic ultrasound-guided biopsy (EUS-FNA) procedures to screen patients with symptoms of pancreatic duct obstruction who later had at least two years of clinical follow up, including surgical resection in cases of pancreatic neoplasia (n = 36) or at least one follow up clinic visit to confirm resolution of symptoms. Blood samples were uniformly collected, processed, and banked per ISEV recommended guidelines. Uniform machine (FACSymphony) settings to standardize light scatter and fluorescence detection were based on commercially available beads (eg. Megamix). Samples were coded and randomized for testing and results were reported as the mean cell- and size-specific EV events/ul of plasma.
Results: Clinical outcomes confirmed 10 cases of cancer and 26 screen negative controls. Principle component analysis suggested that a number of different cell- and size-specific EVs were significantly more common in the cancer cases (adjusted p-value < 0.05, with AUCs >0.80), including Epcam+/CD63+ events likely from cancer cells and CD41+/CD62p+/CD9+ microvesicles from platelets, among others.
Summary/Conclusion: In this proof of principle study employing an EV-Lyoplate design and nanoscale flow cytometry, we could reliably discriminate the EV-biomes in patients with cancer from negative controls. Ongoing studies will determine whether these discriminators will be validated in larger cohorts and provide at least non-inferior predictive value compared with the current gold standard clinical testing assay (EUS-FNA).
Funding: Knight Cancer Institute, Oregon Health & Science University

OF16.2

Analysis of surface markers as biomarkers for small cell lung cancer

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Introduction: Small Cell Lung Cancer (SCLC) is an aggressive tumour type, usually metastatic at diagnostic leading to poor overall survival. Interestingly, SCLC tumours are composed by distinct subpopulations of cells that cooperate as an ecosystem to drive tumour survival. Since the subtype of SCLC may have prognostic significance, the aim of this study was to identify surface marker proteins as biomarkers of SCLC.
Methods: A linear discriminant analysis (LDA) model, implemented in Python via Sci-kit learn, was used to choose the best 4 markers for distinguishing subtypes. This analysis was based on RNA-seq data from a previous study. In order to identify EV-based biomarkers that would identify SCLC EVs and not normal EVs, we excluded from this analysis proteins without a verified transmembrane domain and proteins associated with EVs expected to be present in white and red blood cells, and endothelial cells (according to Exocarta and Vesiclepedia databases). We also prioritized proteins that could be pan markers for SCLC and that might have prognostic significance. To validate our findings,

we performed Western blotting and Flow cytometry in SCLC cell lines from different subtypes.

Results: Our RNA analysis indicated that the best 4 surface markers to distinguish SCLC subtypes were CEACAM5, FAM174A, LRFN1, EPHA2. Immunoblot analysis validated CEACAM5 and EPHA2 but not FAM174A or LRFN1. We also found that NCAM1, a commonly used SCLC marker, only marks some of the subtypes. For further analysis, we chose proteins with antibodies validated for flow cytometry as our chosen biomarker platform. Flow cytometry analysis of CD24 is suitable as a pan-SCLC marker. However, the expression of NON-NE cell lines was decreased compared to RNA-seq data.

Summary/Conclusion: Protein analysis of CEACAM5 and EPHA2 corresponded to RNA-seq data. NCAM was not detected as a pan marker for all SCLC subtypes. However, we could see CD24 expression in all SCLC subtypes, indicating it may be a useful pan marker for SCLC. Future studies will be performed to validate the expression of other surface markers in cells, purified EVs, and plasma of SCLC patients.

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OF16.3

Leukobiopsy – exploiting extracellular vesicle-mediated leukocyte sequestration of cancer-specific signatures

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Introduction: In cancer, extracellular vesicles (EVs) act as a unique exit mechanism for mutant and oncogenic macromolecules (proteins, RNA and DNA) en route from malignant cells to blood1. While this process has inspired major liquid biopsy efforts, the biology of circulating EVs that carry oncogenic mutations (oncosomes)2 is still poorly characterized. It is also unclear what part (if any) of the tumour-related cell free DNA (ctDNA)3,4, a major liquid biopsy analyte, is linked to circulating EVs and what is their fate, receptacles and biological activity.

Methods: We employed as series of cancer cell lines carrying mutations in major oncogenes (HRAS, HER2, EGFRvIII). EV-DNA was analysed by digital droplet PCR (ddPCR), along with nuclear anomalies in donor cells (DAPI, Electron Microscopy) and transfer of DNA to recipient cells of endothelial (HUVEC, MMBEC), astrocytic (NHA) or myeloid (HL60) origin. Blood underwent fractionation into red blood cells (RBC), white blood cells (WBC), platelets (PLT), EVs (100,000g ultracentrifugation) and soluble plasma (SUP)5.

Results: HRAS-mediated cellular transformation (in RAS-3 cells) triggers profound changes in the structure of nuclear chromatin, which is driven into the cytoplasm and released as cargo of EVs. Oncogenic DNA is detectable in blood fractions of tumour bearing mice. While EVs, ctDNA and PLTs contain intermediate levels of mutant DNA, RBCs contain only traces of this material. The highest HRAS copy number per ml of blood is found in WBCs (monocytes and neutrophils), which contain more cancer DNA/cell than liver, spleen and bone marrow. Depletion of neutrophils using anti-Ly6G antibody results in an increase in EV- and ctDNA-associated mutant DNA in blood, suggesting the role of these cells in regulating the circulating levels of cancer cell-derived particles. Uptake of DNA-containing EVs impacts the phenotype of myeloid cells, which adopt thrombo-inflammatory properties. These cells also retain cancer-specific transcripts and other cargo. Finally, normal astrocytes treated with oncogenic EVs also exhibit phenotypic changes and signs of genomic instability including formation of micronuclei.

Summary/Conclusion: We propose that the process of leukocyte sequestration of circulating particles containing tumour-related nucleic acids renders these cells potentially usable as a novel liquid biopsy platform (LEUKOBIOPSY) in cancer. 1. PMID:18425114; 2. PMID:27680302; 3.PMID: 25086355; 4. PMID:31469961; 5. PMID:29971917

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OF16.4

Whole-transcriptomic profiling of circulating small extracellular vesicles derived RNAs for early detection of colorectal carcinoma and adenoma

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Introduction: Early diagnosis of colorectal cancer (CRC) and precancerous adenoma patients is of vital importance. Previously we profiled small extracellular vesicles (sEVs) derived miRNAs isolated from plasma, proposed a new promising biomarker category of CRC patients. Here we further gave a full landscape of circulating sEVs derived RNAs to explore and evaluate sEVs based RNA biomarkers for early detection of both CRC and adenoma patients.

Methods: Plasma sEVs were isolated from 60 participants, including 31 early-stage CRC patients, 19 adenoma patients, and 10 normal controls (NC), and characterized according to MISV2018 guideline. The total sEVs derived RNA expression profile of all participants was investigated by next-generation sequencing (NGS). Weighted gene coexpression network analysis (WGCNA) was performed to categorize differentially expressed RNAs, and t-distributed stochastic neighbour embedding (tSNE) was adopted to distinguish CRC, adenoma, from NC samples with the top-ranked genes in WGCNA modules. RT-qPCR validation was performed in a cohort of 120 additional participants.
Results: A total of 1615 RNA species (including miRNAs, mRNAs, and lncRNAs) were found differentially expressed between plasma sEVs in CRC and NC participants. Additionally, 888 RNA species were differentially expressed between plasma sEVs in adenoma and NC participants. 1160 RNA species were differentially expressed between plasma sEVs in CRC and adenoma participants. WGCNA categorized all RNAs into 6 modules, which exhibited different expression trends during the carcinogenesis of CRC. A 60-gene combined tSNE model consists of the top 10 genes in each module could perfectly classify CRC, adenoma, and NC samples. A 6-gene combined tSNE model consists of the top 1 gene in each module could roughly distinguish CRC and adenoma from NC, with only 1 sample misclassified. RT-qPCR assays also confirmed the potential classification ability of those genes in another validation cohort of 120 participants.
Summary/Conclusion: Circulating sEVs have a distinct RNA profile in both CRC and adenoma patients as compared to normal controls. sEVs derived RNA biomarker combination could for Early Detection of Colorectal Carcinoma and Adenoma.
Funding: This work was supported by grants from the Beijing Nova Program (Z191100001119128); National Natural Science Foundation of China (81702314); Beijing Municipal Science and Technology Project (Z191100006619081); Beijing Municipal Administration of Hospitals' Youth Programme (QML20180108); The Digestive Medical Coordinated Development Center of Beijing Municipal Administration of Hospitals (XXZ0201).

OF16.5

RNA signatures from blood and urine EVs display tissue-lineage from prostate cancer patients

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Introduction: Although the concept of systematic "liquid biopsy" using bodily fluids is simple and elegant, the path of clinical reality has been challenging. Recently, numerous tissue-specific biomarkers have been discovered in EVs derived from blood, urine, cerebrospinal fluid, cell culture media, and a variety of other fluids. However, tracing the lineage of EVs to their tissue of origin remains challenging due to their minute amount of cargo and unavailability of matching biopsied tissue and bodily fluids from the same patient.

We recently demonstrated in three separate publications (Dogra et. al; Smith et. al; Murillo et. al), a new device (nanoDLD) for EV isolations, it's comparison with current technologies, bioengineered vesicles, and a detailed study of RNA types present in small/large vesicles, lipoproteins, and Ago2 protein in different biofluids. In the present study, we aim to investigate the lineage of prostate derived EVs in biofluids.
Methods: Using our chip technology, we have isolated exosomes from prostate cancer cell lines and patient tissue, blood and urine samples. After exosome isolation, small RNA libraries were prepared, and sequencing is carried out at Icahn School of Medicine and New York Genome Center using illumine sequencer HISeq2500. Our nanofluidic pillar array is manufactured in an SiO2 mask using optical contact lithography and deep ultraviolet lithography.
Results: Our study revealed i) RNA markers, which are exclusive to their prostate tissue of origin and are secreted in EVs; ii) approximately 1–3% of prostate tissue-specific RNA were discovered in EVs; iii) Over 77% (17 of 22 RNA) of literature curated prostate-specific RNA signatures were detectable in serum and urine EVs from PCa patients; iv) EVs contained over 50–70% of noncoding RNA (40–60% was miRNA), while tissue predominantly yielded rRNA (>60%); v) Finally, gene set analyses generated that over 90% of EVs RNA were enriched for signalling pathways, yielding miRNA-associated, non-canonical Wnt signalling, and androgen receptor pathways. This study enables us to noninvasively monitor prostate tissue-specific biomarkers, identify tumour-specific RNA, and potentially may benefit in liquid biopsy by avoiding unnecessary surgical procedures.
Summary/Conclusion: In summary, we have investigated patient matched tissue, serum, and urine derived EVs in prostate cancer. We present a set of 68 prostatic

RNA in EVs, which are enriched in noncanonical Wnt signalling, and androgen receptor pathways. This study enables us to noninvasively track prostatic biomarkers, identify tumour specific RNA, and potentially may benefit in liquid biopsy by avoiding unnecessary surgical procedures.

OF16.6

A multi-model, liquid biopsy approach for diagnosing and staging pancreatic adenocarcinoma

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is the third largest contributor to cancer-related death in the USA. Since there is not yet a feasible technology to diagnose PDAC early in the disease, 80% of patients are diagnosed at an advanced stage. Moreover, for patients with confirmed PDAC, standard imaging method has low sensitivity to detect early metastatic disease, which complicates the selection of therapy.

To address these challenges, there has been great interest in developing minimally-invasive, extracellular vesicle (EV) based blood tests for PDAC. To this end, we have integrated measurements of tumour derivedEV RNA cargo with circulating proteins and cell free DNA (cfDNA), and use machine learning algorithms to distill this multiplexed diagnostic to 1. diagnose PDAC patients from healthy and disease controls and 2. distinguish PDAC patients with distance sites of metastasis to guide their treatment. We make use of our lab's magnetic nanopore isolation technique to specifically enrich for tumour derived EVs directly from patient plasma.

Methods: We have developed a high throughput nanofluidic sorting platform, which immunomagnetically isolates individual EVs from plasma using magnetic nanostructures. However, our architectures is uniquely designed for massive parallelization allowing high throughput, robust processing of mL of plasma in minutes. We performed sequencing on a discovery set of patients and controls (N = 29). Subsequently, we trained our panel of biomarkers using a training set of N = 47. Finally, we validated the performance of our platform using an independent blinded test set of N = 145.

Results: The results of a blinded test set achieved an accuracy = 92% and an AUC = 0.95 on binary classification of PDAC patients versus those that were healthy or disease controls. In addition, we achieved an AUC = 0.85 and accuracy = 89% with sensitivity of 90% and specificity of 88% on detecting occult metastasist.

Summary/Conclusion: We developed a highly sensitive pancreatic cancer diagnostics by combining our nanomagnetic isolation platform for tumour-derived EV isolation, RNA sequencing, and machine learning. We isolated tumour-derived EVs and profiled their RNA cargo, combined with cfDNA and CA19-9 for pancreatic cancer diagnosis. The predictive panels successfully distinguished non-cancer patients from PDAC patients, and no-distant metastasis patients(M0) from distant metastasis patients(M1) for appropriate treatment. The resulting AUC and accuracy from the independent blinded test set outperformed any individual biomarker, showing both the benefits and the robustness of combining multiple orthogonal biomarkers for PDAC diagnosis.

OF17

Symposium Session 17: Heart, Lung, and Vessels

Chair: J. Brian Byrd – University of Michigan

OF17.1

The proteome of circulating large EVs in diabetes and hypertension

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Introduction: Both hypertension and diabetes exhibit significant molecular changes to the vasculature that are associated with increased cardiovascular risk. Here we examined the protein composition of large EVs (L-EVs) isolated from the plasma of hypertensive, diabetic and healthy mice to identify common and disease-specific molecular changes.
Methods: We examined circulating L-EVs isolated from transgenic mice expressing active human renin in the liver (TtRhRen, a model of hypertension), OVE26 type 1 diabetic mice, and their wild-type (WT) littermates. At 20 weeks of age mice were sacrificed and blood samples were obtained by cardiac puncture. L-EVs were isolated from platelet-free plasma via differential centrifugation and protein content was assessed via mass spectrometry (MS).
Results: TtRhRen mice exhibited increased blood pressure compared with OVE26 mice or their WT littermates. (144.2 ± 7.6 vs 123.5 ± 4.9 [OVE26] vs. 114.6 ± 5.7 mmHg [WT], $p < 0.05$). MS identified 297 independent proteins with at least 2 peptides per protein. Of these, 163 proteins were found in all groups studied, 48 were exclusive to WT mice, 23 were exclusive to OVE26 mice and 4 were exclusive to TtRhRen mice. In addition, 22 proteins were observed with >1.5 fold change (FC) compared to wild-type mice, and 68 proteins were reduced by >50%. Amongst the top ten differentially expressed proteins, fibrinogen was upregulated in both OVE26 and TtRhRen mice compared with wild-type controls. Similarly Trem-like transcript 1, sarcoplasmic/endoplasmic reticulum calcium ATPase 3 and junction plakoglobin were all downregulated in both OVE26 mice and TtRhRen mice suggesting molecular changes common to both conditions. Conversely, arginase was up-regulated in diabetic, but not hypertensive mice while carboxypeptidase was upregulated in hypertensive but not diabetic mice.
Summary/Conclusion: Taken together, these results show that the protein composition of circulating L-EVs is altered in diabetes and hypertension and that both common and disease-specific changes may be detected. Further analysis of these changes may lead to the identification of novel pathways associated with the pathogenesis of vascular injury in hypertension and diabetes.
Funding: This study was supported by grants (to DB) from the Canadian Institutes of Health Research, an Ontario Early Researcher Award, and the Canada Foundation for Innovation.

OF17.2

Understanding the role of endothelial cell-derived apoptotic bodies in inflammatory signalling and cell clearance in an atherosclerosis model of inflammation.

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Introduction: Apoptotic bodies (ApoBDs) are a class of large (~1-5um) EVs formed during apoptotic cell disassembly, that are becoming increasingly recognized as potential mediators of intercellular communication, e.g. via the transfer of proteins and other cargoes to target cells. During the inflammatory vascular disease atherosclerosis, endothelial cell (EC) apoptosis contributes to loss of barrier function and promotes the formation of plaques in regions of EC damage. Although, experimentally, ECs generate an abundance of ApoBDs, a specific role for EC-derived ApoBDs (EC-ApoBDs) in the progression of atherosclerosis remains poorly defined.
Methods: In the present study, a detailed in vitro characterization of EC disassembly was performed via flow cytometry, confocal live cell imaging and cytokine profiling, followed by function analyses of EC-ApoBDs using a murine in vivo model of dead cell clearance.
Results: Characterization of EC disassembly revealed that ApoBD formation in ECs is regulated by rho-

associated, coiled-coil-containing protein kinase 1 (ROCK1), a process that can be pharmacologically inhibited using a ROCK-1 inhibitor, thereby providing tools for functional in vivo studies. The specific cargo and role in clearance of EC-ApoBDs were then investigated. Profiling of EC-ApoBDs was performed via cytokine antibody array to reveal that EC-ApoBDs generated under inflammatory conditions contain high levels of pro-inflammatory cytokines including MCP-1 and IL-8, suggesting a potential role for EC-ApoBDs in the propagation of inflammation during vascular disease. Furthermore, the ability of EC-ApoBDs to be cleared from the vasculature via phagocytosis was investigated, revealing that EC-ApoBDs can travel to distal organs to undergo clearance.

Summary/Conclusion: These findings provide important insights into the potential functions of EC-ApoBDs generated under both non-inflammatory and inflammatory conditions and may contribute to future studies involving the therapeutic targeting of EC disassembly for the treatment of atherosclerosis.

Funding: This work was supported by grants from the National Health & Medical Research Council of Australia (GNT1141732, GNT1140187)

OF17.3

Adipose mesenchymal stromal cell derived EVs foster cardio-renal protection in the DOCA-salt hypertensive rat model

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Introduction: Cardio-renal syndromes (CRS) are disorders of the heart and kidneys whereby "acute or chronic dysfunction in one organ may induce dysfunction of the other". Stem cell-derived extracellular vesicles (EVs) mediates the protection of the kidney from development of chronic kidney disease (CKD). We here investigated the potential of adipose-mesenchymal stromal cells derived EVs (ASC-EVs) as therapeutic tools for the treatment of CRS.

Methods: Adult Wistar rats were uninephrectomized and treated with a high-Na+ diet and deoxycorticosterone-acetate (DOCA-salt) for 8-weeks (038/15; A02/16-61-15). EVs were isolated by ultracentrifugation method. EV dimension, concentration and surface markers were characterized by NTA, cytofluorimetric analysis and transmission electron microscopy. To characterize the role of EVs in CRS, DOCA-salt rats were injected weekly with ASC-EVs. Systolic blood pressure was measured by the tail-cuff method. Plasma creatinine and urinary protein excretion were determined by colorimetric assays and microalbuminuria by immune turbidimetric assay. qRT-PCR and western blot were conducted to evaluate fibrosis and inflammatory-related genes and proteins in the kidney and heart of DOCA-salt rats. Immunohistochemistry was used to confirm matrix accumulation (a-SMA) and immune infiltrate (CD68+ cells).

Results: Multiple administration of ASC-EVs in DOCA-salt rats induced a protective effect on the kidney, by reducing tubular and vascular damage. Kidney function was also conserved by EV treatment as detected by the normal glomerular filtration rate and the absence of proteinuria with respect to DOCA-salt untreated rats. EV administration significantly decreases the pro-inflammatory molecules MCP-1 and PAI1 and reduce the recruitment of macrophages in the kidney. The mitigation of the inflammatory response by ASC-EV infusion consequentially affected the development of fibrosis, as detected by the decrease in collagens (Col1A1, Col4A1) and fibronectin (FN) expression in respect to DOCA-salt animals. ASC-EVs were able to act in multiple organs, preventing fibrosis and inflammation also in the heart, therefore alleviating blood pressure rise during the 8-weeks of treatment in DOCA-salt rats.

Summary/Conclusion: Our results indicate that ASC-EV administrations in hypertensive-induced CKD rats promote protection from renal damage, reduction of the inflammatory response and prevention of interstitial fibrosis in the kidney. ASC-EVs are also able to protect the cardiac tissue and to control blood pressure increase, displaying complex and multiorgan beneficial effects.

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OF18

Symposium Session 18: EV Biogenesis II

Chair: Suresh Mathivanan – La Trobe University

Chair: Clotilde Théry, PhD – INSERM U932, Institut Curie, PSL Research University

OF18.1

Glycolytic restraint in alveolar macrophages is critical for vesicular secretion of suppressor of cytokine signalling 3

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Introduction: Alveolar macrophages (AMs) tonically secrete extracellular vesicles (EVs) containing suppressor of cytokine signalling 3 (SOCS3) protein. Uptake of SOCS3-containing EVs by alveolar epithelial cells is critical for restraint of cytokine-induced Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) signalling to promote homoeostasis in the distal lung. At steady state, AMs exhibit suppressed glycolytic activity, a metabolic phenotype that promotes homoeostatic function. Whether this glycolytic restraint is critical for AM secretion of SOCS3 is unknown. In fact, to our knowledge, metabolic control over release of any EV cargo has never been explored in any cellular context.
Methods: Immortalized mouse AMs (MH-S) were treated with various doses of 2-deoxy-D-glucose (2-DG) and oligomycin, inhibitors of glycolysis and oxidative phosphorylation, respectively. Primary rat AMs collected by lung lavage were treated with an aqueous extract of cigarette smoke (CSE) with or without 2-DG. Metabolic activity was measured by Seahorse assay, EVs were quantified by nanoparticle tracking analysis, and vesicular (>100-kDa) SOCS3 secretion was determined by western blot of conditioned medium. Additionally, AMs collected from wild-type (WT) and LSL-KrasG12D mice bearing lung tumours 16 weeks after intrapulmonary Ad-Cre were cultured ex vivo in the presence or absence of 2-DG. Vesicular (>100-kDa) SOCS3 secretion was measured by ELISA.
Results: In a dose-dependent manner, oligomycin inhibited, whereas 2-DG enhanced, SOCS3 and EV release by MH-S cells. Treatment of rat AMs with CSE (1%) attenuated secretion of SOCS3, an effect that coincided with increases in glycolytic activity, and co-treatment of AMs with 2-DG abrogated the inhibitory effect of CSE on SOCS3 release. Finally, AMs collected from LSL-KrasG12D mice exhibited a deficiency in SOCS3 secretion relative to WT AMs, an effect that was reversible by overnight culture in the presence of 2-DG.
Summary/Conclusion: In tandem, our data generated using in vitro and in vivo approaches demonstrate that AM secretion of vesicular SOCS3 is down-regulated by glycolysis. We speculate that metabolic control over release of EV cargoes is a phenomenon of broad biologic relevance within and outside of the lung.
Funding: National Science Foundation Graduate Research Fellowship DGE 1256260 (MH) and National Institutes of Health R35 HL144979 (MP-G)

OF18.2

Cyanobacterial extracellular vesicles are an alternative secretion mechanism to deal with copper-induced stress

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Introduction: Bacterial extracellular vesicles (EV) are described to play roles in defence and resistance, pathogenesis and stress responses. Cyanobacteria pioneered oxygenic photosynthesis, and are the ancestors of modern chloroplasts. We previously described that by deleting the gene encoding TolC (ΔtolC) in the model cyanobacterium Synechocystis sp PCC6803 (S6803), a key player in protein-mediated secretion systems, a hyper-vesiculating phenotype could be obtained. The goal of this work was to understand why ΔtolC hyper-vesiculates.

Methods: Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) was used for quantitative proteomic analyses of total cell extracts. EV were isolated as follows: cells were separated from the extracellular medium (EM) by centrifugation (4400 g, 10 min) and filtration (0.2 μm pore-size filters). Cell-free EM was concentrated using centrifugal filters (MWCO of 100 kDa), and later ultracentrifuged for 3 h at 100000 g. The final EV fraction was suspended in growth medium. EV characterization was performed using TEM, DLS, Nanosight, and by the detection and quantification of LPS (lipopolysaccharides). Detection of specific proteins in EV was carried out by Western blot. Copper (Cu) levels were quantified by atomic absorption spectrometry (AAS).

Results: A large-scale quantitative proteomic analysis was performed, resulting in the identification of several metal-related proteins with differential regulation in S6803 ΔtolC. Both wild-type (WT) and ΔtolC cells were then challenged with different metals. Compared to the WT, ΔtolC showed impaired growth only when exposed to Cu, a co-factor for several proteins with roles in primary metabolism. The intracellular Cu levels were quantified and ΔtolC accumulates threefold more Cu than WT cells. We then asked whether the hyper-vesiculating phenotype observed could be linked to the stress induced by Cu accumulation. In EV isolated from ΔtolC we detected the metallochaperone CopM, a periplasmic Cu-binding protein involved in Cu-resistance mechanisms in S6803. In addition, Cu could also be detected in isolated ΔtolC-EV. In addition, more EV were detected when S6803 WT cells were challenged with Cu, in a Cu-concentration dependent manner.

Summary/Conclusion: These results support the idea that bacterial EV represent an alternative Cu-secretion mechanism to deal with Cu-induced stress.

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OF18.3

Ciliary EV cargo sorting and biogenesis in living animals

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Introduction: Extracellular vesicles (EVs) function in intercellular communication. Despite their physiological importance and biomedical relevance, knowledge of EV fundamental biology is not well understood, in part due to a lack of tractable animal systems. Our analysis of environmentally-released C. elegans ciliary EVs provides strong evidence that nematodes package cargo in EVs that mediate inter-organismal communication, in analogy to intercellular signalling in mammals. We predict that conserved mechanisms underlie EV cargo sorting, biogenesis and signalling.

Cilia act as cell towers to both receive extracellular signals and to send information via ciliary EVs. Ciliary defects result in human ciliopathies including autosomal dominant polycystic kidney disease (ADPKD). ADPKD is a life-threatening disease that affects 1/800 and is caused by mutations in PKD1 and PKD2, which encode polycystin-1 and −2. In C. elegans and humans, the polycystins are architecturally similar, act in the same genetic pathway, function in a sensory capacity, localize to cilia, and are shed in EVs, suggesting ancient conservation. Moreover, ciliary EV biogenesis and shedding is an evolutionary conserved process from algae to worms to humans. By studying how cilia make and receive EVs, we aim to uncover fundamental principles of how cells communicate using EVs.

Methods: To study ciliary EV cargo sorting and biogenesis, we use genetically-encoded fluorescent-tagged EV cargo and superresolution Zeiss Airyscan confocal microscopy in living animals.

Results: We find that cargoes are sorted into distinct populations. In cilia, kinesin-2 motors and kinesin-3 KLP-6/KIF28 transport different EV cargoes to the ciliary tip and generate an EV cargo enrichment zone. From here, EVs are shed and released into environment in a spatially and temporally regulated manner. Ciliary EV biogenesis and release is regulated by mechanical pressure and pH. Our work reveals – at the single cell level – that different EVs are made in response to environmental stimuli, which may be important for EV signalling properties.

Summary/Conclusion: Cells exploit the spatially-restricted cilium and its sophisticated transport system to generate distinct populations of ciliary EVs. How these ciliary EV communicate cellular messages awaits decoding.

Funding: NIH DK059418 & DK116606 (MB), KUMC PKD Center (JW)

OF18.4

Comparative proteomics from different cancer cell types reveals novel mechanisms of Rab11-exosome subtype regulation

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Introduction: We recently demonstrated that recycling endosomes marked by Rab11a generate exosome subtypes distinct in cargos and functions from late endosomes, which we collectively term Rab11-exosomes. These exosomes are preferentially released from cancer cells in response to metabolic stress and promote adaptive changes in a xenograft model. Here we use comparative EV proteomics in HCT116 colorectal and HeLa cervical cancer cell lines to identify Rab11-exosome signature proteins and screen for functional effects.
Methods: We analysed EV preparations by mass spectrometry using Tandem Mass Tag® labelling to identify changes in EV protein cargo in response to glutamine depletion. Candidate genes were subsequently knocked down in Drosophila secondary cells, which permit visualisation of Rab11-exosome biogenesis using fluorescence microscopy, and in human cancer cell lines.
Results: We show that accessory ESCRT-III proteins, CHMP5, CHMP1 and IST1, are enriched on glutamine-depletion-induced EVs and play a selective and conserved role in generating Rab11-exosomes. They are, however, not required to traffic ubiquitinated cargos into late endosomes and lysosomes. ESCRT-0 components, thought to regulate trafficking of ubiquitinated cargos into intraluminal vesicles, are also required to make Rab11-exosomes. In flies the ESCRT-0, Hrs, localises to the limiting membrane of Rab11-endosomes. Comparative proteomics reveals other proteins enriched in Rab11-exosomes, which also appear to be needed to mediate this novel exosome formation mechanism.
Summary/Conclusion: We conclude that Rab11-exosome subtypes are formed via a distinct mechanism requiring accessory ESCRT-III components, suggesting a route to selectively target these exosomes.
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OF18.5

Three-dimensional culture models to elucidate exosomal biology

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Introduction: The tumour microenvironment consists of a complex network of host cells embedded within extracellular matrix. Communication between these cellular compartments is critical for tumour progression and exosomes have emerged as important regulators of intercellular communication. While a number of studies have implicated exosomes in cancer progression, mechanisms controlling exosome transfer are not well understood. We developed three-dimensional (3D) culture models to evaluate the role of cues provided by the extracellular matrix in exosome release and uptake.
Methods: Exosomes were isolated from cells in two- and three-dimensional culture via ultracentrifugation and characterized by Nanosight, Qubit protein quantification, and flow cytometry analysis of exosome markers. Exosomes were labelled with fluorescent lipophilic dyes and uptake in recipient cells quantified by flow cytometry.
Results: Cells cultured in 2D display decreased exosome release and increased uptake compared to 3D cultured cells. Exosome release in 3D culture was inhibited with the exosome release inhibitors brefeldin A and GW4869, but was not significantly altered by knockout of Rab27B. In addition, disruption of polarity signals provided by 3D culture did not impact exosome release or uptake in 3D, but induction of oncogenic HRAS increased both secretion and uptake of exosomes through activation of PI3 K signalling.
Summary/Conclusion: Release and uptake of exosomes is altered in 3D environments. These studies help provide insight into exosome production and uptake in vivo and have potential implications for therapeutically targeting exosome release and the development of exosome based therapeutic delivery vehicles.

OF18.6

Extracellular vesicles derived from R345 W-Fibulin-3 retinal pigment epithelial cells promote epithelial-mesenchymal transition

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Introduction: Previous studies in our lab found that expression of R345 W-Fibulin-3 induces RPE to undergo EMT. The purpose of current study was to characterize the extracellular vesicles (EVs) in RPE cells expressing WT-Fibulin-3 versus RPE cells expressing R345 W-Fibulin-3 and investigate the effects of these EVs on RPE cell differentiation.

Methods: ARPE-19 cells were infected with lentivirus with luciferase-tagged wild-type (WT)-Fibulin-3 or luciferase-tagged R345 W-Fibulin-3. EVs were isolated from the media of ARPE-19 cells by conventional ultracentrifugation or density gradient ultracentrifugation. Transmission electron microscopy (TEM) and Cryogenic electron microscopy (cryo-EM) were performed to study the morphology of the EVs. The amount and size distribution of EVs were analysed by Nanosight Tracking Analysis (NTA). EV protein concentrations were quantified using the DCTM Protein Assay (Bio-Rad). EV cargo were analysed by unbiased proteomics using LC-MS/MS with subsequent pathway analysis (Advaita). Migration ability was evaluated in ARPE-19 cells with or without the exposure of EVs by conducting scratch assays.

Results: Morphologically, TEM imaging showed concave-appearing vesicles and cryo-EM imaging showed spherical vesicles with two subpopulations of EVs: a small group with diameters around 30 nm and a large group with diameters around 100 nm. Moreover, TEM and cryo-EM showed an increased amount of small EVs (~30 nm) in the mutant group compared to the WT group. This result was further confirmed by NTA showing that, in the mutant group, the particle size distributions were smaller than the WT EVs. No significant differences were shown in EV protein concentrations per particle between WT and mutant groups. Our previous data suggest that the expression of R345 W-fibulin-3 causes RPE cells to undergo EMT as evidenced by upregulated EMT drivers and an increased migration ability. Proteomic studies showed that EVs derived from ARPE-19 cells overexpressing WT-fibulin-3 contain critical members of sonic hedgehog signalling (SHH) and ciliary tip components, whereas EVs derived from RPE cells overexpressing R345 W-Fibulin-3 contain EMT mediators, indicating that EV cargo reflects the phenotypic status of their parental cells. EV transplant studies showed that exposing native RPE cells to mutant RPE cell-derived EVs containing EMT drivers, including TGF-β-induced protein (TGFBI), VIM, and SMAD4, leads to an enhanced migration ability of RPE cells in a dose-dependent manner.

Summary/Conclusion: The expression of R345 W-Fibulin-3 promotes EMT in RPE cells and leads to the secretion of EVs containing EMT drivers. EVs derived from RPE cells overexpressing R345 W-Fibulin-3 are sufficient for inducing EMT in native RPE cells.

OP2 = PF17

Oral with Poster Session 2: Cancer and Technology

Chair: Lizandra Jimenez – Postdoctoral Research Fellow, Vanderbilt University

Chair: Susmita Sahoo – Cardiovascular Research Center, Icahn School of Medicine, Mount Sinai

OP2.01 = PF17.01

Development of scalable processes to produce therapeutic mesenchymal stromal cell-derived extracellular vesicles and their characterization

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Introduction: Despite of high expectations, mesenchymal stromal cell (MSC)-based therapies still lack efficacy, partially due to loss of cell viability and function upon administration. MSC-derived extracellular vesicles (MSC-EV) emulate the regenerative potential of MSC, shifting the field towards cell-free therapies. Clinical applications require the establishment of a scalable and GMP-compliant processes for the production and isolation of MSC-EV, combined with robust characterization platforms.
Methods: To develop a well-established process for the production of therapeutic MSC-EV, we compared different MSC sources (bone marrow, adipose tissue, umbilical cord matrix), culture media compositions (DMEM supplemented with foetal bovine serum (Thermo Fisher Scientific), DMEM supplemented with human platelet lysate (AventaCell Biomedical) and StemPro MSC SFM Xeno Free medium (Thermo Fisher Scientific)) and culture parameters (oxygen tension and shear stress) in two different culture platforms (2D static tissue culture flask vs 3D dynamic spinner vessels). Subsequently, MSC-EV were isolated by ultracentrifugation or a commercially available isolation kit and characterized according to ISEV guidelines.
Results: MSC derived from different sources/donors were able to grow under normoxia and hypoxia in 2D T-flasks and 3D spinner vessel culture systems, while maintaining their immunophenotype and differentiation potential, according to the minimal criteria defined by the ISCT. The time point for pre-conditioning and collection of conditioned medium for MSC-EV isolation was also optimized for both 2D and 3D culture systems. MSC-EV were characterized according to MISEV 2018 guidelines, using techniques as NTA, protein and lipid quantification, western blot, imaging and Fourier-Transform Infrared Spectroscopy (FTIR). The results indicate that MSC-EV derived from different sources/donors have similar size distribution, however, EV yields tend to be higher for the 3D culture system. Of notice, several spectral regions were identified by FTIR, enabling the detection of differences in the biomolecules present in MSC-EV, MSC-conditioned media and cells produced under different conditions.
Summary/Conclusion: In summary, this study contributes to the establishment of a scalable process for MSC-EV production.

OP2.02 = PF17.02

Evaluation of three different isolation methods for small extracellular vesicles from human plasma in prostate cancer diagnosis

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Introduction: Extracellular vesicles (EVs) have great potential in prostate cancer (PCa) diagnosis and progression monitoring to complement the inaccurate prostate specific antigen (PSA) screening and invasiveness of tissue biopsy. However, current methods cannot isolate pure EVs and therefor EVs characteristics remain largely unknown. In order to develop an accurate approach for EV isolation, we aimed to compare three emerging methods with different characteristics of small EVs (sEVs) from human PCa plasma samples and to choose the best one for diagnostic and functional studies
Methods: PCa patients and age-matched healthy controls (HC) plasma (n = 6 in each group) were used to

isolate sEVs with 3 different isolation methods including commercial ExoQuick Ultra Kit, qEV 35 and qEV 70 size exclusion chromatography (SEC). Isolated sEV were characterized by nanoparticle tracking analysis, immunoblotting, cyrogenic electron microscopy, flow cytometry (FC) and proteomics analysis. For FC characterizing surface marker expression, the sEVs were further purified by CD9 and CD81 commercial immunoaffinity magnetic beads . Lipoprotein was captured by streptavidin biotinylated ApoB magnetic beads to measuring the lipoprotein contamination

Results: The sEV size, morphology, surface protein and protein cargo with proteomics were analysed between the three isolation methods. sEVs isolated from SEC methods had a lower particle size, protein amount, protein/sEV marker ratio and ApoB+/sEV marker ratio than those from ExoQuick Ultra method. In addition, sEVs isolated from qEV35 demonstrated a significantly higher sEV content, more up-regulated and down-regulated PCa proteins from proteomics but lower sEV marker/protein ratio and a higher protein contamination than those from qEV70. Furthermore, sEV marker signal also showed a good correlation with particle numbers instead of protein content in all the methods

Summary/Conclusion: qEV 70 method demonstrated better performance in isolating relatively pure sEVs from human plasma; qEV35 has the better performance in isolating samples with higher sEV content; ExoQuick Ultra isolated samples with closely sEV content to the qEV35 but with the highest non-sEV protein contaminations. People can choose higher sEV content or higher sEV purity according to the downstream analysis

Funding: St. George Hospital Cancer Research Trust Fund, UNSW Sydney-University International Postgraduate Award, Cancer Institute NSW Early Career Fellowship

OP2.03 = PF17.03

Multiplexed surface protein profiling of tumour-derived extracellular vesicles by an electrokinetic sensor

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Introduction: Small extracellular vesicles (sEVs) (30–200 nm in diameter) are secreted by most cells, including tumour cells. They have attracted interest as biomarker for cancer diagnostics based on liquid biopsies, because they are abundant in body fluids and their content (proteins, RNAs and other cargos) reflects their parent cells. Moreover, sEVs may also be used for treatment monitoring, as recent studies suggested that the expression levels of certain markers may change during therapy, reflecting tumour response. For cancer diagnostics and therapeutic purposes in clinical settings, it is important to have a device which allows multiplexed measurements, in order to scan a large number of markers simultaneously and compare the expression levels of different patients, or same patients at different treatment stages, in a time efficient manner.

Methods: Herein, we propose a multiplexed platform for label-free detection and surface protein profiling of sEVs. The technique is based on the electrokinetic phenomena of streaming current and zeta potential (\zeta*) and measures the\zeta* change upon sEV binding on functionalized microcapillary surfaces. For the purpose, we used sEVs derived from lung cancer cells. In its current form, the platform can measure up to 5 channels simultaneously, however, it can be further expanded.

Results: Having demonstrated that our electrokinetic sensor successfully detects sEVs in a specific way, we tested its ability to measure the expression level of membrane proteins. The analysis showed that it could detect differences in the expressions of EGFR on sEVs, with a sensitivity of 10%. We then extended the platform for multiplexed analysis, by connecting and measuring four capillaries, functionalized with different capture probes, simultaneously. For the purpose, we targeted specific tumour markers, i.e. EGFR, and exosomal tetraspanin family proteins, such as CD9 and CD63. The results showed successful multiplexed EV detection.

Summary/Conclusion: Being the sensor suitable for multiplexed sEV detection, we shall present our investigation on a set of pleural effusion samples collected from a cohort of lung-cancer patients with different genetic makeup.

Funding: Erling Persson Foundation

OP2.04 = PF17.04

Optimized immunocapture methods for the direct detection of EV tumour associated proteins in biological fluids: playing around with biophysics

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Introduction: Extracellular vesicles (EVs) are released to biological fluids from different tissues and organs and they contain molecules proposed as biomarkers for multiple pathological conditions. However, most EV biomarkers have not been validated due to the lack of sensitive techniques compatible with high-throughput analysis required for routine screenings. Using immunocapture techniques, combining antibodies against tetraspanins and candidate tumour-specific markers we have recently optimized several assays that greatly facilitate EV characterization.
Methods: We have improved flow cytometry and ELISA assays, increasing substantially the sensitivity for EV detection. Using DLS, EM and analytical ultracentrifugation, we have characterised the biophysical basis of this enhancement. The final methodology can be performed in any laboratory with access to conventional flow cytometry or ELISA reader.
Results: Using combinations of antibodies specific for the tetraspanins CD9, CD63 and CD81, it is possible to detect EVs in minimal volumes of urine and plasma samples without previous enrichment. Additionally antibodies against other less abundant markers, like the epithelial marker EpCAM, have been used to capture and identify EVs directly in minimal volumes of urine or plasma with sensitivity higher than Western Blot analysis of isolated EVs. Furthermore, we demonstrate that additives altering the biophysical properties of an EV suspension, increased detection of tumour antigens in these immune-assays.
Summary/Conclusion: The development of sensitive, high-throughput methods, easily translatable to clinical settings, as ELISA and flow cytometry described here, opens a new avenue for the systematic identification of any surface marker on EVs, even scarce proteins, using very small volumes of minimally processed biological samples. These methods will allow the validation of EV biomarkers in routine liquid biopsy tests.
Funding: MINECO, IMMUNOTHERCAN, TENTACLES, Immunostep

OP2.05 = PF17.05

Normalized extravesicular protein expression profiles on antibody microarrays reveal protein associations in EVs of organotropic and metastatic breast cancer cell lines

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Introduction: When EV subpopulations are enriched on antibody microarrays and probed for their surface proteins, the detection signal is biased towards abundant subpopulations as it is dependent on both the protein expression level and the number of EVs captured. To address this challenge, we developed a novel normalization approach allowing: 1) the estimation of a target signal independent of EV subpopulation size through dye-based EV quantification, and 2) the assessment of subpopulation target enrichment relative to the population average by leveraging TIM4 as an unbiased, lipid-based EV capture. Here, we investigated the expression of cancer-associated proteins, particularly metastasis-associated integrins (ITGs), in breast cancer EVs with varying metastatic potential and organotropism.
Methods: The relative protein enrichment profiles for various EV subpopulations were established from EVs of SkBr3 (HER2+), T47D and MCF-7 (ER+PR+), BT549 and MDA-MB-231 (triple negative) breast cancer cell lines, as well as five MDA-MB-231-derived cell lines of four different organotropisms (brain, bone, lung, liver) using our custom antibody microarrays with our normalization approach.
Results: As expected, HER2 was broadly detected in HER2+ SkBr3 EVs. Interestingly, HER2- T47D and MCF-7 EVs also expressed HER2 where it was highly enriched in its EpCAM+ subpopulations. ITG α6, β3 and β4 were only found in triple negative and organotropic EVs with ITG β3 and β4 differentially enriched based on the organotropism. The population average of MDA-MB-231 and lung-tropic EVs had high expression of ITG β4, where subpopulations of CD44+ EVs showed positive enrichment while CD9+ and CD63+ EVs showed negative enrichment. ITG α5, β3 and β4 were absent in the bone-tropic CD81+ EV subpopulation, a profile atypical in other organotropisms. Lastly, EGFR was negatively enriched in Tetraspanin+ subpopulations in MDA-MB-231 EVs, but positively enriched in these subpopulations in organotropic EVs, especially for brain-tropism.
Summary/Conclusion: Following normalization, we were able to quantify specific protein associations, uncovering a multitude of co-enrichment profiles that characterize specific metastatic and organotropic cell lines. Notably, we found enrichment signatures that distinguish between different organotropisms derived from the same parental cancer line.
Funding: This research was supported by the Natural Sciences and Engineering Research Council of Canada

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OP2.06 = PF17.06

Heparan sulphate proteoglycans are required for EV-mediated delivery of multiple growth factors

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Introduction: The tissue microenvironment surrounding tumours is complex and the cross-talk between cancer and non-cancer cells is essential for tumour growth and progression. We have previously shown that heparan sulphate proteoglycans (HSPGs), on the surface of prostate cancer EVs, are required for delivery of TGFβ and initiation of a disease-supporting fibroblast phenotype. However, HSPGs are known to bind numerous growth factors, so here we have explored the repertoire of such proteins tethered to EVs by HSPGs.
Methods: EVs were isolated from DU145 prostate cancer cell conditioned media by ultra-centrifugation onto a sucrose cushion. Vesicular HSPGs were modified either by removal of heparan sulphate (HS) glycosaminoglycan (GAG) chains using the enzyme Heparinase III (HEPIII), or attenuation of HSPG core protein expression using shRNAs to knockdown specific HSPGs within the parent cell. Differences in proteins present in control vs modified EVs were identified by a sensitive protein array, based on proximity-ligation technology, and selected targets validated by ELISA. Functional delivery of growth factors by EV-associated HSPGs to recipient fibroblasts is being explored using a variety of in vitro techniques.
Results: Proteome analysis identified 49 targets that bind to HS-GAG chains, and also 108 different proteins that showed altered expression following the loss of one or more HSPGs from EVs. Using ELISA, we have been able to quantify selected candidates on wild type vesicles, some of these are lost following HS-digestion. We were also able to validate proteins on HSPG-deficient vesicles. Gene ontology analysis suggests that EV HSPG-mediated delivery of growth factors is important for control of processes such as angiogenesis, tumour invasion and immune regulation. Functional validation of proteins identified is ongoing.
Summary/Conclusion: Here we demonstrate that HSPGs play a key role in loading of EVs with a complex assortment of growth factors, and therefore subsequent EV-mediated growth factor delivery. We anticipate that loss or damage of EV-associated HSPGs will result in attenuation of EV induction of a tumour-supporting fibroblast phenotype.
Funding: Cancer Research Wales

OP2.07 = PF17.07

Robust exosomal biomarker panel discovery in ovarian cancer using machine learning approaches and studying miRNA & miRNA-target interactions

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Introduction: Ovarian cancer (OC) is the fifth leading cause of cancer-related death in women, partly due to difficulty in early diagnosis. Extracellular vesicles (EVs) show promise for use in early diagnostics of OC. Here, EVs from cervical mucus (CM) of ovarian cancer patients were used for discovery of OC biomarkers for diagnostics. Machine learning was used to mine EV miRNA data to develop an OC biomarker panel (validation via The Cancer Genome Atlas). Examination of the miRNA targets reveal that the panel is a sufficiently accurate predictor of OC.
Methods: EVs from the CM of 48 patients (15 high-grade serous, 24 low-grade, 7 benign) were isolated for small RNA-sequencing. The top differentially expressed miRNAs were used in a random forest and "voom" (variance modelling at the observational level) model. Unsupervised approaches were used and then vetted against patient symptomology data. A TCGA ovarian cancer dataset (n = 100) was used for validation.
Results: An OC biomarker panel of 10 microRNAs (voom: 96.55% accuracy; random forest: 88% accuracy) was generated. The panel consists of members from the mir-200 family and the mir-16 family, among others. The miRNA targets are associated with molecular functions and pathways specific in OC progression.
Summary/Conclusion: Our method has identified EV miRNA biomarkers that may be crucial for early, non-invasive detection of OC. Data science has been used to develop a feedback system integrating biochemical experiments, smaller datasets, and previously available data to identify and verify a biomarker panel for OC diagnostics.
Funding: Support from the National Science Foundation, Eppley Foundation for Scientific Research, Gibson Foundation, Prisma Health System and ITOR Biorepository are gratefully acknowledged.

OS19

Symposium Session 19: Neurologic Mechanism

Chair: Efrat Levy – Center for Dementia Research, Nathan S. Kline Institute

Chair: Shanthini Sockanathan – Johns Hopkins University

OS19.1

Extracellular vesicle associated microRNA-29a elicits microglial activation and synaptodendritic injury with chronic methamphetamine exposure

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Introduction: Methamphetamine (MA) and related amphetamine compounds, which are potent psychostimulants, are among the most commonly used illicit drugs. Neuroimaging studies have revealed that chronic MA abuse can indeed cause neurodegenerative changes in the brains of human MA abusers including prominent microglial activation throughout the brain. It is still unclear how chronic inflammation caused by MA abuse leads to long-term damage to the brain. With this in mind, we are particularly interested in studying the role of extracellular vesicles (EVs) in eliciting chronic inflammation in MA exposed brains. In the present study, we focus on the role of a miRNA, miR-29a-3p (miR-29a) in chronic MA exposure. Here, we present novel data that shows for the first time how chronic MA impacts not only the biogenesis but also the EV associated miRNA cargo thereby affecting the overall health of the neurons and glial cells in the brain.
Methods: – Density gradient centrifugation for isolation of Brain-derived Vesicles

- Characterization of BDEs by Western Blotting, Nanoparticle tracking analysis and Transmission Electron Microscopy
- Quantitative RT-PCR
- Digital droplet PCR
- Confocal Imaging of dendritic spines and synapses

Results: In the present study, we show from both in vivo and in vitro studies that chronic methamphetamine (MA) treatment alters EV biogenesis and microRNA (miRNA) cargo. Brain-derived EVs (BDE) isolated from frontal grey tissue of rhesus macaques that were administered MA in a chronic regimen revealed a significant increase in both number and size. Further analysis revealed increase in biogenesis genes and increased levels of miRNA, miR-29a-3p (miR-29a). In situ hybridization of the frontal brain area revealed that miR-29a was exclusively expressed in microglia and neurons. Further, in vitro studies revealed that EV associated miR-29a elicited not only neuronal damage but also was able to activate microglia to release pro-inflammatory cytokines thereby inducing a chronic inflammatory cycle. Finally, we show that an anti-inflammatory drug was able to rescue inflammation, miR-29a levels and synaptodendritic injury.
Summary/Conclusion: In summary, our results present for the first time show that chronic MA exposure in the brain affects EV biogenesis and miRNA expression. We further confirm that miR-29a can serve as potential marker to diagnose synaptic deficits for chronic MA addiction in humans. Finally, we reveal that anti-inflammatory drug could rescue the EV biogenesis and reduces the secretion of miR-29a, thereby rescues synaptodendritic injury. Our data further supports the use of the anti-inflammatory drugs as therapeutic interventions for MA addiction.
Funding: NIDA funding # R01DA042379

OS19.2

Blood-borne and brain-derived ectosomes/microparticles in morphine-induced anti-nociceptive tolerance

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Introduction: Opioid pain treatment is impeded because chronic administration decreases analgesia, a condition called tolerance that prompts dose escalation contributing to morbidity and mortality. Inflammatory interleukin (IL)-1β is required for tolerance

development, so we hypothesized that pro-inflammatory extracellular vesicles (EVs) play a role.
Methods: EVs with opioid administration were assayed in mice and humans. Annexin V-positive, 0.1–1 μm diameter microparticles (MPs) were assessed by flow cytometry in murine and human blood and in murine deep cervical lymph nodes that drain brain glymphatics. Blood-borne exosomes (<100 nm) were assayed by tunable-resistance pulse sensing (TRPS). Anti-nociceptive tolerance following morphine administration to mice was assessed by speed of tail removal from warm water.
Results: Repetitive morphine dosing of mice to induce anti-nociceptive tolerance increased blood-borne MPs by eightfold, and by tenfold in cervical lymph nodes. MPs expressed proteins specific to neutrophils, microglia, astrocytes, neurons and oligodendrocytes. IL-1β content of MPs increased 68-fold. Administration of an IL-1β antagonist to mice diminished blood and glymphatic MPs elevations and abrogated tolerance induction. Intravenous polyethylene glycol Telomer B that lyses MPs and intraperitoneal methylnaltrexone that binds peripheral opioid-mu receptors and myeloid differentiation factor-2 to inhibit toll-like receptors, inhibited MPs elevations and tolerance. Neutropenic mice did not develop anti-nociceptive tolerance, elevations of blood-borne MPs or cervical node MPs expressing microglial proteins. Elevations of blood-borne exosomes were not identified based on TRPS analysis. Patients entering treatment for opioid use disorder exhibited similar MPs elevations as do tolerant mice.
Summary/Conclusion: Neutrophil-derived MPs containing IL-1β are required for morphine-induced anti-nociceptive tolerance.
Funding: This project was supported by Grant N00014-16-1-2868 from the Office of Naval Research and an unrestricted grant from the National Foundation of Emergency Medicine.

OS19.3

EVs are a conveyor of toxic dipeptide repeat proteins in C9orf72 ALS/FTD models

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Introduction: Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motor neurons. In ALS, motor symptoms initiate focally and then progress gradually, distal from the initial focus. Abnormal forms of ALS-associated proteins are physically exchanged between neuronal cells. Pathogenic ALS proteins like SOD1, FUS and TDP43 are transmitted between cells by assisted mechanisms, mainly extracellular vesicles (EVs), spreading toxicity and misfolding of native proteins within the recipient cells. An intronic G4C2 aberrant nucleotide repeat expansion in C9orf72 gene is the most common genetic cause of ALS. Translation of this expanded region occurs by a process called repeat associated non-AUG (RAN) translation that produces five dipeptide repeats proteins (DPRs), polyGA, polyGP, polyGR, polyPA and polyGA. PolyGA, polyGR and polyPR are associated with toxicity in neurons. In this work we study the recruitment of these aberrant proteins into extracellular vesicles (EVs) and the potential role of these EVs in spreading toxicity between cells of the central nervous system.
Methods: To isolate the EVs from cell culture media we isolated by ultracentrifugation the larger vesicles at 21,000xg and the smaller EVs at 100,000xg. Number, size and fluorescence of the vesicles were analysed by fluorescent nanotrack analysis (F-NTA) and by Cytoflex. The protein content of the vesicles was analysed by western blot (WB). To evaluate the potential toxicity of the EVs, a transwell system (TW) was employed. Neuron viability was assessed using live imaging techniques.
Results: NSC34 were transfected with reporter constructs expressing DPRs tagged with GFP protein. By F-NTA, Cytoflex and WB analysis we assessed that all the five DPRs were loaded in both the large and the small vesicles isolated from cell culture medium. By TW, NSC34 transfected with the DPRs were put in contact with primary cortical neurons (CNs) transfected with synapsin driven Td-Tomato for live imaging purposes. We observed that polyGR+ NSC34 were able to cause a significant decrease in CNs viability. We also observed that polyGR+ EVs associated toxicity was directly dependent on polyGR length. This effect was reverted reducing the number of polyGR+ EVs treating NSC34 with GW4869. To understand the downstream effect of polyGR+ EVs in recipient cells we studied TDP43 mislocalization, RAN-translation and activation of the integrated stress response, finding a dysregulation of all these potentially toxic pathways in neurons treated with polyGR+ vesicles.
Summary/Conclusion: Concluding, DPRs are actively secreted in EVs and polyGR+ vesicles cause the activation of toxic mechanisms in the recipient cells, possibly contributing to the spreading of ALS.
Funding: NIH 080–19250-S31201

OS19.4

Amniotic fluid stem cell derived extracellular vesicles modulate pathogenic immune responses in experimental autoimmune encephalomyelitis

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Introduction: Pregnancy is the a condition that profoundly mitigates symptoms of multiple sclerosis (MS) a complex disease characterized by immune dysfunction and neurodegeneration affecting 2.3 million people worldwide. Serum exosomes, released by specific cells during pregnancy, modulate the immune and central nervous system function and contribute to pregnancy-associated suppression of experimental autoimmune encephalomyelitis (EAE), an induced preclinical model of MS. Extracellular vesicles (EVs) are the new means for communication among cells. The aim of our study was to characterize the ability of Human Amniotic Fluid Stem Cells-derived EVs (HASC-EVs) to antigen presenting cell function thus correcting immune dysfunction in EAE.

Methods: Amniotic Fluids were obtained from human 16–17-week pregnant women. HASC-EVs were collected by ultra-centrifugation. EVs were characterized for their specific proteins, lipids and nucleic acids expression. The ability of EVs to modulate immune responses was performed in vitro, testing the ability of EVs to induce a tolerogenic phenotype in mouse bone marrow derived dendritic cells, and in vivo for their potential to suppress EAE, induced by immunization C57/B6 female mice with MOG35-55 peptide.

Results: We found that HASC-EVs expressed high levels of Galectin-1 and promoted a significant increase of the immunoregulatory enzyme indoleamine 2,3-dioxygenase-1 enzyme in DCs. Moreover in in vivo experiments administration of HASC-EVs significantly reduced disease severity in EAE. Such effect was associated with reduced neurological deficits and suppression of pathogenic T helper 17 (Th17) cells, and increased percentage of regulatory T cells (Treg-Foxp3+) cells.

Summary/Conclusion: Our findings unravel immunoregulatory effects of EVs secreted by HASCs. EVs may represent a novel cell-free immune regulatory and regenerative therapeutic approach that can potentially mitigate immune dysfunction and promote remyelination.

OS19.5

Association of neuronal-derived extracellular vesicles cargo with cognitive decline in late middle life

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Introduction: Alzheimer's disease (AD) is characterized by a long preclinical stage during which phosphorylated Tau pathology spreads in the brain leading to clinical symptoms. Pathogenic Tau spreads, in part, via Extracellular vesicles (EVs). We and others have demonstrated that Tau cargoes of neuronal-derived EVs (nEVs) from blood can serve as biomarkers for AD. We aimed to examine whether nEV Tau cargo can predict cognitive decline in late middle age by leveraging samples from participants in the Wisconsin Registry for Alzheimer's Prevention (WRAP) study.

Methods: We blindly immunoprecipitated nEVs using antibody against neuronal L1 cell adhesion molecule (L1CAM) from serum samples of 146 WRAP participants who were cognitively unimpaired at baseline (mean age 62.4 ± 6.3 years old; 71.2% females; 42.5% APOE4 carriers), of whom half subsequently developed cognitive decline. We measured phosphorylated (p181 and p231) and total Tau in nEVs using electrochemiluminescence assays. We used linear regression models to identify differences between cognitive status groups including age, sex ApoE status and the cognitive status*age interaction in the model.

Results: At baseline, we found trends for higher p181- ($p = 0.07$) and p231-Tau ($p = 0.05$) levels in future decliners compared to stable participants. Further, there were significant cognitive status*age interactions for pTau231 ($p < 0.01$), total Tau ($p < 0.001$) and pTau181 ($p < 0.05$) with higher levels with increasing age in future decliners

Summary/Conclusion: nEV Tau cargo differs between late middle-aged individuals at risk for AD with and without future cognitively decline even before decline occurs, presumably due to subclinical spread of Tau

pathology. Further nEV biomarker development may allow preclinical AD diagnosis.

Funding: This research was supported in part by the Intramural Research Program of the National Institute on Aging, NIH, and the WRAP grant R01AG027161.

OS19.6

Extracellular vesicle release from the choroid plexus visualized using ExoMap-2 Mice

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Introduction: In the brain, circulating extracellular vesicles (EVs) in the cerebrospinal fluid (CSF) contain a variety of signalling factors, including proteins, enzymes, and RNA transcripts. While EVs have been implicated in many cell-to-cell signalling contexts, the vast majority of these studies are based on findings derived from cell culture conditions. Thus, the ability to identify cell type-specific EV release from cellular subpopulations within the brain represents a critical barrier in the field.

Methods: To address this knowledge gap, we utilized a novel transgenic mouse model to determine the release of cell-type specific EVs. Here we report the ExoMap-2 mouse, which is designed to express an exosomal green fluorescent protein in response to expression of Cre recombinase. Specifically, the ExoMap-2 transgene was inserted at the mouse H11 locus and consists of (i) a broadly expressed CAG promoter/enhancer, (ii) a floxed ORF encoding MTS-tdTomato, (iii) an ORF encoding the exosomal protein AcylTyA fused to mNeonGreen (mNG), and (iv) a 3′ UTR containing the WPRE element and polyadenylation signal from the bovine growth hormone gene.

Results: Intracranial ventricular injections of the viral vector AAV-TTR-Cre, which drives Cre recombinase expression from the choroid plexus-specific promotor of the transthyretin gene, leads to AcylTyA-mNG expression in the choroid plexus. Moreover, we observed that these mice released mNeonGreen-positive EVs into the cerebrospinal fluid and also visualized the vesicles in the blood. Furthermore, these mice displayed an accumulation of AcylTyA-mNG fluorescence in the medial habenula.

Summary/Conclusion: The results indicate that choroid plexus-derived EVs are trafficked to the CSF and the medial habenula, and more generally, that the ExoMap-2 mouse can be used to follow the trafficking of tissue-specific EVs into biofluids and between tissues in vivo.

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OS20

Symposium Session 20: Cancer: Pathogenesis and Treatment

Chair: Mary Bebawy, PhD – The University of Technology Sydney

Chair: Janusz Rak – McGill University

OS20.1

Extracellular vesicles containing oncogenic mutant β-catenin activate Wnt signalling pathway in the recipient cells

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Introduction: Large-scale colorectal cancer (CRC) sequencing studies have shown that 93% of all tumours had at least one mutation in proteins implicated in the Wnt signalling pathway. Mutations in

β-catenin have often been associated with the constitutive activation of Wnt signalling pathway and has been established as a major driver of CRC. One of the proposed mechanisms of activating Wnt signalling involves extracellular vesicles (EVs) as cellular couriers to transfer Wnt ligands from one cell to another. However, the association of oncogenic mutant β-catenin with EVs has not been studied. Subpopulations of cancer cells with different mutational loads and behavioural variations lead to intra-tumour heterogeneity

Methods: Integrative proteogenomic analysis showed the secretion of mutant β-catenin via EVs. EVs were isolated by ultracentrifugation and OptiPrep density gradient centrifugation. SILAC-based quantitative proteomics analysis, immunofluorescence, biochemical analysis, qPCR and xenograft models were employed to unveiling the role of EVs carrying mutant β-catenin.

Results: An integrative proteogenomic analysis identified the presence of mutated β-catenin in EVs secreted by colorectal cancer (CRC) cells. Follow up experiments established that EVs released from LIM1215 CRC cells stimulated Wnt signalling pathway in the recipient cells with wild type β-catenin. SILAC-based quantitative proteomics analysis confirmed the transfer of mutant β-catenin to the nucleus of the recipient (RKO CRC) cells. In vivo tracking of DiR labelled EVs in mouse implanted with RKO CRC cells revealed its bio distribution, confirmed the activation of Wnt signalling pathway in tumour cells and increased the tumour burden.

Summary/Conclusion: Overall, for the first time, this study reveals that EVs can transfer mutant β-catenin to the recipient cells and promote cancer progression.

OS20.2

Salivary exosomes – Carrier of high risk human papillomavirus causing oropharyngeal cancer

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Introduction: There has been a significant increase in incidence of human papillomavirus 16 (HPV16) driven oropharyngeal cancer (OPC) in developed countries. There is evidence that HPV alters the molecular cargo of exosomes released by OPC. Emerging evidence suggests that HPV integration within the human genome is associated with both genomic and transcriptomic alterations. Consistent with previous studies, the genomic viral–cellular junctions were identified using DIPS-PCR method in 15 (88%) saliva samples collected from HPV16-driven OPC.

Methods: Morphology and molecular features of exosomes derived from three different saliva sampling methods: unstimulated saliva; acid-stimulated saliva; and salivary oral rinses were examined using Transmission electron microscopy (TEM), nanoparticle tracking (NTA) and western blot analysis. HPV-16 DNA detection in salivary exosome was determined by using qPCR method. Proteome profile of salivary exosomes derived from both cancer-free controls and HPV16-driven OPC patients was characterized using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS).

Results: We demonstrate that unstimulated saliva had greater abundance of exosomes when compared to the other sampling methods. Three common exosome markers (CD9, CD63 and CD81) were higher in unstimulated saliva. Only salivary exosomes derived from HPV-driven OPC patients had a detectable level of HPV-16 DNA. The proteomic signature of salivary

exosome was significantly ($p < 0.01$) different between cancer-free controls and HPV-driven OPC. We found elevated protein abundance of five main glycolytic enzymes (i.e. Phosphoglycerate Kinase 1 (PGK1), Glyceraldehye-3-phosphate dehydrogenase (GAPDH), Aldolase (ALDOA) and Lactate dehydrogenase A (LDHA) in salivary exosomes derived from OPC patients, suggesting a functional role of salivary exosome in the reciprocal interplay between HPV-driven OPC and glucose metabolism.

Summary/Conclusion: Our data suggest that the development of a low-cost non-invasive saliva-based test using both salivary exosomal DNA and protein may offer an opportunity to detect HPV-driven OPC, that may be clinically useful in managing these patients.

Funding: Cancer Australia Grant

OS21

Symposium Session 21: EV Signalling

Chair: Wei Guo – University of Pennsylvania

OS21.1

Continuous in vivo release of mast cell derived extracellular vesicles from an implanted device spreads pro-inflammatory response in mice

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Introduction: Mast cells are important players of the immune system and they secrete a wide range of mediators during bacterial infections. Mast cells are also able to release extracellular vesicles (EVs). Here, we report that mast cells communicate with each other in vivo by EVs.

Methods: We isolated bone marrow-derived and peritoneal mast cells from GFP-transgenic and wild type mice. EVs were separated from the conditioned media of these cells cultured in the presence or absence of lipopolysaccharide (LPS). EVs were characterised according to the MISEV2018 guidelines by flow cytometry, electron and fluorescent microscopy, TRPS, the SPV lipid and the BCA protein assays. Separated EV-s were cultured with naïve mast cells, and tumour necrosis factor (TNF)-α production was tested by ELISA and intracellular flow cytometry. GFP+ mast cells were seeded in diffusion chambers which were implanted into the peritoneal cavities of mice enabling us to investigate the continuous in vivo release of EVs. Uptake of GFP+ EVs and TNF-α expression of peritoneal mast cells were tested by flow cytometry and fluorescent microscopy.

Results: Here, we showed that bacterial LPS-sensing mast cells release EVs that in turn, induce TNF-α expression in resting MCs in vitro. Moreover, we confirmed that EVs are transmitted to other peritoneal mast cells in vivo spreading the pro-inflammatory response by inducing TNF-α secretion in peritoneal mast cells.

Summary/Conclusion: EV communication between members of the mast cell network, play an important role in spreading and escalating pro-inflammatory responses to immune stimuli. Our data may provide an explanation how the relatively rare tissue resident mast cells can play key roles in diseases such as autoimmune arthritis.

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OS21.2

Small extracellular vesicles modulated by the αVβ3 integrin reprogramme recipient cells towards an aggressive neuroendocrine cancer phenotype

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Introduction: The ability of small extracellular vesicles (sEVs) to reprogramme cancer cells is known. Integrins, receptors for extracellular matrix proteins, are major players in mediating sEV functions. Previously, we have reported that the αVβ3 integrin is detected in sEVs of prostate adenocarcinoma (PrCa) cells and transferred into recipient cells in a paracrine fashion; however, its role and expression have never been explored in the most aggressive forms of PrCa, such as neuroendocrine PrCa (NEPrCa). NEPrCa does not express androgen receptor (AR) but does express neuron-specific proteins, such as Aurora kinase A, Synaptophysin and Neuron specific enolase, that activate pro-tumorigenic pathways independently from the AR.

Methods: We isolated sEVs from PrCa C4-2B cells using iodixanol density gradients and characterized them by immunoblotting and ExoView. The experiments were performed in vivo by injecting subcutaneously, in nude mice, DU145 cells treated with sEVs expressing or lacking the αVβ3 integrin, and in vitro, by testing anchorage-independent growth of different cell lines treated with the same sEVs. Discarded human tissues from PrCa metastasis were analysed by immunohistochemistry (IHC).
Results: We demonstrate that a single treatment of PrCa cells with sEVs significantly stimulates tumour growth and anchorage-independent growth. Moreover, we show that one treatment with sEVs, shed from C4-2B cells that express αVβ3, but not from the control cells that lack αVβ3, induces differentiation of PrCa cells towards a neuroendocrine phenotype and downregulates AR. Finally, our IHC analysis shows co-expression of αVβ3 integrin and Synaptophysin in NEPrCa metastatic lesions.
Summary/Conclusion: In conclusion, our current study shows, for the first time, that αVβ3 integrin expression in donor cells generates sEVs that reprogramme recipient cells towards an aggressive tumour phenotype.
Funding: This study was supported by NCI-P01-140043, R01-224769 to LRL.

OS21.3

Exosomes in filopodia formation

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Introduction: Exosomes are small extracellular vesicles (SEVs) that carry a variety of cargoes and have been shown to promote tumour cell motility and metastasis. Cell motility is influenced by dynamic formation and stability of filopodia: actin-rich protrusions that extend from the leading edge and perform directional sensing. Filopodia regulators such as fascin are upregulated in multiple epithelial cancers and can promote invasive phenotypes. However, how filopodia are induced and controlled by extracellular factors is poorly understood. Here, we describe a role for SEVs in regulating filopodia formation and tumour cell motility.
Methods: We utilized B16F1 melanoma cells and HT1080 fibrosarcoma cells for fixed- and live-cell imaging to quantify filopodia numbers and dynamics in control and exosome-deplete conditions. iTRAQ proteomics was used to identify SEV protein cargoes that contribute to filopodia formation. In vivo experiments were performed using a chick embryo model for metastasis.
Results: Inhibition of exosome secretion in cancer cell lines, via Rab27a or Hrs knockdown, led to decreased filopodia numbers. Specificity to SEVs was demonstrated by rescue experiments in which purified SEVs but not large EVs rescued the filopodia phenotypes of exosome-inhibited cells. Live imaging of Hrs-KD cells revealed that exosome secretion regulates formation and stability of filopodia. Proteomics data and molecular validation experiments identified the TGF-beta coreceptor endoglin as a key SEV cargo regulating filopodia formation, cancer cell motility, and metastasis.
Summary/Conclusion: In this study, we identified exosomal endoglin as a regulator of filopodia formation and in vivo metastasis. These data are relevant to cancer as endoglin expression is altered in many cancers. In addition, endoglin is the disease gene for hereditary haemorrhagic telangiectasia, and may influence angiogenesis. Overall, our data implicate SEV-carried endoglin as a key cargo regulating filopodia.

OS21.4

Astrocyte-derived EV-mediated blood-brain barrier disruption

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Introduction: The breach of the Blood-brain barrier (BBB), resulting in ensuing neuroinflammation, is a key feature of HIV-associated neurological disorders (HANDs). While combination antiretroviral therapy (cART) has successfully suppressed peripheral viraemia, cytotoxicity associated with the presence of viral Tat protein in tissues such as the brain, remains a significant concern. Our previous study has demonstrated that HIV-1 Tat can induce disruption of BBB by downregulation of tight junction (TJ) proteins in Human Brain Microvascular Endothelial cells (HBMECs) and that this is regulated by the autophagic pathways.
Methods: EVs were isolated from HIV Tat-stimulated mouse/human primary astrocytes using the standard differential ultracentrifugation method and characterized by transmission electron microscopy, NanoSight &

western blot analyses. Among the various miRs dysregulated in HIV Tat -stimulated astrocyte EV cargo, miR-7 was found to be upregulated by realtime PCR. Confocal microscopy identified uptake of astrocytic EVs by HBMECs. Functional assessment of astrocytic EV uptake by HBMECs involved cell permeability using transepithelial electrical resistance as well as trans-well endothelial cell monolayer permeability assays.

Results: HIV-1 protein Tat-mediated induction of microRNAs (miRs) in astrocyte-derived extracellular vesicles (ADEVs) regulated the permeability of BBB by targeting the expression of TJ proteins in the HBMECs. Exposure of HBMECs to Tat-ADEVs resulted in down-regulation of the tight junction protein Claudin 5, resulting in increased endothelial cell monolayer paracellular permeability. Microarray data of Tat-ADEVs demonstrated upregulation of several miRs compared to that of controls, among which upregulated miR-7 was identified to target the TJ proteins using ingenuity pathways analysis. Increased expression of miR-7 was validated in Tat exposed astrocytes and Tat-ADEVs. ADEVs loaded with miR-7 oligos showed similar effects as that observed with Tat-ADEVs in inducing permeability in HBMECs. Increased expression of miR-7 with downregulation of claudin-5 was also recapitulated in microvessels isolated from the brains of Doxycycline-inducible HIV-1 Tat transgenic mice (iTat) mice and in lysates isolated from the frontal cortices of SIV+ macaques/HIV+ autopsied brains.

Summary/Conclusion: Our findings demonstrated that Tat-ADEVs containing miR-7 as an important mediator underlying Tat-mediated disruption of the BBB.

OS22

Symposium Session 22: EVs as Delivery Vehicles

Chair: Steven M. Jay – Assistant Professor, University of Maryland, College Park

OS22.1

Single particle Raman spectroscopy: an emerging tool to quantify composition of engineered extracellular vesicles

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Introduction: Endogenous exosomes and related extracellular vesicles (EVs) are potent nanoparticles released by all cells tested to date. The exploitation of their unique scaffolding for engineering next-generation drug delivery systems represents a major area of academic and commercial interest. The lag in exploiting this potential is in part due to our inability to measured extent and efficiency of modification, e.g., composition and drug loading. Here we report a robust pipeline of optical tweezing combined with Raman spectroscopy to molecularly characterize engineered EVs and quantitatively assess extent of drug loading at single particle resolution.
Methods: EVs derived from cell culture and isolated by ultracentrifugation were fused with synthetic liposomes to create engineered EVs (eEVs). These eEVs were formed via well-established vesicle fusion techniques, namely (1) mechanical extrusion, (2) freeze-thawing, or (3) probe-tip sonication. Prior to formation, calcein was encapsulated in the liposomes and used as a surrogate for soluble drug loading. Laser trapping Raman spectroscopy (LTRS) was used to optically trap single EVs, before and after synthetic manipulation. Raman spectral analysis was used to assess trapped eEVs compared to pure standards to quantify ratiometric variation in chemical composition.
Results: Raman laser trapping experiments confirmed that each formation method results in largely varying (1) extent of fusion between EVs and synthetic calcein-loaded liposomes, (2) efficiency of calcein loading, and (3) particle size. We could also quantify the molar amounts of liposome vs. EV molecules for single particles, revealing a great amount of variation from particle to particle. Functional membrane proteins we left intact to varying degree across fusion methods.
Summary/Conclusion: Given the rising importance of analytical tools able to characterize extent of molecular loading for engineered EVs, we believe this technology will be very useful, thus warrants further investigation for eEV characterization across a variety of clinical applications.
Funding: Randy Carney, PhD was supported by a Research Scholar Grant, RSG-19-116-01-CDD, from the American Cancer Society.

OS22.2

Extracellular vesicles containing host restrictive factor IFITM3 inhibited Zika virus infection of foetuses in pregnant mice through trans-placenta delivery

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Introduction: Zika virus (ZIKV) infection can lead to neurological complications and foetal defects, and has attracted global public health concerns. Effective treatment for ZIKV infection remains elusive and a preventative vaccine is not available yet. Therapeutics for foetus need to overcome blood brain barriers to reach placenta and require higher safety standard.
Methods: In the present study, we engineered mammalian extracellular vesicles (EVs) to deliver a host restrictive factor, interferon-induced transmembrane protein 3 (IFITM3), for the treatment of ZIKV infection.
Results: Our results demonstrated that the engineered IFITM3-containing EVs (IFITM3-Exos) were overall safe to the animals and suppressed ZIKV viraemia by 2 log10 s in the pregnant mice. Moreover, the engineered EVs effectively delivered IFITM3 protein across placental barrier and suppressed overall ZIKV viraemia in the foetuses to the basal level with significant reduction of viraemia in key foetal organs as measured by Q-PCR. Mechanistic study showed that IFITM3 was delivered to the endosomes/lysosomes where it inhibits viral entry to the host cells.

Summary/Conclusion: Our study demonstrates that exosomes can act as a cross placenta drug delivery vehicle to foetus and IFITM3, an endogenous restriction factor that is highly expressed in placenta, is a potential treatment for ZIKV infection during pregnancy.

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OS22.3

Engineering extracellular vesicles with altered cellular tropism for targeted payload delivery in vivo

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Introduction: Extracellular vesicles (EV) are natural and abundant nanoparticles capable of transferring complex molecules between neighbouring and distant cell types. Translational research efforts have focused on co-opting this communication mechanism to deliver exogenous payloads to treat a variety of diseases. Important strategies to maximize the therapeutic potential of EVs include payload loading, functionalization of the EV surface with pharmacologically active proteins, and delivery to target cells of interest.

Methods: Through comparative proteomic analysis (LC/MS) of purified EVs, we identified several highly enriched and EV-specific proteins, including a transmembrane glycoprotein (PTGFRN) belonging to the immunoglobulin superfamily. Leveraging PTGFRN as a scaffold for surface display, we generated EVs with functional targeting ligands, including single domain antibodies (sdAbs), single chain variable fragments (scFvs), single chain Fabs (scFabs), and receptor ligands, on the surface to direct EV uptake to cell types of interest. Biological activity of these engineered EVs was assessed in an array of in vitro and in vivo assays and compared to untargeted controls.

Results: We engineered EVs displaying anti-Clec9A scFabs to target conventional type 1 dendritic cells (cDC1s), anti-CD3 scFabs to target T cells, and CD40 ligand to target B cells. In mice, systemic administration of anti-Clec9A EVs resulted in a 75% increase in the percentage of cDC1 cells that take up EVs over controls. Anti-CD3 EVs resulted in both an increase in the percentage of EV positive T cells (3.75 and 3-fold for CD4+ and CD8+) and the number of EVs per cell (15 and 7-fold for CD4+ and CD8+) in the blood. Furthermore, in primary mouse dendritic cells, anti-Clec9A EVs loaded with STING agonist achieved a 15-fold greater pathway induction compared to untargeted controls. Preliminary in vivo data suggest that anti-Clec9A EVs reduce the required STING agonist dose 10-fold to achieve efficacy and induce anti-tumour responses, compared to control EVs.

Summary/Conclusion: These results demonstrate the potential of our EV engineering platform to generate novel EV therapeutics targeted to cell types of interest for pharmacologic payload delivery.

OS22.4

A novel method for the delivery of cell-free therapy to foetuses with congenital anomalies: a proof of principle study

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Introduction: Antenatal cell-based therapies are currently considered invasive for the foetus. A promising cell-free strategy that holds great regenerative potential for several organs is the administration of stem cell derived EVs, whose cargo contains bioactive molecules that epigenetically regulate target cells. Herein, we aimed to 1) assess the ability of EVs to reach foetal organs when administered to the mother intravenously or intra-amniotically; 2) compare these administration routes on normal foetuses and foetuses with a congenital anomaly.

Methods: EVs were isolated from rat amniotic fluid stem cell conditioned medium using ultracentrifugation. EVs were assessed for size (nanoparticle tracking analysis), morphology (TEM), and expression of CD63, Hsp70, Flo-1, and TSG101 (Western). We injected rat dams with EVs stained by ExoGlow™-Vivo or saline (control) via maternal tail vein (IV) or intra-amniotically (IA) at E20.5. IA and IV injections were performed on dams carrying normal foetuses or foetuses exposed to nitrofen to induce congenital diaphragmatic hernia. After 24h, dams and pups were sacrificed. 3D high-sensitivity optical reconstructions of whole foetuses or micro-dissected foetal organs were imaged using the IVIS® Spectrum imaging system. EV fluorescence signal was compared between normal (n = 27) and nitrofen-exposed (n = 45) foetuses.

Results: Both IV and IA injection routes were successful in delivering EVs to foetal organs. No fluorescent signal

was detected in saline only control. IA injections yielded higher signal than IV, and EVs reached more organs with IA than IV injections. IA injected EVs were detected in the lungs, gastrointestinal, and urinary tract of normal and nitrofen-exposed foetuses. Nitrofen exposed foetuses had higher signal than normal foetuses.

Summary/Conclusion: This proof of concept study shows that antenatal administration of stem cell EVs is feasible with different routes. Although maternally administered EVs cross the placenta, IA injection is more effective at reaching foetal organs. Further studies are underway to reproduce these findings in experimental models of various congenital anomalies.

Funding: CIHR-SickKids Foundation grant

OS22.5

Microalgae as a novel and renewable bioresource of extracellular vesicle

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Introduction: Safe, efficient and specific nano-delivery systems are essential to the current cosmetic, nutraceutical and therapeutic medicine sectors. The ability to optimise the bioavailability, stability, and targeted cellular uptake of bioactive molecules while mitigating toxicity, immunogenicity and off-target/side effects is of the utmost priority. VES4US is a European project, which aims to develop an innovative platform for the efficient production of extracellular vesicles (EVs) from microalgae, which constitute a promising renewable bioresource (www.ves4us.eu). Here we present characteristics of EVs from several microalgal lineages, which offer the opportunity for a potentially developing a new and scalable tailor-made biogenic nanotechnology.

Methods: We cultivated a number of EV-producing microalgal species and developed protocols for EV isolation both at laboratory (differential ultracentrifugation) and pilot scales (tangential flow filtration). The physico-chemical characterization of microalgal EVs was carried out according to the minimal information for studies of extracellular vesicles 2018 (MISEV-2018 guidelines): biochemical methods to verify the presence of specific EV-biomarkers, tuned for microalgal EVs; dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) to assess the particles number and size distribution; electronic scanning microscopy (SEM), atomic force microscopy (AFM), and cryo transmission electron microscopy (cryo-TEM) for imaging analyses; bilayer-specific fluorescence staining (F-NTA) to test the purity of EV preparation.

Results: We identified microalgae as a novel natural source of EVs that could constitute a cost-effective and sustainable way of mass-producing them. We screened 20 strains of microalgae and generated an "EV Identity Card" for each, which contained a variety of EV features relating to their biophysical, biochemical and biological characteristics in line with the MISEV-2018. Our approach will next focus on the scalable production, surface functionalization and bio-engineering of selected microalgal EVs. At the same time, their bioactivity will be explored using both in vitro and in vivo biological models.

Summary/Conclusion: The VES4US consortium is investigating the potential of microalgae as novel EV bioresources. This research will attempt to bioengineer novel naturally-derived nanocarriers, microalgal EVs, suitable for the development of future cosmetics, nutraceutical or therapeutic formulations.

Funding: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 801338.

OS22.6

Sequence-specific RNA trafficking to Extracellular Vesicles is conserved across cell types

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Introduction: Extracellular vesicles (EV) are an attractive biological vehicle for drug delivery, such as therapeutic RNA. Loading of cargo RNAs into EV during their biogenesis can be achieved by hijacking the physiological pathways of intracellular RNA trafficking.

Several sequences have been identified that act as a zipcode for preferential RNA targeting into EV (EV-tropic) or for retention in parental cells (cell-tropic). In this work, we aimed to compare the EV-tropic capacity of specific RNA sequence motifs in promoting loading into EV, across different cell models representing the main cell types found in the body.

Methods: Immune, epithelial and mesenchymal cell lines were transiently transfected with xenogeneic C. elegans microRNAs (miRNAs) containing EV-tropic or cell-tropic sequences and grown in culture. EV were isolated from the supernatant by differential (ultra)centrifugation. RNA was extracted from both cell pellets and isolated EV fraction, and target miRNAs were quantified by digital droplet PCR. Distribution of cargo miRNA across cells and EV was also analysed for chimeras of EV- and cell-tropic sequences.

Results: The miRNAs containing an EV-tropic sequence were highly enriched on the EV fraction, with 1000–10,000 higher levels than in parental cells. Contrarily, cell-tropic miRNAs were only 10–100 times higher in EV. No significant differences were observed in the EV loading efficiency for the various EV-tropic motifs tested. Mutations in the EV-sorting motif resulted in reduced EV loading. EV-tropic sequences consistently promoted miRNA loading into EV across all the cell models evaluated, suggesting conserved biological mechanisms.

Summary/Conclusion: We showed that RNA loading into EV is dependent on the presence of defined EV-tropic RNA motifs, and that sorting mechanisms are conserved across the major cell types tested. The highest loading efficiencies resulted in 0.001 miRNA copies per particle on average, suggesting a limited scope for EV-tropic motifs for therapeutic RNA loading into EV.

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OS23

Symposium Session 23: EVs in Immunology and Inflammation

Chair: Hang Yin – Tsinghua University

Chair: Bahnisikha Barman – Postdoctoral Research Fellow, Vanderbilt University

OS23.1

Pro-inflammatory cytokine-mediated alterations in beta cell extracellular vesicle cargo and function enhance activation of the CXCL10-CXCR3 axis in diabetes

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Introduction: Coordinated activity between pancreatic islet cells is critical for the regulation of glucose homoeostasis. Chronic exposure to diabetogenic factors such as pro-inflammatory cytokines, perturb islet cell crosstalk and β-cell function in diabetes. Extracellular vesicles (EVs) derived from cytokine-exposed β-cells modulate physiological and pathological responses to β-cell stress. However, the mechanisms governing this process remain largely unknown. We set out to test the hypothesis that β-cell failure in diabetes is mediated in part through β-cell autocrine release of pro-inflammatory EVs which promote inflammation and inhibit β-cell function.

Methods: Pro-inflammatory cytokine-exposed EVs (cytoEVs) were generated using conditioned media from mouse Min6 β-cell line treated with diabetogenic cytokines (TNFα, IL-1β, IFNγ, 48h). EVs were also isolated from human type 2 diabetic (T2DM) and lean non-diabetics (LND) plasma. GW4869 (N-SMase inhibitor) was used in the presence of cytokines to determine the effect of reduced EV concentrations on the restoration of β-cell function. Proteomic and RNA-Seq analysis was conducted on Min6 β-cell cytoEV (vs. control EV) and cytoEV treated mouse islets, respectively.

Results: Assessment of EV concentrations from cytoEV and human T2DM plasma revealed a ~ twofold increase ($p < .05$, vs. control (ctl) and LND EV). Immunofluorescence staining of CD9 and CD63 expression was significantly elevated in human T2DM pancreas ($p < .05$, vs. LND). While acute inhibition of EV formation with GW4869 (5 μM) showed significant restoration in β-cell function (glucose stimulated insulin secretion assay, GSIS) in cytokine-exposed mouse and human islets (~7 and 2 fold vs. cytokines alone, $p < .05$). Moreover, functional assessment of mouse islets exposed to cytoEV (48h) resulted in suppression of GSIS (~55%, vs. untreated, $p < .05$). Identification of cytoEV content through proteomic analysis revealed a significant upregulation of the chemokine, CXCL10 (~40 fold vs. ctlEV) and RNA-Seq analysis of cytoEV treated mouse islets depicted a marked upregulation of transcripts associated with CXCL10-CXCR3 signalling ($p < .001$) and downstream pathways (e.g. NFκB; $p = .016$ and JAK/STAT; $p = .021$). Furthermore, inhibition of cytoEV (GW4869) with cytokines markedly decreased CXCL10 (~30%) and CXCR3 receptor (~65%) expression in Min6 β-cells.

Summary/Conclusion: These data suggests that cytokines elevate CXCL10 expression in β-cell EV to enhance inflammation-induced diabetes. This is mediated through EV-autocrine release of CXCL10 consequently activating CXCR3 signalling and downstream pathways to impair β-cell function in diabetes.

OS23.2

Synergy between 15-lipoxygenase and secreted PLA2 promotes inflammation by formation of TLR4 agonists from extracellular vesicles

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Introduction: Damage associated molecular patterns (DAMPs) are endogenous ligands that induce innate immune response, thus promoting sterile inflammation. During oxidative stress, stress-derived EVs (stressEVs) were found to activate Toll-like receptor 4 (TLR4), but the activating ligands were not fully

determined. Additionally, several enzymes, among them 15-lipoxygenase (15-LO) and secreted phospholipase A2 (sPLA2) are induced during inflammation and were suggested to promote DAMP formation.
Methods: StressEVs were produced from HEK293 cells exposed to 10uM A23187 and isolated with ultracentrifugation. 20:4 lysoPI was oxidized for 10 min with 15-LO. Additionally, synEVs were prepared from phospholipids (PLs), oxidized with 15-LO and hydrolysed with sPLA2. Activity was measured by qPCR and ELISA on wt and TLR4-KO macrophages. 15-LO oxidized 20:4 lysoPI was analysed by mass spectrometry. sPLA2 activity was measured in synovial fluid from rheumatoid and gout patients using fluorometric assay. K/BxN serum transfer induced arthritis model on wt and TLR4 KO mice (C57Bl/6 mice) with sPLA2-IIA injection was used (approval no. U34401-14/2019/8 by MKGP of Slovenia).
Results: StressEVs released after oxidative stress were found to activate TLR4 with a gene profile different from bacterial lipopolysaccharide (LPS). StressEVs, 15-LO oxidized synEVs, but only 15-LO oxidized lysoPLs activated cytokine expression through TLR4/MD-2. Hydroxy, hydroperoxy and keto products of 20:4 lysoPI oxidation were determined by MS and they activated the same gene pattern as stressEVs. Furthermore, sPLA2 activity, which we detected in the synovial fluid from patients, promoted formation of TLR4 agonists after 15-LO oxidation. Injection of sPLA2-IIA into mice promoted K/BxN serum induced arthritis in TLR4-dependent manner.
Summary/Conclusion: Both 15-LO and sPLA2 are induced during inflammation, therefore these results imply the role of oxidized lysoPLs in stressEVs in promoting sterile inflammation through TLR4 signalling. The formation of TLR4 agonists is enzyme driven so it provides an opportunity for therapy without compromising innate immunity against pathogens.
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OS23.3

Monocytes traffic extracellular vesicles to damaged muscle and adopt a novel immunophenotype to support muscle regeneration

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Introduction: Extracellular vesicles (EVs) are secreted membrane vesicles that carry bioactive molecules such as miRNAs, mRNAs, proteins, and lipids to modify recipient cell behaviour. We recently demonstrated EVs secreted by cardiosphere-derived cells (CDC-EVs) augment endogenous muscle regeneration in mdx mice, a model of Duchenne muscular dystrophy, when delivered intravenously. In parallel, macrophages preferentially accumulate surrounding small regenerating myofibers in CDC-EV treated mdx muscle. However, it is currently unclear how intravenous CDC-EVs home to dystrophic muscle and exert their therapeutic bioactivity.
Methods: Fluorescently-labelled and unlabelled CDC-EVs were infused into the contralateral femoral vein of wild-type mice with unilateral muscle injury induced by BaCl2. Injured and uninjured muscles were dissected 24h following infusion and subjected to optical imaging, immunohistochemistry, and confocal microscopy. This experiment was repeated using clodronate liposomes to deplete endogenous monocytes/macrophages. Next, RNA-seq was preformed on bone marrow-derived M1, M2, and CDC-EV (MCDC-EV) polarized macrophages from mdx mice. Conditioned media (CM) from these macrophages were tested in an in vitro model of myogenesis. Lastly, small RNA-seq was performed on EVs secreted by M1, M2, and MCDC-EV macrophages.
Results: When delivered intravenously, CDC-EVs naturally home to injured, but not uninjured, skeletal muscle. CDC-EVs were detected in the interstitium adjacent to non-muscle cells, macrophages, and within surviving myofibers. After depletion of monocytes/macrophages by clodronate liposomes, the presence of CDC-EVs in the injured muscle was attenuated. Bioinformatic analyses indicate CDC-EVs confer a novel immunophenotype to mdx macrophages with features of both M1 and M2. Indeed, MCDC-EV CM promotes myoblast proliferation and supports myogenic differentiation. Interestingly, MCDC-EV EVs have a unique miRNA signature and contain several miRNAs with known roles in myogenesis.
Summary/Conclusion: These data indicate circulating monocytes traffic CDC-EVs to damaged muscle where they adopt a novel immunophenotype to support muscle regeneration. We propose MCDC-EV macrophages mediate their pleiotropic effects via paracrine factors, possibly including EVs.
Funding: NIH R01HL124074 to EM

OS23.4

Microglial derived extracellular vesicles activate autophagy and mediate multi-target signalling to maintain cellular homoeostasis

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Introduction: Microglia, the immunocompetent cells of the CNS, play an important role in maintaining cellular homoeostasis in the CNS. These cells secrete immunomodulatory factors including nanovesicles and participate in the removal of cellular debris by phagocytosis or autophagy.

The contribution of microglial-derived extracellular vesicles (M-EVs) to the maintenance of CNS homoeostasis is unclear. In addition, knowledge of canonical signalling pathways of inflammation and immunity gene expression patterns in human microglia exposed to M-EVs is scarce.

Methods: Here, we analysed the effects of M-EVs produced in vitro by either TNFα-activated or non-stimulated microglia BV2 cells. We showed that M-EVs are internalized by both mouse BV2 and human C20 microglia and that the uptake of M-EVs in microglia induced autophagic vesicles at various stages of degradation including autophagosomes and autolysosomes. Consistently, exposure of microglia to M-EVs increased the protein expression of the autophagy marker, LC3B-II, and promoted autophagic flux in live cells. To elucidate the biological activities occurring at the transcriptional level in C20 microglia exposed to M-EVs, the gene expression profiles, potential upstream regulators, and enrichment pathways were characterized using targeted RNA sequencing.

Results: Inflammation and immunity transcriptome gene panel sequencing of both activated and normal microglia exposed to M-EVs showed involvement of several canonical pathways and reduced expression of key genes involved in neuroinflammation, inflammasome and apoptosis signalling pathways compared to control cells.

Summary/Conclusion: We demonstrate that in vitro produced microglial EVs are able to influence multiple biological pathways and promote activation of autophagy in order to maintain microglia survival and homoeostasis.

Funding: This work was financed by Hasselt University and by EFRO through the Interreg V Grensregio Vlaanderen Nederland project Trans Tech Diagnostics.

OS23.5

Evaluation of plasma extracellular vesicles as biomarkers for longevity

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Introduction: Extracellular vesicles (EVs) have emerged as key indicators and effectors of ageing. Although plasma concentrations of EVs decline with age, the EV biomarkers associated with ageing and longevity are not fully understood. Recently, our group found an age-related decline of plasma EVs associated with immune cells during normal human ageing. Our study aims to evaluate the association of plasma EVs with longevity.

Methods: Plasma samples were selected from the Established Populations for Epidemiologic Studies of the Elderly study subjects (n = 48): half dying within 2 years (short-lived group) and half surviving ≥10 years (long-lived group) after the blood draw; all matched for age (median age 77.3 ± 1.7 years, range 72–80), gender (50% female), and race (50% White/50% Black). The samples were acquired under donor consent and IRB approval of Duke University. EVs were separated from the plasma samples, and profiled based on the surface markers of haematopoietic stem cells (HSCs), mesenchymal stem cells, immune cells, skeletal muscles, cardiac muscles and adipocytes (CD81, CD9, CD29, CD63, CD8, CD4, CD68, CD14, CD56, CD15, CD19, CD235a, CD41a, CD34, CD31, HLA-ABC, HLA-G, HLA-DRDPDQ, CD90, CD73, CD105, M Cadherin, RYR1, RYR2, FABP4, DLK1). The percentages of EVs expressing each tested molecule were determined using a high-resolution multicolour BD LSR Fortessa X-20 Flow Cytometer as we recently reported. GraphPad Prism 8.0 software was used for statistical analysis.

Results: We found significantly increased percentages of CD9+, HLA-ABC+, CD31+ and CD41a+ large EVs (1000–6000 nm) in the long-lived compared to the short-lived group. None of the tested surface marker expressing medium (100–1000 nm) or small (<100 nm) EVs showed differential percentages between the short- and long-lived groups.

Summary/Conclusion: EVs carry surface markers from their parent cells. CD9 is expressed by HSCs and immune cells. CD9 regulates homing of human cord blood CD34+ HSCs, and delivers a potent CD28-independent costimulatory signal to activate T cells. HLA-ABC, the key human immunogen, is expressed

by nucleated cells and platelets. CD31 is expressed by HSCs, immune cells and epithelial cells, and CD31 + plasma EVs declined with age in healthy people. CD41a is expressed by HSCs, megakaryocytes and platelets, and is functionally relevant for HSC maintenance and haematopoietic homoeostasis. Our preliminary data suggest that HSCs and immune cell associated plasma EVs (CD9+, HLA-ABC+, CD31+, CD41a+ large EVs) inform on health status related to longevity.

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OS24

Symposium Session 24: Advances in Separation and Concentration II

Chair: Lei Zheng – Department of Laboratory Medicine, Nanfang Hospital, Southern Medical University

OS24.1

Scaling-up the manufacturing of well-characterized mesenchymal stromal cell-derived extracellular vesicles for biomedical applications

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Introduction: It is anticipated that stem/progenitor cells-derived extracellular vesicles (SPC-EVs) will rapidly progress towards clinical studies, and the development of reproducible, efficient, scalable and cost-effective process for their production is expected to boost the therapeutic applications of EVs-based products. In addition, the use of defined serum-/xenogeneic(xeno)-free culture medium formulations could result in substantial improvements for SPC-EVs production in terms of reproducibility, stability and quality, while ensuring the approval of regulatory agencies. The main goal of this work is to develop a full-controlled manufacturing platform for the SPC-EVs production.

Methods: Human mesenchymal stromal cells (MSC) were expanded in a xeno-free microcarrier-based bioreactor culture system operating in fed-batch feeding mode and after 10 days the conditioned medium was collected. Different methods for SPC-EV isolation/purification from the MSC-derived conditioned medium, including chromatography were compared and the the quality of the final product obtained was characterized by different methods according to MISEV, including nanoparticle tracking analysis, lipidomics and Western blot. Moreover Fourier-Transform InfraRed (FTIR) spectroscopy was evaluated in terms of its implementation as a standard technique for the identification and characterization of EVs.

Results: After 10 days of MSC expansion under dynamic conditions, we collected 1.3 L of conditioned medium with approximately 0.5 million EVs/MSC. A combination of a pretreatment with a nuclease for the digestion of DNA/chromatin with a purification using strong anion exchange chromatography led to the best results so far in terms of EVs isolation. Of notice, by FTIR spectroscopy, it was possible to define ratios of spectral bands, that can be used as biomarkers, enabling the discrimination of EVs chemical fingerprint in function of the culture conditions tested.

Summary/Conclusion: The platform established herein could be applied to the production of well-characterized SPC-EVs targeting their biomedical use in different settings (e.g. as drug delivery systems), as well as EVs from other parental cells lines (i.e. dendritic cells) in therapeutic settings as cancer.

Funding: Fundação para a Ciência e Tecnologia (SFRH/PD/BD/128328/2017, PTDC/EQU-EQU/31651/2017, UIDB/04565/2020).

OS24.2

Ultrasensitive protein detection for quantification of extracellular vesicles in human biofluids enables comparison of isolation techniques

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Introduction: Extracellular vesicles (EVs) are released by all cells into biofluids and hold great promise as reservoirs of disease biomarkers. One of the main challenges in studying EVs and using them in diagnostics is a lack of suitable methods to quantify EVs that are sensitive enough and can differentiate EVs from similarly sized lipoproteins and protein aggregates. We propose using ultrasensitive single molecule array (Simoa) assays to quantify EVs by immunoisolating and detecting EV transmembrane proteins in microwell arrays.

Methods: We developed single molecule array (Simoa) assays using the Quanterix HD-X Analyser for the quantification of EVs using the tetraspanins CD9, CD63, and CD81. Simoa allows for the detection of single proteins using arrays of femtoliter wells, turning ELISA into a digital immunoassay. We then used these assays, together with an additional assay for albumin, to compare commonly used EV isolation methods from plasma and cerebrospinal fluid (CSF): ultracentrifugation, precipitation (ExoQuick), and size exclusion chromatography (SEC) using the Izon qEV columns. We further used these assays to rapidly optimize and improve SEC by comparing different SEC resins and column dimensions in both plasma and CSF.
Results: In comparing our Simoa assays to traditional ELISA with the same antibodies, we found that the Simoa assays were more than 100 times more sensitive, detecting the tetraspanins in samples where the proteins were undetectable by ELISA. Given the high dynamic range and high-throughput capabilities of Simoa, we were able to comprehensively compare relative EV yields and EV purity for different isolation methods of EVs from plasma and CSF. We provide average tetraspanin and albumin levels to directly compare the methods. We also tested different SEC resins and provide data for custom SEC columns that outperform Izon qEV and allow for fine tuning of different ratios of EVs to albumin.
Summary/Conclusion: Our results highlight the utility of quantifying EVs using ultrasensitive Simoa assays for tetraspanins. We were able to rapidly Simoa to rapidly evaluate different EV isolation methods in CSF and plasma. In general, the experimental framework we present could be easily applied to evaluate new EV isolation methods, or applied to any other biological fluid. Thus, we think Simoa is a powerful new tool for relative EV quantitation.
Funding: Open Philanthropy Project (OPP)

OS24.3

Combinatorial antibody microarray profiling of intra- and extravesicular proteins in colorectal cancer cell line extracellular vesicles

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Introduction: The protein profile of extracellular vesicle (EV) subpopulations has been shown to contain valuable disease information, notably in cancer. Currently, techniques aiming to find EV proteins that associate together mainly focus on transmembrane proteins, while methods that also probe cytosolic proteins generally resort to a combination of affinity capture, elution, and lysis, which limits throughput. To allow the high-throughput analysis of both membrane and cytosolic EV proteins, we optimized a Total Extracellular Vesicle Antibody Microarray (tEVAM) incorporating fixation and heat-induced epitope retrieval (HIER), then leveraged it to perform combinatorial protein profiling of EVs from colorectal cancer (CRC) cell lines HT29 and SW403.
Methods: Arrays of IgGs targeting surface protein markers were incubated overnight with EVs purified from cancer cell line supernatants. HIER optimization was carried out through variation of buffer contents, presence or absence of prior permeabilization, as well as incubation time and temperature, for a total of 38 conditions. A431 EVs, previously profiled with other methods, were used as a model during the optimization. Cytosolic protein HSP90 and membrane marker EGFR, both with high expression in A431 EVs, were probed and the results used to compare HIER conditions. Following HIER treatment, protein targets were detected through incubation with primary antibodies and fluorescent secondary antibodies or streptavidin. The resulting optimized tEVAM workflow was used to phenotype HT29 and SW403 EVs through probing of trios of surface (2) and internal (1) protein targets.
Results: The selected tEVAM protocol successfully maximized HSP90 signal while minimally affecting EGFR detection, enabling simultaneous analysis of surface and internal proteins. Profiles of more than 450 combinations, featuring integrins, claudins, cytokines, and other key actors of cancer-relevant pathways, were obtained for HT29 and SW403 EVs, revealing co-expression patterns that highlight the biomolecular heterogeneity both within and between CRC cell line EVs.
Summary/Conclusion: Using tEVAM, intra- and extravesicular proteins can be detected simultaneously in EVs immobilized based on surface protein content, yielding extensive combinatorial protein profiles with significance for health and biomarker research.
Funding: This work was supported by the Fonds de recherche du Québec – Nature et technologies (FRQNT) and the Genome Canada Disruptive Innovation program.

OS24.4

Characterization of EVs using orthogonal techniques identifies discrete EV populations from a mouse dendritic cell line

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Introduction: Extracellular vesicles (EVs) have the potential to serve as valuable biomarkers for patient response to cancer therapy. However, development of robust EV-based clinical assays relies on knowledge of EV concentration and diameter distribution. Many different methods exist to measure the size and concentration of EVs, and each method exhibits strengths and limitations. It is important to use orthogonal methods for determination of these important properties of EV preparations. Here, we use dendritic cell-derived EVs to demonstrate that some EV analysis methods can give a biased interpretation of both diameter and concentration. Through comparison, we highlight why orthogonal assays are essential in providing measurement reliability.
Methods: DC2.4 mouse dendritic cells were cultured in flasks containing a total of 1.2 L of EV-depleted media (10% FBS, centrifuged 18 hr. x 100,000 g.) When cells reached 80% confluency, conditioned media was collected, depleted of debris with two 10 min. x 2,500 g spins, and concentrated down to ~5 mL using a Pall Jumbosep 100 kDa MWCO filter. The EV concentrate was purified from protein using an Izon qEV-10 column, with 5 mL fractions collected. The protein content of the EV-containing fractions was analysed by A280, Pierce BCA, and bioanalyzer. The diameter distribution of the EVs was determined by nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS), flow cytometry (FCM), and electron microscopy (EM.) Concentration was compared using NTA, RPS, and FCM. EVs were further analysed by protein mass spectrometry and RNA sequencing.
Results: We have identified two distinct populations of EVs with our DC2.4 preparation, one highly abundant population with a power-law distribution, whose peak diameter is below 60 nm, and a second, less abundant population with a peak diameter at approximately 140 nm. These two distinct populations and their relative concentration were not detectable with all analysis techniques. Based on cross-platform measurements, these populations appear to have distinct compositions that warrant further investigation.
Summary/Conclusion: The use of orthogonal methods allowed the detection of two discrete populations of EVs which was not possible on some platforms and would have resulted in a biased perspective of the sample composition. This work has highlighted the need for orthogonal measurements to be conducted by pairing techniques that do not have the same biases.
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OS24.5

Hybrid plasmonic biomaterial nanofilter scaffold for cancer EV diagnostics based on surface-enhanced Raman scattering (SERS)

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Introduction: Extracellular vesicles (EVs) are nano-sized vesicles shed by all cells that serve vital roles in cell-to-cell communication. Tumour-associated EV subpopulations vary in molecular content (lipids, proteins, nucleic acids, small molecules), enabling minimally invasive spectroscopic analysis for a wide variety of cancers. Here, we use surface-enhanced Raman spectroscopy (SERS) in combination with a novel plasmonic substrate for global chemical composition analysis of cancerous and non-cancerous populations of EVs to determine distinguishing surface characteristics.
Methods: EVs were isolated from ovarian cancer (OvCa) patient serum samples by differential ultracentrifugation. A new hybrid nanoplasmonic scaffold comprised of a microscale biosilicate diatoms embedded with silver nanoparticles (AgNPs) was used for SERS measurements. The substrate was incubated with cysteamine to positively-charge the AgNPs (responsible for the SERS enhancement) so that EVs could attach (EVs are naturally anionic). In a typical experiment, 40 μL of ~108 particles/mL EVs per sample were incubated with the porous substrate surface, which was inverted on a glass cover slip for Raman

interrogation. Principle component analysis (PCA) was used to compare the spectra and determine distinguishing characteristics between populations from tumour and non-tumour sources. We also trypsinized EVs before SERS analysis to see the extent of influence the surface molecules play in localizing the EVs to the AgNP "hot spots."

Results: A total of 8 clinical samples (7 OvCa and 1 non-malignant control) were tested in combination with OvCa SKOV-3 cell line EVs. Simple PCA was able to separate clinical samples according to disease subtype and major peaks were identified to provide chemical content analysis. Each sample exhibited inherent heterogeneity but clustered together in a distinguishable way from the others.

Summary/Conclusion: Despite innate heterogeneity within single samples (i.e., EVs isolated from a single patient sample), EVs isolated from clinical samples could be easily distinguished from each other using our hybrid SERS substrate, with minimal sample processing, a label-free approach, and only a few microlitres of sample. Our study using this novel plasmonic material demonstrates its potential for use as a component in next-generation diagnostic platforms.

Funding: Ovarian Cancer Education & Research Network (OCERN)

OS24.6

Laser Trapping Raman spectroscopy (LTRS) of single vesicles can distinguish extent of lipoprotein contamination

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Introduction: Single-particle analysis is critical for understanding extracellular vesicle (EV) heterogeneity. Yet such techniques remain technically challenging due to low detection sensitivity and presence of variable amounts of "contaminants," including lipoproteins. The high degree of structural similarity between EVs and lipoproteins in size, density, and chemical composition, results in their co-isolation using any of the standard EV isolation techniques. Here we introduce Laser Trapping Raman spectroscopy (LTRS) as a well-suited, label-free, and non-destructive tool to distinguish EVs from various lipoprotein species at single particle resolution.

Methods: EV samples were isolated from SKOV-3 cell culture supernatant by differential ultracentrifugation and their Raman spectra measured. As the most abundant lipoproteins in EV isolations from human biofluids are sub-micron low density lipoprotein (LDL), very low density lipoprotein (VLDL), and high density lipoprotein (HDL) particles, these were purchased as pure components and also measured by LTRS. LDL and VLDL were then spiked-in to isolated EVs to mimic "contaminated" post-isolation EV samples. Raman spectra were analysed by principal component analysis (PCA) using a custom MATLAB script.

Results: LDL and VLDL have been observed to adhere to EV surfaces in vitro after standard isolation techniques. We could readily distinguish pure VLDL, LDL, and HDL standards according to their Raman spectra. PCA revealed distinction of SKOV-3 EVs from both LDL and VLDL. PCA also differentiated SKOV-3 EVs incubated with LDL from SKOV-3 EVs incubated with VLDL. Extent of LDL and VLDL adherence to EVs could be observed and quantified.

Summary/Conclusion: Through Raman and PCA, classes of lipoprotein and EVs can be identified and quantified when co-incubated. LTRS is a quantitative single-EV analysis technique that can be used to differentiate between lipoprotein classes and EVs when incubated together. This technique allows for analysis of EVs where standard isolation methods fall short.

OS25

Symposium Session 25: Neurologic and Ageing Mechanism

Chair: Nicole Noren Hooten, MD, PhD – National Institute on Ageing, National Institutes of Health

Chair: Cathryn L. Ugalde – Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, Australia

OS25.1

Fibroblast growth factor 2-mediated regulation of neuronal exosome release depends on VAMP3/cellubrevin in hippocampal neurons.

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Introduction: Extracellular vesicles (EVs) are endogenous membrane-derived vesicles that shuttle lipids, proteins or nucleic acids between glia and neurons, thereby promoting neuronal survival and plasticity in the CNS and contributing to neurodegenerative conditions. Although EVs hold great potential as CNS theranostic nanocarriers, the specific molecular factors that regulate neuronal EV uptake and release are currently unknown.
Methods: We used a combination of patch-clamp electrophysiology and pH-sensitive dye imaging to examine stimulus-evoked EV release in individual neurons in real time.
Results: Whereas spontaneous electrical activity and the application of a high-frequency stimulus (HFS) induced a slow and prolonged fusion of multivesicular bodies (MVBs) with the plasma membrane (PM) in a subset of cells, the neurotrophic factor bFGF (basic fibroblast growth factor) greatly increased the rate of stimulus-evoked MVB-PM fusion events and, consequently, the abundance of EVs in the culture medium. Proteomic analysis of neuronal EVs demonstrated bFGF to increase the abundance of the v-SNARE vesicle-associated membrane protein 3 (VAMP3, cellubrevin) on EVs. Conversely, knocking-down VAMP3 in cultured neurons attenuated the effect of bFGF on EV release.
Summary/Conclusion: Our results determine for the first time the temporal characteristics of MVB-PM fusion in hippocampal neurons and reveal a new function for bFGF signalling in controlling neuronal EV release.
Funding: Parkinson Fonds Deutschland
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OS25.2

The well-chaperoned extracellular vesicle: their presence in neuropathologies

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Introduction: Heat shock proteins (HSPs) function as chaperones under both normal and pathologic conditions. As chaperones they assist in protein folding, in holding protein complexes for current or future activation, and in degradation of senescent proteins for recycling of components and display for immune surveillance. During stressful situations, HSP quantities and/or activities increase as cells and tissues seek protection from insults. These insults can result in the cell surface display of HSPs, which can then lead to the surface display of HSPs on extracellular vesicles (EVs). HSPs present on the cell surface or in the extracellular space are regarded as "danger signals" in an ancient biologic paradigm. HSP-accessorized EVs may act as "danger boli", carrying not only the HSPs, but hundreds of components of the stressed parental cell, capable of prompting differential responses depending on the status of the recipient cell.
Methods: Clarified/filtered plasma from patients suffering from neurologic maladies (cancer, brain injury, multiple sclerosis) was incubated with peptides designed to bind HSPs. The EVs congeal under these conditions and are pelleted (microfuge) and washed with increasing-stringency buffers. We lysed the EVs

and subjected them to metabolomic analyses (focused on lipids) or assayed them on phosphokinase arrays.
Results: We show that EVs from the blood of patients suffering from brain tumours, or from TBI, or from MS, possess distinct metabolomes compared to blood EVs from healthy donors. We found hundreds of differentially-expressed lipids amongst the patients vs the healthy donors. The levels of annotation and identification for these compounds ranges from level 4 (low, no matches in databases) to level 2 (high, annotation matches to known database components). In addition, we found differences in phosphorylated kinases as cargo in these EVs between patients with matched primary vs recurrent gliomas, and among TBI/stroke patients compared to healthy donors.
Summary/Conclusion: HSP-accessorized EVs present different metabolomic and phosphokinase content which may serve as biomarkers in a "liquid biopsy" setting, but may also play roles in the pathobiology of neurologic diseases.
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OS25.3

Methamphetamine use disorder uniquely plasma extracellular vesicle miRNA expression

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Introduction: Methamphetamine (MA) has deleterious effects to both peripheral organs and the central nervous system. The rewarding properties and addictive potential of MA are correlated with increased synaptic dopamine availability following alterations in dopamine and vesicular monoamine transporter function. In rodents, MA alters brain miRNA expression and the miRNA content of serum extracellular vesicles (EV). Here we examined plasma EVs isolated from human subjects actively using MA (MA-ACT) for size, concentration, protein markers, and miRNA content.
Methods: Plasma samples from 10 MA-ACT, and 10 controls (CTL) were obtained from the Methamphetamine Abuse Research Center. Plasma EVs were evaluated by vesicle flow cytometry (VFC) for size, concentration, and surface protein markers. VFC antibodies included markers for a pool of tetraspanins (CD9, CD63, and CD81), platelet EVs (CD41), pro-coagulant EVs (AnnV), and red blood cell EVs (CD235). Next plasma EV isolated by size exclusion chromatography were analysed by qPCR on TaqMan® Array Human MicroRNA A + B Card Set v3.0. Fold change was calculated by ΔΔCq between MA-ACT and CTL for miRNA expressed in ≥ 60% of samples in at least 1 group. We identified the top 20% of ranked miRNA by F-statistic; of these, the miRNA of interest for MA-ACT were identified by at least a (i) 1.2 fold change in expression, (ii) area under the receiver operating characteristic curve of 0.75, and (iii) Glass's Δ of 1. For miRNA of interest correlations to additional MA variables were conducted, along with Ingenuity Pathway Analysis of predicted gene targets. Tobacco use was controlled for.
Results: VFC data show that the size (~110 nm) and concentration (~7.5 x 1010 particles/ml) of all plasma EVs is comparable between MA-ACT and CTL groups. In addition, the plasma EVs primarily consist of tetraspanin+, AnnV+, or CD41+ EVs, and to a much lesser extent CD235+ EVs. Of the 207 miRNA expressed in MA-ACT and/or CTL plasma EVs, there were 110 miRNA that have at least a 1.2 fold increase or decrease in MA-ACT. 10 miRNA were identified to be of interest in MA-ACT based on fold change, effect size and diagnostic potential, compared to CTL. Further, 5 of the 10 miRNA correlate with a MA associated variable, including frequency of use and age of first use. Together the 10 miRNA best cluster subjects based on MA-ACT status, not tobacco use. Finally, the predicted gene targets of the 10 miRNA are associated with canonical pathways linked to MA.
Summary/Conclusion: EV miRNA expression in MA-ACT subjects was unique to CTL participants, suggesting that MA may affect EV communication among cells. The differential miRNA expression also implicates a role for EVs in behavioural and physiological effects specific to MA, and suggests that there may be changes in expression of miRNA that are relevant to specific drugs of addiction, as well as to a spectrum of drug-mediated addiction disorders.

OS25.4

Bone marrow-derived extracellular vesicles may alter the ageing phenotype of murine haematopoietic stem cells.

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Introduction: Extracellular vesicles (EVs) have been implicated in many age-related diseases, but their role

in natural ageing of haematopoietic stem cells (HSCs) is unclear. We tested the hypothesis that bone marrow-derived EVs (BM-EVs) can modulate the ageing HSC phenotype.

Methods: We flushed bone marrow from old (24–26-month old) and young (6-8-week old) C57/BL6 mice and collected BM-EVs by differential centrifugation (2000 × g for 30 min, supernatant collected and centrifuged 100,000 × g for 1 hour, BM-EV pellet collected and quantified by Nanoparticle Tracking Analysis). We injected old mice with 2 x 10^9 young BM-EVs via tail vein, daily x 3 days. Control mice were injected with age-matched BM-EVs or vehicle alone. We euthanized the mice one month post-injection, harvested whole bone marrow (WBM) and highly purified HSCs (Lineage negative/c-Kit+/Sca-1+/CD150+; LSK-SLAM) and tested stem cell function in competitive bone marrow transplants (4–5 recipients/group).

Results: At 6 months post-transplant, WBM from old mice exposed to young BM-EVs exhibited a statistically significant decrease in engraftment when compared to WBM exposed to age-matched BM-EVs (percent average donor chimerism ± SEM: 15% ± 5% (young EVs) vs. 61% ± 14% (old EVs)). For LSK-SLAM from old mice, we observed a trend towards decreased engraftment when exposed to young BM-EVs and a trend towards increased engraftment potential when exposed to old BM-EVs (percent average donor chimerism ± SEM: 7% ± 4% (young EVs), 27% ± 10% (old EVs), 15% ± 2% (vehicle)). These findings are consistent with our previous data showing that, in contrast to highly purified HSCs which develop impaired stem cell function with ageing, total un-separated old WBM actually has increased engraftment capacity when compared to young WBM. Of note, we found that the classic myeloid skewing by old LSK-SLAM was partially reversed by exposure to both young and old BM-EVs. Finally, consistent with the known increase in highly purified HSCs with age, our preliminary data showed that old mice exposed to young BM-EVs had an approximately 7-fold decrease in the number of LSK-SLAM cells in marrow, indicating that BM-EVs may influence age-related changes in HSC number.

Summary/Conclusion: These preliminary data suggest BM-EVs may play a role in modulating HSC ageing phenotypes, potentially altering engraftment capacity, lineage fate, and LSK-SLAM population size. Future studies delineating the molecular mechanisms underlying these EV-mediated effects could provide key insights into normal haematopoietic ageing.

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OP3 = PS15

Oral with Poster Session 3: Neurological & ID

Chair: Andrew Hoffman, DVM, DVSc – University of Pennsylvania School of Veterinary Medicine

Chair: Sophie Rome – INRAE, Department of Human Nutrition

OP3.01 = PS15.01

Mitovesicles: a new extracellular vesicle of mitochondrial origin altered in ageing and neurodegeneration

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Introduction: Brain extracellular vesicles (EVs) are heterogenous and include previously described microvesicles and exosomes. Herein we characterized a formerly unappreciated population of mitochondria-derived EVs that we term "mitovesicles". Mitochondrial dysfunction is a well-established hallmark of ageing and neurodegenerative disorders as Down syndrome (DS). Hence, we examined mitovesicle levels and cargo under these conditions to characterize in vivo mitovesicle biology and responsiveness to mitochondrial stressors.

Methods: Employing a high-resolution density gradient, distinct and novel populations of EVs were isolated from murine and human DS and diploid control post-mortem brains or from cell media. Morphometric EV features were analysed by nanoparticle tracking analysis and cryogenic electron microscopy, while EV constituents were characterized by Western blotting, mass spectrometry, lipid profiling and mitochondrial RNA qPCR.

Results: We identified a population of double-membrane, electron-dense brain EVs containing multiple mitochondrial markers ("mitovesicles") that are highly distinct from microvesicles and exosomes. Proteomic data show that mitovesicles contain a unique subset of mitochondrial proteins while lacking others, such as Tom20. Mitovesicles have a lipid composition that is unlike that of previously described EVs and is consistent with mitochondrial origin. Functionally, the complex-III inhibitor antimycin-A stimulated in vitro mitovesicle release into the cell media, suggesting an interrelationship between mitochondrial dysfunction and mitovesicle biology. In mouse brains, mitovesicle levels increased with age and were found to be higher in DS compared to diploid controls. Mitochondrial RNA and protein levels were also altered in DS compared to diploid controls.

Summary/Conclusion: We describe a previously unidentified type of metabolically competent EVs of mitochondrial origin that we designate mitovesicles. Our data demonstrate that brain mitovesicle levels and cargo are tightly regulated in normal conditions and are modified during pathophysiological processes in which mitochondrial dysfunction occurs, suggesting that mitovesicles are a previously unrecognized player in mitochondria quality control and may have a role in the trans-cellular tissue response to oxidative stress.

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OP3.02 = PS15.02

Reducing extracellular vesicle release with a novel neutral sphingomyelinase 2 inhibitor for the treatment of Alzheimer's disease

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Introduction: Alzheimer's disease (AD) is a devastating neurodegenerative disease leading to progressive memory loss and ultimately death with limited therapeutic options. Growing evidence supports the theory that toxic proteins, like tau and amyloid, may propagate from diseased cells by packaging toxic proteins

into extracellular vesicles (EVs) and releasing them to infect other cells. One enzyme involved in the biogenesis of EVs is neutral sphingomyelinase 2 (nSMase2), which catalyzes the hydrolysis of sphingomyelin to produce phosphorylcholine and ceramide. Several groups have reported improved cognition and reduced tau propagation when nSMase2 is pharmacologically inhibited or genetically knocked down in AD mouse models. Unfortunately, current nSMase2 inhibitors are not suitable for clinical development due to poor solubility and inadequate pharmacokinetic profiles.

Methods: Our group carried out a high-throughput screening campaign followed by extensive medicinal chemistry efforts leading to the discovery of phenyl (R)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo [1,2-b] pyridazin-8-yl) pyrrolidin-3-yl) carbamate (PDDC), an orally active, nM potent inhibitor with excellent selectivity and brain penetration. We tested PDDC's ability to inhibit exosome release in cultured primary glial cells as well as an in vivo model of acute EV release. We then treated 5XFAD mice with 10 mg/kg of PDDC daily for six months and monitored their behaviour in the fear conditioning assay.

Results: PDDC dose dependently reduced EV release from cultured primary glial cells and significantly reduced plasma EV numbers in an in vivo model. Following chronic treatment with PDDC, 5XFAD mice demonstrated significantly improved cognitive function in the fear conditioning assay.

Summary/Conclusion: These promising findings are currently being expanded using mouse models of tau propagation. If successful, these data would support PDDC as a novel compound for targeting the pathological spread of tau as a therapeutic for AD.

OP3.03 = PS15.03

Profiling EVs in the anterior cingulate cortex of individuals with major depressive disorder

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Introduction: Major Depressive Disorder (MDD) is one of the leading causes of disability worldwide, affecting 20% of the population. The environment has been thought to play a role in the disease development, resulting in biological changes mediated by epigenetic mechanisms. MicroRNA's (miRNA) are well known epigenetic regulators that are disrupted in the depressed brain, and they are packaged into extracellular vesicles (EVs). EVs have emerged as means of intercellular communication, a process that is also disrupted in MDD. They are thought to transfer miRNA between cells, which can alter gene expression in recipient cells. Therefore, we hypothesize that EV cargo is altered in MDD subjects compared to healthy controls (HC). The aim is to extract EVs from human post-mortem anterior cingulate cortex, a region previously associated with depression, and profile the miRNA cargo and compare it between MDD subjects and HC.

Methods: Post-mortem human brain tissue from the anterior cingulate cortex of 20 MDD subjects and 20 HC was mildly dissociated in the presence of collagenase type III. Residual tissue, cells, and large vesicles were eliminated, and EVs were isolated using size exclusion chromatography. The quality was assessed by western blots and transmission electron microscopy (TEM). RNA was extracted and a small-RNA library was constructed and sequenced using the Illumina Platform. Differential expression analysis was then performed.

Results: Western blots showed little to no Endoplasmic Reticulum (Calnexin), Golgi (BiP), or mitochondrial (VDAC) contamination, along with enrichment of the exosomal marker CD9. TEM images showed the typical cup-shaped morphology with sizes mostly between 30 and 200 nm. Preliminary sequencing results revealed that miR-33a-5p, which is predicted to target glutamate receptors, is downregulated in EVs from MDD subjects.

Summary/Conclusion: High quality EV extractions can be obtained from post-mortem brain tissue using our method. This will be the first study to profile brain-derived EV miRNA in the context of depression. Future studies will be needed to determine the effect of the different levels of miR-33a-5p. This could provide novel mechanistic insights into the pathophysiology of MDD and will serve as a starting point to examine the potential role of EVs in MDD pathology.

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OP3.04 = PS15.04

Combining nanomagnetic isolation and artificial intelligence to guide the treatment of traumatic brain injury

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Introduction: Traumatic brain injury (TBI) is characterized by diverse primary mechanisms of injury that lead to the development of secondary pathological cascades that drive neurological deficit post-TBI. Inability to separate patients based on the presence of these different endophenotypes represents a major challenge for diagnosis and treatment of TBI.

Extracellular vesicles including exosomes isolated from patient plasma have emerged as promising potential biomarkers for TBI due to their ability to cross the BBB into systemic circulation with molecular cargo intact for analysis. We have developed a novel microfluidic platform for rapid isolation of brain-derived EVs providing a tool with which the biochemical state of neurons and glia can be directly assessed post-TBI. We used the ultra-sensitive, single molecule array (SIMOA) to quantify concentrations of 7 protein biomarkers from the plasma and brain derived EVs from mild TBI patients and controls. By combining multiple protein biomarkers, we could discriminate mTBI patients from controls in both the training and the blinded test set.

Building on this work, we are also characterizing single EV heterogeneity of neuron derived EVs by developing novel droplet based digital assay for single EV quantification at ultra-low concentration. Droplet based assay for single EV analysis would potentially be very informative for early disease diagnosis and therapy decision.

Methods: Our microfluidic platform for EV isolation consists of tracked-etched membranes with millions of nanopores (600 nm), coated with a magnetic film (NiFe) to precisely capture immunomagnetically labelled brain-specific EVs from plasma. Single molecule array (SIMOA) was used to quantify concentrations of the 7 protein biomarkers (Tau, UCHL-1, NFL, GFAP, IL6, IL10, and TNF) in the plasma and brain-derived exosomes of mild TBI (mTBI) patients and controls. To identify single EV, we applied droplet based enzyme-linked immunosorbent assay and encoded the fluorescent signal for single EV quantification within parallelized microfluidic platform.

Results: We report that concentrations of plasma and exosome GFAP, NFL, and UCHL1 were elevated in mTBI patients compared to controls ($p < 0.05$), and that each of these biomarkers are uncorrelated with one another. Discrimination of mTBI patients from controls was most accurate when machine learning algorithms on the panel of biomarkers. Specifically, combining plasma NFL, GFAP, IL6 and TNF- with Tau from GluR2+ EVs showed 88% accuracy with 80% sensitivity and 100% specificity.

Summary/Conclusion: This data suggests that neuron-derived exosomes contain information that characterizes the injured and recovering brain. It also suggests that analysis of a panel of biomarkers from a combination of both blood and exosomal compartments could lead to more accurate diagnosis of mTBIs.

OP3.05 = PS15.05

L1CAM is not associated with extracellular vesicles in cerebrospinal fluid or plasma

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Introduction: Neurons in living psychiatric and neurological patients are inaccessible for cell type specific analysis of RNA and protein. Our understanding of these diseases instead relies upon imperfect sources of biochemical information such as post-mortem brain tissue analysis and animal models. Furthermore, there is a paucity of biochemical assays available to diagnose and manage brain diseases. Extracellular vesicles (EVs) present an opportunity to noninvasively sample the contents of neurons in cerebrospinal fluid (CSF) and plasma. In order to isolate neuron-derived EVs (NDEVs), a cell type specific transmembrane protein is necessary for immunocapture. L1CAM, a protein abundant on the surface of neurons, has been used extensively in the literature for NDEV isolation. However, L1CAM exists in humans in several isoforms without a transmembrane domain, and as such it can be secreted as a free protein. Additionally, the ectodomain of L1CAM can be cleaved off of the cell surface in physiological processes. It remains to be demonstrated whether the L1CAM found in CSF and plasma is EV associated, or if it is instead a spliced or cleaved isoform behaving as a free protein.

Methods: Using Single Molecule Arrays (Simoa), a digital form of ELISA, as well as Western blotting, we quantify EV markers (CD9, CD63 and CD81) as well as L1CAM and Albumin. We use these assays to determine in which fractions of size exclusion chromatography (SEC) and density gradient the L1CAM appears. We also immunocapture L1CAM from CSF and

plasma and perform Western blots for the internal and external domains of L1CAM.

Results: Simoa and Western blot analysis of SEC and density gradient fractions demonstrated that while the EV markers peaked all together, L1CAM eluted in the free protein fractions along with Albumin in both CSF and plasma. When immunoprecipitation was performed, Western blotting revealed different isoforms of L1CAM in CSF and plasma.

Summary/Conclusion: Our data utilize a multitude of distinct techniques that converge to demonstrate that L1CAM is not associated with EVs in CSF or plasma. Furthermore, our data suggest that the isoforms present in CSF and plasma are distinct, which indicates that the L1CAM in plasma is likely not coming from the brain. This data call into question the utility of L1CAM as a NDEV marker and point to the need to find novel candidates for immunoprecipitation of NDEVs.

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OP3.06 = PS15.06

An in vitro and in vivo perspective on the role of erythrocyte-derived extracellular vesicles in Parkinson's disease pathology

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Introduction: In Parkinson's disease (PD), α-synuclein (α-Syn) aggregates known as Lewy bodies (LB) are present in both the central and peripheral nervous system. Furthermore, data showing that α-Syn can spread from PD patients to transplanted tissue has led to a new theory postulating that pathological forms of α-Syn can drive disease by "infecting" healthy cells and corrupting normal proteins. The exact routes and mechanisms involved in such spreading are yet to be fully understood but it is known that α-Syn can be secreted from cells and transported via extracellular vesicles (EV). EV derived from erythrocytes (EEV) are of particular interest in this regard as they have been shown to contain α-Syn.

Methods: We first optimized a protocol for the isolation of fluorescently labelled human EEV. The capacity of these EEV to cross the blood-brain barrier (BBB) was then evaluated in vitro using a Boyden chamber composed of primary human brain endothelial cells. Next, EEV were added to a more complex and physiologically relevant 3D human BBB model including iPSC-derived brain microvascular endothelial cells. In both in vitro protocols, flow cytometry was performed on media collect from each compartment to determine the number of EEV. Immunofluorescence was performed to assess the localization of fluorophore tagged EEV. We are also using an in vivo paradigm for the extraction and testing of EEV spread and an in situ cerebral perfusion (ISBP) model in WT mice to investigate if and how EEV cross the BBB using confocal microscopy.

Results: In both in vitro models, flow cytometry analyses showed that fluorescently tagged EEV added to the luminal side traversed the endothelial cell barrier. Confocal analysis revealed that some EEV could also be found within endothelial cells themselves. Ongoing experiments are being conducted in our newly developed 3D BBB to further confirm these results. Our preliminary in vivo experiments showed that fluorescently labelled beads, similar in size to EEV, used in the ISBP experiments are detectable in the brain parenchyma of injected WT mice using confocal microscopy. Preliminary work also includes ISBP injections of EEV in 6-month-old WT mice, (n = 6/groups) derived from PD patients (at different stage of the disease) and a healthy individual as a control.

Summary/Conclusion: Our preliminary data suggests that EEV can indeed move across the BBB in both in vitro and in vivo experimental setups. Ongoing experiments will determine the dynamics and processes involved in this transport and whether EEV can precipitate and/or exacerbate disease-related features.

Funding: FRQS

OP3.07 = PS15.07

Exosomes from N-Myc amplified neuroblastoma cells induce migration and confer chemoresistance to non-N-Myc amplified cells: implications of intra-tumour heterogeneity

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Introduction: Neuroblastoma accounts for 15% of childhood cancer mortality. Amplification of the oncogene N-Myc is a well-established poor prognostic marker for neuroblastoma. Whilst N-Myc amplification status strongly correlates with higher tumour aggression and resistance to treatment, the role of N-Myc in the aggressiveness of the disease is poorly understood. Exosomes are released by many cell types including cancer cells and are implicated as key mediators in cell-cell communication via the transfer of molecular cargo. Hence, characterising the exosomal protein components from N-Myc amplified and non-amplified neuroblastoma cells will improve our understanding on their role in the progression of neuroblastoma.
Methods: In this study, comparative proteomic analysis, nanoparticle tracking analysis, transmission electron microscopy, RNAi-based knockdown, migration and cellular survivability assays were performed to understand the role of exosomes isolated from cells with varying N-Myc amplification status.
Results: Label-free quantitative proteomic profiling revealed 968 proteins that are differentially abundant in exosomes released by the N-Myc amplified and non-amplified neuroblastoma cells. Gene ontology-based analysis highlighted the enrichment of proteins involved in cell communication and signal transduction in N-Myc amplified exosomes. Treatment of less aggressive SH-SY5Y cells with N-Myc amplified SK-N-BE2 cell-derived exosomes increased the migratory potential, colony forming abilities and conferred resistance to doxorubicin induced apoptosis. Incubation of exosomes from N-Myc knocked down SK-N-BE2 cells abolished the transfer of resistance to doxorubicin induced apoptosis.
Summary/Conclusion: These findings suggest that exosomes could play a pivotal role in N-Myc-driven aggressive neuroblastoma and transfer of chemoresistance between cells.

OP3.08 = PS15.08

Dissecting the heterogeneity of extracellular vesicle sub-populations at single vesicle level

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Introduction: Quantification and characterization of single extracellular vesicles (sEVs) based on surface markers can aid in dissecting the heterogeneous landscape of EV subpopulations. We and others have demonstrated the potential of imaging flow cytometry (IFC) to perform sEV characterization. We recently showed release of protoporphyrin (PpIX) positive sEVs by 5-aminolevulinic acid (5-ALA) dosed glioma cells, in vitro and in vivo. Rickfels et al. also used IFC to demonstrate the enrichment of CD63+/CD81+ EVs in the plasma of glioma patients. Herein, we performed in vitro studies to characterize EV subfractions using 5-ALA as well as EV and CNS specific surface markers.
Methods: We use IFC to characterize EVs released by glioma using 5-ALA, fluorescently labelled EV (CFDA-SE, CD81) and glioma specific (tenascin C and epidermal growth factor receptor vIII, EGFRvIII) markers. Furthermore, we characterized EVs released by EGFRvIII positive glioma cells treated with dexamethasone, a steroid commonly used in glioma patients, to determine the effect of steroids on EV release. EVs were quantified by IFC and results were confirmed by qPCR for the levels of EGFRvIII mRNA.
Results: Firstly, we optimized protocols to label glioma sEVs using fluorescently labelled EV markers (CFDA-SE, CD81) and tumour specific markers (tenascin C and EGFRvIII). Of the total EVs (CFDA-SE), we demonstrate that 58% are tenascin C positive, 2.7% are EGFRvIII positive and 1.6% are 5-ALA positive. There was only a minor overlap (<16%) between the sub-populations. Finally, we show that dexamethasone treated glioma cells release lower total EVs (2.5-fold), tumour specific EVs (2.8-fold; EGFRvIII), EGFRvIII mRNA compared to mock treated cells.
Summary/Conclusion: We demonstrate the potential of IFC to monitor sEVs released by glioma cells exposed to different stimuli. This allows the characterization of EV sub-populations providing a working model to understand the dynamics of tumour EVs at a single vesicle level.
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OP3.09 = PS15.09

Proteomic analysis of EVs from the filamentous fungal plant pathogens Fusarium graminearum and Fusarium oxysporum f. sp. vasinfectum

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Introduction: F. graminearum (Fgr) and F. oxysporum f. sp. vasinfectum (Fov) are severe fungal pathogens of

cereals and cotton, respectively. Fgr and Fov cause economic losses and threaten food and fibre supplies worldwide. Understanding host-pathogen interactions is crucial for developing new strategies for disease control. We are determining whether extracellular vesicles (EVs) have a role in the interaction between fungal pathogens and their host plant.

Methods: We isolated EVs from Fgr and Fov by size-exclusion chromatography and characterized them by NTA and TEM. EVs from Fgr and Fov are between 100–300 nm and have morphology similar to EVs reported for other fungi. We performed label-free quantitative proteomics to describe the protein cargo of EVs from Fgr and Fov, including a comparative study of EVs from Fov grown on different media: Czapek Dox (CD) and Saboraud's Dextrose Broth (SDB).

Results: A total of 658 proteins were detected in Fgr EVs and, according to prediction software EffectorP, 12.5% of these were potential effectors. Similarly, 70% of EV proteins do not contain signal peptide indicating that packaging into EVs is a novel mechanism of secretion for these proteins. Notable Fgr EV proteins include lipases, proteases and synthases for toxins and chitin. Fov produced EVs in similar quantities in both growth media tested, but EV protein cargo differed between them. There was a 39% overlap in proteins identified in the 465 CD and the 658 SBD EV proteins. In general, EV proteins were involved in metabolism, cell wall architecture and oxidoreduction, with 15.4% and 12.9% of potential effectors, respectively. Polyketide and toxin synthases, proteases and effectors were present in both types of Fov EVs.

Summary/Conclusion: This new fungal EV isolation method was rapid, yielded high-quality EVs, and did not submit particles to high centrifugal forces. Our data revealed that both Fgr and Fov produce EVs enriched with proteins that could alter host immune responses or facilitate fungal infection. Furthermore, the protein composition of Fov EVs was dependant on culture conditions. This supports a potential role for fungal EVs in disease progression in plants and provides the foundations to pursue the role of EVs in plant-fungal interactions with the potential to identify new targets for disease control.

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OP3.10 = PS15.10

Trypanosoma cruzi releases different types of extracellular vesicles that distinctly modulate host cells

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Introduction: Extracellular Vesicles (EV) released by infective forms of Trypanosoma cruzi, the agent of Chagas' disease, modulate inflammatory response of macrophages through the activation of Toll 2 receptor (TLR2) via mitogen-activated protein kinase pathway. This induces the production of nitric oxide (NO) and expression of the cytokines TNF-α, IL-12 and IL-6, which could explain the inflammation observed in experimental Chagas' disease, and eventually in the progression of human disease. EVs released by the parasite are heterogeneous and it is unknown which factor, or factors present in the different vesicle populations act during the interaction with host cells. Objectives. The goal of the present work was to characterize and isolate the different populations of EVs released by T. cruzi and test their effects on macrophages.

Methods: EV released by trypomastigotes forms of T. cruzi (Y strain) were purified by Asymmetric flow field-flow fractionation (AF4) and characterized by Nanoparticles tracking analysis (NTA). The different populations of EVs were incubated with host human monocytes cells (THP-1) and cytokines production determined by ELISA and qPCR. The different EV populations were also incubated with LLCMK-2 epithelial cells and the infection by T. cruzi determined.

Results: We found two distinct populations of EVs. A population with 50 to 50 nm (EV1) and another with 100 to 120 nm (EV2). EV1 induced more TNF-alpha, IL-6, IP-10 and CCL20 than EV2. It was also more effective in promoting T. cruzi infection in epithelial cells.

Summary/Conclusion: T. cruzi released two EV populations that affects differently host cells. Identification of these EVs composition might help to better understand the role of EVs in the modulation of T. cruzi infection

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OP3.11 = PS15.11

Commensal bacterial extracellular vesicles act as carriers for norovirus

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Introduction: Human norovirus (HuNoV) are one of the most common causes of gastroenteritis and, along

with inducing morbidity and mortality by diarrhoea, have a massive economic impact resulting in approximately 60 USD billion each year in healthcare costs and missed worker productivity. Development of anti-viral therapies for HuNoV has been hampered by the lack of robust in vitro cultivation systems. Several cell types support viral replication but only produce modest amounts of virus due to unknown reasons, making these systems insufficient for use in drug development and infectivity assays.

Noroviruses are known to attach to gram-negative enteric bacteria and this facilitates infection in vitro. However, the microbiome- norovirus-host communication link is missing. Noroviruses infect immune cells present in lamina propria during acute infection, but bacteria themselves are large enough to cross the mucosal and the tight epithelial barrier which separates gut lumen from lamina propria. We hypothesized that binding of noroviruses to bacteria enhances extracellular vesicles (EV) production. Because commensal bacterial EVs by themselves do not have any detrimental effects on host cells, we believe using EVs in in vitro culture will enhance norovirus infection, thus producing higher titre of viruses for vaccine and anti-viral drug development.

Methods: Attachment assay: Purified norovirus was incubated with Enterobacter cloacae, Lactobacillus acidophilus and Bacteroides thethiotaomicron, and grown to produce EVs. The attachment was confirmed via qPCR.

Isolation of EVs: Clarified media supernatants were subjected to ultracentrifugation at varying speeds and 0.2um filtration. Co-purification of norovirus with the EVs was checked.

EV quantification and characterization: EV total protein content was measured by microBCA. The number of vesicles were quantified by Nanoparticle tracking analysis. Scanning and Transmission electron microscopy was performed to check quality of EV preparation and determine if virus was attached to the vesicles. Internal EV protein content was evaluated using MS-HPLC. The EVs were also check for infectivity via TCID50 assay.

Results: Incubation of noroviruses with commensal bacteria resulted in significant increases in production of EVs compared to uninfected controls. Murine norovirus (MNV), used as a surrogate, was found to be associated with EVs. EM analysis determine association of viruses with the bacteria as well as the MVs, while also showing certain surface structural changes in virus attached bacteria compared to mock bacteria. The EVs were found to cause infection in naive macrophages.

Summary/Conclusion: Changes in EV production and content by bacteria exposed to noroviruses will provide insight into its pathogenesis and possible solutions to the low viral output from HuNoV culture systems.

OP3.12 = PS15.12

Detection of bacterial extracellular vesicles in blood from healthy volunteers

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Introduction: Bacteria constitutively produce biologically active extracellular vesicles (EVs), which contain RNA, DNA, and/or proteins. Bacteria use these EVs for communication with other bacteria and recent research suggests bacterial EVs can also affect host cells. Given these findings, it is necessary to examine the role of bacterial EVs in human disease. Current methods of bacterial EV isolation from human specimens cannot distinguish between bacterial species. However, there is utility in examining EVs from specific species, as bacterial species and their EVs may have unique contributions to human disease. Our objective was to isolate circulating EVs specifically from Escherichia coli (EEVs) and Haemophilus influenzae (HEVs), two known colonizers and pathogens in the gut and airway, respectively.

Methods: Total EVs were isolated from the blood of six healthy volunteers via precipitation and size exclusion chromatography. EVs were then selected via a novel latex bead-based fluorescent antibody construct targeting species-specific outer membrane proteins. We used flow cytometry to evaluate the isolated EVs.

Results: The constructs were saturated with EEVs at an antibody concentration of 11.5 μg/mL of plasma, as geometric means ≥11.5 μg/mL were nearly equal. HEVs were detected at 48 μg/mL of plasma, but saturation is yet to be determined. EEVs were imaged by a FEI Talos F200X electron microscope and measured between 40–90 nm, and HEVs were between 60–160 nm. Both types of EVs were spherical.

Summary/Conclusion: Using this novel technique, we were able to isolate, detect, and visualize EEVs and HEVs. This technique enables the study of specific bacterial EVs. In the future, EV contents will be assayed. Furthermore, this technique will be modified so that specific bacterial EVs from body fluids can be used for downstream functional applications. This is the first time that bacterial EVs from targeted bacterial species have been detected in blood from healthy humans.

OS26

Symposium Session 26: Scientific Collaboration and Outreach

Chair: An Hendrix – Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Belgium; Cancer Research Institute Ghent, Belgium

Chair: Cecilia Lässer – Postdoc, Krefting Research Centre, Institute of Medicine, Sahlgrenska Academy at University of Gothenburg

OS26.1

HEVI NZ: A hub for extracellular vesicle investigations in New Zealand supports research and education

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The University of Auckland, Auckland, New Zealand

Introduction: New Zealand (NZ) has a population of just 4.8 Million people with a remote geographical location in the Pacific Ocean. Its unique culture, food-based industries and ethnic population make NZ an invaluable place for extracellular vesicle research into all areas. However, as for many places in the world, standardization of methodologies, training and access to appropriate equipment is challenging.
Methods: The Hub for Extracellular Vesicle Investigations (HEVI) is a virtual research centre established in 2017 with three-year seed funding from a University of Auckland Strategic Research Initiatives Fund. Two staff members are employed to support training, education and optimization of methods. The HEVI is guided by a governance group providing valuable input from Australasian experts in EVs.
Results: Since 2017 the HEVI has organized 2 research symposia, 2 hands-on training days, hosted 2 international students as well as providing one-on-one training for 41 individuals from universities and institutes across NZ. Training is provided on multiple isolation and characterization methods and tailored to individuals access to essential equipment without bias towards individual manufacturers or techniques. Travel funding has supported 38 people to attend conferences and workshops for the purposes of education, networking and research dissemination. The HEVI also provides support for project design with 10 grants awarded to HEVI members and a number of manuscripts in submission for publication.
Summary/Conclusion: Establishment of a local research hub has aided recruitment of new researchers to the field as well as supporting established researchers through standardization of methodology and provision of expert staff to enable development of new techniques tailored to project needs. Over the next year we aim to secure ongoing funding for this valuable resource, strengthen a national network and link with the Australasian and other EV societies for further training opportunities.
Funding: Vice Chancellors Strategic Research Initiatives Fund, The University of Auckland

OS26.2

EMBO practical course "extracellular vesicles: from biology to biomedical applications"

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Introduction: The EMBO practical course "Extracellular Vesicles: From Biology to Biomedical Applications" is organized each year by a group of 4 researchers active in the EV field in collaboration with the EMBL Advanced Training Center in Heidelberg. The course focuses on training PhD students and postdoctoral researchers who enter or are already active in the field of EV research. Given the large number of methods and protocols that is being used by researchers in the EV field, the organizers aim to provide practical guidance to new researchers and teach them appropriate skills.

Methods: Participants obtain theoretical knowledge and hands-on experience on different EV purification and characterization techniques, such as fluorescent labelling, density gradient centrifugation, size exclusion chromatography, electron microscopy, flow cytometry and nanoparticle tracking analysis and on databases like EV-TRACK and FunRich. In addition, the organizers and invited lecturers from several different research areas explain which strategies are used to understand the role of EV in biomedical applications and give an overview of the current state of the field of EV research.
Results: The course therefore covers a broad range of topics important in the EV field, including heterogeneity in EV subpopulations, mechanisms of EV cargo selection, EV biogenesis, pre-analytical variables, therapeutic and diagnostic use of EV, and in vivo functions of EV. Group discussions are facilitated and stimulated via assignments to analyse data obtained during the practicals and to critically evaluate literature. Participants also have the opportunity to present their own research during poster presentations and ask for feedback from organizers and invited lecturers.
Summary/Conclusion: Among the participants selected for the course, a large geographical distribution is reached, including researchers from the newer EU member states and from outside of Europe, to ensure a broad geographical distribution of the knowledge gained during this course.

OS26.3

1st Lugano ExoDay – technical challenges in exosome research. First extracellular vesicles workshop in the Southern Switzerland

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Introduction: On 25 October 2019, we organized the 1st Lugano ExoDay, first initiative in the Southern Switzerland to bring together resident researchers and European experts in the field of Extracellular Vesicles (EVs). The workshop, centred on "Technical challenges of extracellular vesicle research" aimed to highlight technical requirements and advances in the EVs area, focusing on isolation, characterization and tracking.
Methods: The workshop started with a lecture by Dr. Cecilia Lässer, from the University of Gothenburg. The rest of the workshop was divided in two working groups (WG), each introduced by a keynote lecture followed by presentations by young researchers and a round-table discussion. WG1, introduced by Dr. Mercedes Tkach, from the Institute Curie in Paris, focused on recent advances on EVs characterization and isolation. WG2 was centred on EVs tracking and introduced by Dr. Frédérik Verweij, from the Institute of Psychiatry and Neuroscience of Paris.
Results: Dr. Lässer opened the workshop with a comprehensive review and introduced recent developments in the EVs field. The first WG discussed different isolation methods, focusing on ultracentrifugation, size exclusion chromatography and immunoprecipitation-based techniques. Supported by the keynote speakers, the participants agreed that the best approach to optimize the isolation process consists in the combination of different techniques. WG2 shared insights about new strategies to visualize and tracking EVs, focusing on how to improve the routinely approaches used, defining optimal criteria for EVs labelling and imaging. All the participants had an in-depth overview on the requirements and the state-of-the-art techniques currently in use for the isolation, characterization and tracking of EVs.
Summary/Conclusion: The transferable knowledge acquired during the workshop ensures participants to remain up-to-date with the advances in the field of EVs. As our ultimate goal is to create a competence centre in Southern Switzerland around the field of EVs, the workshop was an invaluable opportunity to intensify collaborations between resident laboratories and broaden the scientific exchange with laboratories of the experts hosted during the event. Given the success of this first workshop we are already working to prepare the second edition and make the event a recurring appointment.
Funding: Supported by the Swiss National Science Foundation

OS26.4

The role of core facilities and emerging technologies in maximizing rigour and reproducibility of EV quantification and characterization and following MISEV guidelines

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Introduction: It remains very clear in the field of extracellular vesicle (EV) research that the rapid rate of increase in publications and expansion of

interdisciplinary clinical EV interest has created the need for increased standardization and access to the appropriate technologies to uphold these standards. As the first core facility in the USA with the sole intention of creating a space where users can both isolate and characterize EVs, we provide a central location for the facilitation of EV research via access to multiple technologies (both established and emerging) such as resistive pulse sensing, nanoparticle tracking analysis, ultracentrifugation, high-performance liquid chromatography, flow cytometric analysis of EVs and additional immune or fluorescence-based EV characteri zation techniques.

Methods: We surveyed a group of leading scientific investigators and researchers in varying stages of their scientific careers in the Mid-Atlantic region of the US. The survey data demonstrate applications of greatest current and future interest to be employed in a shared lab resource.

Results: The current demand is highest for isolation services, ultracentrifugation and NTA, with a gradually increasing demand for immunophenotying analyses such as the ExoView chip array, fluorescent NTA and flow cytometry. We additionally present strategies and data-based examples of how shared resource facilities can facilitate multifactorial and rigorous EV characterization in accordance with MISEV guidelines, and encourage collaboration among EV researchers.

Summary/Conclusion: In order to answer the larger remaining questions in the EV field such as the isolation of specific EV subsets, EV tracking between cells and the use of EVs for biomarker discovery and drug delivery, it is essential that shared resource facilities interact not only with investigators, but with each other to integrate the necessary resources to progress.

OS26.5

Programme to assess the rigour and reproducibility of extracellular vesicle-derived analytes for cancer detection

Matthew Young and Sudhir Srivastava

National Cancer Institute, Rockville, USA

Introduction: Cancer cells release more EVs than normal cells and EVs secreted from tumour cells can promote tumour progression, survival, invasion and angiogenesis. The EV cargo may mirror the altered molecular state of the cell of origin. Therefore, EVs have potential for the development of non-invasive markers for early detection of cancers. EVs and their cargo also have the potential to be multiplexed with other molecular markers or screening modalities (e.g., imaging) to develop integrated molecular-based computational tools for the early detection of cancer.

One challenge with using EVs as a biomarker is the lack of robust and reproducible methods for the isolation of a pure vesicular population. There is a lack of clear consensus for an optimal method of isolation of a pure EV population that is devoid of contamination with similar-sized vesicles of different origins. There is also a lack of standards to ensure rigour reproducibility.

Methods: The current funding opportunity announcement (FOA), PAR20-053, is promoting research on the isolation and characterization of extracellular vesicles (EVs) and their cargo for the discovery of biomarkers to predict cancer and cancer risk.

Results: The previous cycle of this FOA, PAR16-267/277, successfully funded 7 R01 and 4 R21 grants. These awards are focused on proteomics profiling of EVs, effect of methodological and biological variability, asymmetric-flow field-flow technology, therapeutic monitoring, LSS and SERS lab on a chip optical spectroscopic, EVs in obesity-driven hepatocellular carcinoma, nanoscale structure and bio-molecular heterogeneity, urinary EV DNA, and EV markers in paediatric cancers.

Progress from these awardees have shown separation of two discernible exosome subpopulations and identified a distinct nanoparticle, the exomere (Nature Cell Biology, 2018); and have shown that large-EVs contain the entire genome of the cell of origin, including cancer-specific genomic alterations (Journal of Extracellular Vesicles, 2019). Protocols that critically evaluate and refine the existing methodologies to improve utilization of EVs in clinical use have been shared (Nature Protocols, 2019).

Summary/Conclusion: Drs. Sudhir Srivastava and Matthew Young are the program directors for the PAR which began accepting applications on 5 January 2020. This and other EV funding opportunities will be discussed.

Funding: This is a Funding Opportunity Announcement offered by the National Cancer Institute

Late-Breaking Oral Presentations

OS27

Symposium Session 27: Late Breaking: EV Biology and Biomarkers

Chair: Xiaomei Yan – Professor, Xiamen University

Chair: Eduard Willms – Post Doc, La Trobe University

OS27.1

Detecting tumour-associated membrane receptors on extracellular vesicles via immuno-PCR with Affibody-DNA-Conjugates

Christiane Stiller[a], Petra Hååg[b], Elizabeth Paz Gomero[a], Vasiliki Arapi[b], Kristina Viktorsson[b], Rolf Lewensohn[b] and Amelie E. Karlström[a]

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Introduction: Early detection of cancer as well as monitoring cancer treatment are important to improve cancer care. Diagnostics for cancer are mainly based on tissue biopsies and re-biopsy during treatment is challenging. Moreover, current diagnostics are expensive, time-consuming and have low-throughput. Therefore, liquid biopsies are expected to bring the next breakthrough in cancer diagnostics. In liquid biopsies tumour-secreted material is isolated from body fluids and subsequent analyses thereof allow for non-invasive diagnostics. One type of tumour-secreted materials are extracellular vesicles (EVs), which are schedded from tumour cells. EVs are surrounded by a lipid bilayer, whichs composition resembles the plasma membrane of their parental cell. As many tumours are driven by over-expression or upregulation of transmembrane proteins e.g. growth factor receptors, detection of the later on EVs holds promise for early tumour detection and treatment monitoring.
Methods: For the immuno-PCR EVs were first affinity-captured on magnetic beads, allowing immobilization of purified EVs as well as EVs secreted into cell culture medium or spiked into plasma. Afterwards each sample was divided and affibody-DNA-conjugates directed against different targets were added. Affibodies are small affinity proteins, which often are developed as high affinity binders for tumour imaging, making them suitable probes in the presented assay. After washing, the bead-EV-affibody-DNA-complexes were analysed for the immobilized DNA-amount via qPCR.
Results: Via the presented immuno-PCR EVs secreted from the non-small cell lung cancer cell line H1975 as well as the ovarian cancer cell line SKOV3 were analysed. The immuno-PCR method allowed the detection of the tumour-associated membrane receptors epidermal growth factor receptor (EGFR), receptor tyrosine-protein kinase ERBB2/Her2 and insulin-like growth factor 1 receptor (IGF1R). Different levels of membrane receptors depending on the EV source and concentration were detected.
Summary/Conclusion: The presented immuno-PCR showed to be a comparably fast and robust method for detection of tumour-associated membrane receptors on EVs derived from cancer cell lines with medium through-put and is currently further developed into a method for liquid biopsy for non-small cell lung cancer patients.
Funding: Erling Persson Foundation. Swedish and Stockholm Cancer Society

OS27.2

Heterogeneity in EV cargo expression measured by single vesicle flow cytometry

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Introduction: Introduction. EVs produced by cells can originate from different cellular compartments and EVs in complex biofluids may originate from many different cell types. Traditional biochemical analysis, which reports on the total composition of all EVs in a sample can't adequately resolve this heterogeneity. Single vesicle analysis methods can, if they have the necessary specificity, sensitivity and speed. Flow cytometry (FC) is capable of rapid and quantitative analysis of individual particles, but conventional FC-based assays lack the specificity and sensitivity to measure individual EVs. Assays that combine sensitive instruments with EV-selective sample staining can measure individual EVs with accuracy and precision. To better understand the nature and origins of EV diversity, we

used single vesicle FC (vFC) to quantitatively measure vesicle number, size, and surface cargo expression on individual EVs.

Methods: Methods. EVs in culture supernatants (293 T, A431, U87, THP-1, SH-SY5Y) were used neat or enriched by standard methods including differential centrifugation or ultrafiltration. EVs from platelets (PLT) and red blood cells (RBC) were induced by ionophore treatment of washed cells, and measured in diluted supernatant. EVs were stained with a membrane-selective dye and fluorescence-labelled antibodies using a commercial vFC assay kit (Cellarcus Biosciences), measured using a commercial flow cytometer (CytoFlexS, Beckman Coulter), and data analysed using FCS Express v6 (De Novo Software). Vesicle size, fluorescence intensity, and antibody binding were calibrated using appropriate vesicle and bead-based standards and essential controls performed as recommended by the MIFlowCyt-EV reporting guidelines.

Results: Results. To assess the compositional heterogeneity of EVs, we first characterized the expression of tetraspanins (TSs; CD9, CD63, CD81, CD82, CD151, CD53, CD231) on EVs released from cultured cell line and primary cell cultures. We find quantitative differences in the expression of TS on EVs from different cell types that generally reflected the expression on the cell of origin, with most EV types expressing detectable amounts at least one of the common TS molecules (CD9, CD63 or CD81) but generally not all three. In EVs from some cell types, TS expression was uniform across the EV population (CD9 on EVs), but EVs from other cell types differentially expressed TSs, with some EVs expressing no detectable TS (RBC EVs). Intracellular cargo labelled genetically using fluorescent proteins (eGFP or mNeonGreen) or fluorogenic enzyme substrates (CFSE) were measured in individual EVs and revealed distinctive associations between EV surface and internal cargo.

Summary/Conclusion: Conclusions. High resolution measurement of cargo on/in individual EVs can help interpret EV heterogeneity in terms of cell of origin, signals carried, and effects on target cells.

OS27.3

Integrated omics reveal conserved and divergent modulation of cardiovascular disease by tissue-entrapped extracellular vesicles

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Introduction: Fewer than 50% of patients develop both vascular and valvular calcification, implying differential pathogenesis. While circulating extracellular vesicles (EVs) act as biomarkers of cardiovascular diseases, tissue-entrapped EVs are implicated in early mineralization but their contents and function are unstudied. We developed an innovative method to isolate and study EVs from fibrocalcific tissue and investigated entrapped EV cargoes in human cardiovascular diseases.

Methods: Human carotid artery endarterectomies and stenotic aortic valves were obtained from 27 donors under IRB-approved informed consent. Tissues underwent enzymatic digestion, ultracentrifugation, and a 15-fraction OptiPrep density gradient. Global proteomics was performed on intact tissue, each OptiPrep fraction, and EV-enriched pooled fractions; the latter also underwent miRNA-seq. Fractionated samples were also studied by CD63 immunogold electron microscopy (TEM) and nanoparticle tracking analysis (NTA). High confidence miR targets were predicted by TargetScan, pathway analyses utilized the BioCarta/KEGG/Reactome databases, and protein-protein interaction networks were built in STRING.

Results: Vesicle-associated pathways were increased 5.1x ($p < 0.01$; 15/30 vesicle-related top GO terms) in proteins common to intact arteries and valves ($n = 1{,}411$). Proteomics found 24 EV markers to be highly enriched in the four least-dense OptiPrep fractions of arteries and valves, while extracellular matrix and mitochondria were predominant in denser fractions, as confirmed by TEM/NTA. Proteomics and miRNA-seq of tissue EVs quantified 1,104 proteins and 123 miR cargoes linked to 5,182 target genes. Pathway networks of proteins and miR targets common to artery and valve tissue EVs revealed a shared regulation of Rho GTPase and MAPK intracellular signalling cascades. 179 proteins and 5 miRs were

significantly altered between artery and valve EVs ($q < 0.05$); multi-omics integration found that EVs differentially modulated cellular contraction and p53-mediated transcriptional regulation in vascular and valvular tissue.

Summary/Conclusion: Our findings delineate a novel strategy for studying tissue-entrapped EV protein and miR cargoes and identify critical roles that tissue-resident EVs play in mediating cardiovascular disease.

Funding: This study was supported by a research grant from Kowa Company (MA) and NIH grants R01 HL136431, R01 HL141917 and R01 HL147095 (EA).

OS27.4

MiR-20a regulates exogenous CD82 expression on proliferation, invasion, migration and angiogenesis of gastric cancer

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Introduction: To investigate the possible mechanism of MiR-20a regulating the expression of exosome CD82 on proliferation, invasion, migration and angiogenesis of gastric cancer, and to study the application value of CD82 in the early diagnosis and prognosis of gastric cancer.

Methods: The gastric cancer cell line MGC-803 was used as the research object. The exosomes were extracted from the culture supernatant of MGC-803 by exosome extraction kit. The extracted exosomes were identified by transmission electron microscopy and Western blotting. The expression of CD82 in exosomes was detected by ELISA. The expression of CD82 in exosomes and CD82 in whole blood and serum were detected by Western Blot. They were randomly divided into blank group (MOCK) and miR-20a lentivirus experimental group (miR-20a group). The lentivirus control group (miR-20a/Con) was transfected into cells. qRT-PCR was used to verify the status of miR-20a after transfection; Western-blot was used to detect the expression of CD82 and downstream ERK1/2, AKt and m TOR proteins; MTT assay, cell colony formation assay, Transwell migration assay for cell proliferation, invasion, and migration. A nude mouse xenograft model was constructed to observe the growth of transplanted tumours,microvessel density (MVD) was detected by immunofluorescence, and distant metastasis was recorded.

Results: The expression of CD82 in exosomes was detected by ELISA and Western Blot. The expressions of CD82, Akt, ERK1/2 and m TOR in miR-20a group were significantly lower than those in miR-20a/Con and MOCK groups. CD82 protein is positively correlated with downstream protein levels.The growth rate and cell invasion ability of miR-20a group were significantly lower than those of miR-20a/Con group and MOCK group. The weight of the nude mice in the MOCK group and the miR-20a/Con group decreased, while the weight loss in the miR-20a group was not significant. The tumours in the miR-20a/Con group and the MOCK group showed invasive growth accompanied by abundant microvessels, while the miR-20a group had smaller tumour volume and uniform cell distribution. Only a small amount of microangiogenesis was observed, and no obvious necrotic area was observed.

Summary/Conclusion: miR-20a affects the proliferation, invasion, migration and angiogenesis of gastric cancer mediated by Akt/ERK/m TOR signalling pathway by regulating the expression of exosome CD82.

OS27.5

Streamlined detection and quantification of plasma extracellular vesicles and their protein cargo by high-performance nanoscale flow cytometry and label-free mass spectrometry

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Introduction: Nanoscale flow cytometry (FC) and mass spectrometry (MS) are useful for profiling EV surface proteins and performing bulk EV proteomics, respectively. This study sought to develop pre-analytical and analytical pipelines for EV protein profiling that are applicable to clinical studies.

Methods: To optimize plasma EV detection and quantification by FC, modifications of instrument settings and serial dilutions of platelet-free plasma (PFP) and antibodies were tested for improved separation of signal from noise and reduction of event coincidence and swarming. The high-performance flow cytometry (HPFC) platform was used to assess the effect of time (1, 2, 3, 5, 9, 24, 48 or 120hrs) between blood draw (into ACD, NaCit, EDTA or heparin) and blood processing, on ex-vivo release of EVs from blood cells. Label-free MS was used to examine the intensity and breadth of identified proteins in plasma EVs purified using several density and size separation methods,

either manually or automated, along with various buffer conditions.

Results: EV event aborts were minimized at a PFP dilution, prior to staining, of 1:10 and by using a narrow cytometer window extension. Target EV signals were distinct from noise and were Triton X-100 labile. The most significant changes in plasma EVs were associated with platelet-derived fractions, use of heparin and >5-hour delay before blood processing. Yet, platelet EV numbers did not significantly change for up to 24 hrs in citrated and EDTA plasma. Higher overall coverage of known EV proteins and a fivefold increase in number of uniquely identified proteins were observed in MS profiling of EVs prepared by a combination of ultracentrifugation (UC) and manual size-exclusion chromatography (SEC) compared to preparation by FPLC on Capto Core 700/Superose 6 resins. UC/SEC was better than direct SEC at reducing contamination by excipient plasma proteins. Column buffers with trehalose increased EV protein recovery while adding protease inhibitors had minimal effect.

Summary/Conclusion: With our optimized HPFC protocol, we established that blood EV numbers remain stable for up to 24 hrs in ACD or EDTA and that UC+SEC with trehalose-containing buffer result in high canonical EV protein recovery. We are applying these workflows to investigate cancer-associated changes in plasma EV protein cargo.

Funding: This project was supported by funding from the Cancer Early Detection Advanced Research (CEDAR) center at Oregon Health & Science University's Knight Cancer Institute.

Support for this project also came from the Proteomics Shared Resources of Oregon Health & Science University

OS27.6

The value of exosomes as a potential biomarker for Devil Facial Tumour Disease.

Camila Espejo, Richard Wilson, Greg Woods and Bruce Lyons

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Introduction: The Tasmanian devil (Sarcophilus harrisii), the largest living carnivorous marsupial is endangered because of two transmissible cancers: Devil Facial Tumour Disease (DFTD) one and two. Current efforts to manage DFTD are hindered by the lack of a preclinical diagnostic test for DFTD. Detecting DFTD infection is only possible once tumours are noticed, too late to stop DFTD progression. A preclinal test could tell us about unknown components of DFTD pathogenesis, such as latent period and host-tumour dynamics.

Exosomes are extracellular vesicles released by most types of cells under both physiological and pathological conditions. Exosomes have utility as diagnosis and prognosis biomarkers in a range of diseases, including cancers. The aim of this study is to investigate exosomes-based approaches towards a preclinical and progression biomarker for DFTD 1 and 2 in Tasmanian devils.

Methods: Exosomes were isolated from three different DFTD-1, DFTD-2 and devil fibroblast cell lines by size-exclusion chromatography. Likewise, exosomes were isolated from plasma of healthy and diseased devils. To determine the size and morphology of exosomes, samples were imaged with transmission electron microscopy. Exosomes isolated from cell lines and devil plasma were analysed with mass spectrometry to characterise proteins and determine their differential expression between the cell origins, and healthy and diseased animals.

Results: This study identified the presence of myelin proteins in exosomes from DFTD cells relative to fibroblasts, which are diagnostic of DFTD. Additionally, we found that exosomes derived from DFTD-2 abundantly express the inhibitory checkpoint molecule CD200 relative to exosomes from DFTD-1 cells and devil fibroblasts, indicating a potential candidate for a differential diagnosis between tumours. Moreover, exosomes from DFTD cells present a greater amount of proteins related with metastasis in comparison with fibroblast exosomes, such as integrins. Finally, we report the protein expression profile of exosomes from healthy and diseased devils, showing clear differences between them and the presence of immunosuppressive and metastasis proteins in animals in late stages of the disease.

Summary/Conclusion: DFTD-exosomes may provide a non-invasive diagnosis tool to detect early stages of DFTD in Tasmanian devils to facilitate the prevention of the disease. Furthermore, DFTD-exosomes may have utility as a prognosis biomarker, determining late stages of the disease using a simple a blood test, which would facilitate monitoring of wild populations. This project will provide long-term benefits for the future of the devils and encourage exosome-based solutions for other future wildlife disease outbreaks.

Funding: National Geographic Early Career Grant
Holsworth Wildlife Research Endowment Grant
Dr Eric Guiler Tasmanian Devil Research Grant

OS28

Symposium Session 28: Late Breaking: Therapeutics, Biogenesis and Biodistribution

Chair: Charles Lai – Ministry of Science and Technology, Taiwan

Chair: Ann M. Wehman – Junior Group Leader, Rudolf Virchow Center at the University of Würzburg

OS28.1

Absolute quantification of Extracellular vesicles in vivo by a sensitive bioluminescence system.

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Introduction: Despite the increased understanding of EVs, from involvement in disease pathophysiology to therapeutic delivery, improved molecular tools to track biodistribution are largely lacking. Current approaches used for EV labelling lacks sensitivity and specificity. Here, we have explored bioluminescent labelling of EVs to achieve a highly sensitive system for absolute in vivo quantification and tracking of exogenous EVs at low cost and in a high throughput manner.
Methods: EV-producing cells were genetically engineered to express various tetraspanin-luciferase fusion proteins. EVs purified by UF-SEC from these cells were characterized by NTA, multiplex bead-based array, TEM and WB, followed by luciferase assay to determine the labelling efficiency. For in vitro applications cell lysate from treated cells or the conditioned medium were subjected to luciferase assay. For in vivo applications two different methodologies were applied to determine biodistribution; either by non-invasive real time in vivo imaging using IVIS or by luciferase assay on harvested tissues for absolute quantification of injected EVs.
Results: We initially performed a systematic comparison of five different luciferases for endogenous labelling of EVs and identified NanoLuc and ThermoLuc as lead candidates. We applied this technology to monitor in vitro cellular uptake and observed cell type differences in cellular uptake of engineered EVs. In addition, we also observed an effect of different culturing conditions on exocytosis kinetics. For in vivo application, we applied the NanoLuc labelling strategy to determine the pharmacokinetics and effect of different routes of injection on EV distribution. Our results indicated a rapid uptake profile of administered EVs in different tissues with liver, spleen, and lungs being the primary recipients. We also observed similar results upon tracking in vivo biodistribution in real time immediately after administration. Finally, we show how different subpopulations of EVs differ in their in vivo biodistribution.
Summary/Conclusion: Overall, NanoLuc and ThermoLuc labelling of EVs holds great potential for various in vivo and in vitro applications. In addition, it can enable the simultaneous detection of different subpopulations of EVs in vivo, which may aid in our understanding of different sub-populations and their behaviour in vivo. Apart from monitoring therapeutic EVs, with one simple modification this platform offers great potential for tracking tumour derived EVs both in vivo and in vitro and thus could aid in the development of anti-tumour therapies.

OS28.2

Biofunctional peptide-modified extracellular vesicles for cell targeting, macropinocytosis induction, and effective intracellular delivery

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Introduction: [INTRODUCTION] In our research group, developing therapeutic techniques based on extracellular vesicles (exosomes, EVs) by effective usage of peptide chemistry to deliver therapeutic/diagnostic molecules into targeted cells has been focused. In this presentation, modification techniques using biofunctional peptides such as arginine-rich cell-penetrating peptides [1], artificial coiled-coil peptides with receptor targeting [2], and cell-penetrating sC18 peptides [3] derived from cationic antimicrobial protein, CAP18 for cancer targeting with macropinocytosis induction, on the EV membranes will be introduced.

I will also show effects of lyophilization of the peptide-modified EVs on their biological activity [4].

Methods: [METHODS] CD63 (EV marker)-GFP-fusion protein expressed EVs were used for cellular EVs uptake assessments. All biofunctional peptides were synthesized by Fmoc solid-phase method.

Results: [RESULTS] Macropinocytosis with actin reorganization has been shown to be crucial for cellular EV uptake [5]. We developed the methods for modification of arginine-rich CPPs or sC18 peptides on EV membranes using chemical linker techniques, and for example, arginine-rich CPPs modification can induce proteoglycan-clustering (e.g. syndecan-4) and macropinocytosis signal transduction [1]. The artificial leucine zipper peptide-modified EVs recognize the peptide-tagged epidermal growth factor receptor (EGFR) on targeted cells, leading to macropinocytotic cellular EV uptake [2]. In addition, lyophilization is a useful technique for long term storage, however, we found that lyophilization negatively affected biological functions of encapsulated proteins in the EVs after their cellular uptake [4].

Summary/Conclusion: [CONCLUSION] These techniques and findings will contribute to development for the EV-based intracellular delivery systems.

Reference: [1] Sci. Rep. 6, 34937 (2016), [2] Chem. Commun. 53, 317 (2017), [3] ChrmMedChem. 12, 42 (2017) [4] Anticancer Res. 39, 6701 (2019), [5] Sci. Rep. 5, 10300 (2015)

OS28.3

Multi-compartmented microvesicles: novel extracellular secretory organelles that release exosomes and extracellular vesicles

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Introduction: Extracellular vesicles (EV) bud from the plasma membrane (PM) as microvesicles (MV) or arise from the fusion of multivesicular bodies (MVB) with the PM to release intralumenal vesicles (ILV) as exosomes. The variety of bioactive molecules carried by EV imparts diverse functionality to EV in intercellular signalling. The biogenesis and extracellular release of these specialized messenger organelles is not well understood. To investigate, we studied endothelial cells that line the inside of blood vessels, known to release EV that support angiogenesis.

Methods: Cultured human umbilical vein endothelial cells (HUVEC) were examined by thin-section electron microscopy (EM), serial sectioning and immunogold labelling to study the structure and composition EV release sites. To obtain optimal views of cellular ultrastructure, cells were preserved by fast-freezing and a freeze-substitution.

Results: A potential release site was identified in EM thin sections as a discrete domain, up to several microns long, on the otherwise smooth HUVEC PM, where numerous bulbous membrane protrusions with thin necks were clustered. The cytoplasm in these protrusions was enriched with MVB and other vesicles and appeared to be on the verge of pinching off to release multi-compartmented MV (MCMV). Consistent with this notion, in the neighbouring extracellular space, a plethora of MCMV of 300–1200 nm with ultrastructural features matching the bulbous protrusions were observed, supporting the concept that MCMV bud from the release site. Serial sections confirmed that these extracellular MCMV were independent of cells and not linked by nanotubes or other processes. Remarkably, fusion of MVB with the MCMV membrane was directly observed, presumably caught in the act of releasing ILV (exosomes) from the MCMV. Immunogold labelling for EV markers is being used to identify proteins enriched at release sites and on released MCMV.

Summary/Conclusion: In summary, 1) MCMV bud from localized sites on the endothelial PM, 2) MCMV contain MVB, and 3) fusion of MVB to MCMV to release exosomes occurs extracellularly. MCMV can now be evaluated as a potential source of exosome and EV release that occurs after budding from the cell of origin, adding new layers of regulation to when, where and how EV are assembled and released.

Funding: This work was supported by the Division of Intramural Research of the NIH.

OS28.4

One size does not fit all: overcoming barriers to successful discovery and scaled manufacturing of therapeutic extracellular vesicles

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Introduction: Extracellular vesicles have tremendous intrinsic therapeutic potential. However, the limited availability of production cell lines presents a barrier to scaled EV production and novel EV discovery. Indeed, EV sources have been largely confined to a handful of cell types with the vast majority consisting of MSC EVs. To overcome this limitation, we developed a diverse library of hundreds of clonally pure and scalable progenitor cell lines that provides an alternative resource for EV drug discovery and production.
Methods: We harnessed the capacity of human pluripotent stem cells (hPSC) to differentiate into virtually any cell type by subjecting hPSC to a wide variety of media and culture conditions to maximize the diversity of partially differentiated cells. The resulting heterogeneous "candidate cultures" were plated at clonal density and further selected for self renewing and scalable clones. Transcriptomic analysis indicated >200 distinct progenitor lines. Cell fate potentials were mapped by screening for cell type specific marker expression in various differentiation conditions. EVs were produced using cGMP methods (TFF and SEC) and characterized by NTA, TRPS, surface marker analysis, RNA and protein content. Bioactivity assays included proliferation, migration, vascular tube network formation, senolysis, and oxidative stress.
Results: The progenitor library contained >200 distinct lines with diverse lineage fates including various types of bone, cartilage, muscle,and fat cells, as well as all blood vessel cell types. The lines displayed much longer replicative lifespans (70–100pd) than primary cell lines like MSC. Clonal purity minimized phenotypic drift resulting in maintenance of cell identity, genome integrity, differentiation capacity and bioactive EV production over extended culture. EVs were highly diverse in their RNA and protein cargo and bioactivity displaying various degrees of migratory, proliferative, angiogenic and senolytic activity. Library screening identified EVs with higher angiogenic potency than primary adult stem cell EVs.
Summary/Conclusion: We demonstrated scalable and stable production of bioactive EVs from a large progenitor cell library. Library screening resulted in discovery of novel angiogenic and senolytic EVs having diverse RNA and protein cargo. We are currently creating a corresponding library of progenitor cell EVs to accelerate discovery of novel EVs and their production cell lines.
Funding: The initial establishment of the cell library was funded in part by grants from the California Institute of Regenerative Medicine and National Institutes of Health.

OS28.5

Structural insight to interactions of extracellular vesicles and membrane active anticancer-antimicrobial peptides

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Introduction: Besides extreme potential in biomedical applications, extracellular vesicles (EVs) are also promising candidates to expand biophysical understanding of membrane active biomolecules. Their complex bilayer composition allows the better understanding of adsorbed proteins and protein coronas as well, which sets of macromolecules will likely be key for advanced EV targeted delivery. Considering cargo, membrane active peptides are interesting as these can be both drugs to be delivered, but can also facilitate cargo insertion through lipid bilayers. However, at present very little is understood regarding interactions between the peptides and the EV lipid bilayer, and between peptides and membrane associated proteins on EVs.
Methods: We have recently demonstrated, that EV membrane adsorbed proteins and their interactions can be studied by techniques such as polarized light spectroscopy, microfluidic resistive pulse sensing measurements and freeze-fraction transmission electron microscopy [1]. Furthermore, initially we studied several peptides with known antimicrobial properties and found that these strongly interact with the EV surface proteins, resulting in efficient removal of some from the lipid bilayer [2].
Results: Here we present investigation of further EV-peptide interactions also focusing on anticancer peptides, which may be promising drug candidates for targeted delivery. These studies allowed to gain insight to novel functions of several peptides, such as melittin, magainin, buforin, lasioglossin, temporin, but also provide a more detailed understanding on how EV protein coronas, or EV bilayers are affected, to such extent that they cannot exert their potential function as delivery systems.
Summary/Conclusion: The above interactions are expected to be interesting both for applicability, i.e. for selecting suitable compounds for EV processing, and also for curiosity-driven understanding of peptide functions, and EV-biomolecule interactions. Based on these we promote that peptide – EV interactions will receive increased focus in EV-engineering.

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OS29

Symposium Session 29: Late Breaking: Central Nervous System EVs

Chair: Saumya Das – Massachusetts General Hospital, Harvard Medical School

Chair: Mikin Patel – Graduate student, Department of Biological Sciences, Vanderbilt University

OS29.1

A specific non-coding RNA in extracellular vesicles from induced neuronal cells and epigenetic regulation of neurotransmission

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Introduction: Our late-breaking finding is the identification of a non-coding RNA (ncRNA) in extracellular vesicles (EVs) from neuronal cells that is a natural antisense transcript for the DBH gene and associated with epigenetic changes and gene silencing. DNA methylation in neurons is involved in memory and neurological disorders (Science 2018 361 (6409)). Earlier work found that during chronic brain infection with Toxoplasma gondii induced a decrease in norepinephrine levels and expression of the host DBH gene; and the decrease is correlated with behaviours linked to noradrenergic signalling (Infect Immun. 2019 87(2); Infect Immun. 2016 84(2861)). DBH catalyzes the production of norepinephrine from dopamine in noradrenergic neurons. We found that EVs from infected cultures suppress transcription of the DBH gene and hypermethylation of the gene in noradrenergic cells in vitro. In this study, we identify a ncRNA in the EVs from infected neuronal cells.

Methods: Neuronal cells were induced by infection with Toxoplasma gondii and EVs purified on sucrose gradients. EVs were characterised by electron microscopy and used to treat rat and human neuronal cells and expression levels of DBH mRNA and nascent DBH gene transcription were measured. Induced EVs were injected into the locus coeruleus of rats and DBH gene expression was monitored. RNA purified from EVs was screened for natural antisense transcripts (NATs) by strand-specific RT-PCR.

Results: We found that EVs purified from infected neuronal cultures induced transcriptional gene silencing (TGS) and DNA methylation of DBH in recipient neuronal cells. The induced EVs down-regulated DBH gene expression >200-fold and induced DNA hypermethylation of the DBH gene. This could be induced in the brains of recipient rats by intracerebral injection of EVs. Using a panel of strand-specific primers, antisense transcripts for the DBH gene were identified in infected cells. This permitted us to examine the RNA in purified EVs and identify a lncRNA in EVs selective for EVs from infected cultures.

Summary/Conclusion: This is the first study to find a specific neurotransmitter antisense lncRNA in EVs associated with transcriptional gene silencing and epigenetic changes in the gene. This represents a different type of neuron-to-neuron signalling than the classic chemical and electrical neurotransmission. The findings will enhance our understanding of neurological disorders (ie. schizophrenia, epilepsy, drug addiction) and how memory works.

OS29.2

Human CD4 + T regulatory-derived extracellular vesicles and associated microRNAs: role in cell-to-cell communication and involvement in the loss of immune tolerance during multiple sclerosis

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Introduction: An impairment of immune tolerance is a determining factor in multiple sclerosis (MS) and

dysregulation of CD4 + T regulatory (Treg) cell function is believed to be a major pathogenic factor. MicroRNAs (miRNAs) released by Treg cells in association with extracellular vesicles (EVs) have been shown to participate in the block of pathological immune responses by inhibiting the growth and cytokine production of CD4 + T conventional (Tconv) cells, but the molecular mechanism is still poorly characterized. Aim of the present work was to evaluate whether Treg cell-derived EV-associated miRNA signature is dysregulated in MS and whether this defect may play a role in the development of autoimmunity.
Methods: Human Treg cells isolated from blood of naïve to treatment relapsing-remitting MS patients and healthy controls were in vitro stimulated and released EVs were isolated by size exclusion chromatography and characterized by nanoparticle tracking analysis, electron microscopy and flow cytometry. EV-associated miRNAs were quantified by traditional RT-qPCR and droplet digital PCR for absolute quantification. The actual EV-mediated passage of RNA molecules from cell to cell was followed through RNA-specific fluorescent staining and Treg-derived EV effect on Tconv cell transcriptome was evaluated by RNAseq.
Results: In healthy conditions, the treatment of Tconv cells with Treg-derived EVs was shown to cause the specific repression of genes involved in the proteasome-dependent proteolytic process, known to be crucial for T cell activation. In MS, Treg-derived EVs may have lost this capability as a direct consequence of a significantly decreased expression of miR-142-3p, able to target key factors of the proteasome system.
Summary/Conclusion: Our results unveil a novel molecular mechanism for Treg-mediated maintenance of self-tolerance based on EV-associated miR-142-3p and its potential alteration in human autoimmunity.
Funding: Fondazione Italiana Sclerosi Multipla, FISM, # 2016/R/10 and # 2018/R/4

OS29.3

Revealing the proteome of Brain Derived Extracellular Vesicles isolated from human Amyotrophic Lateral Sclerosis post-mortem tissues.

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Introduction: Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterised by the deposition of misfolded proteins in the motor cortex and motor neurons. Although a multitude of ALS-associated proteins have been identified, few have been associated with extracellular vesicle (EV) trafficking, a form of inter-cellular communication. Additionally, the role of EVs in ALS is undetermined, specifically in relation to pathogenic stress granule formation, a response to cellular stress involving aggregation of non-coding RNAs and their RNA binding proteins. Therefore, this study aimed to determine the proteome of brain derived small extracellular vesicles (BDEVs) isolated from ALS subjects and identify novel ALS-associated deregulated proteins and their potential contributions to pathogenic pathways in ALS.
Methods: BDEVs were isolated from human post-mortem ALS (n = 10) and control (n = 5) motor cortex brain tissues through an ultracentrifugation protocol (Vella et al., 2017). Following thorough characterisation, BDEVs that successfully met the minimum criteria required by The International Society for Extracellular Vesicles were classified as EVs. The BDEVs subsequently underwent mass spectrometry analysis on the Thermo Scientific Q-Exactive HF with Ultimate 3000 RSLCnano. Proteins identified to be statistically significant differentially expressed then underwent validation by western blotting.
Results: A panel of 16 statistically significant differentially packaged proteins were identified in the ALS BDEVs. This included several up-regulated RNA binding proteins and a down-regulated cell adhesion molecule; DHX30, STAU1 and VCAM1, respectively. Pathway analysis revealed that the BDEVs were enriched in proteins associated with stress granule dynamics, exosomal and lysosomal pathways.
Summary/Conclusion: The identification of the RNA binding proteins in the ALS BDEVs suggests there may be a relationship between ALS-associated stress granules and ALS BDEV packaging. The packaging of stress granule associated RNA binding proteins into ALS BDEVs may be an attempt by the cells to compensate for lysosomal dysfunction caused by stress granule accumulation, a feature of ALS. Thus, these results highlight a potentially novel role for EVs in the pathogenesis of ALS.
Funding: Australian Government Research Training Program Scholarship

National Health and Medical Research Council Program Grant

Motor Neuron Disease Grant

OS29.4

Separation of microglial EVs from human brain-derived EVs

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Introduction: Microglia cells are the resident macrophages of the central nervous system (CNS) and maintain tissue homoeostasis under physiological conditions. During chronic inflammation and neurodegenerative diseases, this balance is lost. Studying microglia in the CNS is difficult in part because microglial markers like Iba1, CD11b, CD68 and CD45 are also found on macrophages. Recently, TMEM119 was reported be exclusively expressed by microglia cells in both mouse and human CNS. We have sought to determine whether brain-derived extracellular vesicles (bdEVs) display Tmem119 and betray the state of microglia of origin.

Methods: bdEVs were separated from human cortical samples and characterized per MISEV2018 guidelines by Western blot, NanoView, transmission electron microscopy and nanoflow (NanoFCM). Tmem119 antibodies were tested by immunocytochemistry (ICC) of microglia SV40 cells and differentiated U937 macrophage-like cells. LPS treatment was used to mimic inflammatory conditions. A TMEM119 antibody was then chosen for microglial EVs capture from total bdEVs. Efficiency was cross-validated by Western blot.

Results: bdEVs from human cortex had typical EV morphology and size distribution and expressed abundant CD9 and CD81 but lower CD63. Cellular marker GM130 was depleted in EVs vs tissue. Particle counts number range from 10e7-10e8 particles/100 mg tissue ICC revealed that TMEM119 was upregulated during inflammation by microglial cells but not by macrophage-like cells. A TMEM119-EV+ population was successfully separated by immunoaffinity.

Summary/Conclusion: An efficient and reproducible protocol was used for bdEV isolation, and TMEM119 could be used to separate EVs of presumed microglial origin. Further optimization is underway, with the goal of using TMEM119 to identify microglial EVs and their informative cargo in the periphery.

Funding: Michael J. Fox foundation 00900821

Poster Presentations

PT01: EVs in the Central and Peripheral Nervous Systems

Chair: Seena Ajit, PhD – Drexel University College of Medicine

PT01.01

miR-451a highly expressed in extracellular vesicles secreted from Human foetal mesencephalic neural progenitor cells is one of key therapeutic factors for Parkinsonism multiple system atrophy

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Introduction: We have successfully developed human foetal mesencephalic neural progenitor cells (hmNPCs) for long-term cultivation . The whole cultivation process of tissue preparation, cultivation, and cryopreservation has been established using strict serum-free conditions under a good manufacturing practice. Long-term-cultivated hmNPCs retained stemness and hmNPCs have excellent differentiation efficiency into dopaminergic neurons. hmNPCs reversed impaired motor function in a rodent model of parkinson's disease (PD). Based on the promising results in animal experiments, the clinical trial is under way (NCT01860794).

Multiple-system atrophy (MSA) is one of fatal neurodegenerative diseases with a combination of progressive autonomic nervous system disorders, Parkinson's syndrome, and cerebellar pyramid syndrome. There are three types of MSA such as MSA-A, MSA-C, and MSA-P. In case of a MSA-P type, it is difficult to diagnose due to the similarity of symptoms with Parkinson's disease (PD).

Methods: In Vitro and In Vivo animal MSA Model were established and Rotational behavioural was performed.

NPC cells were isolated and cultured based on Moon et al.

miRNA sequencing (BGI) was performed and several bioinformatics analyses were done.

Results: Based on the finding that hmNPCs exhibited therapeutic effects on PD, we hypothesize that hmNPCs will have a therapeutic effect on MSA-P, where sympotoms are largely common with PD. As expected, transplanted hmNPCs survived, integrated, and differentiated in to dopamine neurons in the host brain, consequently leading to the functional recovery in the MSA-P model. To further investigate the therapeutic key factors of hmNPCs in MSA-P, miRNA sequencing of the extracellular vesicles (EVs) secreted from hmNPCs was performed.

We found that miR-451a highly expressed in the NPC-derived EVs is one of key regulators of inflammatory response via NFkB pathway. We further experimentally demonstrated that miR-451a had anti-inflammatory effect on cells of MSA-P condition such that the level of CX3CL1 expression and its receptor, CX3CR1 were both decreased in the MSA-P modelled cells and in severe inflammatory environment in MSA brain.

Summary/Conclusion: Our study first showed that miR-451a in hmNPCs-EVs is one of key therapeutic factors for the recovery of brain damage through immuno-modulation in MSA-P.

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PT01.02

Patterns of apolipoproteins J, D and oxidative markers in circulating extracellular vesicles from MCI and Alzheimer's patients

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Introduction: Oxidative insults are known to be involved in the pathophysiology of Alzheimer's disease (AD). We have previously demonstrated that some blood-based redox-signature were associated to the cognitive scores in mild cognitive impairment patients and in AD (Perrotte et al., 2019). The aim of this study was (1) to evidence the presence of some oxidative markers in circulating extracellular vesicles (EVs), and (2) to compare to their plasma levels.

Methods: Plasma samples from healthy, MCI and AD patients were from the Memory Clinic of Sherbrooke (Québec, Canada). AD patients were stratified in three

groups (moderate, mild and severe) according to the MMSE and MoCA scores. Total plasma extracellular vesicles (pEVs) were isolated from plasma with the Total Exosome Isolation reagent (Invitrogen™ by Life Technologies Inc.). pEVs were then characterized by electronic microscopy, NTA, DLS and Western Blot. Antioxidants apolipoprotein J, D (apo J, ApoD), the glyoxalase-1 and protein carbonyls were determined by Western blot.

Results: In pEVs, we found that apo D levels were higher in MCI patients but not in AD patients. Protein carbonyls levels were higher later, in pEVs from moderate and severe AD while apo J levels were not different in pEVs from the five groups of patients.

In plasma, the pattern of apo J and apo D was different. The levels of apo D was not different in the five groups of patients while apo J levels were elevated in MCI and in all AD groups. Protein carbonyls were higher earlier from mild AD group, earlier than in pEVs. The levels of the detoxifying enzyme glyoxalase-1 were higher in pEVs than in plasma and were significantly decreased in early AD as compared to control subjects and MCI

Summary/Conclusion: These results demonstrate a differential regulation of redox homoeostasis in plasma and in pEVs from AD patients.

Funding: Acknowledgements: This work was supported by the Chaire Louise & André on Alzheimer's disease, Foundation Armand-Frappier (CR) and CIHR grant (TF).

PT01.03

Extracellular vesicle biomarkers of complement activation and synaptic loss in Multiple Sclerosis

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Introduction: Multiple Sclerosis (MS) is a neurological disorder characterized by white matter demyelination and extensive synaptic pathology. Recent studies have shown synaptic loss in the grey matter of MS brains in the absence of demyelinating lesions which could account for disease progression independent of demyelinating episodes. Opsonization of synapses with complement components is a mechanism by which phagocytic cells normally prune synapses, but, when occurring in excess, it may underlie pathologic synapse loss. We sought to identify blood-borne biomarkers of hypothesized complement-mediated synaptic loss in MS using circulating neuronal-enriched and astrocytic-enriched extracellular vesicles (NEVs and AEVs).

Methods: NEVs and AEVs were immunocaptured in parallel from the plasma of 60 MS patients (45 with Relapsing Remitting, 15 with Progressive MS) and 31 healthy controls, targeting the neuronal-specific marker L1CAM and the astrocyte-specific marker GLAST, respectively. We measured the protein levels of pre- and post-synaptic proteins synaptopodin and synaptophysin in NEVs using ELISAs and multiple complement cascade components (C1q, C3, C3b/iC3b, C4, C5, C5a, C9, Factor B, Factor H) in AEVs using a Luminex array.

Results: Synaptopodin and synaptophysin protein levels in NEVs of MS patients compared to controls were markedly reduced (2.5-fold; $p < 0.0001$ for both), whereas multiple complement components in MS AEVs were markedly increased (C1q: 2.5-fold change; C3: 1.25-fold change; C3b/iC3b: twofold change; C5: 1.4-fold change; C5a: 1.4-fold change; Factor: 1.5-fold change; $p < 0.0001$); differences were not observed in total circulating EVs or neat plasma. Strikingly, we found the NEV-associated synaptopodin/synaptophysin and the AEV-associated complement levels to be negatively correlated in people with MS (synaptopodin vs: C1q, $r = -0.7$ and $p < 0.0001$; C5, $r = -0.6$ and $p < 0.0001$; Factor H, $r = -0.46$ and $p < 0.0002$/synaptophysin vs: C1q, $r = -0.75$ and $p < 0.0001$; C5, $r = -0.65$ and $p < 0.0001$; Factor H, $r = -0.52$ and $p < 0.0002$), but not in controls.

Summary/Conclusion: Circulating EVs provide markers of synaptic loss and complement activation in MS and suggest a link between astrocytic complement production and synaptic decline.

Funding: This research was supported in part by the Intramural Research Program of the National Institute on Ageing, National Institutes of Health.

PT01.04

Methylglyoxal and glyoxal affect the protein cargoes in neuronal-derived extracellular vesicles

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Introduction: Advanced glycation end-products (AGEs) and their receptor RAGEs are known to be involved in the pathogenesis of Alzheimer's disease (AD). Methylglyoxal (MG) or glyoxal (GO) are the

precursors of AGEs and particularly N-(1-carboxymethyl)-L-lysine (CML), the most abundant AGEs. MG induced tau hyperphosphorylation and causes hippocampal damage and memory impairment in mice.

The aim of our study was to analyse the effects of MG and GO on the neuroprotective, neurotrophic factors, inflammatory and neurodegenerative markers in the human cell line SK-N-SH and their release into the neuronal derived-EVs.

Methods: Briefly, SK-N-SH cells were incubated in FBS free media with MG and GO (0.5 mM) for 24 hours. Neuronal derived-EVs (nEVs) from culture media were isolated as previously described (Haddad et al. 2019). nEVs were characterized by electronic microscopy, NTA and by Western Blot.

Cellular and nEVs concentrations of BDNF, PRGN, NSE, APP, MMP9, ANGPTL-4, LCN2, PTX2, S100B, RAGE, DJ-1 and alpha synuclein were determined by a Luminex assay from R&D Systems, Inc. Aβ1-40, Aβ1-42, pTau T181 and total tau levels were measured also with luminex assay from EMD Millipore Corp.

Results: We found that both AGEs precursors, at non toxic concentration, reduced the neuronal levels of NSE with no effect on BDNF, PTRX-2, LCN-2, DJ-1, on neurodegenerative markers and on CML. GO decreased the levels of PRGN, APP, ANGPL-4 while the expressions of MMP-9 and ANGPL-4 were, respectively lower and higher in the presence of MG.

MG and GO greatly reduced the release of LCN-2 by neuronal cells in nEVs. BDNF and PRGN in nEVs were reduced in the presence of GO. Both MG and GO did not modify the release of NSE, APP, MMP9, AGNTL-4, PTX-2, DJ-1, Aβ, pTau and CML in nEVs.

Summary/Conclusion: Our data demonstrated that MG and GO differently affect the content of some protein cargoes in nEVs and suggest that targeting MG and GO may be a promising therapeutic strategy to prevent neurodegeneration.

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PT01.05

Cigarette smoke extract alters extracellular vesicle release and circular RNA expression

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Introduction: Peripherally circulating brain-derived extracellular vesicles (EVs) and their encapsulated RNAs may serve as biomarkers for HIV-associated neurocognitive disorders (HAND). However, rates of cigarette smoking are significantly higher in HIV+ individuals than the general population, and smoking can modulate the expression of these markers. To better understand how cigarette smoke might modulate RNA expression and EV release, we examined several CNS-derived cell lines, representing astrocytes (U87 MG), microglia (SV40), and oligodendrocytes (HOG).

Methods: Cigarette smoke extract (CSE) was prepared by bubbling through culture medium using a standardized and published method. All cell types were exposed to either 0% or 50% CSE for 24 hours. Cell viability was assessed by MuseTM Cell Analyser, and EVs were isolated from culture conditioned media (CCM) by size exclusion chromatography. The void (fractions 1–6), EV (7–10), and protein (11–14) enriched fractions were pooled and concentrated. EVs were characterized by transmission electron microscopy (TEM), microfluidic resistive pulse sensing, and Western blotting. Total RNA was isolated from cells and circular RNA (circRNA) expression was assessed with a circRNA Microarray.

Results: In response to CSE exposure, cell viability was only slightly reduced for all cell types. TEM images validate the presence of vesicles in the EV fractions, and their absence in the void and protein fractions. Spectradyne particle counts indicated CSE exposure substantially increased the CCM particle count in the EV fraction when compared with control. The presence of expected EV markers (CD63, CD81, and TSG101) in the EV fractions, and their absence in the void and protein fractions was observed via Western blot. Intracellular circRNA expression was significantly altered in all three cell lines.

Summary/Conclusion: CNS cells display physiologic responses to CSE that include vesiculation pathways and significant alterations in circRNA expression. We are now studying the effects of CSE exposure on circRNA expression in released EVs.

Funding: This work is supported by DA040385, DA047807, and AI144997.

PT01.06

A method for exosomal RNA extraction from paired human brain and blood specimens

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Introduction: Diagnosis and treatment of neuropsychiatric disorders has made little progress in the last half-century likely in large part due to the absence of a scalable technique to profile the complex biological activity of the brain in a living person. Exosomes are nanovesicles 30–150 nm in size that mediate intercellular communication and contain proteins, lipids, and nucleic acids. It has been shown that brain derived exosomes can be found in peripheral blood, but determining whether peripheral exosomes truly reflect ongoing brain processes has to date not been possible due to the absence of paired living brain and blood specimens. Here, we present a novel method for paired sampling of the dorsolateral prefrontal cortex (DLPFC) and peripheral blood from living human subjects for exosomal RNA profiling.
Methods: Informed consent, approved by the IRB at the Icahn School of Medicine at Mount Sinai, was obtained for patients undergoing Deep Brain Stimulation (DBS). Paired brain and blood specimens were collected from 8 patients at two deep brain stimulation (DBS) electrode implantation procedures: left hemisphere followed by right hemisphere (total of 30 samples). We developed protocols to profile RNA from exosomes of brain tissue extracellular matrix (ECM) and peripheral blood. Exosomes were isolated via our in-house protocol using ultracentrifugation. RNA was then extracted from the exosomes using the Qiagen miRNeasy Mini Kit protocol. Quality control (QC) was performed to determine whether RNA obtained was sufficient for next-generation sequencing.
Results: We demonstrate the safety of a novel procedure to sample the brain in living human subjects. Bioanalyzer traces and QC data show a mean total RNA of 14.83 ng (range 1.72–137.17 ng) and no samples fell below the threshold required for library preparation and sequencing (10 pg) determined by in-house optimization on the SMART-seq v4 Ultra low input kit.
Summary/Conclusion: To our knowledge, we have performed the first study to sample pairs of DLPFC and blood from living human subjects for exosomal RNA for subsequent next-generation sequencing. Ongoing analyses by our group promise to establish peripheral exosomal RNA transcripts reflective of brain activity. This non-invasive approach to probing neurobiology in the living human brain may facilitate the development of exosome-based diagnostics for neuropsychiatric disorders.

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PT01.07

MicroRNAs from adipocyte-derived small extracellular vesicles are associated with neurodegeneration

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Introduction: The relationship between obesity and dementia is complex. While obesity in middle age triples the risk of dementia 30 years later, many patients with Alzheimer's Disease (AD) are cachectic, and a decline in adiposity portends progression of dementia. This suggests adipose-derived factors are important to nervous system homoeostasis. We previously showed that adipocyte-derived small extracellular vesicles (ad-sEVs) induce pathologies critical to developing obesity-related diseases and may provide a mechanistic link between adiposity and dementia. We hypothesized that altered expression of ad-sEV microRNAs involved in neurodegenerative pathways is associated with more severe cognitive impairment
Methods: We studied serum and cerebrospinal fluid (CSF) from 19 participants with AD and 14 non-AD controls. Ad-sEVs were isolated from samples by precipitation and immunoselection. Ad-sEV microRNA expression was profiled in both biofluids and compared.
Results: Serum and CSF microRNA expression correlated strongly (r2 = 0.98). In serum, 189 microRNAs were differentially expressed by a Fold Change $\geq|1.1|$ in the AD and control groups ($p \leq 0.1$) and 251 microRNAs were differentially expressed in CSF. Using Ingenuity Pathways Analysis, we identified mRNAs expressed in nervous system tissue that are targeted by the differentially expressed microRNAs. The mRNAs map to 145 diseases and functions; neuronal cell death, neurodegeneration, and neuronal growth and developmental pathways are highly represented. Of the 189 differentially expressed microRNAs in serum, 6 were moderately correlated with participants' score on the Mini-Mental State Exam, a test of cognitive function ($rs = |0.488–0.609|$). As validation, RenCell Cx cortical derived neuronal stem cells had decreased doubling time when exposed to ad-sEVs from obese adipose tissue in vitro.

Summary/Conclusion: These findings support our hypothesis that altered expression of circulating ad-sEV microRNAs are involved in neurodegenerative pathways associated with cognitive impairment. These findings support using serum ad-sEVs as a surrogate for CSF ad-sEVs. Functional validation is underway to define the connection between ad-SEVs and AD. Understanding the link between obesity and AD is crucial as the population ages and the global obesity epidemic grows.
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PT01.08

Expression of extracellular vesicles after acute traumatic brain injury: an exploratory flow-cytometry study

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Introduction: Coagulation derangements related to disseminated intravascular coagulation (DIC) are common after TBI and contribute to secondary neural injury. Extracellular vesicles (EVs) are released from all cell types, including platelets, endothelium, and lymphocytes, which are responsible for DIC. We hypothesized that specialized flow cytometry techniques could identify a unique EV signature of DIC in acute TBI.
Methods: Using a modified flow cytometry instrument for detection of small particles, fluorescence panels were created to assess for EVs from endothelial cells (CD144, CD105), platelets (CD31, CD62p, CD41a, CD42b), and erythrocytes (CD235) as well as brain biomarkers (S100b, UCHL-1, GFAP, tau and NSE) and T-lymphocytes (CD3, CD4, CD8, CD31). Samples were prepared in Trucount tubes to determine volume and treated with triton to confirm presence of EVs.
Results: 13/17 study patients and 16/20 controls were male. 76% of study patients presented with a Glasgow Coma Scale of 15. In the hypercoagulability panel, of the 10 subsets with statistically significant differential expression, 4 involved S100b+ and were elevated in patients. Platelet-derived CD41a EVs and UCH-L1 EVs were significantly elevated in controls in 7 EV subsets identified in the brain-specific panel. Finally, CD3+/31+ EVs, derived from T-cells and identified in the endothelial/T cell panel, are significantly lower in patients suggesting CNS recruitment.
Summary/Conclusion: Endothelial and platelet/erythrocyte EVs may be elevated early after TBI. S100B-carrying EVs are significantly elevated in circulation of TBI patients; if reproducible, this signature profile may be informative for diagnosis and risk stratification. Further study is warranted to evaluate whether this expression correlates with secondary microvascular brain injury.
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PT01.09

Enrichment of miR-451a in CNS extracellular vesicles following impairment of the blood brain barrier

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Introduction: Extracellular RNAs (exRNAs) are present in essentially all biofluids and include all types of RNA including miRNA. To enhance their stability outside of the cell, exRNAs are bound within ribonucleoprotein complexes or packaged into extracellular vesicles (EVs). The blood brain barrier (BBB) is a dynamic interface between the systemic circulation and the CNS and is responsible for maintaining a stable extracellular environment for CNS cells. The intent of this study was to determine if EVs and their contents are transferred from the peripheral circulation to the CNS under conditions of an impaired BBB.
Methods: The BBB of mice was disrupted by hyperosmolar mannitol injections. To validate that the BBB has been disrupted with mannitol, intravenously-dosed [13 C]-sucrose was increased in the forebrain by 14-fold with mannitol compared to sham treated mice. EVs were isolated from the forebrain, hindbrain and spinal cord following gentle tissue lysis and differential ultracentrifugation. EVs were validated by NTA, TEM and western blotting. miR-451a, a miRNA that is highly abundant in erythrocytes, was measured in the EVs by qPCR.
Results: qPCR showed that miR-451a in CNS tissue EVs increased with mannitol treatment in the forebrain, hindbrain and spinal cord by 15-, 1.6- and two-fold respectively. qPCR analysis of mRNA from reported miR-451a target genes showed reduced target gene expression with mannitol.
Summary/Conclusion: We demonstrate that EVs containing miR-451a, a highly abundant miRNA present

within erythrocytes and erythrocyte EVs, is enhanced in the CNS upon BBB disruption.

PT01.10

Astrocyte-derived extracellular vesicles in morphine tolerance

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Introduction: Opiates, such as morphine are used extensively in the clinical setting owing to their beneficial effects. Paradoxically, however, the prolonged use of morphine often results in the development of tolerance, drug addiction, and ultimately leading to various comorbidities associated with drug abuse. Although great efforts have been made, at present there is no treatment. The sonic hedgehog (SHH) plays a key role in brain development, and brain cells fine-tuning processes such as their proliferation, patterning, and fate specification Recent findings have demonstrated that inhibition of the SHH signalling prevents morphine tolerance in rodent models. We thus hypothesize that extracellular vesicles (EVs) derived from morphine exposed astrocytes and their cargo such as SHH are critical for the development of morphine tolerance.
Methods: Mice were received either saline or chronic morphine injection with escalating doses of morphine for 5 days (subcutaneously; 10 mg/kg, day 1, 20 mg/kg days 2–3, and 40 mg/kg days 4–5). The development of tolerance was assessed by measuring the tail-flick latency using Tail Flick Analgesia Metre (LE7106, Harvard Apparatus). EVs were isolated using either differential ultracentrifugation from astrocyte conditioned media or gradient ultracentrifugation from brain tissues. Western blotting and qPCR were performed to determine the expression/activation of SHH signalling pathway components.
Results: Our data showed that the levels of SHH protein were upregulated in morphine exposed astrocyte-derived extracellular vesicles (morphine-ADEVs). Furthermore, SHH containing morphine-ADEVs activated SHH signalling in astrocytes. Our in vivo study further demonstrated the upregulation of SHH, as well as the activation of SHH signalling, in astrocytes of morphine-administered mice.
Summary/Conclusion: These findings thus demonstrated an autocrine mechanism for SHH pathway activation in astrocytes associated with morphine tolerance. These findings could pave the way for the development of SHH signalling pathway targeted strategies in the prevention and treatment for substance use disorders.

PT01.11

Biophotonics-based platforms for the evaluation of circulating extracellular vesicles as biomarkers of neurodegeneration in Alzheimer's disease

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Introduction: In the search for novel and non-invasive biomarkers of Alzheimer's disease (AD), both circulating brain-derived Extracellular Vesicles (EVs) and whole serum represent a valuable integration of the currently used classification system. To face the technological challenge of EVs and serum analysis, we propose the use of biophotonics techniques as reliable, sensitive, fast and label free methods, potentially useful in tailoring pharmacological and rehabilitation treatments.
Methods: Circulating EVs, isolated by SEC, and serum samples were collected from 10 healthy subjects (HC) and 10 AD patients. All subjects were asked to complete Montreal Cognitive Assessment scale and MRI examination. Surface Plasmon Resonance (SPR) was performed in order to detect EVs coming from neurons, astrocytes, oligodendrocytes and microglia and to characterize each of them for the amount of ganglioside M1 (GM1), Aβ and TSPO expressed on their surface. Serum analysis was performed using a Raman microscope through the Surface Enhanced Raman Spectroscopy (SERS) effect by mixing serum with Ag nanoparticles. The Pearson's correlation index was used to assess the linear correlation between SPRi data and clinical, MRI data and data obtained from multivariate analysis (MVA) of SERS spectra.
Results: The SPR analysis of EVs showed that the selected bioactive molecules are differently loaded on neural EV populations and that their amount is increased on total EVs in AD patients compared to HC. We observed a significant correlation between MVA data from SERS and the presence of Aβ on neuronal and microglial EVs and of TSPO on neural EVs, measured with the SPR array.
Summary/Conclusion: Thanks to our methodological innovation we have verified the potentiality of EVs as AD biomarkers, correlating biophotonics blood-based analysis with clinical data. This platform could provide a powerful tool for the evaluation of AD neurodegeneration.

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PT01.12

Raman profiling of extracellular vesicles as new blood-based biomarker for brain disorders: focus on Parkinson's disease

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Introduction: Extracellular vesicles (EVs) play a pivotal role in brain homoeostasis and intercellular communication in both physiological and pathological conditions. In Parkinson's disease (PD), EVs are key players in the transfer of α-synuclein, with blood EVs reported to undergo proteomic modifications. Nonetheless, the detection and characterization of the EV cargo is technologically challenging, limiting the use of EVs as biomarkers so far. Herein, we propose Raman spectroscopy for the label-free, bulk characterization of blood EVs in PD patients.

Methods: EVs were isolated by SEC and ultracentrifugation from the serum of 18 healthy subjects (HC) and 22 PD patients. In all patients, the severity of PD was evaluated with the Unified Parkinson's Disease Rating Scale (UPDRS) part III and with Hoehn and Yahr scores (HY). After proper EV characterization following MISEV2018 guidelines, Raman analysis was performed. The Raman microspectroscope was used with a 532 nm laser in the spectral ranges 600–1800 cm-1 and 2600–3200 cm-1. Data from HC and PD patients were compared by multivariate statistical analysis (PCA-LDA).

Results: The Raman analysis of EVs highlighted differences in the biochemical profile of the two groups, with the main variations in the spectral regions related to proteins, lipids and saccharides. A preliminary estimate of the accuracy of Raman profiling of blood EVs for PD diagnosis was obtained, demonstrating an accuracy of 71%. Even more interestingly, we demonstrated the correlation between the Raman spectra and the clinical scales (UPDRS and HY) used to stratify PD patients.

Summary/Conclusion: In conclusion, the biochemical signature of blood EVs can be detected by Raman spectroscopy in PD patients and the EV spectral modifications can be related to their clinical status. These data suggest the possibility to use the Raman profile of circulating EVs as a biomarker for brain disorders, complementary to other specific molecular markers.

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PT01.13

Impact of circulating extracellular vesicles on brain functions and behaviours

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Introduction: Peripheral immune alterations have been described in psychiatric disorders such as schizophrenia, depression, and autistic spectrum disorders. In addition, behavioural changes have been observed in various immunodeficient animal models. However, the mechanisms by which peripheral immune system influences brain development and function are not well understood. In this study, we explored the mechanisms by which circulating extracellular vesicles (EVs) mediate immune-brain communication and influence mouse behaviours.

Methods: Mice deficient for Rag1 or Rag2 gene (Rag KO mice) were used as a model to study the effects of loss of adaptive immune cells (T and B cells) on brain cellular phenotypes and behaviours. Circulating EVs were collected from their sera and analysed by using electron microscopy, nanoparticle tracking assay, and Western blotting. Brain cellular phenotypes were assessed by immunofluorescent staining and gene expression analysis. Behavioural phenotypes of Rag KO and WT mice were examined in social interaction test. In vivo transfer of EVs was performed to see its effects on behavioural alterations of Rag KO mice.

Results: Rag KO mice displayed social behavioural deficits, accompanying by enhance c-Fos immunoreactivity and altered microglia morphology in the medial prefrontal cortex (mPFC). Circulating EVs were also affected in these mice and lacked the expression of

markers for T cells. A set of microRNAs (miRNAs) in circulating EVs were diminished in Rag KO mice. In vivo transfer of circulating EVs rescues the social behavioural deficits of Rag KO mice and ameliorate the c-Fos immunoreactivities in mPFC of Rag KO mice.

Summary/Conclusion: Our data showed that circulating EV profiles were altered in mice lacking adaptive immune cells and, accordingly, showing social behavioural deficits. Notably, our in vivo experiments suggest that circulating EVs may contribute to social behaviours. Further study will provide a novel biological insight into the mechanisms underlying peripheral-to-brain immune communication via EVs.

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PT01.14

MicroRNA profile of circulating extracellular vesicles are associated with upregulation of neuroinflammatiory signalling pathway in aged animals

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Introduction: The involvement of neuroinflammation on ageing process is widely recognized. Extracellular vesicles (EVs), such as exosomes, are able to cross the blood-brain barrier and were related to neuroinflammation. In this context, EVs have been considered a potential mechanism of spreading molecules, including microRNAs (miRNAs) that can promote mRNA degradation or inhibit translation of their targets. Our aim was to investigate the miRNA profile of circulating total EVs during ageing process and their impact on canonical pathways.

Methods: The Local Ethics Committee (Comissão de Ética no Uso de Animais – UFRGS; n 29818) approved all animal procedures and experimental conditions. Plasma was obtained from Wistar rats (3 and 21 months-old) and total EVs were isolated. EV microRNA isolation and microarray expression analysis was performed to determine the predicted regulation of targeted mRNAs.

Results: The analysis of global microRNA expression revealed 48 differentially expressed microRNAs ($p < 0.05$; fold change of $\geq |1.1|$); 18 miRNAs were up-regulated and 30 were down-regulated in circulating total EVs from aged animals compared to young-adult ones. A conservative filter was applied on Ingenuity Pathway Analysis (IPA) and only experimentally validated and highly conserved predicted mRNA targets were used. IPA showed that neuroinflammation signalling is ranked among the top canonical pathway impacted by differentially expressed microRNAs and is upregulated in aged animals ($p < 0.0001$; z-score: 3.413). The differentially expressed miRNAs impacted 32 molecules in the neuroinflammation pathway. Interestingly, the ion channel GRIN2B is predicted to be up regulated and is a target of many EVs miRNAs; in accordance with our results GRIN2B was previously related to neurodegenerative diseases. Moreover, let-7a-5p is predicted to be downregulated and target all the 32 molecules of the neuroinflammation signalling pathway. Previous studies have correlated let-7a-5p and neurodegenerative diseases.

Summary/Conclusion: Our data suggest that circulating total EVs cargo, specifically miRNAs, are altered by ageing and impact neuroinflammation pathway, suggesting the involvement EVs miRNA on ageing-induced susceptibility of neurodegenerative diseases.

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PT02: EVs in Dermatology

Chair: My Mahoney – Thomas Jefferson University

Chair: Fabio Quaglia – Thomas Jefferson University

PT02.01

Keratinocyte-derived exosomal packaging drives conversion of injury-site macrophage in granulation tissue of murine wound

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Introduction: Bidirectional cell-cell communication via paracrine mechanisms is critical for wound healing. A new paradigm involving exosome-borne distinctive repertoire of cargo such as miRNAs has emerged as a predominant mechanism of cellular communication at the site of injury. Unlike other shedding vesicles of similar size, exosomes selectively package miRNA by sumoylation of heterogeneous nuclear ribonucleoprotein (hnRNP).

Methods: Keratinocyte-derived exosomes (Exoκ) were genetically labelled with fluorescent reporter (GFP) using tissue nanotransfection. Purified, GFP-labelled Exoκ were isolated from dorsal murine skin and wound-edge tissue by differential ultracentrifugation followed by affinity selection using magnetic beads. Distributions of intact exosome were analysed using a prototype Jarrold-geometry charge-detection mass spectrometer to directly measure differences in particle mass and charge distributions. Complementary MS and ion mobility spectrometry (IMS)-MS experiments have been used to characterize surface glycans and glycopeptides. To selectively inhibit miRNA packaging within the Exoκ in vivo, pH-responsive targeted siRNA functionalized lipid nanocarriers (TLNκ) were designed using materials that have prior history of FDA approval for human use.

Results: An increase in mass/charge ratio with glycan binding sites on the surface of wound-edge Exoκ were observed compared to dorsal skin Exoκ. Wound-edge Exoκ were selectively taken up by the macrophages in the granulation tissue (n = 6). Keratinocyte targeting siRNAhnRNP functionalized lipid nanocarriers (TLNκ) were designed with encapsulation efficiency of 94.3%.

Application of TLNκ encapsulating siRNA of hnRNP (TLNκ/si-hnRNP) to murine dorsal wound-edge significantly inhibited the expression of hnRNP by 80% in epidermis compared to control (TLNκ/si-control)(n = 10). Moreover, mice treated with TLNκ/si-hnRNP showed impaired barrier function, with significant presence of macrophage in granulation tissue at day 10, suggesting impaired conversion of macrophage in the granulation tissue.

Summary/Conclusion: This work provides a novel insight wherein exosomes of keratinocyte lineage are recognized as a major contributor that directs macrophage conversion in granulation tissue for wound healing.

PT02.02

Multifaced effects of Milk-exosome (Mi-Exo) as modulator of scar-free wound healing

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Introduction: Recently, milk exosome (Mi-Exo) has been focused particularly on the possibility of oral distribution for therapeutic agents. However, studies related to the cosmeceutical effects associated with Mi-Exo are fairly limited. The purpose of this study is to suggest the anti-oxidant and anti-inflammatory effect of Mi-Exo and possibility that can be induced by scar free healing by micro RNA in Mi-Exo.

Methods: The characteristics of the extracted Mi-Exo were verified by size measurement, morphological characteristics through Cryo-EM and western blot. For antioxidant experiments, an ABTS assay was performed. Next, mRNA expression through four major cytokines (TNFα, IL-6, COX-2, iNOS) was used to evaluate anti-inflammatory effects. Finally, cell migration assay was performed to confirm the effect of scar-free healing and the detection of miR-30b in Mi-Exo and VEGF mRNA expression confirmed.

Results: Mi-Exo using 1% acetic acid extraction showed the highest yield. The average size of the exosomes is approximately 110 nm, confirmed the typical double membrane vesicle. As a result of antioxidant experiments, it was confirmed that the treatment of exosomes of 10^10 particles showed about 65% antioxidant activity. When 10^10 particles were treated, RNA expression of cytokines showed about 2 times more inhibitory effect than control. ELISA test results also confirmed that the concentration-dependent decrease. The activation of the raw cell less proceeded as the treated Mi-Exo increased. The cell scratch assay cells did not close the cells as the number of milk exosomes increased (wound closing % of 10^10 particle = 4.7%). and miR-30b in milk exosomes was detected at Ct value = 22.

Summary/Conclusion: The antioxidant and anti-inflammatory effects of Mi-Exo showed the greatest efficacy when 10^10 particles were treated. In addition, it induced to scar free healing rather than wound healing. Mi-Exo has great potential as a superior natural material in the future cosmeceutical field.

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PT02.03

Extracellular vesicles in human milk expose tissue factor and promote coagulation

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Introduction: Tissue factor (TF), a transmembrane protein, initiates coagulation by binding and activating coagulation factor VII (FVII). TF is associated with extracellular vesicles (EVs) in saliva and urine, but it is unknown whether also human milk (HM) contains EVs exposing coagulant TF.

Methods: HM was collected from six healthy nursing women with informed consent. EVs were isolated by ultracentrifugation and size exclusion chromatography (SEC). The presence of TF antigen exposing EVs was studied by Western blot, flow cytometry, cryo-electron microscopy (cryo-EM), and surface plasmon resonance imaging (SPRi). The ability of TF exposing EVs to trigger coagulation was investigated with a plasma fibrin generation test (FGT), performed in the absence or presence of antibodies against TF or FVII(a).

Results: Addition of HM to plasma shortened the plasma clotting time, even when HM was highly diluted. After ultracentrifugation of HM, both TF antigen and TF activity were detected in the EV-containing pellet. After SEC, TF antigen and TF activity were present in the EV-containing fractions 8 and 9. The presence of TF-exposing EVs in these SEC fractions was confirmed by Western blot (CD9, CD63 and TF), flow cytometry, SPRi, and FGT. In addition, the presence of EVs in HM was confirmed by cryo-EM.

Summary/Conclusion: We demonstrate the presence of highly procoagulant TF-exposing EVs in HM.

Funding: Y.H. was supported by a scholarship from the China Scholarship Council (CSC).

J. T. was supported by an unrestricted travel grant from the International Society on Thrombosis and Haemostasis.

PT02.04

Scalable isolation of EVs from different probiotic strains with potential as cosmetic ingredients

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Introduction: Extracellular vesicles (EVs) are increasing their application in a number of fields. Recently, it has been shown that skin health may be affected not only by commensal skin bacteria, but also by the EVs that they secrete. However, because most of the efforts have been directed to the characterization and evaluation of EVs, the scaling up of the production process remains a bottleneck at the industrial level. In this work, the goal was to evaluate the potential applications of EVs produced by different probiotic strains commonly used in the cosmetic field, considering the economic and technical viability of the process.

Methods: To meet our goal, a standardized workflow was defined to isolate EVs from probiotic strains such as Lactobacillus and Bifidobacterium species, that have demonstrated cutaneous immuno-regulatory effects.

The different bacterial strains were produced under standard culture conditions. To isolate the secreted bacterial EVs, different chromatographic techniques were performed starting from clarified growth medium. Then, EVs were evaluated in vitro for a number of biological effects related with skin health.

Results: The EV yields obtained after downstream processing were calculated for each strain and isolation technique by means of nanoparticle tracking analysis (NTA) and total protein content. Moreover, EVs were visualized by electron microscopy. The in vitro evaluation of isolated EVs was based on changes in the expression of five biomarkers related with anti-ageing, anti-inflammatory and whitening effects using distinct skin cell types to identify possible cosmetic claims that could be associated to each probiotic source.

Summary/Conclusion: The potential of EVs obtained from probiotic strains as cosmetic active cell-free ingredients was preliminarily assessed with this work, where the process yield and cosmetic function were evaluated. However, additional experiments will be needed in order to increase and optimize the productivity of each step of the EV manufacturing process.

PT02.05

Acerola derived exosome-like nanovesicles enhances the repair of ultraviolet B-induced DNA damage in cultured skin fibroblasts

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Introduction: Acerola (Melpighia emarginata DC.) is a fruit is known to contain not only high amounts of ascorbic acid but also various nutritional components such as carotenoids and polyphenols. Previous reports showed the acerola juices are able to confer protection against Ultraviolet radiation B (UVB), to improve barrier function of skin. UVB is the main cause of DNA damage in epidermal cells, generating several types of pro-mutagenic lesions, like cyclobutene prymidine dimers (CPDs) and prymidine (6–4) prymidinone photoproducts (6–4PPs): if not repaired, this DNA damage leads to skin cancer. In this study, we investigated the biological property of the acerola derived exosome-like nanovesicles (ADENs), aiming to clarify the involvement of ADENs in repair of UV-induced DNA damage.

Methods: Normal human dermal fibroblasts (NHDFs) were purchased from Lonza Inc. The exosome-like nanovesicles were isolated from acerola juices using exoEasy Maxi kit (Qiagen). The morphology and size distribution of ADENs were checked by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA, NanoSight LM10, Malvern). NHDFs were exposed to UVB (1 mJ/cm2) with pre- or post-ADENs. Effect of UVB was assessed by examining cell viability, cell morphology, and DNA damage levels through biochemical assays, microscopy and protein expression studies.

Results: Purified ADENs were compatible with NTA or TEM for assessing the nanovesicle size range and concentration (200–400 nm). When NHDFs were added with ADENs and incubated at 37°C for 48 h, there was no effect of ADENs on cell proliferation of NHDFs. We found that ADENs treatment to UVB exposed NHDFs significantly reduced CPDs and 6–4PPs DNA adduct formation. Present results showed that ADEN treatment prevented UVB induced DNA damage in NHDFs.

Summary/Conclusion: We confirm that ADENs have the effect of repairing DNA damage caused by UVB. These results provide that ADENs can be a new source to protect human skin from UV-induced skin cancer.

PT03: Engineering and Loading EVs

Chair: André Görgens – Department of Laboratory Medicine, Clinical Research Centre, Karolinska Institutet

PT03.01

EV-mediated HOTAIR delivery for increased angiogenesis

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Introduction: Introduction: Despite the development of a variety of therapies, complex wounds resulting from disease, surgical intervention, or trauma remain a major source of morbidity. Extracellular vesicles (EVs) derived from mesenchymal stem/stromal cells (MSCs) have been shown to improve wound healing, especially via enhanced wound angiogenesis. However, despite their clearly established potential, EVs have limitations that may limit clinical relevancy, such as low potency.

Hypothesis: Increased expression of pro-angiogenic lncRNA HOTAIR within MSC EVs enhances their pro-angiogenic effects and thus their wound healing properties.

Methods: Methods: HOTAIR was overexpressed in human dermal microvascular endothelial cells (HDMECs) to determine any molecular or functional pro-angiogenic effects. Anti-angiogenic miRNAs and angiogenic mRNA levels were quantified by RT-qPCR. Effects of HOTAIR on proliferation of HDMECs was also determined. HOTAIR was then loaded into MSC EVs by delivering a CMV-based HOTAIR plasmid to MSCs for endogenous loading via a concentration gradient. EVs were collected by differential centrifugation. HOTAIR content within EVs was confirmed by gel electrophoresis and RT-qPCR. Effects on migration of HDMECs by HOTAIR-loaded MSC EVs were determined using a scratch assay.

Results: Results: Overexpression of HOTAIR decreased miR-29 c and miR-107, while increasing VEGF and HIF-1a. HDMEC proliferation was also increased in HDMECs overexpressing HOTAIR ($p < 0.01$). HOTAIR was visually confirmed in HOTAIR-loaded MSC EVs by gel electrophoresis, but was undetectable in unmodified MSC EVs. RT-qPCR confirmed a 900-fold increase of HOTAIR compared to control MSC EVs. HDMECs showed a more statistically significant rate of gap closure when treated with HOTAIR-loaded EVs ($p < 0.01$) than compared to control MSC EVs ($p < 0.05$).

Summary/Conclusion: Summary: Loading lncRNA HOTAIR into MSC EVs is achievable by a concentration gradient-dependent method and offers potential to enhance the angiogenic properties of MSC EVs.

PT03.02

Nanomaterial labelling of exosomes for cell biology

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Introduction: Exosomes are vesicles secreted by many, if not all, cell types and have been known about for decades. Among larger micro vesicles that are produced directly from the cell membrane, the small (30–150 nm), exosomes are similar in size to a virus surrounded by a lipid bilayer. We and others have demonstrated that exosomes contain proteins, lipids, RNA, and DNA, making them promising materials for diagnosing and treating diseases, including many cancers such as brain cancer. In addition, exosomes from neurons and glial cells represent a novel type of intercellular communication. However, their size makes them hard to track with traditional fluorescence microscopy. To address this, we developed photothermal microscopy (PTM), which uses gold nanomaterial labelling to track exosomes' interaction with and effect on cells/tissue.

Methods: Exosomes secreted by tumour cells and general exosomes found in the blood were isolated using differential ultracentrifugation or a commercially available kit (Invitrogen). Next, the exosomes were characterized by (TEM), (NTA), and western blotting to determine shape, size, morphology and the protein profile in the exosomal membrane. After characterization, the exosomes were labelled with gold

nanoparticles via sonication. Next, the samples were washed, and the exosomes were labelled with fluorescence dye to stain the membrane. After staining and labelling, the exosomes were added to U87 cells in culture and incubated for 3 h. They were then fixed by 4% paraformldehyde and imaged by PTM.
Results: PTM found that exosome-cell interactions are exosome-type dependent, as U87 cells took up exosomes from other U87 cells but not human serum exosomes. This suggests that exosome uptake is a selective process and depends on the source of the donor cells.
Summary/Conclusion: Exosomes can be labelled with gold nanoparticles via sonication then successfully tracked by PTM to study the effect of exosome source on exosome-cell interactions and communication. Cells incubated with U87 exosomes took the vesicles up rapidly, while cells incubated with serum exosomes had little uptake. PTM will help us design selective exosome-based strategies to treat different conditions, including brain cancer and CNS damage.
Funding: NSF EPSCoR RIII Award 1457888.

PT03.03

Loading of goat´s whey extracellular vesicles with spiked microRNA and curcumin as an strategy for developing new nanocarriers for acellular therapies

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Introduction: Extracellular vesicles (EV) are involved in cell signalling and are present in a variety of cell secretions such as milk, from which enormous amount of EV can be purified, thus milk is an attractive raw material for scaling up EV production for therapeutic, cosmetic or other uses. Here we isolated EVs from the whey fraction of goat´s milk and demonstrated that such EVs can be loaded with molecules like polyphenols and miRNA.
Methods: To achieve this, milk was collected from lactating goats and fractionated by acidification and centrifugation into whey and caseins. EVs were purified from the former fraction by serial centrifugation and precipitation with commercial kit (Total Isolation/ Thermo Fisher) and characterized by Electron Transmission Microscopy (TEM), Western Blot to identify surface markers and measurement of size through Nanotracking Analysis. Once isolated, EVs were loaded with different concentration of a spiked synthetic miR39 or with the polyphenol curcumin. miRNA or curcumin were co-incubated over night with EVs at 4oC, precipitated and purified as described above, with an additional washing and precipitation for curcumin. Concentration of miRNA uploaded by EVs was quantified using miR39 specific qPCR. Curcumin was measured using a spectrophotometer at 420 nm.
Results: EVs isolated from whey had an average size of 120 nm, were positive for HSP70, CD9 and Alix. In TEM, EVs were identified with their natural conformation and corresponding size to exosomes. qPCR showed a significant difference of expression of miR39 in relation to control (loaded with shame) and the negative control ($p < 0.05$). Curcumin presence was also confirmed after washing and precipitacion.
Summary/Conclusion: In conclusion, milk EVs and exosomes can be loaded with miRNA and a polyphenol and can be used as alternative nanocarrier for acellular therapies.
Funding: Supported by CONICYT grant 21161052 to PS.

PT03.04

Development of novel tool for purification and characterization of extracellular vesicles in ageing and disease

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Introduction: Extracellular vesicles (EVs) are cell-derived lipid membrane nanoparticles that serve as messengers of intercellular communication, transferring bioactive molecules to recipient cells. EVs have a natural therapeutic potential with high flexibility and biosafety for employing natural and synthetic biomolecules as therapeutic delivery vehicles. Considering the importance of EVs, their isolation methods are still a bottleneck. To get insights into the tissue-specific cargo in vivo for complete exploitation of EVs as therapeutic, biomarker and diagnostic tools, EV purification methods are critical. The aim of the study was brought about to develop an efficient EV purification method both in vitro and in vivo and to further investigate function of EVs in cellular senescence.

Methods: To isolate tissue- specific EVs in vivo we developed recombinant EVs by genetically fusing snorkel-tag to the CD81. The snorkel-tag enables on-column protease treatment for purifying EVs which does not rely on traditional immunoaffinity purification protocols using low pH or high salts solutions.
Results: We systematically evaluated the purification of EVs harbouring snorkel-tag by employing different methodologies. Our findings suggest that EVs harbouring snorkel-tag indeed can be purified at high purity without altering EV biophysical properties. Furthermore, we expressed CD81-snorkel-tag under p16ink4a promoter and were able to purify EVs derived from senescent cells.
Summary/Conclusion: Finally, we are developing an in vivo model with CD81-snorkel-tag under p16ink4a promoter. This will provide us detail insights into the EV cargo secreted from senescent derived cells, by purifying EVs harbouring snorkel-tag under pathophysiological conditions, allowing us to develop biomarkers and therapeutic tools. Summarized, we have here developed novel tool for studying content and function of EVs in the context of ageing and disease. This tool will now pave the way for studying the molecular mechanisms underlying these EV functions in vivo.
Funding: This work was funded by the Austrian Science Fund PhD Program BioToPeBiomolecular Technolgy of Proteins (W1224).

PT03.05

Engineering exosomes with GATA-4

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Introduction: Exosomes, are small vesicles (30–150 nm) secreted from cells that can transport and deliver of their components such as lipids, proteins, DNA, mRNA, and miRNA to target cells. GATA-4, a cardiac transcription factor, has been shown to regulate differentiation, proliferation, and survival of a wide range of cell types. Delivering GATA-4 protein into ischaemic tissues may be one of the most straightforward approaches to improve cardiac function following myocardial infarction. Here, exosomes were engineered with GATA-4 by infusing GATA-4 with exosome targeting peptide.
Methods: The open reading frame of mouse GATA-4 cDNA was ligated to XPACK lentivirus vector (XPACK-GATA-4) and pLVX-EF1-IRES-Pouro lentivirus vector (pLVX-GATA-4), respectively. HEK 293 cells were transduced by lentivirus, then exosomes were isolated from conditioned medium of HEK293 cells using ultracentrifugation. Exosomes were identified using transmission electronic microscope (TEM), and the expression of GATA-4 was semi-quantified using western blot. The internalization of exosomes was tracked via treating bEnd3 cells with exosomes pre-labelled with PKH26.
Results:

(1) DNA sequencing confirmed the open reading frame of GATA-4 cDNA in frame with exosome target peptide.
(2) Isolated exosomes from HEK293 cells transduced with XPACK-GATA-4 and pLVX-GATA-4 appeared as diverse round-shaped entities and sized about 25–160 nm under TEM.
(3) GATA-4 was expressed in both cell lysis of HEK293 cells which were transduced with XPACK-GATA-4 and pLVX-GATA-4. However, GATA-4 was only expressed in exosomes isolated from HEK293 cells transduced with XPACK-GATA-4, and not in exosomes from HEK293 cells transduced with pLVX-GATA-4.
(4) Exosomes expressing GATA-4 can be internalized by bEnd3 cells.

Summary/Conclusion: Exosomes can be directly engineered with GATA-4 and internalized by bEnd3 endothelial cells, which may be a potential effective approach for delivery of GATA-4 to target cells.
Funding: NIH: HL140962

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PT03.06

Chinese hamster ovary cells engineered for production of GFP-loaded extracellular vesicles

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Introduction: Chinese hamster ovary (CHO) cells have dominated as the mammalian cell host for the manufacture of humanized biologics, in part owing to their genomic plasticity and robust growth in suspension culture. There is great interest surrounding the use of extracellular vesicles (EVs) as novel therapeutics owing to their capacity to deliver bioactive molecules. However, much remains unknown about the mechanisms involved in EV cargo loading, limiting their development as novel biologics. To this end, we have

engineered CHO cells to stably express constructs enabling loading of GFP into EVs.

Methods: Tetraspanins are established markers of EV identity. Accordingly, CD81 was selected as a tethering point to generate EVs with GFP cargo and constructs were generated via golden gate assembly. CHO cells were stably transfected by electroporation and expression was verified with fluorescence microscopy and western blotting. Growth in batch culture was monitored to establish maximum viable cell densities for EV harvest and recovered EVs were characterized by nanoparticle tracking analysis (NTA). Finally, uptake of GFP-EVs was studied using time-lapse fluorescence imaging in co-culture experiments.

Results: Strong localization of CD81-GFP was observed at the cell membrane and blotting confirmed intact tetraspanin fusion present at the expected molecular weight. Additionally, cells were confirmed to retain high GFP expression post-cryopreservation. Stable cell pools were able to reach viable densities greater than 7 million cells/mL in batch culture and NTA allowed for detection of GFP cargo even prior to EV isolation. EV-mediated transfer of functional GFP to recipient cells was found to occur over a period of hours.

Summary/Conclusion: Collectively, our findings indicate that tetraspanins can be used as targets to package recombinant protein cargo into mammalian derived EVs. Moreover, CHO cells overexpressing cargo destined for EVs can reach high cell densities and produce functional EVs to facilitate yield challenges often associated with EV recovery.

Funding: Funded by the UK Engineering & Physical Sciences Research Council (EPSRC) Centre for Doctoral Training in Emergent Macromolecular Therapies hosted at University College London (UCL).

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PT03.07

Manufacturing extracellular vesicles derived from human mesenchymal stromal cells (MSC) in bioreactors for cancer therapy

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Introduction: Extracellular vesicles (EVs) are considered promising for therapeutic applications. EVs resemble the cell membrane, allowing high biocompatibility to target cells, while their small size makes them ideal candidates to cross biological barriers. Despite the promising potential of EVs for therapeutic applications, robust manufacturing processes that would increase the scalability and consistency of EV production are still lacking.

Methods: In this work, EVs were produced by mesenchymal stromal cells (MSC), isolated from different human tissue sources (bone marrow, umbilical cord matrix and adipose tissue). MSC were selected as these cells allow for a scalable production of EVs, while displaying low immunogenicity. A Vertical-Wheel™ bioreactor system was implemented for the production of MSC-derived EVs and compared with traditional static systems. The obtained EV products were characterized by nanoparticle tracking analysis, atomic force microscopy, zeta potential and Western blot.

Results: The bioreactor system allowed to obtain EVs at higher concentration and productivity, as well as more homogeneous size distribution profiles, when compared to traditional static culture systems.

Functional studies were performed using breast cancer and lung cancer cell lines. Proliferation assays allowed to determine the dose-response profiles of these cell lines when exposed to MSC-derived EVs. A bell-shaped profile was observed for most cases, since raising the EV concentration lead to increased cell proliferation until a certain point (25–50 μg/mL), after which cell proliferation was attenuated with increasing EV concentrations.

Summary/Conclusion: The bioreactor culture system allowed a substantial improvement in the production of MSC-derived EVs, while the obtained dose-response profiles will be valuable to determine the most appropriate EV concentrations for anticancer drug delivery. Overall, we demonstrate that this culture system is able to robustly manufacture human MSC-derived EVs in a scalable manner towards the development of novel therapeutic products such as anticancer drug delivery systems.

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PT03.08

Biodistribution and cellular location of inhaled exosomes and liposomes in the lung

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Introduction: Increasing evidence reveals the potential role of extracellular vesicles, such as exosomes and liposomes, in lung regenerative medicine for the treatment of lung diseases. Encapsulation and delivery of potential RNA and microRNA targets into liposomes and exosomes are attractive drug delivery methods, but remain difficult to deliver to the pulmonary parenchyma to reach target lung cell types. Here, we demonstrate effective delivery and cellular uptake of exosomes and liposomes to the pulmonary parenchyma via inhalation treatment in a murine model of idiopathic pulmonary fibrosis.
Methods: Human lung stem cells (LSCs) were generated and expanded from healthy whole lung donors. LSC-exosomes were purified via ultrafiltration and DiI-labelled using Vybrant□ labelling solution according to the manufacturer's instructions. DsRed-labelled liposomes were generated using Lipofectamine™ RNAiMAX Transfection Reagent and BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control according to the manufacturer's instructions. LSC-exosomes and liposomes were delivered via nebulization to CD1 mice with bleomycin-induced pulmonary fibrosis. Exosome and liposome delivery and biodistribution were visualized 4- and 24-hours post-treatment through histological analysis. The study was approved by the Institutional Animal Care and Use Committee of North Carolina State University and complied with all national and state ethical standards.
Results: Exosome and liposome delivery to the pulmonary parenchyma was confirmed by the presence of DiI and DsRed fluorescence in lung histological sections that penetrated the mucus-lined respiratory epithelium. More exosomes and liposomes surpass mucus-lined surfaces 24-hours post-treatment compared to 4-hours post-treatment. Fluorescent colocalization of exosomes and liposomes with alveolar type I cells, alveolar type II cells, basal lung cells, and CD68 + macrophages was observed through immunohistochemistry analysis. More exosomes and liposomes colocalize with these cell types 24-hours post-treatment compared to 4-hours post-treatment.
Summary/Conclusion: LSC-exosomes and liposomes penetrate the mucus-lined respiratory epithelium and reach the pulmonary parenchyma through inhalation treatment. LSC-exosomes and liposomes are uptaken by alveolar epithelial cells, basal cells, and interstitial macrophages with improved biodistribution 24-hours post-treatment.
Funding: This study was supported by the NC State Chancellor's Innovation Fund.

PT03.09

Transfection reagent artefact accounts for some reports of extracellular vesicle function

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Introduction: Extracellular vesicle (EV) functions are frequently investigated by transiently transfecting cells with plasmid DNA to produce EVs modified with protein(s) or nucleic acid(s) of interest. However, EVs and the DNA-complexes used to transduce cells are physically similar, raising the possibility that they may co-purify during differential ultracentrifugation, the most common EV isolation procedure. Activities attributed to EVs may therefore be due to contaminating DNA -transfection reagent complex.
Methods: EV producing cells were transiently transfected with plasmid DNA encoding gene-editing or split enzymes fused to EV-targeting protein sequences. Differential and density gradient ultracentrifugation were used to purify EVs from cell culture supernatant or DNA lipoplexes from cell-free culture media. Protein expression and localization to EVs was confirmed by Western blot. Cell lines stably expressing fluorescent or luminescent reporters were used to assess functional enzyme delivery in recipient reporter cells.
Results: Reporter cells treated with ultracentrifuge pellet material (UCP) from media of transiently transfected cells showed robust and reproducible signal, however fractionating the UCP with an iodixanol density gradient revealed that reporter activity was associated with high-density fractions that were depleted in EVs. UCP isolated from identical transfection conditions, but lacking cells (and exosomes), showed identical biological activity levels and distribution in iodixanol gradients, suggesting that the activity was due to contaminating transfection reagent complexes and not EVs. Serial media changes on EV producing cells post-transfection did not significantly reduce UCP activity on reporter cells. Treatment with nucleases did not digest complexed DNA, did not significantly reduce DNA levels in the UCP as measured by qPCR, and did not decrease activity in reporter cells treated with UCP from either transfected cells or no-cell controls.

Summary/Conclusion: We find that DNA-transfection reagent complexes are not separated from EVs using differential ultracentrifugation and that common approaches to remove such complexes, including media exchanges and nuclease treatment, are ineffective. Due to the pernicious nature of the DNA-complex in these cellular assays, it is likely that some reports of EV function are likely artefacts produced by contaminating DNA-complexes. We find that density gradient centrifugation can effectively separate EVs and DNA-complexes, highlighting the importance of validating elimination of contaminating transfection reagent complexes when using transient transfection to interrogate EV function.

PT04: EV Protein Biomarkers

Chair: Suresh Mathivanan – La Trobe University

PT04.01

Cancer stem cell-derived exosomes: potential biomarkers for early diagnosis and prognosis in pancreatic cancer

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Introduction: Pancreatic cancer (PaCa) is the most deadly manlignancy, due to late daignosis and early metastatic spread, which prohibits surgery. it is urgently for relaible, early detection. Research shows that tumour-derived exosomes, which had been present in the blood in the early stage of tumour formation and before metastasis, is the vanguard forces of tumour formation and metastasis; Cancer stem cell-derived exosomes (CSC-Exos) has stronger migration ability, so the detection of blood CSC-Exos for early diagnosis and monitoring of progress for PaCa has great research potential and the value of application.
Methods: Protein markers were selected according to expression in exosomes of PaCa cell line culture supernatants, but not healthy donors' serum-exosomes. According to these preselections, serum-exosomes were tested by flow cytometry for the pancreatic cancer stem cell marker CD44v6 and Tspan8.
Results: The majority (95%) of patients with PaCa and patients with nonPa-malignancies reacted with anti-CD44v6 and anti-Tspan8. Serum-exosomes of healthy donors' and patients with non-malignant diseases were not reactive. Recovery was tumour grading and staging independent including early stages.
Summary/Conclusion: Thus, the evaluation of pancreatic CSC-derived exosomes awaits retrospective analyses of larger cohorts, as it should allow for a highly sensitive, minimally-invasive PaCa diagnostics.
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PT04.02

Characterization of Extracellular vesicles separated from biospecimens of former national football League players at a risk for chronic traumatic encephalopathy

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Introduction: Chronic Traumatic Encephalopathy (CTE) is a tauopathy that affects individuals with a history of mild repetitive brain injury frequently seen in contact sports. Initial neuropathologic change of CTE include perivascular deposition of phosphorylated tau (p-tau) in cortical neurons and, in later stages, the formation of neurofibrillary tangles in neurons throughout the brain. Extracellular vesicles (EV) are known to carry neuropathogenic molecules in neurodegenerative disease and able to cross the blood brain barrier. We therefore examined the protein composition of EV separated from cerebrospinal fluid (CSF) and plasma in former national football League (NFL) players with cognitive dysfunction, and an age-matched control group with no history of contact sports.
Methods: EVs were separated from CSF and plasma from former NFL players (n = 4, 14) and controls (n = 5, 12) by affinity separation method or size exclusion chromatography, respectively. The EV protein profiling was characterized by SIMOA for tau and p-tau and mass spectrometry. The protein data was analysed for EV enrichment, differentially expressed proteins, pathway analysis and correlation with cognitive function, head impact and tau/p-tau levels by biostatistics and bioinformatics.
Results: The level of total tau and p-tau in CSF EVs was not significantly changed, but significantly elevated in plasma EVs from former NFL players. The 95 proteins were commonly identified between the paired plasma-CSF from the same patients, but there was no significant correlation with disease status. Collagen

alpha-3(VI) chain (COL6A3), −1(VI) chain (COL6A1) and Reelin (RELN) were differentially expressed in former NFL players' plasma EVs. A combination of these 3 proteins in plasma EV can distinguish former NFL players from controls with 85% accuracy by machine learning.

Summary/Conclusion: The interacting plasma-CSF EV proteomes provide an original resource to EV biomarker development for neurodegenerative disease, and COL6A3, RELN and COL6A1 in plasma EVs can be potential biomarker for monitoring the CTE development.

PT04.03

Density-based fractionation of urine to unravel the proteome landscape of extracellular vesicles in prostate cancer

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Introduction: Current diagnostic tests are unable to discriminate indolent from aggressive prostate cancer (PCa), leading to overdiagnosis and overtreatment, and an intense interest in biomarkers to improve clinical decision making. Urine is considered an ideal proximal fluid for biomarker identification in PCa due to its direct contact with the urogenital system. The discovery and translation of extracellular vesicle (EV) content into PCa biomarkers remains challenging due to the difficulty of obtaining urinary EV (uEV) with high specificity.

Methods: We developed a step-by-step protocol to separate uEV by orthogonal implementation of ultrafiltration and bottom-up density gradient centrifugation (BU-ODG). We implemented complementary particle and protein measurements to identify uEV (lower density) and protein rich fractions (higher density) and assess the performance of BU-ODG (specificity, efficiency and reproducibility). Using mass spectrometry-based proteomics we interrogated uEV and protein rich fractions from matched urine and radical prostatectomy tissue samples from PCa patients (n = 4), and urine from men with PCa prior to (n = 12) and after local treatment (n = 10), benign prostatic hyperplasia (n = 12) and other urological cancers (n = 11).

Results: BU-ODG separated uEV from soluble proteins and Tamm-Horsfall Protein (THP) complexes with high specificity and reproducibility, outperforming differential ultracentrifugation, ExoQuick and size-exclusion chromatography. Comparison of the uEV proteome from men with benign or malignant prostate disease, allowed us to expand the known human uEV proteome and identify a PCa specific uEV proteome not uncovered by the analysis of the protein rich fraction. Proteomic analysis of EV separated from prostate tumour interstitial fluid and matched uEV confirmed PCa specificity of the uEV proteome. Analysis of the uEV proteome from patients with bladder and renal cancer provided additional evidence of the selective enrichment of protein signatures in uEV reflecting their respective cancer tissues of origin.

Summary/Conclusion: We identified hundreds of previously undetected proteins in uEV of PCa patients and developed a powerful toolbox to map uEV and protein rich fractions, ultimately supporting biomarker discovery for urological cancers.

PT04.04

Immunoglobulin A coating of faeces-derived bacterial vesicles as a marker of inflammatory bowel disease in humans

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Introduction: IgA is the most abundant antibody in mucosal secretions and plays a crucial role in maintaining the balance between the host and the gastrointestinal microbiome. Recent studies suggested that pronounced IgA coating is especially prominent among inflammatory commensals which drive intestinal disease. Membrane vesicles (MVs, nano-sized particles released by bacteria) have also been found to interact with the host and modulate development and function of the immune system. However, their interaction with IgA has not been studied yet. Here we developed a method to isolate and characterize the MVs from faecal samples and checked for possible differences in IgA coating patterns of MVs in health and disease.

Methods: MVs were isolated by using a combination of ultrafiltration and size exclusion chromatography from faecal samples of 6 healthy controls (HC), 6 patients with active Crohn disease (CD) and 6 CD patients in a

remissive state. Quantification and verification have been done with tunable resistive pulse sensing (TRPS-based analysis) bead-based flow-cytometer (BBFC) and transmission electron microscope (TEM). MVs were selected with specific antibodies for capturing (Gram +: LTA, Gram-: OmpA) followed by PE-conjugated anti-human IgA antibodies as detection.

Results: We could successfully isolate 1*109-5*109 particles/ml from 500 mg of faeces. BBFC in combination with TRPS provide a valuable method for (semi-)quantitative measurements of mixed populations. Intriguingly, remarkable differences were found between IgA coating MVs derived from healthy controls and active and remissive CD patients as MVs derived from healthy controls were significantly more coated compare to both CD patient groups. In details, for selected G-ve derived MVs: 60% of the total population of MVs derived from HC were coated, 20% from remissive CD patients, and <5% of active CD patients; and for selected G+ ve derived MVs: 55% of the total population of MVs derived from HC were coated, 34% from remissive CD patients, and 25% of active CD patients. (Data are represented as the mean).

Summary/Conclusion: Here we demonstrate for the first time that MV isolated from the faecal samples are also coated with IgA, and surprisingly MVs from healthy volunteers were more densely coated than MVs from diseased patients. The possible consequence of this difference remains to be determined in future studies.

PT04.05

Monitoring altered tetraspanin and PSMA expression in prostate cancer derived extracellular vesicles via Advanced Image Flow Cytometry (ISX)

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Introduction: New diagnostic and therapeutic options for patients with prostate cancer are urgently needed. Prostate-specific membrane antigen (PSMA)-based imaging and therapy are increasingly used for prostate cancer management. Unfortunately, as a membrane protein, PSMA is not found as a soluble protein in the blood and therefore has limited utility as a diagnostic biomarker. However, PSMA has reportedly been observed as a cargo protein of prostate cancer-derived extracellular vesicles (EVs).

Methods: We demonstrate altered PSMA expression on EVs derived from prostate cancer cell cultures (C4-2, LNCaP) in response to novel next-generation androgen receptor inhibitor (enzalutamide), a standard chemotherapy agent (docetaxel), a novel experimental nonsteroidal antiandrogen (Epi-001) that binds covalently to the N-terminal domain of the androgen receptor and dihydrotestosterone (DHT). Additionally, EVs were isolated from the plasma of prostate cancer patients who participated in the proCOC biobank campaign at the USZ. Plasma was taken and stored from patients both pre- and post- prostatectomy.

Results: Transmission electron microscopy, nanoparticle tracking analysis and simple Western (WES) analysis show stable size distribution and amount of EVs produced by treated and non-treated cells. Using advanced image-based flow cytometry, altered tatraspanin and PSMA expression could be detected in EVs isolated from cell culture supernatants of LNCaP and C4-2 prostate cancer cells following their treatment.

Summary/Conclusion: Measuring PSMA expression on extracellular vesicles might pave the way to use image flow cytometry of EVs to develop a blood based diagnostic test for prostate cancer patients with a wide range of possible applications including: 1) monitoring response to therapy and, 2) early indications of potential relapse.

Funding: Vontobel Fondation.

PT04.06

Proteomic profiling of human neural cells derived extracellular vesicles to identify human brain cell-type specific markers

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Introduction: Alzheimer's Disease (AD) is a common neurodegenerative brain disease which affects appropriately 30 million patients worldwide. One of the major challenges in AD is to develop reliable biomarkers for early diagnosis and disease-modifying therapies, especially before the clinical symptoms. Extracellular vesicles (EVs) carry cargos of proteins, lipids and nucleic acids. There was no comprehensive characterization of EVs isolated from specific brain cell types, which may be useful for cell type-specific biomarkers. The purpose of this study is to isolate EVs

from human induced pluripotent stem cell (iPSC)-derived brain cells for proteomic profiling and characterization of cell type-specific molecules.

Methods: Human iPSCs-derived neurons, microglia and primary cultured astrocytes were differentiated in EV-depleted media. The EVs were isolated by differential centrifugation combined with size exclusion chromatography, followed by characterization using nanoparticle tracking analysis and mass spectrometry. The proteomic data were subjected to bioinformatics analysis

Results: We identified 153 proteins from neuron-derived EV (NDE), 215 proteins from microglia-derived EV (MDE) and 380 proteins from astrocyte-derived EV (ADE) by proteomics. Gene ontology analysis indicated that most of these proteins are associated with EVs. Furthermore, 15, 48 and 251 proteins are present individually in NDEs, MDEs and ADEs. Among them, high levels of ATP1A3 and SYT1 in NDEs, ITGAM and CD300A in MDEs, and EAAT1 and GFAP in ADEs were found, all of which are typically and highly expressed in the original cells.

Summary/Conclusion: Our results provide us the potential candidates for cell-type specific EV markers, which will be helpful to develop non-invasive tools to enrich EV originating from specific brain cells and may lead to the development of new biomarkers for neurodegenerative disorders.

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PT04.07

Quality control for bacterial EVs

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Introduction: The MISEV guidelines of 2018 (DOI: 10.1080/20013078.2018.1535750) are a tremendous resource for extracellular vesicle (EV) research, but they are heavily focussed on mammalian EVs, i.e. EVs from humans and laboratory animals, where protein cargoes are well characterised, and a wide selection of antibodies are commercially available. Protein markers can be used to identify and define the types of mammalian EV and to determine the presence of any contaminants that might confound functional studies. Similar resources are not as readily available for bacterial EVs as these are not as well characterised, commercially available antibodies are much less abundant and immunological variation between different bacterial species (and there are 1 trillion bacterial species on planet Earth!) means that each species, strain, or group of related species may require different antibodies.

Methods: To identify quality markers for bacterial EVs, we have characterised the proteome of cells, crude EVs (ultracentrifuge pellet from cell free culture supernatant) and size exclusion chromatography or density gradient centrifugation purified EVs from two different (pathogenic vs probiotic) strains of Escherichia coli grown under two different environmental conditions, and one strain of Mycobacterium marinum grown in one medium.

Results: Our results identify a selection of proteins enriched in purified EV preparations, and proteins that are depleted after purification steps.

Summary/Conclusion: Our results allow the identification of potential markers for EV purity and non-EV contaminants, but also highlight the variability in bacterial EV preparations and suggest potential targets that can be used to investigate the heterogeneity of bacterial EV populations.

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PT04.08

Relationship of extracellular vesicle cargo with clinical markers of mortality and race

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Introduction: Recent findings indicate an increase in mid-life mortality rates in the USA and persistent, significant race-related health disparities exemplified by differential mortality rates. This suggests that exploring new molecular markers that may be linked to mortality could provide novel insights into factors that are driving mortality rates. Accumulating data suggests that extracellular vesicles (EVs) circulating in

blood may be potential biomarkers of age-related disease. EVs are nano-sized membranous vesicles that bear molecular cargo and mediate intercellular communication between different cells and tissues. Little is known about whether EV characteristics differ by race or whether EVs are associated with clinically relevant mortality risk factors.

Methods: In this cross-sectional study, plasma EVs were isolated from middle-aged African American (AA) and white males and females.

Results: We report no significant differences in EV size or concentration with race or sex. There were significantly higher EV levels of phospho-p53, total p53, cleaved caspase 3, ERK1/2 and phospho-AKT in whites compared to AAs. Higher EV levels of phospho-IGF-1R were found in females compared to males. We examined EV characteristics and protein cargo in the context of well-established clinical mortality risk factors. EV concentration was significantly, and positively, associated with several mortality markers including, high-sensitivity C-reactive protein (hsCRP), homoeostatic model assessment of insulin resistance (HOMA-IR), alkaline phosphatase, pulse pressure, body mass index, and waist circumference. The relationship of EV concentration and cargo with mortality markers differs by race.

Summary/Conclusion: Our data show that EV-associated proteins can differ by race and sex and are associated with mortality risk factors. This study provides insight into the characterization of EVs in middle-aged AAs and whites, which may aid in the development of EV-based diagnostics.

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PT04.09

Repurposing specialised cell-free DNA blood collection tubes for extracellular vesicle isolation

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Introduction: Liquid biopsies offer a minimally invasive approach to patient disease diagnosis and monitoring. However, many plasma processing protocols have been designed with a single biomarker in mind. Here we investigate whether specialised DNA blood stabiliser tubes could be repurposed for the analysis of extracellular vesicles (EVs).

Methods: Peripheral blood (n = 3) was collected into K3-EDTA, Roche or Streck cell-free DNA (cfDNA) blood collection tubes and processed using sequential centrifugation immediately or after storage for 3 days. MicroEV were collected from platelet poor plasma by 10,000 g centrifugation and NanoEVs isolated using size exclusion chromatography. Particle size and counts were assessed by Nanoparticle Tracking Analysis, protein by BCA assay and dot blotting for blood cell surface proteins.

Results: Major variations in Micro and NanoEVs were seen with delayed time to processing. NanoEV counts did not change with processing delay or tube collection type but the associated protein amount increased, indicative of cell lysis or activation. The protein was predominantly derived from from platelets (CD61) and red blood cells (CD235a). The increase in associated protein was seen more in the K3-EDTA and Streck tubes indicating that the Roche tubes may offer improved cell stability. Conversely, MicroEVs increased in both quantity and protein content with delay to processing indicative of both lysis and cell activation, irrespective of tube type. Epithelial cell surface marker EpCAM abundance remained the same across conditions in both Micro and NanoEVs demonstrating that EpCAM+ EVs were stable.

Summary/Conclusion: Specialised cfDNA collection tubes can be repurposed for Micro and NanoEV analysis, however simple counting or using protein quantity as a surrogate of EV number may be confounded by pre-analytical processing. The EVs would be suitable for disease selective EV subtype analysis if the molecular target of interest is not present in blood cells.

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PT04.10

Role of exosomes in the management of cellular disease in the genetic susceptible participants in the Chilean population

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Introduction: Nutrigenomics and nutrigenetics have been defined as the effect of nutrients on gene expression and genetic variation on dietary response, respectively. Here, we propose the isolation and characterization of exosomes from donors carrying

different alleles of HLA-DQA1 and HLA-DQB1, to investigate their involvement in coeliac disease (CD) management.

Methods: A Chilean population (n = 30) was investigated for SNPs mutations in HLA Class II alleles associated to CD predisposition (as well as other mutations related to other food intolerances), using the GenoChip Food Technology. Exosomes have been isolated from donors' serum by ultracentrifugation and characterized by SDS-PAGE, Western Blotting (CD63 and CD9), and transmission electron microscopy. Exosomes were also studied for their interleukins (IL-6 and IL-1ra) content.

Results: Among the studied population, 86% present at least one of the alleles leading to CD development and 60% carry alleles encoding for α- and β-chains heterodimers associated with very high risk to develop CD. In parallel, isolated exosomes from donors with low to extremely high risk for CD showed high IL-1ra content (108.8 ± 15.91 to 148.8 ± 12.37), as the persons were not following any treatment. However, values of IL-1ra decrease in exosomes isolated form persons receiving treatment for CD. A relationship between exosomes' content and genetic susceptibility for CD has been observed, which may suggest their possible use as biomarkers for CD as the diagnostic of this disease is still a big issue.

Summary/Conclusion: Until this point of this underway project, we demonstrate the existence of a relationship between the exosomes' content in IL-1ra and genetic susceptibility for CD. Furthermore, the genetic predisposition to CD could also modulate the gut colonization process, another important player in intestinal homoeostasis. In the next step, extracellular vesicles from gut microbiota will be isolated and analysed to determine their role in CD management.

PT04.11

Subpopulations of EVs in serum and plasma

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Introduction: The ability to isolate extracellular vesicles (EVs) from blood is vital in the development of EVs as disease biomarkers. Both serum and plasma can be used but few studies have compared them in terms of amount and type of EVs. We have previously developed a method to isolate EVs from plasma with minimal contamination of lipoprotein particles (Karimi et al 2018). The aim of this study was to compare the presence of different subpopulations of EVs in plasma and serum.

Methods: Blood was collected from healthy subjects, from which plasma and serum were isolated. EVs were isolated using a combination of density cushion and size exclusion chromatography (SEC) (protocol 1) or a combination of density cushion and density gradient (protocol 2) or immune-capturing (anti-CD63, anti-CD9 and anti-CD81 beads) (protocol 3). Purity and yield of EVs were determined by nanoparticle tracking analysis (NTA), Western blot, electron microscopy (EM), ExoView, flow cytometry and mass spectrometry (LC-MS/MS).

Results: As determined by NTA and protein measurement more EVs could be isolated from plasma with protocol 1 and the majority of the vesicles were CD9/CD41a positive as determined with ExoView and Western blot. Additionally, flow cytometry and Western blot showed that more CD9/CD41a positive EVs where also identified with protocol 3. Furthermore, Western blot showed increased amount of CD41a in plasma samples in protocol 2. When labelled EVs were spiked in freshly collected blood, no difference in recovery was seen for plasma and serum.

Summary/Conclusion: This study shows that a larger amount of EVs could be isolated from plasma compared to serum when three different isolation methods were used. Firstly, this suggests that more EVs are present in plasma. Secondly, it suggests that these vesicles are probably released by platelets and that EVs are not trapped in the clot during serum formation. Future studies are needed to answer how this affects the use of blood-derived EVs as biomarkers from serum and plasma.

PT04.12

Tumour-derived extracellular vesicles contain distinct integrin proteins

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Introduction: Cargo profiling, including proteomic analyses, of tumour cell-derived extracellular vesicles (EVs) may provide ripe opportunities for further understanding cancer growth, drug resistance, and metastatic behaviour. Accumulating data suggest that cancer-derived EVs contain membrane-bound integrin proteins which may aid in cell detachment, migration,

and homing to future metastatic niches. We have previously published an extensive proteomic profile of secreted vesicles from the NCI-60 panel of human cancer cells.

Methods: Here, we further examine the distinct integrin components in these cancer-derived EVs, and additionally profile EVs released from benign epithelial cells by liquid chromatography and tandem mass spectrometry for comparison.

Results: We demonstrate the enrichment of integrin receptors in cancer EVs compared to vesicles secreted from benign epithelial cells. Total EV integrin levels, including the quantity of integrins α6, αv, and β1 correlate with tumour stage across a variety of epithelial cancer cells. In particular, integrin α6 also largely reflects breast and ovarian progenitor cell expression, highlighting the utility of this integrin protein as a potential circulating biomarker of certain primary tumours. Other integrins including α4, αL, and β2 are enriched in vesicles derived from leukaemia cells, and may provide a means to distinguish haematopoietic cell-derived EVs.

Summary/Conclusion: This study provides preliminary evidence of the value of vesicle-associated integrin proteins in detecting the presence of cancer cells and prediction of tumour stage. Differential expression and selective packaging of integrins into EVs may contribute to further understanding the development and progression of tumour growth and metastasis across a variety of cancer types.

PT04.13

Effect of nicotine and menthol on cytochrome P450 and antioxidant enzymes in rat plasma-derived extracellular vesicles

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Introduction: Tobacco products such as e-cigarettes pose potential adverse health effects caused by direct exposure to aerosolized nicotine, flavorants such as menthol, and other particulates. Here, we aimed to study the hypothesis that whether nicotine and menthol modulate nicotine-metabolizing cytochrome P450 2A6 (CYP2A6), antioxidant enzymes (AOEs), SOD1 and catalase in plasma extracellular vesicles (EVs). Modulation of these enzymes would eventually lead to nicotine-induced toxicity and HIV-1 pathogenesis via EVs-based cell-cell interactions.

Methods: We isolated and characterized EVs from rat plasma before and after nicotine self-administration (NIC) with audiovisual cue (AV) and menthol and characterized using EV markers according to the ISEV guidelines. Protein associated with CYP2A6, SOD1, and catalase were quantified by western blot.

Results: We measured size, total protein, and acetylcholine esterase activity of EVs and found no significance difference in these characteristics before and after NIC. To investigate the effect AV, menthol alone or in combination in the absence and presence of NIC, first we evaluated the expression of EV markers CD9 and CD63. The results showed menthol and AV together increased the levels of CD9 ($p \leq 0.05$), the marker of small vesicles, in the presence of NIC. The NIC with menthol and AV showed a pattern of increased levels of small vesicle but could not reach to significance. Next, we demonstrated that the NIC with AV increased the level of SOD1 ($p \leq 0.05$), which showed a pattern of increased levels of catalase and CYPA6, though statistically non-significant. The expression of nicotine receptor did not change under any conditions used. The results showed an increased level of CYP2A6 ($p \leq 0.01$), SOD1 ($p \leq 0.05$), and catalase ($p \leq 0.05$) in plasma EVs in the menthol-NIC group compared to menthol group only. NIC group with a combined AV and menthol, showed further increase in the levels of CYP2A6 ($p \leq 0.01$), and catalase ($p \leq 0.05$). Further analysis of plasma EVs on inflammatory cytokines/chemokines in these groups, and the effect of plasma EVs on nicotine-induced toxicity and HIV pathogenesis are underway.

Summary/Conclusion: Nicotine administration increased, though not statistically significant, the levels of circulatory EVs. Moreover, the study provided evidence that nicotine in the presence of menthol, AV, and/or menthol+AV increased nicotine-metabolizing CYP2A6 in all the groups and AOEs in specific groups.

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PT04.14

Proteomic signatures in breast cancer exosomes and cell lines

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Introduction: Biomarker discovery in breast cancer (BC) is a clinical need for therapeutics and non-invasive diagnostics. Tumour exosomes are involved in pre-metastatic niche formation and drug resistance and represent a source of non-invasive biomarkers. The identification of tumour exosomal biomarkers provides, not only, the possibility to discriminate patient groups also potential targets to control cancer progression that could be exploited to develop innovate BC therapeutic strategies.
Methods: We have performed a comparative differenti. al proteomic profile of four BC cell lines and their derived- exosomes, representative of the most relevant BC subtypes in clinic to search non-invasive biomarker candidates. Then, we have carried on two bioinformatics approaches: 1) protein association network analysis interaction (STRING) and 2) pathway inference analysis (Hipathia), to characterize the functional profiling for each BC subtype.
Results: We have found differentially-expressed proteins, in both cells and exosomes, that include indicators of invasion, metastasis, angiogenesis and drug resistance. Exosome proteome profile reflects their different BC cell origin suggesting potential indicators of BC subtype. Further, bioinformatics analysis reveals a differential role of exosomes in BC signalling pathways in recipient cells, according to their protein cargo and cell origin.
Summary/Conclusion: Our results show a set of cells and exosome proteins that highly discriminate BC subtypes and may significantly contribute to further studies for the design of BC biomarker predictor to stratify BC patients and the development of novel therapeutic strategies.
Funding: A set of potential biomarkers to discriminate breast cancer subtypes.

PT04.15

Circulatory EVs as potential biomarkers of HIV-drug abuse interactions and neurological dysfunction in HIV-Infected subjects and alcohol/ tobacco Users

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Introduction: Abuse of alcohol and tobacco can exacerbate HIV pathogenesis and its associated complications. Further, the diagnosis of neurocognitive disorders associated with HIV infection and drug abuse using CSF or neuroimaging are invasive or expensive methods, respectively. Therefore, extracellular vesicles (EVs) can serve as reliable non-invasive markers due to their bidirectional transport of cargo from the brain to the systemic circulation. Hence, we aimed to study the specific EVs proteins, which are altered in both HIV and drug abusers to identify a physiological marker to indicate the immune status and neuronal dysfunction of HIV-positive drug abusers.
Methods: EVs were isolated from plasma of the following subjects: a) Healthy b) HIV c) Alcohol drinkers d) cigarette Smokers e) HIV+alcohol drinkers f) HIV +cigarette smokers. Quantitative proteomic profiling of EVs was performed by mass spectrometry and potential EV proteins associated with neuronal dysfunction were quantified by westernblot.
Results: The EVs were characterized according to the ISEV guidelines. A total of 343 proteins were detected in EVs of all the study groups. Comparison of proteins among all the study groups revealed that hemopexin was significantly altered in HIV+drinkers compared to drinkers and HIV subjects. Further, our study is the first to show properdin expression in plasma EVs, which was decreased in HIV+smokers and HIV+drinkers compared to HIV patients. Though we couldn't identify the few other CNS-specific proteins, G-FAP and L1-CAM, associated with neuronal dysfunction in plasma EVs by Mass spectrometry, we could detect those by westernblot. The protein expression of GFAP ($p < 0.01$) was significantly enhanced in plasma EVs obtained from HIV-positive subjects and drinkers compared to healthy subjects, suggesting enhanced activation of astrocytes in those subjects. The L1CAM expression was found to be significantly elevated in smokers ($p < 0.05$). Both GFAP and L1CAM levels were not further elevated in HIV+smokers compared to HIV+nonsubstance users.
Summary/Conclusion: The present findings suggest that hemopexin, and properdin show potential as markers for HIV-drug abuse interactions. Further, astrocytic and neuronal-specific markers (GFAP and L1CAM) can be packaged in EVs and circulate in plasma, which is further elevated in the presence of HIV infection, alcohol, and/or tobacco and thus may represent as potential biomarkers for neurological dysfunction in those subjects.
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PT04.16

Electrochemical detection of miRNA-21-5p

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Introduction: MicroRNAs (miRNAs) are small, single-stranded, non-coding RNA species that regulate gene expression post-transcriptionally, and are transported by extracellular vesicles (EVs). They play an essential role in biological processes, such as development, cell proliferation, apoptosis, stress response and tumorigenesis. Thus, miRNAs are considered relevant biomarkers in health. More particularly, miRNA-21-5p is expressed in neurons after traumatic brain injury, being expectably transported to peripheral fluids by brain EVs that cross the blood-brain barrier. The main goal of this work is to develop an electrochemical biosensor for the detection of miRNA-21-5p in serum.

Methods: Overall, the experimental assembly of the biosensor was made in three stages. The first one consisted in the electrodeposition of AuNPs, the second one in the incubation of anti-miRNA21-5p on the carbon screen-s printed electrodes and the final stage in the incubation of mercaptosuccinic acid for blocking unspecific bindings. The probe was hybridized with the target miRNA21-5p by a consecutive incubation of several standard solutions. Each modification was evaluated with cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV). The electrochemical behaviour of the biosensor was followed in all steps by monitoring the electron transfer features of a standard redox system. The redox probe selected for this purpose was [Fe(CN)6]4-/[Fe(CN)6]3-.

Results: The results indicated that the electrodeposition of gold was more effective for −1.5 V for 600 s and could lead to better signals upon anti-miRNA-21-5p hybridization.

Summary/Conclusion: In general, the experiments showed increasing charged transfer resistance upon the incubation of higher concentrations of miRNA-21-5p.

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PT05: Advance in EV Quantitation

Chair: Malene M. Møller – Department of Clinical Immunology, Aalborg University Hospital

Chair: Rienk Nieuwland, Ph.D. – University of Amsterdam

PT05.01

Accurate EV concentration is critical for experimental rigour and reproducibility

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Introduction: Extracellular vesicles (EVs) are studied for their potential as powerful therapeutics and biomarkers of health and disease. Key to success in this research are rigorous experiments to quantify the cargo and biological activity of different vesicle preparations. In these experiments, EV concentration is a critical variable that must be carefully controlled to ensure scientific rigour and reproducibility: Without controlling for concentration (dose), experimental outcomes will exhibit excess variability that could mask important biological discoveries.

In this study, three orthogonal methods are compared for accuracy in EV quantification: Microfluidic Resistive Pulse Sensing (MRPS) and Nanoparticle Tracking Analysis (NTA) were compared to each other and relative to the gold standard method, Transmission Electron Microscopy (TEM). The ability of NTA to accurately measure particle concentration is shown to depend on the polydispersity of the sample itself. Results validate the accuracy of MRPS and emphasize the importance of using orthogonal techniques to quantify EVs.

Methods: Reference urinary vesicles were prepared and analysed with the three methods and the relative concentration accuracy of NTA and MRPS were compared as a function of particle size. The hypothesis that NTA concentration accuracy was impeded by sample polydispersity was tested using polystyrene bead mixtures having a range of polydispersity. A theoretical argument based on fundamental physics explains the experimental observations.

Results: TEM and MRPS measurements of the EVs were in excellent agreement and showed a broad, polydisperse particle size distribution with no peak on the measured size range (50 nm – 400 nm diameter). NTA differed significantly from TEM and MRPS by reporting a steep decrease in measured concentration below about 150 nm that resulted in a peak in the reported particle size distribution. Bead measurements confirmed the hypothesis to be tested: Sample polydispersity significantly affects the ability of the NTA method to accurately measure concentration, even for particles as large as 150 nm diameter.

Summary/Conclusion: These experiments validate MRPS as an accurate method for quantifying EVs and highlight the importance of using orthogonal measurement methods in accordance with MISEV2018 guidelines.

PT05.02

Clinically relevant synthetic reference materials to standardize concentration measurements of extracellular vesicles: state-of-the-art and future prospects

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Introduction: There is an unmet need to standardize concentration measurements of extracellular vesicles (EVs). Flow cytometry is the clinically most applicable method, but the currently available reference materials for calibration are insufficient. For example, the refractive index (RI) between standard particles and EVs substantially differs, whereas concentration and fluorescence calibration particles are too bright. The goal of this study is to ascertain the most desired properties of reference materials to standardise EV measurements.

Methods: An online survey was prepared within the MEVES II project to measure the desired size, concentration range, optical properties, choice of fluorochromes, and stability of synthetic EV reference materials for flow cytometry (FCM) measurements. Besides the desired properties of EV reference particles, also the available instrumentation was assessed in the survey, which was sent to the members of the Stakeholder Committee of METVES II project and members of the EV Flow Cytometry Working Group.
Results: The most desired size, concentration, and RI range for EV reference particles is 50 nm to 500 nm, 10E7 to 10E10 1/mL, and 1.35–1.45, respectively. Based on Mie-theory evaluation of the sensitivity of the available instruments, none of the respondents would be able to detect 50 nm particles with RI = 1.35 with their current instruments. Regarding fluorescence intensity, the most desired range according to the responses is from 10 molecules of equivalent soluble fluorochromes (MESF) to 10 000 MESF. Considering the sizes of EVs and fluorescent labels, the maximal MESF that can be obtained for EV reference particles with 50 nm diameter and high molecular mass fluorescent dyes is in the range of several hundreds. Typical antigen densities on EVs fall below 100 copies per EV with 50 nm diameter, i.e. MESF values above 100 are probably not physiologically relevant in this size range.
Summary/Conclusion: A part of the desired properties of EV reference materials precludes either their physical feasibility of production or their detection at most currently available FCMs, meaning that the intended reference materials will be future-proofed.
Funding: This work was supported under 18HLT01 METVES II project by the European Metrology Programme for Innovation and Research (EMPIR). The EMPIR initiative is co-funded by the European Union's Horizon 2020 research and innovation programme and the EMPIR Participating States.

PT05.03

Comparison of production and activity of amniotic fluid stem cell extracellular vesicles from 3D hollow fibre bioreactor and 2D culture.

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Introduction: EV clinical translation is limited by scale-up of EVs production. Hollow fibre bioreactors (HFBR) support culture of large numbers of cells at high densities. Culture conditions may affect EV composition and potency. Here we compare production, potency, identity and therapeutic potential of EVs collected from cells grown in culture dish (2D) versus HFBR (3D).
Methods: Human clonal AFSC were derived from patient-consented amniotic fluids. 1x10e6 hAFSC were seeded in 2D (145cm2), and 1.6X10e7 hAFSC on a small 20kd MWCO HFBR (FiberCell-C2025D, 450cm2) with fibronectin coating; both cultured in Chang medium with 20% of ES-FBS, starved for 24 hr and then EVs collected. The effect of harvest frequency was tested (8hrs, 24 hr, 72hrs,1 wk). 2D-EVs and 3D-EVs were compared by Nanosight, potency assay (by WB), identity (by Exoview analysis) and therapeutic effect (in vivo in an animal model of kidney disease, Alport Syndrome).
Results: 2D production was ~5.5X10e9 EV/ml/24 hrs while 3D was ~2.8X10e10 EV/ml (first four 24 hrs) and ~4.4X10e10 EV/ml (two days of hourly harvests). Very little difference in EV concentration and very similar size distribution (~130 nm) were observed during harvest intervals; possibly indicating either significant EV re-uptake or inhibition of EV secretion dependent upon free EV in the supernatant. 3D-EVs trapped VEGF (an in vitro established potency assay) as efficiently as 2D-EVs, and expressed CD9, CD81, CD63, CD80, CD86 and VEGFR1 as 2D-EVs.
Summary/Conclusion: 3D-EVs had comparable properties and bio-activity to 2D-EVs, but the HFBR produced 10x more EVs. HFBR cell culture conditions for hAFSC still need optimization, however an available 1.2 m2 cartridge provides a 50X scale up potential. The HFBR, a cGMP closed system, can produce sufficient numbers of EV to support pre-clinical and clinical applications with at least similar properties to EVs produced by conventional 2D methods.
Funding: – Intramural funding
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PT05.04

Demonstration of High Gain mode in combination with Imaging Flow Cytometry for improved EV analysis

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Introduction: Extracellular vesicles (EVs) are membrane-derived structures that include exosomes, microvesicles, and apoptotic bodies. In recent years, the importance of EVs has become apparent, as they are key mediators of intercellular communication.

However, quantifying and characterizing EVs in a reproducible and reliable manner is challenging due to their small size – exosomes range from 30 to 100 nm in diameter. It is well-known that flow cytometers were originally designed to measure and detect cells, and due to the quantitative power flow cytometry offers, there has been a push to quantify and characterize EVs using flow cytometric methods. However, these systems have not been designed to measure objects smaller than a cell.

Methods: Here, we describe the use of High Gain mode on the Amnis® ImageStream® Imaging Flow Cytometer to address the challenges of measuring small particles. In this new High Gain mode, the charge-coupled device (CCD)-camera is manually adjusted to higher gain settings, increasing the signal obtained from the EV. Object thresholds and masking have also been adjusted to better identify and detect small particles.

Results: Preliminary results using murine leukaemia virus-sfGFP reference particles have shown up to a fivefold increase in the number of GFP-positive objects collected in High Gain mode, when compared to standard gain on the ImageStream System.

Summary/Conclusion: In this study, we demonstrate improved small particle detection, including EVs, using this new High Gain mode on the ImageStream Imaging Flow Cytometer.

PT05.05

Distance-controlled accelerated catalysed hairpin DNA circuit for multiple and sensitive detection of exosomes-associated miRNAs

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Introduction: Sensitive and simultaneous monitoring of multiplexed exosome-associated RNAs is of great value for early cancer diagnosis remains a challenge.

Methods: Here, we report a simple, multiple and sensitive exosomes-associated multiplex miRNAs detection method that uses distance-controlled accelerated catalysed hairpin DNA circuit (CHDC) system without any complex operation or enzymatic amplification. The distance-controlled accelerated CHDC can directly enter the plasma exosomes to generate fluorescent signal quantitatively by specifically targeting miRNAs without any transfection means.

Results: We show that distance-controlled accelerated CHDC strategy with signal amplification capability could selectively and sensitively identify low level RNAs in serum EVs, distinguishing patients with early- and late-stage breast cancer from healthy donors and patients with benign breast disease.

Summary/Conclusion: This simple, accurate, sensitive, and cost-effective liquid biopsy by the distance-controlled accelerated CHDC method is potent to be developed as a non-invasive breast cancer diagnostic assay for clinical applications.

PT05.06

Impact of isolation methods on biophysical heterogeneity of single extracellular vesicles

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Introduction: Current biophysical analysis of extracellular vesicles (EVs) typically encompasses particle density and size distribution determinations using various techniques. However, variabilities in EV isolation methods and the structural complexity of these biological-nanoparticles (sub-100 nm) necessitate more rigorous nanoscale biophysical characterization of single EVs to facilitate more reliable and comparable EV-based assays.

Methods: Combining atomic force microscopy (AFM), super-resolution optical and conventional particle sizing light scatter and microfluidic techniques, we compared the unique sub-nanometre scale biophysical properties of breast cancer cell-derived EV isolates obtained using different isolation methods.

Results: AFM and dSTORM particle size distributions showed coherent unimodal and bimodal particle size populations in centrifugation and immune-affinity isolates respectively. More importantly, AFM imaging revealed striking differences in nanoscale morphology, surface undulations, and vesicle-to-non-vesicle ratios among EV isolates from different isolation methods. Our findings demonstrate the effectiveness of orthogonal high-resolution biophysical characteristics of single EVs, not discernable via particle size distributions and counts alone.

Summary/Conclusion: The identified nanoscale biophysical characteristics of EV isolates represent a strategic and complementary framework to resolve

differences in the heterogeneity and purity of EVs from different cell types and isolation techniques.

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PT05.07

Ready-to-use, pre-labelled and stable human EVs for validating new standardization procedures of EV concentration measurements

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Introduction: Extracellular vesicle (EV) concentrations measured by flow cytometry are incomparable. To improve comparability, the METVES II consortium is developing traceable reference materials and procedures, which require validation by test samples. In previous interlaboratory comparison studies, however, a main source of variation was introduced by pre-analytical variables and measurement artefacts introduced by test samples. To minimize variation introduced by test samples, our aim is to develop off-the-shelf biological test samples containing pre-labelled EVs.

Methods: Human urine and plasma were collected from healthy donors. EVs were labelled with lactadherin-FITC, isolated by size-exclusion chromatography to remove free dye and minimize swarm detection, and mixed with dimethyl sulphoxide (DMSO), ExoCap or trehalose, frozen in liquid nitrogen and stored at −80° C. After thawing, EV concentrations were measured by a calibrated flow cytometer (Apogee A60-Micro).

Results: Compared to the EV concentrations measured in fresh plasma and urine, the concentrations decreased 27% in plasma ($p = 0.04$; mean of the 3 cryopreservation agents) and 35% in urine ($p = 0.05$) after one day of storage. After 5 months of cryopreservation, the concentration of plasma EVs decreased 2% (DMSO and Exocap) and 8.5% (trehalose) compared to one day of storage, whereas the concentration of urine EVs decreased 6% (Exocap) and 18% (DMSO and trehalose).

Summary/Conclusion: We have developed ready-to-use, pre-labelled human EVs that are stable up to 5 months and dedicated for use in interlaboratory comparison studies. To further increase stability, other cryopreservation agents will be tested. Our biological test samples will be key to validate the new reference materials and procedures developed by METVES II in 2021.

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PT05.08

Understanding intracellular fate of EV-delivered content

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Introduction: Despite much work performed on evaluating the potential effects of extracellular vesicles (EVs), the functional uptake of their cargo is still controversial. This project aimed to demonstrate that EV content (protein and mRNA) is protected and can be subsequently transferred with functional activity into recipient cells, while also developing a tool to assess and quantify functional EV uptake.

Methods: Fusion proteins used were mitochondrial localized coxVIII-CFP-nanoluc(Cox) and nuclear localized H2B-RFP-nanoluc(H2B).

Results: HEK293 T cell-derived EVs protected Cox proteins from proteinase K digestion while demonstrating significantly improved efficiency of uptake when compared to free protein, as measured by bioluminescence that was still detectable in recipient cells 96 hrs post-EV-exposure. To confirm functional uptake, recipient cells exposed to EVs containing H2B for 72 hrs were imaged and some recipient cells manifested fluorescent red nuclei. To demonstrate the presence of functional mRNA within EVs, producer cells were transfected for such a duration as not to have detectable levels of protein in the EVs while still containing detectable levels of mRNA (qPCR) even after RNaseA treatment. Transfer of these EVs to HeLa cells showed an increase in expression of H2B which was blocked by

cyclohexamide, confirming translation of the mRNA (2.2kb). To determine if recycling of EV delivered proteins occurs, recipient HeLa cells were exposed to EVs containing Cox for 72 hrs. All extracellular EVs were removed and cells were trypsinized (0.25% for 30 min) to remove any non-internalized Cox protein. 48 hrs later, EVs (CD63+ and CD9+) released from cells contained Cox suggesting recycling of protein or possibly recycling of entire EVs. Lastly, an assay was developed to measure functional EV uptake. Nanoluc protein was split in two and fused to mTurquoise2 (N65) or mScarlet-I(66 C). Expression of each fragment alone exhibited non-detectable levels of luminescence while expressing both together had a significantly increased signal. Delivery of either fragment within an EV to a cell expressing the corresponding fragment worked as confirmation and quantification of EV uptake (HEK293, U87, HeLa cells).

Summary/Conclusion: This study robustly demonstrates EV delivery of functional mRNA and protein to cells, while also establishing a simple assay to quantify and validate functional EV uptake.

PT05.09

Theoretical model of EV losses due to adsorption on the tube walls. Application for immunomagnetic detection of the vesicles

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Introduction: Short-term storage of unfrozen samples of vesicles, mainly at 4°C, overnight or during a couple of days is rather common laboratory practice. However, it was found to lead to significant losses of vesicle concentration supposedly due to adsorption on the walls of the tube. The present work develops a theoretical model intended to describe the vesicle adsorption process. The experimental validation of the model was made using method of immunomagnetic precipitation.

Methods: The theoretical model considers the "diffusion-limited" case of vesicles storage. The maximal adsorption capacity of the surface of contact between the tube and the solution is given as the number of vesicles in hexagonally packed monolayer.

For experiment, the vesicles were purified from HT29 cell culture supernatant by differential centrifugation, aliquoted and kept at −80 C. Further the aliquots were consequently unfrozen, and placed into the tubes with different surface treatment and kept at +4 C. The kinetics of vesicles loss was measured by anti CD9 immunomagnetic capturing followed by CD81, EpCam and CD166 staining and flow cytometry.

Results: The model allows the estimation of the adsorption-associated losses as dependent on initial vesicles concentration, volume of the solution, tube geometry, the storage temperature and duration case of quiet vesicles storage (without mixing) and also accounts an expected effect of active agitation of the solution (EV-beads complexes formation).

Theoretical calculations were illustrated by analysis of EV at different storage conditions and during reaction of immunomagnetic precipitation of the vesicles.

Summary/Conclusion: It was demonstrated that application of tubes surface treatment allows increasing sensitivity of immunomagnetic precipitation method to 2x10^5 for CD81+, 5x10^5 for EpCam+ and 2x10^8 for CD166+ vesicles.

PT05.10

Videodrop by Myriade: the use of interferometric microscopy to quantify vesicles in complex samples such as faecal filtrates

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Introduction: It is now largely accepted that the intestinal microbiota plays a key role in Intestinal Bowel Diseases (IBD). An imbalance in the composition and diversity of the intestinal microbiota (i.e. dysbiosis) of patients has been repeatedly pointed out by several teams. There are also indications that extracellular vesicles produced by bacteria and exosomes produced by epithelial cells might be increased in this family of diseases.

Methods: In order to differentiate healthy and IBD faecal samples on the basis of their vesicle profiles, we want to develop a means to enumerate rapidly particles in faecal samples, based on interferometric microscopy. The Videodrop technology, developed by Myriade, relies on the creation of single beam interferences between two signals from the same light path by nanoparticles such as small vesicles. It will permit to compare on large scales the viral load of healthy subjects and IBD patients.

Results: This fast and easy-to-use device was compared to the NTA on several types of eukaryotic and prokaryotic vesicles and our preliminary results are encouraging.

Summary/Conclusion: If IBD patients indeed have an increased number of vesicles in their stools, the Videodrop could be a new diagnosis tool for such conditions.

PT06: EVs in Stem Cells (including Cancer Stem Cells)

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Chair: Gareth R. Willis – Harvard University & Boston Children's Hospital

PT06.01

Characterization of small extracellular vesicles produced by human mesenchymal stromal cells in an improved extracellular vesicle-free medium

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Introduction: Small extracellular vesicles (sEVs) produced by mesenchymal stromal cells (MSC-sEVs) may be useful in cell-free therapies for immunomodulation and tissue regeneration.
Methods: To characterize MSC-sEVs produced ex vivo, human bone marrow MSCs were cultured in MesenCult-ACF Plus (MACFP), an EV-free and animal component-free culture medium for 3 days and spent medium collected to isolate sEVs by ultracentrifugation (UC). Analyses of sEVs were performed by Nanoparticle-tracking analysis (NTA), western blot (WB), and human umbilical vein endothelial cell (HUVEC) tube formation assay.
Results: Analysis of fresh uncultured MACFP by UC, NTA and WB for CD63, CD81, and CD9 confirmed the absence of sEVs. MSC-sEVs isolated from spent MACFP by UC ranged from 80–150 nm in size and were positive for CD63, CD9, and CD81 proteins. These sEVs could be stored at −80°C for >4 months in solution or lyophilized with minimal loss based on NTA and WB analysis. The MSC-sEVs contained the MSC-associated microRNAs let7a, miR21, and miR26a as per qPCR analysis. The biological function of ex vivo isolated MSC-sEVs was assessed using a human umbilical vein endothelial cell (HUVEC) tube formation assay. HUVECs treated with MSC-sEVs generated tubes as early as 6 h after seeding, which were not observed in control HUVEC cultures until 15 h. Moreover, the number of branch points present in such tube structures was >fourfold higher in HUVEC cultures (n = 5) supplemented with MSC-sEVs versus control, with the former lasting >60 h and the latter lasting <50 h in culture. Direct comparison of the performance of MACFP medium to media containing non-depleted or EV-depleted foetal bovine serum demonstrated that only MSCs cultured in MACFP (n = 3) were able to expand robustly with a doubling time of 1.1, 2.1 and 8.9 days in these media, respectively. Lastly, methods for isolating sEVs using newly developed EasySep-EV™ magnetic separation kits and size exclusion columns will be presented.
Summary/Conclusion: Taken together, these data demonstrate that MSC-sEVs can be produced in high yield in MACFP medium and that these possess similar physical, phenotypic and functional characteristics as sEVs in vivo.
Funding: This work was privately funded by STEMCELL Technologies Inc.

PT06.02

Comparison of classical and imaging flow cytometry platforms for the characterization of stem cell-derived extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are heterogeneous group of small vesicular structures released by different types of cells, including stem cells (SCs). As recent studies demonstrate that they may enclose bioactive content and transfer it into the target cells, growing interest is placed on the utilization of EVs in the field of biomedical research. However, there is still lack of standardized methods of EVs characterization. As an example, typical flow cytometry-based protocols, commonly used for cells phenotyping, may be inadequate for the characterization of EVs as particles with size close to the detection limit of conventional

cytometers. Thus, the aim of this study was to optimize and compare the use of different flow cytometry platforms for the multiparameter analysis of EVs isolated from different types of SCs populations.

Methods: EV samples were obtained by ultracentrifugation of conditioned media collected from selected SCs types, including human induced pluripotent SCs (iPS) and mesenchymal SCs (MSCs). Next, several high resolution flow cytometry systems: Cytoflex, Apogee (A50 and A60 Micro-Plus) and Image Stream Mk II were employed to compare their sensitivity and resolution, as well as influence of "swarm" effect. Furthermore, we examined EVs phenotype, including expression of tetraspanins and other surface markers.

Results: Our results have revealed that tested flow cytometry systems may be utilized for the phenotypic characterization of EVs secreted by SCs populations. However, the conventional staining and gating strategy protocols have to be thoroughly optimized. Additionally, depending on a type of tested cytometer, we have demonstrated the difference in a "swarm" effect and its influence on obtained results regarding EVs phenotype. Finally, imaging flow cytometry platform was also employed to visualize EVs on the single particle level.

Summary/Conclusion: In conclusion, we have demonstrated that tested high-resolution flow cytometry platforms are convenient methods for the multiparameter characterization of EVs produced by different types of SCs populations. However, careful selection of particular measurement parameters should be performed depending on a type of employed system.

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PT06.03

Evaluation of ATCC's exosomes from cell culture supernatant as reference standards in research and development.

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Introduction: Exosomes are subcellular particles 30–150 nm in size released from cells through a fusion of multicellular bodies with the plasma membrane. Exosomes are stable carriers of cell-free cargo in the form of DNA, RNA, and proteins, thereby making them an attractive candidate for diagnostic and therapeutic applications. However, isolating a consistent population of exosomes can be challenging and there is an unmet need for highly characterized exosomes for use as reference standards in extracellular vesicle research (EV).

Methods: Exosomes were isolated from cell culture supernatants of different ATCC cell lines including stem cells and cancer cell lines representing the most prevalent cancer types -prostate, colorectal, breast, lung, cervical and glioblastoma, using Tangential flow filtration (TFF). These exosomes underwent sterility and mycoplasma tests as a part of their quality control. The morphology and size distribution of these exosomes were evaluated through multiple strategies including Nanoparticle tracking analysis (NTA), Asymmetrical flow field-flow fractionation (AF4), Cryo-electron microscopy (Cryo-EM) and Spectra DyneTM particle analyser. Exosome surface markers were also analysed through multiple strategies such as electro chemiluminescent ELISA, flow cytometry and western blotting. Also, stem cell exosomes and cancer exosomes were further evaluated for functionality through in vitro functional assays including migration assay, angiogenesis and anchorage independent growth assay.

Results: Our optimized TFF method resulted in high yields of > 1 × 1010 exosomes/mL and average protein equivalent of more than 1 mg/mL. More than 90% of the exosomes population had an average size distribution of 50–200 nm and median size of 110 nm confirmed through a number of different size distribution instruments. Although cell line dependent, we were able to obtain similar expression levels of different cell surface markers including tetraspanins (CD63, CD81, CD9) when evaluated through different methods. Our functional data demonstrated stem cell exosomes were functionally active in promoting cell migration and tubule formation. Additionally, cancer cell exosomes were found to promote a malignant phenotype in an anchorage independent growth assay.

Summary/Conclusion: Collectively, we demonstrated our ability to reproducibly manufacture production-scale batches of exosomes from multiple different cell types. Our purified exosomes are of high yield, meet well-established quality control specifications, and are robust in maintaining size distribution, surface marker expression, and functionality in vitro. Therefore, they can serve as ideal reference materials that can support different EV-based research applications.

PT06.04

Exo-cise: Extracellular vesicles enriched from plasma post-exercise promotes myogenesis and neurogenesis

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Introduction: Physical activity brings about a widespread physiological response and elicits the beneficial adaptation of several tissues and organs. Furthermore, regular participation in physical activity reduces the risk of developing major non-communicable diseases such as cardiovascular disease, diabetes, cancer, osteoporosis, and dementia. Two important processes known to occur following physical activity are myogenesis and neurogenesis; both of which involve the activation and proliferation of specialised tissue-resident stem cells. The molecular mechanisms regulating these processes following exercise are poorly understood to date. Here, we investigated the contribution of extracellular vesicles, which are released into the circulation after exercise, to benefit adult myogenesis and neurogenesis.
Methods: Small extracellular vesicles were enriched from the blood of healthy participants before and following maximum and moderate intensity exercise. Differentiation and proliferation using a range of methods was measured following vesicle treatment onto primary myoblasts and neuronal primary ex-vivo stem cells. Activation of key cellular pathways were measured.
Results: We show significant proliferation and differentiation changes of both stem cell types. This is independent of extraction method, extracellular vesicle depleted fractions and is interestingly conserved across mammalian species. Remarkably, we see an age-related effect.
Summary/Conclusion: This advocates that short single bouts of exercise may promote myogenesis and neurogenesis via systemic signalling of extracellular vesicles which opens an interesting field in endogenous EV therapies.
Funding: The Royal Society and Oxford Brookes University Nigel Groome Studentship.

PT06.05

Extracellular vesicle characterization in human induced pluripotent stem cell-derived retinal pigment epithelium

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Introduction: Clinical-grade induced pluripotent stem cell-derived retinal pigment epithelial cells (hiPSC-RPE) show promise as a cell-based therapy for retinal degeneration. While clinical trials are ongoing, the potential of extracellular vesicles (EVs) as biomarkers for monitoring eye health and disease is not well studied. This study characterized the EV surface profile and cargo of hiPSC-RPE to offer a baseline assessment in normal and disease conditions. Moreover, we evaluated the importance of PNPLA6, a gene involved in membrane integrity and when mutated causes retinal degeneration, in EV biogenesis and secretion.
Methods: EVs were isolated from serum-free culture medium of hiPS-RPE and identified with nanoparticle tracking analysis, transmission electron microscopy, and immunoblot analysis of exosomal markers, including Alix, TSG101, and CD63. Surface marker detection and proteomic profiling were completed using an EV surface marker kit and mass spectrometry, respectively. Small interfering RNA targeting PNPLA6 was used to knockdown the expression in hiPSC-RPE and EVs were characterized.
Results: Nanoparticle tracking analysis confirmed the presence of both microvesicles (>150 nm) and exosomes (<150 nm) by size distribution and the concentration of EVs (1x108 particles/ml) from RPE. TEM displayed typical morphological characteristics of EVs. The presence of known EV markers, Alix, TSG101, and CD63 was confirmed via immunoblot and flow cytometry. Surveillance of EV surface markers revealed enrichment of epithelial markers (CD326) and stem cell markers (CD133/1) that depict donor cell origin and functional proteins including integrin-binding (CD29) and TGF-beta receptors (CD105). In addition, proteomic analysis revealed regulators of inflammation and RPE function, including hemopexin, clusterin, complement factor I, and pigment epithelium-derived factor. Furthermore, reduction in PNPLA6 expression reduced vesicle secretion and vesicle size compared to non-targeting controls.
Summary/Conclusion: HiPSC-RPE expresses a population of EVs reflective of normal RPE function. The knockdown of PNPLA6 negatively impacts vesicle secretion and suggests a role for EVs in retinal health. Future studies will elucidate the role of EVs in retinal maintenance and disease states.
Funding: NEI Intramural Funding and Knights Templar Eye Foundation, Inc

PT06.06

Increased VEGF secretion from MSC cell culture in normoxic condition

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Introduction: Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and was first described as an essential growth factor for vascular endothelial cells. VEGF plays a role in normal physiological functions such as bone formation, haematopoiesis, wound healing, and development. Mesenchymal stem cell (MSC) was found to secretes potential growth factors such as VEGF when cultured in vitro. However there are some beliefs that Foetal Bovine Serum (FBS) which usually used as serum in cell culture content VEGF.
Methods: MSC seeded in in 96-well plate in with concentration of 5,000 cell/well. Cells were incubated for 24 hours and fasted for another 16 hours using only DMEM. Cells were treated with complete medium consist of DMEM and 10% FBS. Culture medium were collected after 5, 12, and 24 hours after treatment. Cell were culture in 37°C dan 5% CO2. VEGF concentration was detected using ELISA technique.
Results: VEGF concentration was not found in FBS which do not contact with MSC. An increasing of VEGF concentration in time-dependent manner was shown when culture medium was used in MSC cell culture in normoxic condition. The result of VEGF concentration when culture 5, 12, and 24 hours were 74.022 pg/mL, 76.67 pg/mL, and 93.58 pg/mL, respectively. The mechanism of MSC release growth factor is still under investigated. However, the Classic growth factors and cytokines serves paracrine control molecules which were important in regenerative medicine. VEGF was found to be an important molecules in angiogenesis process and determine the fate of cells.
Summary/Conclusion: MSC secreted VEGF and concentration increased in time-dependent manner.

PT06.07

Isolation and characterization of exosomes from canine stem cells

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Introduction: Unlike induced disease models using laboratory animals, naturally occurring disease models display pathophysiologic attributes that are more similar to human diseases. Unfortunately these models are underutilized in translational regenerative medicine research. This is partly due to the slow development of species-specific experimental therapeutics to investigate comparative efficacy. Thus, we set out to isolate and characterize exosomes from canine adipose-derived mesenchymal stem cells (cAD-MSC) to use as a comparative therapeutic in dogs. To accomplish this, we optimized an isolation and purification strategy and characterized their molecular properties.
Methods: Exosomes were isolated by sequential centrifugation and subsequent ultrafiltration. The proteome was characterized by tandem mass tag (TMT) mass spectrometry and the miRNA cargo was identified using a canine specific PCR array with subsequent target and enrichment analysis using TargetScan and the Panther platform, respectively. Also, nanoparticle tracking analysis and transmission electron microscopy were used to determine exosome size and structure. To investigate bioactivity, we measured the ability of exosomes to inhibit collagen production in an in vitro model of fibrosis.
Results: Exosomes were purified by ultrafiltration using a 100 KDa cut-off. Proteomic analysis by TMT mass spectrometry identified 1262 unique proteins. 90% of the ExoCarta top 100 were identified from this list. Additionally, we identified the miRNA cargo within exosomes and found 27 highly expressed miRNAs. Enrichment analysis identified multiple pathways of probable regulation including angiogenesis (Fold Enrichment = 3.1; $p < 0.0001$) and transforming growth factor-beta (TGFb) signalling (Fold Enrichment = 4.1; $p < 0.0001$). Exosome size was quantified to be 119.7 ± 3.4 nm with a modal average of 98 nm. Lastly, in the presence of exosomes, TGFb stimulated fibroblasts deposited 32.2% less collagen than vehicle controls ($p = 0.036$).
Summary/Conclusion: In summary, cAD-MSCs exosomes display structural and functional features comparable to stem cell derived exosomes from other species. Use of these exosomes in naturally occurring disease canine models may provide superior predictive value for human clinical trials.
Funding: Support provided by the CCAH, School of Veterinary Medicine, UC Davis.

PT06.08

Mesenchymal stem cells-derived exosomes promote in vitro the progression of triple negative breast cancer cells

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Introduction: Mesenchymal stem cells (MSCs) are multipotent stromal cells and have been described as key regulators of different aspects of tumour physiology. In tumour pathogenesis, MSCs can integrate the tumour microenvironment after recruitment and are able to interact with cancer cells to promote tumour modifications by affecting epithelial-to-mesenchymal transition (EMT). It was revealed that exosomes derived from MSCs are critical players in the tumour niche. Exosomes are a novel way of cell-to-cell communication and play crucial roles in the majority of pathways that contribute and affect response to therapy, cell-adhesion molecules and the progression of tumour cells. Because of the known importance of this communication we decided to investigate the implication of MSCs with triple negative breast cancer (TNBC) cell lines as well as exosomal profiles between the experimental conditions.

Methods: The interactions of MSCs with triple negative breast cancer cell lines (MDA-MB-231 and Hs578T) was performed by coculturing MSCs (or TNBC cell lines) with exosomes derived from TNBC cell lines (or MSCs). Physical characterization of isolated exosomes was performed followed by their molecular investigations. Cell proliferation was detected by MTT assay and migration was analysed by wound healing assay using 2D cultures. Moreover, we also used 3D culture to assess the exosomes uptake and to observe their capability of internalization into a 3D structure. The alterations in expression level of some transcripts (mRNAs and miRNAs) and protein profile were investigated by qRT-PCR, western blot and immunofluorescence staining.

Results: We found that MSCs-derived exosomes are actively incorporated by triple negative breast cancer cell lines (2D culture). In coculture, in TNBC cells the expression level of mesenchymal markers and EMT markers (E-cadherin, vimentin) at mRNA and at protein levels, as well as miRNA-derived exosomes targeting mesenchymal genes were significantly affected. Using bioinformatics tools, we highlighted the important biological processes which were activated by promoting tumour modifications. In addition, using 3D culture we provided a comprehensive understanding regarding exosomes internalization in 3D structures, which closely mimics in vivo conditions, compared to 2D culture.

Summary/Conclusion: In this work, we focus on the investigation of MSCs-derived exosomes in order to highlight their implication in several biological processes, including tumour proliferation and progression of triple negative breast cancer cells. All these alterations affect the response to therapy and should be considered for developing efficient therapeutic strategies.

PT06.09

Natural killer cell-derived extracellular vesicles have a potent anti-leukaemic effect and selectively target the cancer stem cell subpopulation

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Introduction: Natural killer (NK) cells of the immune system recognize and kill tumour cells. Extracellular vesicles (EVs) secreted from NK cells are capable of killing tumour cells independent of the cell to cell contact required for NK cell activation. Cancer is a leading cause of death, primarily due to metastasis and recurrence. Cancer stem cells (CSC) within tumours are resistant to chemotherapy and immune attack, and cause metastasis and relapse. Identification of the cancer types killed by NK EVs is limited, and the effect of NK EVs on CSCs has not been described. Here we determine whether NK-derived EVs kill a myeloid leukaemia cell line and its CSC subpopulation.

Methods: NK EVs were isolated from our NK cell line, NK3.3, derived from normal human lymphocytes. NK3.3 EVs were characterized by immunoblotting, proteomics, and next generation RNA sequencing. Human K562 leukaemia cells were treated with NK3.3 EVs in vitro and analysed for proliferation and markers of cell death.

Results: NK3.3 EVs contain EV-associated proteins Alix, CD63, HSP70, and Tsg101, NK effector molecules perforin, granzymes A and B, granulysin and NKLAM/RNF19b, an E3 ubiquitin ligase required for maximal NK cytotoxicity, and tumour suppressor miR-186. NK3.3 EV treatment of K562 significantly decreased its expression of proliferation markers CD71 and Ki67, and increased the frequency of apoptotic and necrotic cells, paralleled by elevated levels of active caspases −3 and −7. Non-tumorigenic cells were unaffected by NK EV treatment. Most notably, NK3.3 EV treatment significantly reduced the frequency of K562 cells highly expressing ALDH, a CSC marker.

Summary/Conclusion: NK3.3-derived EVs have a robust anti-tumour effect on K562 myeloid leukaemia cells and selectively target the CSC population, suggesting they may circumvent the evasion and resistance

mechanisms used by CSCs. NK3.3 EVs therefore have the potential to be a safe alternative, or synergistic partner, to current cancer therapeutics.

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PT06.10

Scalable manufacturing system for MSC-EV generation

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Introduction: Due to their potential as a key bioactive agent in regenerative medicine applications, MSC-derived extracellular vesicles (MSC-EVs) are increasingly being investigated as a clinical therapy. Manufacturing that generates enough EVs for product development and clinical doses is currently a limitation in the field and clearly a scalable manufacturing solution will be necessary for successful translation. Moreover, a complementary approach that increases the EV productivity, i.e. the number of EVs produced per cell, could further help to accelerate the development of MSC-EVs as a therapy.

Methods: We developed a process that leverages a series of new cell culture reagents to couple to our established cell-media system for scalable manufacturing of MSC-EVs. Briefly, human bone marrow- or umbilical cord-derived MSCs were rapidly expanded under xeno-free conditions (i.e. >150X expansion within 10 days). Cultures were then switched to our proprietary EV collection medium and EVs were harvested for up to three additional days. At the end of culture, the EVs in the conditioned media were concentrated using a tangential flow filtration (TFF) system. To increase the productivity of MSCs, two medium supplements were developed that increased EV yield by either increasing the number of EVs generated per cell in a shortened culture process or increasing the number of collected EVs by lengthening the EV collection culture period.

Results: This scalable MSC-EV manufacturing method was implemented in both 2D flask and 3D bioreactor culture and generated over 2,000 particles per cell in 2D and over 4,000 particles per cell in 3D. With the addition of a medium supplement to increase EVs produced per cell, the EV productivity was increased >2x after 24 hrs. Alternatively, EV productivity was also increased >2x by addition of the medium supplement that extended EV collection culture period.

Summary/Conclusion: MSC-EV success in clinical translation will be reliant on a manufacturing method that can scalably and reliably generate large amounts of EVs. These results present one such solution. Furthermore, increasing EV productivity, for instance by medium supplements that increase EVs per cell or lengthen culture times could further address the limitation of generating the EVs required for development and translation of clinical therapies.

PT06.11

Simplifying scalable MSC EV production in a microcarrier-based bioreactor system

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Introduction: The growpt0ing numbers of MSC-EV clinical applications drives the need for a scalable MSC-EV production platform. While most MSC-EVs are generated while cells are attached to tissue culture plastic, such 2D cultures cannot be scaled up to meet the yields necessary for commercialization of EV-based therapeutics. We have shown that 3D bioreactors can be used to generate MSC-EVs and that paradigm can be scaled directly in terms of yield from the 3 to 15 L scales. The technical expertise of seeding cells onto microcarriers for expansion in bioreactors, however, requires technical expertise not available to all those in the EV field. Therefore, our goal here is to simplify and expedite the EV collection process in bioreactors by cryopreserving cells on microcarriers, such that end users can merely thaw and then collect MSC-EVs.

Methods: MSCs were expanded in 2D and then seeded on three different microcarriers and cultured in a bioreactor for 5 days. When confluent, cells on microcarriers were cryopreserved. To evaluate the microcarriers and the cryopreservation protocol, the cells-microcarriers were thawed, cultured in a bioreactor in growth media for 24 hours, then in EV collection media for 3 additional days. Cell recovery and EV production upon thaw was evaluated and compared to EV collection from fresh, non-cryopreserved cells.

Results: Total cell counts 24 hrs post thaw were comparable to those before cryopreservation and to fresh samples prior to EV collection. Following 3-day EV collection, concentration of particles collected from cryopreserved cells on microcarriers were similar to those collected from the fresh cells (2E9 particles/ml). This process was validated for two different microcarriers using two separate cryopreservation solutions.
Summary/Conclusion: Our results show that cryopreserved hMSCs on microcarriers can support EV collection in a 3D bioreactor process with a particle yield that is comparable to those collected from fresh cells. This cryopreserved product can simplify EV production, reducing cost and time by removing process steps associated with the hMSC expansion, with in a paradigm suitable for scale-up.

PT06.12

The whitening, anti-wrinkle, and wound-healing effects of Extracellular vesicles from Orbicularis oculi muscle-derived stem cells.

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Introduction: Skeletal muscle-derived stem cells possess potent therapeutic activities in the treatment of muscle-related disorders. In our study, we tried to isolate and characterize orbicularis oculi muscle (ORM)-derived stem cells (ORM-SCs) from the discarded human tissues which were obtained from the ocular surgery-subjected patients. We also prepared the natural extracellular vesicles (EVs) from the cultured ORM-SCs and assessed the their therapeutic actitities including the skin whitening, anti-wrinkle, and wound healing effects.
Methods: We isolated the ORM-SCs from the patients subjected to ocular surgery and characterized the ORM-SCs by analysing cell morphology, proliferation, expression levels of the cell surface and stemness-associated markers, and tri-lineage differentiation and colony-forming capacities, confirming the stemness properties of the ORM-SCs. Then, we prepared the natural EVs from the ORM-SCs via the centrifugation and filtration of the media supernatants and their therapeutic activity was investigated.
Results: The isolated ORM-SCs showed spindle-like morphology and positive expression of CD105, CD 90, and CD73, but they were negative in expression of CD45 and CD34. The ORM-SCs showed the capacity of osteogenic, adipogenic, and chondrogenic differentiations. The EVs from ORM-SCs (ORM-SC-EVs) possessed the apparent inhibitory effect on the melanin synthesis in B16F10 cells by blocking the tyrosinase activity, although ORM-SC-EVs treatment did not dramatically change the expression level of melanogenesis-related genes, such as microphthalmia-associated transcription factors (MITF), tyrosinase (TYR), tyrosinase-related protein1(TYRP-1), and TYRP-2. In addition, we confirmed that ORM-SC-EVs could stimulate skin cell migration and increase the expression level of anti-wrinkle related genes and wound-healing properties.
Summary/Conclusion: This study revealed the stem cell property of ORM-SCs and the whitening, anti-wrinkle, and wound healing effects of ORM-SC-EVs, suggesting that ORM-SCs and ORM-SC-EVs can be successfully used for stem cell-based EV therapy and cosmetics, by regulation the melanogenesis, wrinkle, and wound.
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PT06.13

Use of stem cell extracellular vesicles as a holistic approach towards CNS repair

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Introduction: Neurological diseases and disorders are leading causes of death and disability worldwide. Many of these pathologies are associated with high levels of neuroinflammation and irreparable tissue damage. We have previously shown that extracellular vesicles (EVs) from infected cells contain viral by products (non-coding RNAs and proteins) and that these EVs can exert deleterious effects on recipient cells1-3. Therefore, in the context of neurotrophic viruses EVs may contribute to or perpetuate processes relating to neuroinflammation and neurodegeneration. Due to their multipotent properties, stem cells have broad applications for tissue repair; additionally, stem cells have been shown to possess both immunomodulatory and neuroprotective properties. In recent years it has been well-established that stem cell EVs play a critical

role in the functionality associated with stem cells. The diverse biological cargo contained within these vesicles are proposed to mediate their effects and, to date, the reparative and regenerative effects of stem cell EVs have been demonstrated in a wide range of cell types. While a high potential for their therapeutic use exists, there is a gap of knowledge surrounding their characterization, mechanisms of action, and how they may regulate cells of the central nervous system (CNS).

Methods: We have isolated and recovered high yields of EVs from large scale cultures of both induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) using tangential flow filtration. Our EV characterization includes both phenotypic (size, tetraspanin expression) and biochemical assays. EV functionality has also been assessed in vitro utilizing several cell-based assays related to cellular viability, migration, angiogenesis, and immunomodulation in both healthy and damaged recipient cells with relevance to the CNS.

Results: Our data suggests that EVs from different sources of stem cells display unique phenotypes, exhibit differential association with various cytokines, proteins, and long non-coding RNAs, and have the ability to significantly enhance processes that are critical for cellular repair4. Lastly, utilizing an iPSC-derived neurosphere model, we have observed a robust uptake of stem cell EVs and have found that these EVs are able to effectively penetrate these 3D structures.

Summary/Conclusion: Collectively, these results highlight the "holistic" properties of stem cell EVs by demonstrating their ability to partially reverse or reduce damage in various cell types.

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PT06.14

The effect of cell culture media on extracellular vesicle secretion from mesenchymal stem cells and human pluripotent stem cell-derived neurons

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Introduction: Cell culture media and its supplements are known to affect the secretion and isolation of extracellular vesicles (EVs) from cell cultures. Identification of these effects is crucial especially when planning to use EVs as therapeutic agents. Here, we investigated the effect of cell culture media on EV yield from human mesenchymal stem cells (MSCs) and human pluripotent stem cell (hPSC)-derived neurons.

Methods: EVs were collected from cell-conditioned media (CCM) and no cell control (NCC) media using size-exclusion chromatography (SEC). MSCs were cultured in DMEM/F12:neurobasal medium or in Opti-MEM reduced serum medium, both supplemented with exosome-depleted foetal bovine serum (FBS). The EV yield from hPSC-derived neurons was compared at two maturation time points (day 46 and 60), in DMEM/F12:neurobasal or in Opti-MEM, with and without 3-hour KCl stimulation. SEC fractions were analysed by nanoparticle tracking analysis (NTA), protein concentration assay and blinded transmission electron microscopy (TEM).

Results: CCM samples had a clear peak of EVs in SEC fractions 7–10, which was not detected with NCC. Interestingly, a second population of EVs eluted in SEC fractions 13–17 in both CCM and NCC, indicating presence of EVs in exosome-depleted FBS. Moreover, this second population differed largely between used media batches. Culture medium had no significant effect on MSC EV yield (DMEM: 8.30E+08 particles/ml, Opti-MEM: 9.61E+08 particles/ml). With neuronal cultures, no significant differences in EV yield were found between culture media or cell maturation time points. In contrast to earlier findings, 3-hour stimulation of neurons by KCl resulted in significantly smaller EV yield compared to non-stimulated controls (stimulated: 5.28E+10 particles/ml, non-stimulated: 1.43E+11 particles/ml, $p < 0.02$).

Summary/Conclusion: Our results indicate that exosome depleted-media are not entirely devoid of vesicles, which can cause bias in downstream analyses. However, SEC is a good method to separate cell-secreted EVs from the contaminating medium-derived EVs. Culture medium did not affect the number of EVs secreted by MSCs or neurons; instead, we observed larger differences between media batches. This data emphasizes the importance of analysing the NCC as negative control in all cell culture experiments.

PT06.15

Mouse mesoangioblast stem cell extracellular vesicles are able to influence macrophage cell activity

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Introduction: It is largely demonstrated that stem cells release extracellular vesicles (EVs) that are able to modify target cell behaviour. Interestingly, there is a bidirectional signalling exchange between stem cell EVs and damaged cells. Moreover, it is well known that macrophages, could also play a role in wound repair and tissue regeneration. It was also demonstrated that stem cell EVs are involved in immune cell regulation. For this reason, today takes hold the idea that EVs could replace stem cells in regenerative medicine. The aim of our work was to evaluate if EVs released by mouse mesoangioblast stem cells (A6) could have a role in immune cell regulation. Specifically, we have investigated the possible A6 EV effect on murine macrophages (RAW264.7) in terms of cell proliferation, migration and phagocytic ability, and cytokines/chemokine release.

Methods: A6 EVs were collected from conditioned milieu by ultracentrifugation. Raw 264.7 cell proliferation with or without A6 EVs was evaluated via CFSE assay. Scratch test was performed to assay their migration ability. To study RAW264.7 cell phagocytosis they were treated with 2 μm beads. Finally, cytokine array was used to monitor their secretion after EV treatment.

Results: We have found that A6 EVs inhibited macrophage proliferation as proved by a proliferation index significantly reduced after EV treatment. Simultaneously, we have noticed that EVs increases RAW264.7 migration ability. Furthermore, A6 EVs are able to increase macrophage phagocytic activity. As it is known that Hsp70 is involved in for macrophagic activity increase and A6 EVs express Hsp70 on their surface, we performed phagocytosis assays assay by blocking the protein or its receptor TLR2, TLR4 and CD91. Our data demonstrated that A6 EVs increase phagocytosis through Hsp70 and its receptors.

We have also proved that A6 EVs modify the expression pattern of cytokines/chemokines released in the extracellular milieu by RAW264.7 cells. In particular, we observed an increase in anti inflammatory cytokines, and a decrease in some inflammatory ones, suggesting that EVs could polarize macrophages towards an anti inflammatory M2 phenotype.

Summary/Conclusion: In conclusions, our data show that A6 EVs influence macrophage activity and additional studies could provide a new insight into understanding the underlying potential of EVs in tissue regeneration.

PT07: EV Biogenesis (from Prokaryotes to Euraryotes), Component Loading, and Release

Chair: Pascale Zimmermann – INSERM/KU Leuven

Chair: Caitlin McAtee – Vanderbilt University

PT07.01

Analytical ultracentrifugation identifies distinct populations of extracellular vesicles (EVs) carrying the evolutionarily-conserved polycystin-2 protein in C. elegans

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Introduction: Autosomal dominant polycystic kidney disease (ADPKD) affects ~1/800. ADPKD is one of the most common, life threatening human genetic diseases and leading cause of end-stage renal failure in adults and children. 95% of ADPKD cases are caused by mutations affecting the ciliary PKD1 and PKD2 complex (Polycystic Kidney Disease 1 and 2). In a healthy kidney, the polycystins localize to renal cilia. Mutations that abrogate ciliary localization of PKD2 (yet preserve its channel function) also cause cysts. Besides cilia, PKD2 is also found in other subcellular locations including extracellular vesicles (EVs) of human urine. How dysfunction of PKD2 trafficking and localization leads to the kidney pathology remains unknown.

PKD2 is evolutionarily conserved across all members of Eumetazoa. In C. elegans, PKD-2 is exclusively expressed in ciliated male-specific neurons, where it is trafficked to cilia and EVs. GFP-tagged PKD-2-containing EVs play a signalling role in inter-organismal communication between animals. Conservation of polycystin-2 cellular localization between worm and human suggests that their network of molecular interactions may also be conserved. We propose that PKD-2 plays distinct roles in cilia versus ciliary EVs.

Methods: To understand the role of EVs in C. elegans inter-organismal signalling, we aim to identify the PKD-2-associated EV proteome, transcriptome, and metabolome. We established a pipeline for fluorescent labelling and tracking specific EV cargoes in a living animal using super-resolution microscopy. We used fluorescence of the PKD-2 carrying EVs to optimize biochemical procedures for their enrichment.

Results: Our initial analysis revealed two populations of PKD-2-carrying EVs that differ in their densities: 1.11–1.12 versus 1.14 g/mL. We are currently characterizing these two distinct populations using transmission electron microscopy and refining our enrichment procedure for protein identification by mass spectrometry, sequencing of their RNA cargoes and metabolome analysis.

Summary/Conclusion: What function human PKD2 plays within the cilia and within the urinary EVs is not well understood. Identification of molecular mediators of C. elegans PKD-2 EV signalling will inform on the interactome of human PKD2 and its function in cilia versus EVs.

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PT07.02

C. elegans ciliated sensory neurons release distinct subpopulations of extracellular vesicles

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Introduction: Ectosomes play roles in many physiological and pathophysiological processes, and their precise is dependent on molecular cargo and parent cell type. A single cell can release distinct subpopulations of EVs enriched with different molecular cargo, which adds complexity to elucidating cargo sorting and biogenesis mechanisms. In the nematode C. elegans, ectosomes bud from sensory neuron cilia and are released into the environment to modulate animal behaviour.

Methods: C. elegans is genetically tractable and optically transparent, allowing for live imaging of fluorescently tagged EV cargo. We express all tagged cargo at endogenous levels, adding physiological relevancy.

Results: We discovered that the calcium homoeostasis modulator ion channel CLHM-1 localizes to cilia of EV-releasing neurons and observed GFP-tagged

CLHM-1 in ciliary EVs. Using super resolution microscopy, we imaged EVs released from animals co-expressing tdTomato-tagged CLHM-1 and GFP-tagged PKD-2 (another vesicle cargo) in the same neurons. While the two proteins colocalize in the cilia, CLHM-1::tdTomato and PKD-2::GFP rarely colocalize in EVs. This indicates that separate subpopulations of EVs are being released from the same neurons. To determine how the CLHM-1 subpopulation is formed, we are investigating candidate genes. ANOH-1, a homolog of the Ca2+ scramblase TMEM16F, localizes to neuron cilia and induces phosphatidylserine exposure on the outer membrane leaflet. In anoh-1 mutants, the number of CLHM-1::GFP EVs released is significantly decreased but the number of PKD-2::GFP EVs does not significantly change. In addition, I am using FACS to isolate CLHM-1 and PKD-2 containing EVs and analysing the respective proteomes with LC-MS/MS.

Summary/Conclusion: We are elucidating mechanisms that give rise to distinct subpopulations of ciliary EVs in C. elegans and defining cargoes being enriched in these EV subpopulations to gain insight into EV cargo sorting and biogenesis mechanisms in ciliated neurons.

PT07.03

Ceramide accumulation induces exosome secretion through lysosomal protein LAPTM4B

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Introduction: Exosomes, a type of extracellular vesicles originated from multivesicular bodies (MVB), are important carriers of cellular molecules and have critical roles in intracellular communication in both health and disease. Ceramides (Cer) are implicated in biogenesis of exosome, however the molecular machinery that mediates exosome secretion remains obscure. Lysosome-associated protein transmembrane-4B (LAPTM4B) is a lysosome/late endosome-resident transmembrane protein, which has been reported to bind Cer. We demonstrate here that LAPTM4B is involved in the exosome secretion, which are induced by exogenous Cer treatment or lysosomal ceramidase inhibition in cultured neuronal cells.

Methods: Neuroblastoma SH-SY5Y cells were treated with Cer (porcine brain-derived Cer or synthetic d18:1/C2:0~ C24:0 Cer) for 24 h. Exosomes were isolated from the culture supernatants by sequential centrifugation and their amounts were measured using PS Capture exosome ELISA kit. To analyse MVB transport, MVB and recycling endosomes are visualized with GFP-CD63 and Rab11 immunostaining, respectively.

Results: We found that exogenous treatment of Cer, especially those with C16 and C18 fatty acids, resulted in a marked increase in exosome secretion. In addition, lysosomal Cer accumulation induced by acid ceramidase inhibition also accelerated exosome production. Knockdown of LAPTM4B significantly prevented the ceramide-dependent exosome release. In addition, we showed that these Cer loading promoted colocalization of CD63-positive MVB with Rab11-positive recycling endosomes, further demonstrated that LAPTM4B knockdown cancelled the Cer-dependent increase of the colocalization.

Summary/Conclusion: These data suggest that lysosomal Cer binds to LAPTM4B and promote the transport of MVB to plasma membrane, resulting in an increase of exosome secretion in neuronal cells.

PT07.04

Chloroquine-mediated lysosomal inhibition alters composition and function of cancer-derived extracellular vesicles

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Introduction: Small extracellular vesicles (sEV) are signalling entities released by many types of eukaryotic cells. sEV are of special interest in cancer due to their reported roles in modulating the cancer microenvironment and facilitating cancer cell invasion. Macroautophagy (hereafter autophagy) is a catabolic process well-known for the recycling of cytosolic cargos through lysosome-mediated degradation. In this study, we profiled the changes in sEV content and function under lysosome inhibition and investigated the involvement of autophagy machinery in sEV content.

Methods: Chloroquine (CQ) was used to inhibit lysosomal degradation and autophagy turnover in triple-negative breast cancer (TNBC) cell lines. sEV were collected via precipitation after pre-clearing and concentration of conditioned media. Western blotting, NanoSight and transmission electron microscopy were used to profile sEV. Quantitative mass spectrometry was used to characterize CQ-induced changes in the sEV proteome. Antibody-conjugated magnetic beads were used in immunoprecipitation of sEV.

Results: CQ treatment did not substantially alter the physical properties of TNBC-derived sEV. However, CQ treatment altered the sEV proteome and growth effects of sEV on normal and endothelial recipient cells. CQ treatment induced co-localization of mammalian ATG8 proteins with endolysosomal markers in the cytoplasm, which coincided with an enrichment of ATG8 s and their adaptor proteins in sEV. CQ-induced enrichment of ATG8 s in sEV required lipidation, and occured preferentially in one subset of sEV.

Summary/Conclusion: Our study reveals changes in the content and function of cancer cell-derived sEV in response to perturbation of intracellular trafficking pathways, demonstrates the flexibility and heterogeneity of sEV composition, and has implications for CQ efficacy in therapeutic settings.

Funding: CIHR with Avon Foundation for Women-Canada grant OBC127216 and BC Cancer-Pfizer Research Innovation Fund. JX was supported by a CIHR Doctoral Award.

PT07.05

Evaluation of culture conditions on the presence of Ago2 in extracellular vesicles

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Introduction: Introduction: Argonaute 2 (Ago2) is the essential component of the RNA-Induced Silencing Complex (RISC) that binds miRNAs and promotes mRNA degradation. Extracellular vesicle (EV)-carried miRNAs have been shown to influence gene expression and functional phenotypes in recipient cells. Many investigators have found Ago2 in EVs and it is postulated that Ago2 is a major transporter of miRNAs into small EVs (SEVs), such as exosomes. Others have reported extracellular Ago2 that is non-vesicular. We set out to evaluate the effect of growth factor signalling and serum contamination on the detection of Ago2 in SEVs.

Methods: Methods: Wildtype KRAS colorectal cancer cells, DKs8, were conditioned with 3 different culture media (serum-free DMEM, DMEM supplemented with EV-depleted FBS, and Opti-MEM). EVs were purified from conditioned media by cushion-density gradient ultracentrifugation. Western blot analysis of DKs8 total cell lysates, large EVs and density gradient fractions was performed, probing for Ago2 and EV marker proteins. The size and concentration of the EVs were determined by Particle Metrix analysis.

Results: Results: In all conditions, we found the highest abundance of SEVs in fractions 6 and 7, as assessed by Western blot analysis. Ago2 was detected in the same fractions as SEVs in both the serum-free DMEM and Opti-MEM conditions, although the levels of Ago2 was higher in the serum-free DMEM fractions compared to that of Opti-MEM. In contrast, Ago2 was present in both vesicular and non-vesicular fractions in the DMEM supplemented with EV-depleted FBS condition. No significant differences were observed in the size and number of EVs collected in the three conditioning methods.

Summary/Conclusion: Summary/conclusion: The presence or absence of Ago2 in EVs has been controversial. Multiple factors may affect the ability to detect vesicular Ago2, including serum and growth factors in the conditioned media that may provide sources of extravesicular Ago2 and also regulate the trafficking of Ago2 into vesicles.

PT07.06

Extracellular vesicles secreted from melanoma cell lines contain similar gangliosides and associated molecules with lipid rafts on the cellular membrane

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Introduction: Cancer-associated glycosphingolipids have been utilized as tumour markers and targets of cancer therapy. We have investigated roles of gangliosides in cancers, and clarified that cancer-associated gangliosides enhance malignant properties of cells by forming complexes with membrane molecules in lipid rafts. In this study, we analysed contents of gangliosides and membrane molecules on extracellular vesicles (ECVs) secreted from melanoma cell lines.

Methods: Melanoma cell lines with various ganglioside patterns were used for isolation of ECVs. Ganglioside-modified melanomas with genetic engineering were also used. Genetic modification was done by cDNAs of ganglioside synthase genes. ECVs were collected by ultra-centrifugation, or by Tim4-beads. Contents in ECVs were analysed by immunoblotting or flow cytometry. Roles of lipid rafts in the generation and secretion of ECVs were analysed by treating cells with 1 mM methyl β-cyclodextrin.

Results: Using 4 melanoma cell lines, ECVs were isolated by ultra-centrifugation, and their sizes were analysed by NanoSight. All samples showed uniform sizes between 100 and 200 nm. Protein amounts in ECVs were measured, showing heterogeneous levels at 10 ~ 80 μg/100 mL. Then, gangliosides expressed on ECVs from these cell lines were analysed using Tim4-beads and flow cytometry. GD3 and GD2 were detected on ECVs almost proportionally with expression levels of those gangliosides on the cell surface. Then, immunoblotting was performed to analyse integrin levels in ECVs from transfectant cells expressing high levels of GD3, showing increased levels of integrins in ECVs from GD3+ cells compared with those from GD3- cell lines. Integrin levels in cell lysates from these cells (GD3+ and GD3 – cells) were almost equivalent. Treatment of a GD3-expressing melanoma cell line by 1 mM methyl β-cyclodextrin resulted in marked reduction of secreted ECVs and amounts of TSG101 in them.
Summary/Conclusion: Ganglioside expression patterns on melanoma cells were well reflected in the expression of gangliosides on ECVs. These results as well as increased levels of integrins in ECVs from GD3 + cells suggest that gangliosides and lipid rafts are involved in the generation and secretion of ECVs.
Funding: This study was supported by a Grant of JST Basic Research Program (CREST) of Japan.

PT07.07

Hypoxia alters the metabolic and miRNA content of breast cancer-derived exosomes

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Introduction: Hypoxia, or low oxygen tension, is a common feature associated with tumour growth and is known to regulate tumour cell function, especially through rewiring of cell metabolism. However, how hypoxia influences tumour cell interactions with surrounding cells is not fully elucidated. We sought to evaluate how hypoxia alters metabolite and metabolism-associated miRNA packaging in exosomes.
Methods: Exosomes were isolated from 4T1 breast cancer cells cultured in normoxia (21% O2) and hypoxia (5% O2) via ultracentrifugation, Optiprep gradients, and size exclusion chromatography. Exosomes were further characterized by Nanosight, Qubit protein quantification, and flow cytometry analysis of exosome markers. Metabolite and miRNA profiling was performed on exosomes and exosome-producing cells in normoxia and hypoxia.
Results: Secretion of exosomes was increased under hypoxic conditions. Metabolite profiling revealed alterations in metabolites specific to exosomes derived from hypoxic cells. Profiling of exosomal miRNA showed packaging of metabolism-related miRNA into exosomes derived from hypoxic cells.
Summary/Conclusion: Hypoxia alters the metabolite and miRNA profiles of cancer cells, with selective packaging of these molecules into exosomes. We identified metabolites and miRNA that are depleted and enriched in exosomes compared to cells. These studies identify hypoxia-associated shifts in exosome cargo, providing insight into exosome cargo packaging with potential implications for understanding how cancer cell-derived exosomes regulate recipient cell function.

PT07.08

Lysosomotropic agents prompts the release of extracellular vesicles carrying autophagy-associated markers: evidence of a general mechanism of secretion driven by lysosomal impairment

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Introduction: Drug-induced lysosomal storage disorders (LSDs) are due to the transient intracellular accumulation, mostly of phospholipids, into multilamellar inclusion bodies within late endosomal/lysosomal compartment. They represent a major side-effect for many drugs of several pharmacological categories. Most LSDs inducers are cationic amphiphilic drug (CAD), but the molecular mechanisms leading to accumulation of undigested substrates are unknown. Extracellular vesicles (EVs) have been implicated in cell waste disposal, but it is unclear whether they might be involved in extracellular release of undigested substrates.
Methods: To investigate this aspect, we developed Hek cells stably expressing the fluorescent fusion proteins EGFP-CD63 and mCherry-CD63, separated EVs by differential ultracentrifugation and quantified by EV-associated fluorescence and NTA particle count.
Results: EVs released by these models upon treatment with drugs inducing the accumulation of phospholipids (amiodarone) or glycosaminoglycans (tilorone), showed the release of fluorescent medium/large EVs

(10k fraction) and small EVs (100k fraction), whose size and distribution were similar to the same vesicles released by control cells, but enhanced the recovery of medium/large EVs and to a lower extent of small EVs, Analysis of EVs associated markers revealed a dose-dependent increase of autophagy-associated markers in medium/large and small EVs. Similar results were obtained when autophagic flux was impaired by drugs raising lysosomal pH by different mechanisms, such as chloroquine and bafilomycin, but not when autophagic flux was stimulated by drugs such as curcumin or overexpression of the endosomal/lysosomal regulator TFEB.

Summary/Conclusion: Overall results show that impairment of autophagic flux, either by indigested substrates or higher lysosomal pH, is associated with an increased release EVs enriched in autophagy markers, compatible with autophagomes and/or amphisomes, unravelling a connection with secretory autophagy.

PT07.09

Roles of extracellular vesicles for lenalidomide-resistance in multiple myeloma

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Introduction: Multiple myeloma (MM) is a haematological tumour. Last decade, the prognosis of MM has improved by the development of therapeutic drugs; however, MM cells acquire drug resistance by long-term exposure of these therapeutic drugs. One of the possible explanations of drug resistance is that cells with drug resistance transmit information to other MM cells and their microenvironmental cells. Although the elucidation of the mechanism of drug resistance in MM have been desired, it remains poorly understood.

Methods: In order to understand the mechanism of drug resistance in MM, lenalidomide resistant cell lines were established by long-term exposure of low concentration of lenalidomide. Drug resistance was assessed by MTS assay and caspase assay. The amount of EV was measured by ExoScreen, which is ultra-sensitive detection method of EVs by measuring surface protein of EVs, such as, CD9 and CD63 (Yoshioka et al., Nat Commun., 2015). To identify the genes which involved in drug resistance, RNA sequence among the drug-resistant cell lines and their parental cell lines was performed.

Results: Firstly, characterization of these cells was confirmed. We found that all of the lenalidomide resistant cell lines secreted more EVs than their parental cell lines. In addition to this, the size of EV derived from resistant cells are smaller than those of parental cells. Next, we collected EVs from resistant cells and parental cells by using ultracentrifugation, and added them to parental cells in the presence of lethal dose of lenalidomide. Compared with EV derived from parental cell lines, the EVs derived from lenalidomide resistant cell lines increased a number of living parental cells. These results suggested that the EVs derived from lenalidomide resistant cells can affect the lenalidomide sensitive cells.

As a result of RNA sequence, several genes highly expressed in resistant cell line we found, which associated with lysosome pathway. Among them, attenuating the SORT1 and LAMP2 genes could significantly reduce the EV secretion in MM cells, leading to enhance the lenalidomide sensitivity.

Summary/Conclusion: Our results showed that EV secretion via SORT1 or LAMP2 could induce the drug resistance in MM.

PT07.10

Study on biological stimulate mechanism of stem cell-derived exosome generation by nanoparticles

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Introduction: Mesenchymal stem cells (MSCs) are pluripotent stromal cells known to release extracellular vesicles (EVs) containing various growth factors and antioxidants that can positively affect surrounding cells. Nanoscale MSC-derived EVs, such as exosomes, have been developed as bio-stable nano-type materials, but had low yield and were difficult to quantify. We hypothesized that the mechanism of nanoparticle-enhanced exosome production would stimulate intracellular molecules. The aim of this study was to elucidate the molecular mechanisms of exosome generation by comparing the internalization of surface-modified positively charged nanoparticles and exosome generation from MSCs.

Methods: Mesenchymal stem cells (MSCs) were cultured in MEM-alpha with 10% FBS and 1× antibiotics. The positively charged nanoparticles were synthesized by Poly-lactide-co-glicolide (PLGA) and polyethylenimine (PEI) with cy5.5 for tracking nanoparticles. All of the exosome image were identified using an electron microscope. Additionally, it was confirmed the internalization of the nanoparticles by IF. The primary antibodies used were anti-EEA1, anti-Rab7 and anti-GM130. In order to prove the development of exosomes, RT-PCR using autophagy-related mRNA was performed. Real-time RT-PCR was performed using the Applied Biosystems sequence detection system 7900. Lastly, miRNA from MSC-derived exosome analysed automatically in the Affymetrix data extraction protocol using the provided Affymetrix GeneChip® Command Console® Software (AGCC). All statistical testing and visualization of differentially expressed genes was conducted using R statistical language 3.3.3
Results: We determined that Rab7, located in the MVB and autolysosomal membrane, was increased upon exosome expression and was associated with autophagosome formation. These results suggested that nanoparticles migrated to lysosomes during treatment; however, intracellular exosome-forming factors were stimulated during endosomal maturation simultaneously.
Summary/Conclusion: Therefore, MSC-derived exosome research using nanoparticles is useful for increasing exosome yield and the discovery of nanoparticle-induced genetic factors.
Funding: This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI19C1334).

PT07.11

Theoretical description of formation of extracellular vesicles by budding of membrane

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Introduction: Understanding mechanisms of extracellular vesicles (EVs) formation is of utmost importance for their effective use in science, medicine and technology. In particular, the discovery of universal mechanisms explaining the phenomena taking place in vesiculation appears to be crucial and highly warranted. Mammalian erythrocytes and giant phospholipid vesicles have been largely used as model systems to study principles of membrane budding and vesiculation. The mechanisms conveniently studied in these simple systems are then generalized to other types of biological membranes. We present a theoretical description of membrane budding and compare the theoretically obtained shapes with the observed ones.
Methods: In accordance with the fluid crystal mosaic model, membrane is considered as composed of constituents (inclusions) subjected to the local curvature field created by surrounding constituents. Constituents can attain different in-plane orientations in the membrane which correspond to different energies. The thermal motion oposes the complete orientational ordering. The single-constituent energy expresses a mismatch of the curvature of the membrane at the position of the constituent and the intrinsic principal curvatures of the constituent and in – plane orientation of their principal axes. The free energy of the whole membrane is obtained by summing up (integration) the contributions of the constituents and using methods of statistical physics, and minimized by using numerical methods.
Results: To outline the principle of (outward and inward) budding, respective sequences of shapes corresponding to a formation of one (outward and inward) spherical bud were calculated by minimization of the free energy. Also the corresponding shapes observed in EVs (imaged by electron microscopy) and in erythrocytes and giant phospholipid vesicles (imaged by optical microscopy) are shown. It can be seen that theoretically calculated shapes and experimentally observed ones agree well over up to 3 orders of magnitude (the order of the size of giant phospholipid vesicles is between 1 and 100 micro metres, in erythrocytes it is about 5 micro metres and in EVs it is about 100 nanometres).
Summary/Conclusion: Budding of the membrane is an universal mechanism in formation of external and internal vesicles.
Funding: This work was supported by the Slovenian Research Agency under grants P3-0388, J1-9162, J2-8166 and J2-8169 and EU Commission under grant Ves4us. Authors are indebted to Anna Romolo for technical assistance in preparing the manuscript.

PT07.12

Role of Arrdc4-mediated ubiquitination in extracellular vesicle biogenesis and protein trafficking

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Introduction: The release of extracellular vesicles (EVs) from cells is important for many cellular mechanisms both in normal physiology and in disease. Arrdc4 (arrestin domain containing protein 4) is an adaptor protein known to facilitate the ubiquitination of target substrates by Nedd4 family ubiquitin ligases. It also traffics cargo to extracellular vesicles. Previous studies show the involvement of Arrdc4 in the trafficking of the divalent metal ion transporter DMT1 to EVs in a ubiquitin-dependent manner, and we aimed to further understand this mechanism.

Methods: We performed mass spectrometry to identify ubiquitinated lysine residues in Arrdc4. We then generated Arrdc4 WT and lysine mutant clones and expressed these in cells to determine the effect on EV biogenesis and protein trafficking.

Results: Mass spectrometry data identified 5 potential ubiquitinated lysine residues. Out of these, lysine 270 appeared to be the most important for Arrdc4 function. Arrdc4K270 R mutation caused a decrease in the number of EV released by the cell compared to Arrdc4 WT, and a reduction in trafficking of DMT1 to EVs. Furthermore, we also observed a decrease in DMT1 activity and an increase in its intracellular degradation in the presence of Arrdc4K270 R. K270 also appeared to be ubiquitinated with K29 polyubiquitin chains by the ubiquitin ligase Smurf1.

Summary/Conclusion: Our data suggests that K29 polyubiquitin chains are the signal for Arrdc4-mediated EV biogenesis and protein trafficking, and loss of this signal causes cargo to be rerouted to intracellular degradation mechanisms.

PT08: Scientific Outreach and Collaboration

Chair: Tanina Arab – Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine

PT08.01

A 3D-printed model to represent the structure and nature of extracellular vesicles, for public engagement and education events.

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Introduction: Explaining the field of extracellular vesicles to the lay public and young audiences can often be challenging. Whilst diagrams and images of EVs may be helpful, conveying clearly the shape and composition of an EV by these means is not always a success. Whilst many members of the audience may be familiar with concepts of cells and related structures, others will find such discussions very abstract and challenging. In order to aid interactions with lay audiences we embarked on the design of a physical hand-held plastic model, representing a typical EV. Incorporating flexibility in the design allowing the community to adapt it to showcase their own research. The second goal was to ensure manufacturability using widely available 3D-printing technologies.
Methods: The basic model design was conceived by Dr C. Burton, and iteratively developed using Solidworks, 2019, then exported for use in any CAD environment (STL format). A model showing a halved EV hemisphere, with a visible lipid-bilayer was developed. Attachable rings allow trans-membrane-molecules to be represented, current designs include MHC Class-I, HSPGs, integrins, tetraspanins and supported by handouts accompanying the models. Intraluminal cargo is included via removeable "pegs", and examples representing RNA or simple globular proteins, and a template has been created.
Results: The design is free and open source, and available to the community at: https://www.thingiverse.com/thing:3986565. Instructions for 3D printing are available from the UK Extracellular Vesicle Society website; https://www.ukev.org.uk/public-engagement-materials/. Models have been produced using entry-level 3D printers and trialled at engagement events with good early responses.
Summary/Conclusion: The authors hope the community will use and develop this 3D-model design and that the approach provides an additional and helpful tool for educating audiences about the complexities and roles of EVs in biology and disease.

PT08.02

Centrifugal filtration-SEC is promising for extracellular vesicle isolation from D492 and D492HER2+ breast epithelial cell lines

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Introduction: Despite recent developments in breast cancer therapy, there is still need for a more targeted approach. Extracellular vesicles (EVs), endogenous nanovesicles released from human cells, are an attractive choice as nanodrug carriers due to their size, stability and their unique targeting specificity. The aim of this study was to determine if centrifugal filtration (CF) combined with size exclusion chromatography (CF-SEC) would be useful for EV isolation from two epithelial breast cell lines D492 and D492HER2+, representing the tissue of interest, and the amount of cell culture needed to get measurable EV concentrations.
Methods: Cell culture media (without serum) from the immortalized breast epithelial cell lines D492 and D492HER2+ was concentrated with centrifugal filtration (CF) followed by isolation with size-exclusion chromatography (SEC) using HiPrep 16/60 Sephacryl S-400 column run with ÄKTA Start (280 nm), 240 min runs. Each fraction (4–5 ml) was collected with fraction collector. Dulbecco's particle free PBS was used as mobile phase. The resulting particles were analysed with nanoparticle tracking analysis (NTA, NanoSight NS300, camera gain 10, static mode, capture time 90 sec), western blotting (WB), microBCA and transmission electron microscopy (TEM, samples fixed with 2% formaldehyse and stained with 2% uranyl acetate, run at 80kV).
Results: Although SEC did not show any prevalent peaks from early eluting regions previously shown to

contain extracellular vesicles, these fractions (F1-F3, 40–130 min) were collected from D492HER2+ cell culture medium. Interestingly, both NTA and TEM suggest that F2 and F3 contained EVs as the isolated particles measured 65 and 58 nm, respectively and TEM revealed spherical particles 20–50 nm in diameter. WB was unable to detect the EV associated protein Alix (but was present in the whole cell lysate). Soluble proteins and protein aggregates eluted late in the SEC chromatogram (180 min), with protein analysis (microBCA), TEM and WB confirming their presence.

Summary/Conclusion: CF-SEC is a promising method for EV isolation for pharmaceutical applications, but further work is needed to optimize the isolation process using ÄKTA Start for these cell lines.

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PT08.03

Customer stories from the EV Core of University of Helsinki

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Introduction: The EV core, world's first EV-dedicated technology platform established in 2016, is a joint venture of two extracellular vesicle (EV) research laboratories at University of Helsinki. As an academic research/service facility, the EV core provides infrastructure, state-of-the-art and emerging EV-technologies for research groups, hospitals, companies and authorities in the EV-field. The EV core provides EV isolation, purification and characterization services and offers contacts to downstream analyses in other core facilities based on optimized EV protocols. Here, we present and discuss the customer experiences and prospects with the aim to further develop EV core services.

Methods: Our most wanted services are nanoparticle tracking analysis, electron microscopy, EV isolation and RNA isolation and consultation. Currently, the key down-stream analysis methods are (mi)RNA sequencing, metabolomics, flow cytometry and functional assays.

Results: We present the stories from our customers starting with their research questions and need for the EV expertise/consultation and equipment. Next, we show how the projects advanced and what types of EV core -derived or other downstream services helped them to achieve their aims. In the end, we will acknowledge the customers experience and current status of their research.

Summary/Conclusion: Narratives of customer stories are an effective starting point for fruitful discussions about the current status and next developments in the young EV service field.

PT08.04

Recent ISEV Workshops: Open, reproducible and standardized EV research (Ghent, 2019) and EVs in immunology (Buenos Aires, 2020)

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Introduction: Since its founding in 2012, ISEV has sought to further extracellular vesicle research in various ways including scientific meetings. These events encompass annual meetings as well as smaller, topically focused workshops, with the first ISEV Workshop (on RNA and EVs) organized in New York City in October, 2012. In December, 2019, the Workshop "Open, Reproducible, and Standardized EV Research" was held in Ghent, Belgium. In March, 2020, the Workshop, "EVs in Immunology" was held in Buenos Aires, Argentina, with a preceding Education Day.

Methods: The international organizing committees of the 2019 and 2020 ISEV Workshops prepared scientific programs around key themes of EV rigour and standardization (Ghent, Belgium, 2019 workshop) and EVs in immunology (Buenos Aires, Argentina, 2020 workshop). Abstract and application submissions were invited. Applications were reviewed and ranked by panels of EV experts for each event, and participants were invited.

Results: The 2019 and 2020 Workshops assembled a total of more than 100 individuals for talks and discussions around the themes of Rigour and Standardization and EVs in Immunology. The Buenos Aires Workshop was preceded by an Education Day, coordinated by the ISEV Executive Committees for Education and Science

and Meetings. During these two workshops, poster presentations were permitted for the first time, affording additional presentation and interaction opportunities. The Rigour and Standardization Workshop also featured real-time discussant polling to facilitate discussion.

Summary/Conclusion: ISEV workshops such as those addressing Rigour and Standardization (Ghent, 2019) and EVs in Immunology (Buenos Aires, 2020) continue to provide opportunities for focused discussion of small groups of experts on key topics in the field. Often followed by published products, ISEV workshops help to lead and coordinate progress in EV science. For future ISEV workshops, educational activities may again expand the reach of each event, while poster sessions and app-driven real-time responses should be considered for enhanced interactions and participant canvassing.

PT08.05

EV Journal Club: exchanging pizza for a worldwide audience during COVID-19

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Introduction: A monthly journal club focused on extracellular vesicle science was established at Johns Hopkins University in 2016, featuring lunch and presentations by academic and industry participants. When COVID-19 prevented in-person meetings beginning in March, 2020, the journal club was converted to a virtual, weekly format on the popular online meeting app Zoom. The journal club has persisted despite initial problems with online vandalism. Most sessions are also made public on a YouTube channel, https://www.youtube.com/c/ExtracellularVesicleClub.

Methods: Weekly EV Club sessions are arranged by the host. Most focus on a specific manuscript related to EVs, but some weeks feature presentations of published or soon-to-be-published research by the presenting authors. Sessions are advertised one week to several days in advance on social media platforms such as LinkedIn, Twitter, and Facebook, asking interested parties to sign up to join a mailing list via SurveyMonkey. The log-in information is then sent to the mailing list. Upon clicking the link, participants are placed in a virtual waiting room for vetting by the host and volunteers. After admission, all parties but the host and presenter are muted to avoid distractions. Questions and comments may be placed in a chat box. Contributions are monitored and compiled by the host and volunteers to build a question-and-answer session at the end of the presentation. Recorded sessions–with or without editing as needed–are placed on the YouTube channel for additional access.

Results: Despite initial problems with online vandalism known as "Zoombombing," the journal club has continued weekly during the COVID-19 shutdown in the host country (US). An audience of between 80 and 150 individuals is typical. Participants typically ask more questions than can be answered in a one-hour time frame. The online format also allows for debate-style events and polling of the audience.

Summary/Conclusion: This EV Journal Club is an example of how online tools can be used to facilitate international scientific interactions. Further development of such formats could provide alternative approaches for ISEV activities in the Science, Education, and Communication areas.

PT09: EV Immunology, Autoimmunity, and Inflammation

Chair: Heather H. Pua, MD, PhD – Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Centre

Chair: Evan Keller – University of Michigan

PT09.01

Air pollution exposure and allergic rhinitis exacerbation: the role of nasal microbiota and extracellular vesicle communication

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Introduction: Allergic Rhinitis (AR) is a systemic airway disease involving the respiratory tract. Lifestyles and environmental factors (e.g. particulate matter, PM) have a role in disease pathogenesis and recurrence.

The study aim is to assess whether the exposure to PM10 and PM2,5, chosen as paradigmatic environmental stressors, could modify the composition of nasal microbiota (NM) and extracellular vesicle (EV9 signalling network, showing a role in allergic AR exacerbation).

Methods: NM analysis were performed on V3-V4 16 S rRNA gene regions amplified from upper-airway tracts of 25 AR cases and 25 healthy individual controls to perform NM analyses. EV size, concentration and cellular origin for each subject were assessed by nanoparticle tracking analysis (NTA) and flow-cytometry (FC). Information on daily PM10 and PM2,5 concentrations at the municipality of residence in the 7 days preceding nasal sampling (i.e. Day −1 to Day −7) was assigned to each subject by ArcGIS software. Multivariable and logistic analyses were applied on NM, NTA and FC outcomes.

Results: When taxonomy composition was considered, in controls Actinobacteria (50.8%) was the most represented, followed by Firmicutes (34.7%) and Proteobacteria (12.8%) while in cases Proteobacteria were 38.8%, Actinobacteria were 37.1% and Firmicutes were 23.4%.

Cases showed a higher concentration of all the investigated EV types, derived from platelets (CD61+), activated endothelium (CD62e+), monocytes (CD14+), eosinophils (CD294+), neutrophils (CD177+), mastocytes (CD203 c+), epithelial cells (EPCAM+), GRAM+ bacteria (Lipoteichoic Acid+), GRAM- bacteria (LPS+). The effect was greatest in the case of mastocytes EVs which were increased 2.5-fold in cases versus controls ($p < 0.001$). EVs were modified by PM exposure at several time lags. In particular, a negative association between PM10 and eosinophil EVs was observed (beta = −0,016; p-value = 0,017).

As we clustered subjects according to their NM, we observed this variable was a strong effect modifier of the association between PM exposure and EV release.

Summary/Conclusion: Our findings start to provide an insight on the effect of air pollution on EVs, taking into account the effect of NM, in patients with AR. Further research is necessary to disentangle the mechanism exerted by inhaled pollutants in modulating EVs and NM, and therefore AR exacerbation.

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PT09.02

Aryl hydrocarbon receptor activation induces the expression of specific MicrorRNAs in Th17 cells that are release into extracellular vesicules and associated with arthritis

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Introduction: In Rheumatoid Arthritis (RA), an autoimmune disorder characterized by a chronic sinovial inflammation, smoking is a major risk factor

contributing to disease progression, and poor response to therapy. Th17 cell is actively involved in worsening smooking-associates inflammation mediated by Aryl hydrocarbon receptor (AhR), a cytoplasmic transcription factor involved in xenobiotic metabolism. Both, AhR and Th17 cells, has important implications during RA development. Considering that cigarette smoke is a potent epigenetic modifier, we hypothesized that AhR activation, by cigarette components, would transcribe specific microRNAs in Th17 cells as a molecular mechanism to exacerbate inflammation in arthritis.

Methods: MicroRNA expression was evaluated by large-scale approach or real-time PCR. C57/BL6 and AhR null mice were submitted to arthritis experimental models and exposed or not to cigarrete smoke (ethical committee approved 048/2012). Extracellular vesicles (EVs) were isolated by ultracentrifugation, and characterized by western blot and nanosight. RANKL-induced osteoclasts (OCs) differentiation in vitro was stained for TRAP. Inhibition of miRNAs were performed using anti-miRs transfection.

Results: We identified a specific group of miRNAs induced in Th17 cells after AhR activation. During arthritis progression, the microRNAs are expressed and increases after exposure to cigarette smoke. In the absence of AhR their levels were drastically reduced. Interestingly, we found that these microRNAs are released by Th17 cells into EVs, and are able to promote osteoclastogenesis. OCs differentiation in vitro increases in the presence of Th17-derived EVs, and this process is reduced in the absence of microRNAs.

Summary/Conclusion: MicroRNA-mediated gene regulation plays crucial roles in the immune system functions, and their abnormal expression is highly correlated with the pathogenesis of RA. EVs are known to function in cell-to-cell communication and are able to transmit their contents and cause changes in the target cell. Our findings demonstrate a new molecular mechanism by which cigarette smoke could aggravate inflammation in arthritis; through the activation of AhR receptor in Th17 cells, inducing the transcription of specific microRNAs that are released into EVs, and act as pro-inflammatory mediators.

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PT09.03

Extracellular Vesicles released by Trypanosoma cruzi trypomastigotes under different stress conditions

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Introduction: Chagas disease (CD) is caused by the flagellated protozoan T. cruzi. Trypomastigote forms are capable of releasing extracellular vesicles (EVs) that contain the major surface molecules of the parasite. The parasite has a complex life cycle that leads to it a rapid adaptation in the environmental changes in the hosts. However, the effects of stress on on EVs release are not completely understood. Objetive: we evaluated the release of EVs by trypomastigotes incubated under different stress conditions and the immunomodulatory role of these EVs in pre-activated bone marrow-derived macrophages (BMDM).

Methods: Nanoparticle tracking analysis (NTA) and scanning electron microscopy (SEM) showed an increase in EVs releasing by trypomastigotes at 4°C under acidic conditions, EVs released was affected and triggered amastigogenesis process.

Results: Treatment with sodium azide (NaN3) also caused changes in the release of EVs regarding size and concentration. Nitrosative stress caused by sodium nitrite (in culture medium mildly acidic, pH 5.5; in this condition NaNO2 releases Nitric Oxide) stimulated an increase in production of EVs by T. cruzi. When the parasites were treated with 100 nM S-nitrosoglutathione (SNOG), we observed a reduction in size and concentration of vesiculate material by trypomastigotes. At a higher SNOG concentration (100 μM), the concentration of the vesiculate material increased. T. cruzi-derived EVs exposed to stress conditions increased the expression of iNOS, Arg 1, IL-12 and IL-23 genes in IFN-γ and LPS pre-activated BMMs.

Summary/Conclusion: Results suggest that the viability and/or integrity of the parasite are necessary for the EVs releasing. In those in vitro conditions they triggered a proinflammatory response in host cells. This may be a strategy developed by the parasite to favour its establishment in the host.

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PT09.04

Immuno-toxicological evaluation of human mesenchymal stem cell-derived extracellular vesicles

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Introduction: Mesenchymal stem cells (MSCs) have been widely used to the field of autoimmune diseases or tissue regeneration therapy. Recently, many research groups have reported that MSCs showed their ability via secreted paracrine mediators including extracellular vesicles (EVs) rather than cell-to-cell contact. MSCs mainly exist on bone marrow, peripheral blood, umbilical cord and adipose and can mostly secrete EVs. It has emerged that EVs alone are responsible for the therapeutic effect of MSCs in plenty of animal diseases models. Hence, MSC-derived EVs may be used as an alternative MSC-based therapy in regenerative medicine.
Methods: As part of safety programme for human therapeutics, we performed immunotoxicological assessment of EVs obtained from human MSCs (hEVs) in mice and human peripheral blood mononuclear cells (hPBMCs). Firstly, mice were treated intravenously with a negative control, a positive control (LPS; 0.4 mg/kg), or low-dose (1x10e8 paticles/head) and high-dose (1x10e9 paticles/head) of hEVs every other day for 10 days and then analysed lymphocyte subsets from collected spleen by FACS.

Next, we treated the EVs on hPBMCs for 3 days with low conc. (1x10e8 particles/ml), high conc. (1x10e9 particles/ml), PMA/Ionomycin as a cell activator or CPT (10 μM) as an apoptotic inducer. Annexin V/PI and CSFE were analysed by FACS.
Results: As a result, splenic NK cells and B cells were slightly increased about 2 ~ 7% in hEVs- treated mice, without biological significance, compared with a positive control (LPS) as an immunogenicity inducer. And there were no effects on serum levels of inflammatory cytokines in mice. In addition, hEVs had no cytotoxic effect on hPBMCs at both low and high conc. Under the culture medium with EVs-depleted FBS, the hEVs appeared minimal anti-apoptotic effect on hPBMCs. For the CFSE assay, the hEVs showed slight proliferation on hPBMCs and PBMC activation induced by PMA/Ionomycin.
Summary/Conclusion: In conclusion, the hEVs have little immuno-toxicological effects in mice and hPBMCs. Further detailed studies to elucidate immunological response of hEVs for development of human therapeutics are needed.
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PT09.05

Investigation of immune response to mesenchymal stem cell-derived extracellular vesicles in the cancer setting

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Introduction: Mesenchymal Stem Cell-derived extracellular vesicles (MSC-EVs) are thought to be a fingerprint of the secreting cell and therefore may retain the cancer targeting and immune privilege of MSCs. Thus MSC-EVs hold immense potential as tumour-targeted therapeutics for breast cancer. The aim of this study was to determine whether MSC-EV administration in tumour bearing immunocompetent animals would initiate an immune response.
Methods: EVs were isolated from conditioned media of both human and murine bone marrow-derived MSCs through sequential differential centrifugation, microfiltration and ultracentrifugation. EVs were characterized by Nanoparticle Tracking Analysis (NTA), Western Blot and Transmission Electron Microscopy (TEM). 1 x 10(8) human or murine MSC-EVs were administered intravenously into 4T1 breast tumour bearing Balb/c mice (n = 6) and healthy controls (n = 6). Tumour tissue, draining lymph node and spleen were then harvested, dissociated and flow cytometry performed targeting markers associated with a range of immune cells including T-cells, macrophages and natural killer (NK) cells.
Results: EVs were successfully isolated from murine and human MSCs with the appropriate size of small EVs (sEVs: 30–150 nm) and morphology including a lipid bilayer observed by TEM. EVs expressed tetraspanins CD63, CD81, CD82; Cytosolic protein TSG101 and were negative for Calnexin. EV concentrations ranged from 4.08 x 10(9) – 6.6 x 10(9)/ml. In order to study a range of immune cell populations two antibody panels were created using complimentary fluorescent dyes. The proportion of T-cells (CD4+, CD8+, CD25+), Neutrophils (GR-1+, LY-6 C+), Dendritic cells (CD11 c+), Macrophages (CD11b+, MHCI+, MHCII+), NK cells (CD27+) and B cells (CD19+) remained stable in the tumour, draining lymph node and spleen of all tumour-bearing animals that received

either human or murine MSC-EVs, with no significant change observed in any category.

Summary/Conclusion: The data presented supports the hypothesis that MSC-EVs retain the immune privilege of the secretory cell, with human cell-derived EVs illiciting no immune response in mice. This is encouraging and reinforces the potential for use of MSC-EVs in the therapeutic setting.

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PT09.06

Outer membrane vesicles of Mycobacterium avium modulate inflammatory response in monocytes

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Introduction: Mycobacterium avium (M. avium) is a slow growth rate non-tuberculous mycobacterium (NTM). M. avium infection is a severe global health problem. But the mechanisms of pathogenicity of M. avium are poorly understood. Outer membrane vesicles (OMVs) that traverse the cell wall and contain a varied bioactive components inculding DNA, RNA, protein and toxins. Previous studies have suggested that these OMVs are produced in vitro and during animal infection, but the role of OMVs secretion during the interaction of M. avium with host cells remains unknown.

Methods: In this study, M. avium were grown in Middlebrook 7H9 medium (M7H9) supplemented with 10% (v/v) OADC enrichment and 0.5% (v/v) glycero. M. avium OMVs were isolated by ultracentrifugation method. Characterization of OMVs by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). The RAW 264.7 murine macrophages were incubated with the M. avium OMVs to analyse inflammatory response and production of nitric oxide (NO) and reactive oxygen species (ROS) of macrophage.

Results: In this study, we demonstrate by fluorescence microscopy that murine macrophages can phagocytosis OMVs produced by M. avium. Incubation of M. avium OMVs with murine macrophages resulted in increased levels of extracellular tumour necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), terleukin-6 (IL-6) and interleukin-12 (IL-12). Meanwhile OMVs stimulated macrophages produce NO and ROS.

Summary/Conclusion: The above results indicate that the outer membrane vesicles of M. avium could stimulate macrophage function and induce inflammatory immune responses comparable to M. avium infection but do not cause cell apoptosis. Our findings suggest that M. avium secretory outer membrane vesicles have the potential to influence the interaction of M. avium with host cells.

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PT09.07

Sepsis alters the differential expression of microrna in platelet-derived small extracellular vesicles of paediatric patients

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Introduction: Hospital associated venous thromboembolism (HA-VTE) in paediatric patients is the second most common contributor to harm in hospitalized children. Platelet-endothelial interactions are integral to the formation of VTE, especially in inflammatory conditions such as sepsis. Small extracellular vesicles (sEVs) have the ability to reprogramme target cell phenotypes via their microRNA contents and are known to contribute to VTE formation. We hypothesize that sepsis alters platelet-derived sEV microRNAs capable of net upregulation of vascular endothelial procoagulant and downregulation of anticoagulant pathways.

Methods: Using a precipitation solution and size exclusion chromatography, we isolated sEVs from platelet poor plasma of children admitted to the paediatric intensive care unit for sepsis and from healthy controls. We positively selected platelet-derived sEVs using immunomagnetic isolation for CD42B platelet antigen and confirmed selection using flow cytometry. MicroRNA was profiled using Affymetrix GeneChip miRNA 4.0 array.

Results: MicroRNA from 9 sepsis patients (median age 7.0 years; IQR:1.2–13 and 77% female) with a median pSOFA score of 6 (IQR:2.5–10) and from 5 healthy controls (median age 9 years; IQR:6.5–13.5 and 20% female) was isolated and compared. In septic vs. healthy patients 30 miRNAs were differentially expressed (false discovery rate (FDR)<0.05; fold change ≥|1.5|) affecting 921 mRNA pathways. In septic children, pathways affecting chemotaxis and cell movement of leukocytes were predicted to be activated with z-scores ≥2.

Summary/Conclusion: We developed a method to successfully isolate platelet-derived sEVs. Sepsis alters the platelet-derived sEV microRNA profile in paediatric patients with sepsis. These microRNAs are predicted to target chemotaxis and cell movement pathways, important contributors in the formation of HA-VTE. Further analysis into specifically targeted pathways should be conducted as a potential target for the prevention of HA-VTE in sepsis.

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PT09.08

T cell-exosome-derived miR-142-3p impairs glandular cell function in Sjögren´s syndrome

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Introduction: Sjögren´s syndrome (SS) is a systemic autoimmune disease that mainly affects salivary and lacrimal glands. Mechanisms of SS pathogenesis are poorly understood. It is thought that inflammation leads to destruction of exocrine glands, however the triggers of autoimmunity and the mechanisms by which inflammation drives immunopathology are not characterized. Our work identifies T cell-exosome-derived miR-142-3p as a pathogenic driver of immunopathology in SS. MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules that regulate the expression of target genes through translational repression of mRNAs. Through transcriptomic profiling studies our group had previously documented a significant upregulation of miR-142-3p in patient SS tissues and in serum exosomes.

Methods: Structured search for target genes of miR-142-3p involved in salivary gland (SG) physiology was performed with mirDIP 4. SERCA2b, RyR2 and AC9 were selected for further validation and functional analysis. Binding of the miRNA was confirmed by luciferase reporter assays in HSG cell lines and human-derived primary epithelial cells. The mRNA and protein levels of SERCA2b, RyR2 and AC9 were determined by qPCR and Western blot, respectively. To investigate the cell-specific distribution of miR-142-3p in relation to the expression levels of SERCA2b, RYR2, and AC9, a double fluorescent in situ hybridization was performed. Ca2+ signalling and cAMP levels were measured using fluorescent sensor. To isolate exosomes, the T cell medium and serum of SS-patients and healthy volunteers (HV) were collected.

Results: We show that miR-142-3p is over-expression in the SGs of SS-patients. Next, we demonstrated that miR-142-3p is contained in exosomes in serum of SS-patients significantly more than serum of HV. We also show that activated T cells secrete exosomes containing miR-142-3p which transfer into glandular cells and affecting intracellular Ca2+ signalling, cAMP production and protein production by miR-142-3p targets (SERCA2B, RyR2 and AC9).

Summary/Conclusion: This study provides evidence for a functional role of the miR-142-3p in SS pathogenesis and promotes the concept that T cell-activation directly may impair epithelial cell function through secretion of mi-RNA containing exosomes.

PT09.09

Treg-Derived IL35-Coated Extracellular Vesicles Promote Infectious Tolerance

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Introduction: Interleukin-35 (IL35) is an immunosuppressive cytokine composed of Epstein-Barr-virus-induced protein 3 (Ebi3) and IL12-alpha chain (p35) subunits, yet the forms that IL35 assumes and its role in peripheral tolerance, remain elusive.

Methods: We induce CBA-specific, IL35-producing T regulatory (Treg) cells in TregEbi3 WT C57BL/6 reporter mice, and identify IL35 producers by expression of Ebi3TdTom gene reporter, plus Ebi3 and p35 proteins.

Results: Curiously, both subunits of IL35 were displayed on the surface of tolerogen-specific Foxp3 + and Foxp3neg (iTr35) T cells. Furthermore, IL35 producers, although rare, secrete Ebi3 and p35 on extracellular vesicles (EV) targeting a 25- to 100-fold higher number of T and B lymphocytes, causing them to acquire surface IL35. This surface IL35 is absent when EV/exosome production was inhibited, or if Ebi3 is genetically deleted in Treg cells.

Summary/Conclusion: The unique ability of EV to coat bystander lymphocytes with IL35, promoting exhaustion in, and secondary suppression by, non-Treg cells, identifies a novel mechanism of infectious tolerance.

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PT09.10

Unique formulated dual targeting antigen specific and delivered miRNA-150 gene regulating exosomes acting at the immune synapse to induce APC-derived secondary suppressive exosomes

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Introduction: An exosome-APC circuit we uncovered may be applicable beyond skin immunity we study in mice.

Methods: High antigen dose tolerized CD8 + T cells make suppressive antigen-specific exosomes due to chosen surface antibody light chains that enable targeting antigen presenting cells (APC) antigen-specifically for delivery of also chosen inhibitory miRNA-150 to mediate specific functional gene alterations.

Results: Both antigen and gene specificity aspects are lent to naïve but activated exosomes by simple in vitro incubations alone. For mechanism, these primary exosomes bind antigen peptides in MHC on APC that in turn make secondary suppressive exosomes that act peptide/MHC-specifically on the effector T cells at the immune synapse. They transfer another miRNA for strong prolonged inhibition of active delayed-type hypersensitivity (DTH) for days even, when the primary miRNA-150-pos exosomes are administered orally at the height of the in vivo response, in a physiological dose.

Summary/Conclusion: It is shown possible to induce therapeutic exosomes with Ag targeting of choice due to placed Ab on the surface and that also target specific gene functions of acceptor cells due to carriage of a selected miRNA. This dual Ag and gene-specific therapy has applications in treatment of Cancer, Autoimmunity and Allergies.

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PT09.11

The role of Mac-1 and calcium signalling in the formation of neutrophil granulocyte derived extracellular vesicles

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Introduction: Previously, our group characterized distinct populations of extracellular vesicle (EV) released from neutrophilic granulocytes: EV formed spontaneously (sEV) and upon activation with opsonized particles (aEV). The aEV differs in protein cargo and its ability to inhibit bacterial growth. We described that Mac-1 integrin (CR3 receptor) plays key role in the aEV production and extracellular calcium supply is crucial in this signalization. In the present work, our aim was to investigate whether Mac-1 activation or Ca-signalling on their own are sufficient for the initiation of the aEV biogeneis.

Methods: We isolated neutrophil derived EVs from peripheral human blood and murine bone marrow by two-step centrifugation and filtration. We tested the effect of Ca-ionophore and examined the EV production on C3bi coated surface and in soluble form. We quantified the vesicles by flow cytometry and determined their protein content by Bradford assay. We examined their antibacterial effect in parallel with optical density-based measurement and our flow cytometry based method.

Results: On C3bi coated surface, we observed an increased EV production, and these EVs possessed antibacterial capacity. However, in soluble condition, C3bi did not induce further EV production, and these EVs did not show any antibacterial property. We found that Ca-ionophore initiated EV formation, but these EV did not show antibacterial effect. We observed EV production increase after Ca-ionofore treatment both in the presence and in the absence of extracellular Ca. The Ca-ionophore slightly increased the opsonized particle induced EV production, but did not potentiate their antibacterial capacity.

Summary/Conclusion: Mac-1 activation is not just crucial, but sufficient in initiation of the aEV biogenesis. Clustering of this receptor is required. While the Ca-signal is crucial, it is not sufficient in the generation of aEVs.

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PT09.12

Extracellular vesicles and their microRNA cargo in retinal health and degeneration: mediators of homoeostasis, and immune modulation

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Introduction: Photoreceptor cell death and inflammation are known to occur progressively in retinal degenerative diseases such as age-related macular degeneration (AMD). However, the molecular mechanisms regulating these biological processes are largely unknown. Extracellular vesicles (EV) are essential mediators of cell-to-cell communication with emerging roles in the modulation of immune responses. EVs, including exosomes, encapsulate and transfer microRNA (miRNA) to recipient cells and in this way can modulate the environment of recipient cells. Dysregulation of EVs however is correlated to a loss of cellular homoeostasis and increased inflammation. In this work we investigated the role of isolated retinal small-medium sized EV (s-mEV) in the regulation of homoeostasis and immune modulation in both the healthy and degenerating retina.
Methods: Isolated s-mEV from healthy and degenerative (photo-oxidative damaged) mouse retinas were characterized using dynamic light scattering, transmission electron microscopy and western blot, and quantified using nanotracking analysis. Small RNA-seq was used to characterize the miRNA cargo of retinal s-mEV isolated from healthy and degenerating retinas. Finally, the effect of exosome inhibition on s-mEV-mediated immune modulation was investigated using systemic daily administration of exosome inhibitor GW4869 and analysed by in situ hybridization of s-mEV-abundant miRNA. Electroretinography and immunohistochemistry were performed to assess functional and morphological changes to the retina as a result of exosome depletion.
Results: Our results demonstrated an inverse correlation between s-mEV concentration and photoreceptor survival, with decreased s-mEV numbers following retinal degeneration. Small RNA-seq revealed that s-mEVs contained uniquely enriched miRNAs, however no differential composition in s-mEV miRNA cargo following photo-oxidative damage was observed. Exosome inhibition using GW4869 exacerbated photoreceptor degeneration, with reduced retinal function and increased levels of inflammation and cell death seen following photo-oxidative damage. Further, reduced translocation of the photoreceptor-derived s-mEV was demonstrated following exosome-inhibition in photo-oxidative damaged mice.
Summary/Conclusion: Taken together, we propose that retinal s-mEV and their miRNA cargo play an essential role in maintaining retinal homoeostasis through immune-modulation, and have the potential to be targeted using gene therapy for retinal degenerative diseases.

PT09.13

Impacts of agricultural dust exposure on human lung-resident mesenchymal stromal/stem cells and their extracellular vesicles

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Introduction: Agricultural dust is considered a high-risk occupational hazard by the CDC, with impacts reaching throughout the communities surrounding these industries, leading to increased incidence of respiratory illness and disease among individuals within this occupation and these communities. Lung-resident mesenchymal stromal/stem cells (lr-MSC) have an important role in maintaining homoeostasis in the lung, and mediating pro- and anti-inflammatory effects, particularly during exposure to inhaled irritants, like agricultural dust. One way in which these lr-MSC promote lung homoeostasis is through the release of extracellular vesicles (EV), with a variety of cargo that elicit changes among target cells. We hypothesize that exposure to agricultural dust modifies the quantity and cargo of EV released by lr-MSC to promote lung tissue homoeostasis.
Methods: Primary human lung-resident mesenchymal stromal cells were exposed to extracts of dusts collected from swine confinement facilities (DE) for 8 or 48hrs and the media from these exposures were collected and enriched for EV by opti-prep density gradient ultracentrifugation. The quantity of these EV were assessed by nanoparticle tracking analysis. Additionally, cytokine and chemokine release by lr-MSC were analysed by enzyme-linked immuno assays.
Results: As assessed at 48 hr following treatment, DE-exposed lr-MSC released pro-inflammatory cytokines, IL-6 and IL-8, with IL-8 release reaching statistical significance at 0.1%, 0.5%, and 1% DE concentrations

($p = 0.0367$, <0.0001, and <0.0001 respectively) and IL-6 trending a similar dose response but only statistically significant at 1% DE ($p = <0.0001$). DE exposure of lr-MSC also induced changes in the lr-MSC-derived EV populations when compared to vehicle control, where lr-MSC released significantly more EV in the 5 and 10% iodixanol fractions ($p = <0.0001$ and 0.0219, respectively) at 8 hr following DE treatment. Alternatively, there were significantly less EV in the 20 and 40% density fractions in the media of DE-exposed lr-MSC versus vehicle control.

Summary/Conclusion: Following exposure to agricultural dusts, lr-MSC-derived EV populations more likely consist of exosomes and ectosomes, which play an important role in promoting lung tissue homoeostasis during exposure-related pulmonary inflammation.

PT10: Single-EV Analysis

Chair: Patricia M. Ozawa – PhD student, Department of Genetics, Universidade Federal do Paraná

PT10.01

Clinically applicable method to avoid swarm detection during flow cytometry analyses of single extracellular vesicles in human plasma

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Introduction: During analyses of single extracellular vesicles (EVs) by flow cytometry (FCM), particles below the detection limit may exceed the trigger threshold, which is called swarm detection and generates false-positive counts. Serial dilutions are recommended to find the minimal dilution for which swarm detection is absent. However, because particle concentrations in plasma vary, the optimal dilution differs >100-fold between donors, but it is unfeasible to do serial dilutions for each clinical sample. Therefore, our aims are to (1) develop a faster method to avoid swarm detection, and (2) increase the number of detected EVs per second.

Methods: We measured serial dilutions of CD61-stained EVs in platelet free plasma (PFP), with and without spiking of FITC beads, by FCM (Apogee A60-Micro). We triggered either on side scatter or fluorescence.

Results: For scatter triggering with our FCM, swarm detection consistently occurred for plasma samples exceeding a (total particle) count rate of 4,100–4,200 events/s. The CD61+ EVs concentration scaled linearly over 1.5 orders of magnitude of the dilution and most donors required >1000-fold dilution to avoid swarm detection, thereby reducing CD61+ EV counts. For fluorescence triggering, the CD61+ EVs concentration scaled linearly over >3 orders of magnitude of the dilution. For all donors, swarm detection was absent after 32-fold dilution (relative to pure plasma). The count rates of CD61+ EVs were 30–100-fold higher compared to scatter triggering. The spiked FITC beads confirmed that the median signals remained constant.

Summary/Conclusion: We have developed two clinically applicable ways to avoid swarm detection. For scatter triggering, the count rate provides direct feedback on the presence of swarm detection in plasma samples. For fluorescence triggering, swarm detection was absent for all plasma samples diluted ≥32-fold and compared to scatter triggering, count rates of CD61 + EVs were 30–100 fold higher, thereby improving statistical significance.

Funding: Edwin Van der Pol is supported by the Netherlands Organisation for Scientific Research – Domain Applied and Engineering Sciences (NWO-TTW), research programmes VENI 15924.

PT10.02

Benchmarking flow cytometric analysis of nanoparticles: a cross-platform study for single extracellular vesicle detection

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Introduction: Despite flow cytometry being widely used to analyse cells in suspension, most commercial instruments lack sensitivity when measuring nanoparticles (NPs) and extracellular vesicles (EVs). Furthermore, the use of appropriate reference materials (RMs) for calibration and quality control are essential to compare results acquired with different instruments. To work towards successful clinical applications for EV biomarker profiling, benchmarking studies including state-of-the-art flow cytometers are required. We here investigated the ability of three different flow cytometers to detect NPs and EVs.

Methods: The instrument sensitivity of light scattering detection was evaluated by using synthetic NPs of different sizes and refractive indices. Fluorescent calibration was investigated by using molecules of equivalent soluble fluorophores (MESF) beads. Biological recombinant EVs (rEVs) were used to validate the detection and quantification of fluorescent EVs in a side-by-side cross-platform study using an N30 Nanoflow Analyser (NanoFCM), an optimized BD Influx and a CytoFLEX LX.
Results: We found that when light scatter based detection was used, the NanoFCM detected the smallest non-fluorescent NPs, the BD Influx was able to provide reliable FSC information from the smallest detected NPs and the CytoFLEX performance was greatly improved by the use of Violet-SSC. Biological rEVs showed that the NanoFCM could clearly resolve fluorescent EVs while the BD Influx and CytoFLEX were unable to fully resolve rEVs from background, although fluorescence threshold improved detection. In addition, our findings revealed that different concentrations are required to ensure single EV detection in these platforms.
Summary/Conclusion: We identified several strengths and limitations for each platform with respect to single EV analysis. Furthermore, our results showed that proper calibration and RMs are of utmost importance to ensure reliable interpretation of EV flow cytometric data.
Funding: European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie (grant agreement No 722148) and the National Natural Science Foundation of China (grants 21934004 and 21627811).

PT10.03

Caution when using membrane dyes for sequential extracellular vesicle analysis

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Introduction: Confirmation that particles detected by microflow cytometry are actually extracellular vesicles (EVs), or at least membranous in composition, can be achieved through a variety of methods. Positively staining particles with a membrane dye strongly suggest that the particle contains a membrane; loss of stain (or detection) after detergent solubilization of the membrane-dyed particles provides even stronger evidence that the particles were EVs. It is important to recognize that the labelling protocol provided by the membrane dye manufacturer may not be ideal for all types of EV-containing biological samples, such as blood, urine, semen etc.). Removal of excess dye from stained EVs is very difficult and can be impractical depending on the nature of the experiment. However, this means that the potential for excess dye to contaminate subsequent sampling is high. Therefore, it is important to determine optimal working concentrations and labelling conditions when using membrane dyes for EV detection to understand properties that may impact your analyses.
Methods: To assess the utility of membrane dyes, titration curves were generated to determine the optimal working concentrations of membrane dyes for EV detection in conditioned media and human serum samples. Once the optimal concentration was determined the potential of dye carry-over from sample to sample during microflow cytometry detection was evaluated by tracking dye positive (dye+) particles in phosphate buffered saline (PBS) blanks and matched, unlabelled, sample replicates.
Results: We found that optimal concentration of any membrane dye is dependent on sample type. Even with the inclusion of system washes to prevent sample carryover, there was carryover of low amounts of dye+ particles into sequentially analysed PBS blanks. If unstained samples were analysed following a stained sample, excess dye (or at least dye+ events) appeared in the data. A sample concentration effect was also seen; samples of lower concentrations were more susceptible to dye carryover.
Summary/Conclusion: When using membrane dyes to stain EVs in biological samples, especially if an autosampler is employed to run a series of tests, it is critical to determine the optimal concentration of dye for each type of sample, as excess dye can carry over to the next sample in the queue. In addition, determining the necessary steps to clean any excess dye following each sample run will improve the accuracy of EV detection and analyses.
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PT10.04

Correlation between size and protein expression of single exosomes by combined atomic force and fluorescence microscopy

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Introduction: Exosomes are biological nanoparticles released by all types of cells, healthy as well as cancer cells. By travelling in body fluids (blood, saliva, etc.) exosomes carry proteins and genetic cargo between cells in distant organs. Therefore, they are involved in intercellular communication and play an important role in the development of several diseases, such as cancer. For diagnostic and therapeutic purposes, it is fundamental to measure the size and the molecular content of exosomes. Nevertheless, a direct correlation between these two quantities at the single-exosome level has remained elusive mainly due to their small size (~30-200 nm) and their biological/soft and heterogeneous nature.
Methods: In this work, we overcome the challenges in correlative measurements by combining two powerful techniques: atomic-force (AFM) and fluorescence microscopy. AFM allows accurate size-measurement of exosomes in liquid, thus preventing their damage. On the other hand, fluorescence microscopy is used to estimate the amount of surface proteins/markers of exosomes attached on a glass substrate.
Results: Exosomes from HEK293 cell line were first bound onto patterned glass coverslips in a cell containing 1x phosphate buffer saline (PBS). Surface proteins of such exosomes could be labelled with antibodies or alternatively by bioengineering. The AFM, mounted on top of the fluorescence microscope and aligned with the optical axis, allowed combined size-fluorescence measurements on individual exosomes. In particular, to prevent size/shape alteration of the soft nanoparticles, force curve-based (or quantitative) imaging was performed in liquid environment. The correlation data was acquired for different samples/surface proteins/markers.
Summary/Conclusion: In conclusion, we have measured the size and the protein expression level of single exosomes by combining AFM and fluorescence microscopy. This approach, first of its kind for exosomes, allows to accurately estimate both properties at the single-exosome level, thus giving valuable information for diagnostic and therapeutic use of these nanoparticles, as well as for their fundamental knowledge.

PT10.05

Development of a panel of antibodies for capture and labelling of EVs from neural cell types

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Introduction: There are no universal markers of extracellular vesicles, but often they are identified by the presence of tetraspanins in their membrane. Based on this, products have been developed to precipitate or quantify EVs by acting upon CD9, CD81, and CD63. However, EVs also carry proteins from their parent cells, and capturing EVs based their presence allows for a more complete understanding of vesicle heterogeneity from a single cell type, and for EVs derived from specific tissues to be enriched from other biofluids in support of biomarker assessment. For example, EVs derived from the brain could be captured from the general population of serum EVs for better assessment of cargo associated with proteinopathy. The goal of this study was to identify specific antibodies to capture and label EVs bearing the neural markers CD171, SNAP25, α-Synuclein, Tau, and NCAM.
Methods: The targets were overexpressed in HEK293 T cells through transient transfection of plasmids (Origene). Media was conditioned for 24–48 hours, and then centrifuged to remove cell debris. Cell lysates and concentrated conditioned media (CM) were analysed by Western blot. Unpurified CM, or CM after performing size exclusion chromatography (SEC, Izon), were analysed in the ExoView R100 system. Diluted CM was incubated on custom antibody microarray chips overnight. Then the chips were labelled with a cocktail of 3 labelled antibodies, washed and imaged. Vesicles were counted, sized, and phenotyped. Next, commercially available pooled human CSF was analysed in a similar fashion to determine their abundance in a relevant biofluid.
Results: Multiple antibody clones were tested in different combinations for capture and labelling for the five different neuronal enriched proteins of interest, and optimal combinations were identified. Some markers were identified on particles > 50 nm in size that were negative for tetraspanins, while others colocalized with tetraspanins. Through comparing permeabilized and intact EVs with and without SEC to remove non-vesicular proteins, we found that tau could be on the vesicle surface, within the vesicle, and free in solution.
Summary/Conclusion: The ExoView platform can be customized to enable the detection of proteins of interest and to determine whether they are on the EV surface, intravesicular, or non-EV associated.

PT11: EVs in Cardiovascular Diseases and Vascular Disorders

Chair: Saumya Das – Massachusetts General Hospital, Harvard Medical School

Chair: J. Brian Byrd – University of Michigan

PT11.01

Association between circulating extracellular vesicles and thrombogenic risk markers for cardiovascular disease

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Introduction: Elevated numbers of circulating extracellular vesicles (cEVs) have been observed in different diseases, most notably cardiovascular diseases (CVDs). EVs are reported to have thrombogenic properties, but there is very little information about the association between circulating EV numbers and thrombogenic risk markers for CVD. The aim of this research was to investigate whether thrombogenic risk markers for CVD are associated with increased numbers of circulating EVs.
Methods: Forty non-smoking male and female subjects (40–70y) at moderate risk for CVD were recruited for the study. EVs from platelet-free plasma (PFP) were isolated using size exclusion chromatography (SEC). The concentration and size distribution of EVs were measured by Nanoparticle Tracking Analysis (NTA) and flow cytometry (FCM). Three EV markers, including Annexin V for the circulating phosphatidylserine-positive (PS+) EVs, CD41 for platelet-derived EVs and CD105 for endothelial-derived EVs were used for phenotyping. In addition, coagulation and fibrinolysis were assessed using a thrombodynamics analyser (Hemacore). Platelet aggregation to determine platelet function was assessed by a high-throughput platelet function assay with a wide range of concentrations of agonists, including adenine diphosphate (ADP), collagen-related peptides (CRP-XL), epinephrine, thrombin receptor activating peptide (TRAP-6) and U46619. The association between thrombogenic risk markers for CVD and EV numbers was tested by Pearson's correlation coefficient and linear regression model using the statistical program, SPSS.
Results: Circulating EV concentration with threshold of 71 nm, measured by NTA, were positively associated with coagulation-related risk markers, including rate of clot growth ($r = 0.568$; $p = 0.01$) and clot size at 30 min ($r = 0.480$; $p = 0.002$). PS+ EVs derived from endothelial cells, determined by FCM, were negatively associated with lysis onset time ($r = -0.410$; $p = 0.009$), whereas they were found positively correlated with lysis progression ($r = 0.417$; $p = 0.007$). Both mean and mode size of cEVs, detected by NTA, were significantly correlated with U46619-induced platelet aggregation ($r = -0.338$; $p = 0.033$, $r = -0.382$; $p = 0.015$, respectively).
Summary/Conclusion: In subjects at moderate risk for CVD, cEV numbers were positively related to rate of clot growth and clot size and size of cEVs was negatively related to platelet activity. Higher numbers of endothelial cell-derived PS+ cEVs were associated with lower rates of fibrinolysis. This suggests that cEVs promote clot growth and reduce fibrinolysis, and may therefore be an indicator for greater risk of CVD.
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PT11.02

Beyond stem cells: extracellular vesicles from human induced pluripotent stem cells (hiPSC) and hiPSC-cardiomyocytes as therapeutic approaches for heart failure

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Introduction: Heart failure is caused by a variety of underlying diseases, the most common being myocardial infarction. Initially regarded as an alternative to pharmacological approaches, stem cell transplantation has failed to demonstrate clinically meaningful results. Instead, it has become increasingly apparent that the therapeutic effects of transplanted cells are largely mediated by their secretome, while mounting evidence suggests Extracellular Vesicles (EVs) play a major role in cardiac repair. Within this framework, EVs from human induced pluripotent stem cells (hiPSC) and hiPSC-derived cardiomyocytes (hiPSC-CM), hold a tremendous potential to treat cardiovascular disease.

We isolated EVs from conditioned culture media at key stages of the hiPSC-CM differentiation and maturation processes, i.e. from hiPSC (hiPSC-EV), cardiac progenitors (CPC-EV), immature (CMi-EV) and mature (CMm-EV) cardiomyocytes, with the aim of studying their potential role as therapeutics, and whether their effectiveness was influenced by the state of their parent cell.

Methods: hiPSC were differentiated into cardiomyocytes in a 3D culture approach, using the protocols developed by our group. EV isolation was performed on an iodixanol density gradient, and the EVs were characterized in terms of particle size and particle size distribution, presence of EV-specific markers, and imaging through transmission electron microscopy. Functional studies were performed using human umbilical vein endothelial cells (HUVECs) to evaluate EV-uptake, cell migration and angiogenesis.

Results: EVs from all hiPSC and cardiac derivatives presented a typical cup-shaped morphology and expressed CD63 and CD81. EV yield varied along differentiation, with a minimum for CPC and a maximum for CMi. PKH26-labelled EVs were uptake by HUVECs, and colocalized with calnexin, a protein from the endoplasmic reticulum. Wound healing assays showed an increased cell migration in HUVECs treated with cardiomyocyte-derived EVs, in comparison with control EVs isolated from foetal bovine serum.

Summary/Conclusion: Our findings suggest a different EV secretion profile along CM differentiation and maturation, with preliminary assays showing EV functionality. Ongoing work aims at elucidating the possible differences in function and cargo amongst these types of EVs.

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PT11.03

Endothelial cells differentially load and secrete extracellular vesicle-derived microRNAs into apical and basolateral compartments

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Introduction: Endothelial cells (EC) are a major secretory organ that selectively release biologically active molecules. Given that extracellular vesicle (EV)-derived miRNAs mediate cell-cell communication and serve as biomarkers, we hypothesized that they are secreted in a polarized manner in steady and activated states.

Methods: Primary human aortic endothelial cells and human umbilical vein endothelial cells (HUVECs) were cultured in transwells (± apical IL-1β, 10 ng/ml, 4 h). Apical and basolateral EVs were isolated from supernatants by polyethylene glycol precipitation (ExoQuick-TC, System Biosciences, Palo Alto, CA, USA). EV-miRNA profiles were determined by next generation sequencing (HTG Molecular Diagnostics Inc., Tucson, AZ, USA) and differential expression evaluated using Partek Genomics Suite software (version 8.0). EC barrier function was assessed by FITC-dextran flux (40 kDA) and localization of VE-cadherin expression (MAB9381, R&D Systems). Directional miRNA transfer was assessed in miR-39-transfected HUVECs by seeding onto transwells with monocytes (THP-1 cells, apical and basolateral compartment, 24 h).

Results: EV isolation was confirmed with nanoparticle tracking analysis (130 nm ±19.57, n = 12) and protein content-based characterization (CD63 positive, Calnexin negative). EV size and concentration was unaffected by IL-1β. IL-1β-stimulated ECs demonstrated a 1.89 ± 0.098 fold change in FITC permeability ($p < 0.0001$, n = 3), but VE-Cadherin expression was maintained at adherens junctions (n = 2). Sequencing performed on EC-EVs isolated from each compartment showed differential miRNA expression ($p < 0.05$, fold change >1.5; n = 7). Polarized EV-miRNA secretion was maintained after IL-1β stimulation but demonstrated altered miRNA profiles. Selective and directional release of EV-miRNA was demonstrated in a model of cellular communication, whereby ECs transfected with exogenous miRNA-39 preferentially deliver this to monocytes in the apical compartment (274 ± 163 fold increase, n = 2).

Summary/Conclusion: ECs selectively secrete EV-miRNA in a polarized fashion and may therefore participate in selective and directional cell-cell communication. These findings have important implications for discovery of EV-miRNA based biomarkers and therapeutics.

Funding: University Health Network, Division of Vascular Surgery, Peter Munk Cardiac Centre.

PT11.04

Insights into EV subpopulations positive and negative for Annexin A1 from smooth muscle cells and valvular interstitial cells

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Introduction: Annexin A1 associates with extracellular vesicles (EVs) and can mediate vesicle aggregation. This may play a role in microcalcification in calcific aortic valve disease (CAVD), but this is poorly understood. Annexin A1 is thought to be a marker of membrane-derived EVs, but because it can be found on the cytoplasmic or extracellular side of the plasma membrane, its localization within or on the surface of EVs is unclear. The goal of this study was to determine whether annexin A1 is found on the surface of EVs in two cell lines relevant to CAVD, and develop an assay that can be used to determine whether this changes under pathogenic conditions.
Methods: EVs were isolated by differential ultracentrifugation from the conditioned medium (CM) of smooth muscle cells (SMC) and valvular interstitial cells (VIC). Total protein in the cell lysates and EV pellets was analysed by western blot. EVs from cells treated with control siRNA or Anxa1-siRNA were enumerated and phenotyped using the ExoView R100 platform. EVs with surface expression of CD9, CD81, CD63, and annexin A1 were captured using a customized antibody microarray chip. Then EVs were labelled with fluorescent antibodies to assess EV number, size, and colocalization of EV proteins. The knockdown of annexin A1 allowed us to assess the specificity of the selected annexin A1 antibody.
Results: The EV fraction was positive for CD9, and lacked markers of other vesicle types. Western blot on the EV pellet and supernatant in ± EDTA indicated that there is annexin A1 both on the surface of and within the EVs. Using the antibody microarray chips, numerous annexin A1+ EVs were captured on the annexin A1 spots from the control CM, and there was a marked decrease in capture and labelling from Anxa1-siRNA treated cells. Under both conditions, vesicles were also captured on tetraspanin probes, with the greatest number captured on CD9, then CD63 and CD81. There was a significant population of annexin A1+ EVs that was negative for tetraspanins.
Summary/Conclusion: Annexin A1 is found on the surface of EVs. The assay developed in collaboration with NanoView Biosciences is well suited for assessing the number and phenotype of annexin A1+ EVs derived from SMC and VIC cell lines, which could provide a useful method for understanding EV populations in CAVD patient cell lines.
Funding: This work was supported by HL136432 and HL147095.

PT11.05

Possibility of exosomal microRNAs associated with chronic limb-threatening ischaemia, the end stage of atherosclerosis, as a promising biomarker

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Introduction: Chronic limb-threatening ischaemia (CLTI), the end stage of peripheral artery disease (PAD), has poor prognosis and is attributed to life-style disease. With increasing of atherosclerotic disease all over the world, establishment of biomarker for should play a pivotal role for early detection and preventing aggravation of the disease. The aim of this study is to explore the possibility of liquid biopsy for atherosclerotic disease by analysis of CLTI-associated exosomal microRNAs.
Methods: CLTI due to PAD was diagnosed by ankle-brachial blood pressure index, skin perfusion pressure (<40 mmHg) and angiography. Ten preoperative CLTI patients and 10 control patients without PAD were analysed (All patients with diabetes and 50% of patients had end-stage renal failure [ESRD]). To identify biomarkers associated with CLTI, exosomes were extracted from patient's serum after ultracentrifugation and total RNA including small RNA was isolated from the exosomes. The expression profile of exosomal microRNAs associated with CLTI were evaluated using a next generation sequencing.
Results: Forty-three exosomal miRNAs associated with CLTI were identified. Intriguingly, these miRNAs were clearly categorized with ESRD, which was well known as end-stage of life-style disease: these were stratified into 20 microRNAs for ESRD patients and 23 microRNAs for non-ESRD patients. Since ESRD is the most important factor significantly related to patient's prognosis in CLTI, exosomal microRNAs reflected patient's comorbidity onto the expression profile.
Summary/Conclusion: A portion of the expression profile of exosomal microRNAs associated with CLTI was identified. Exosomal microRNA could be a biomarker to stratify patient's condition along with their comorbidities and is very promising for individualized diagnosis in atherosclerotic diseases with risk diversity.

PT11.06

Postoperative plasma exosomal miR-21 and miR-133a signature in patients with left ventricular reverse remodelling after surgical mitral valve repair

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Introduction: Patients with mitral valve regurgitation (MR) show volume overload and progressive heart remodelling with increased left ventricular (LV) end-diastolic volume (EDV). Surgery is recommended for patients with substantial decay of global cardiac function (LV ejection fraction, LVEF) in order to reverse LV remodelling. However, approximately 10% patients show poor recovery. Identification of patients at high risk of post-operative LV remodelling may help preventive strategies. In this scenario, microRNAs delivered by plasma exosomes (pEXOs) might have a predictive value as well as complement the routine clinical measures of surgical outcome.

Methods: Primary MR patients (N = 19; 45–71 y.o.) underwent implantation of a prosthetic mitral ring. LV remodelling was assessed by cardiac magnetic resonance imaging and pEXOs were isolated by optimized ultracentrifugation before surgery (T0) and six months after surgery (T1). Isolated pEXOs were quantified by nanoparticle tracking analysis and miR-1, miR-21, miR-133a, and miR-208a were measured by RT-qPCR. The same analysis was performed on healthy subjects with normal cardiac function (N = 8). Local ethical committee approved the study (EMIGRATE study, approval n°1529) and informed consent was obtained from all patients.

Results: pEXOs levels at T0 were lower (−32%, p = 0.02) in patients with worst postoperative LV function, while they were higher at T1 (+31%, p = 0.03) in patients with reversed LV remodelling after surgery. At T1, the increase in pEXOs levels was associated to decreased heart mass index (−13%, p = 0.02) and higher levels of exosomal miR-21 (+78%, p = 0.02) and miR-133a (+69%, p = 0.05) were detected in patients with improved LV function.

Summary/Conclusion: Higher postoperative levels of pEXOs delivering miR-21 and 133a depict LV reverse remodelling after surgical mitral valve repair. Monitoring of exosomal microRNAs cargo might predict postoperative outcome in patients with MR.

PT11.07

Expression of lipocalin-2 (LCN2) in circulating extracellular vesicles (EVs) and femoral plaque-derived EVs of peripheral arterial disease patients.

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Introduction: Peripheral arterial disease (PAD) is the most prevalent cardiovascular condition globally and it is estimated to increase greatly with the ageing of the population. Extracellular vesicles (EVs) have emerged as potential components of liquid biopsy related to their protein and nucleic acid cargo. The transcriptomic analysis of circulating EVs has revealed the expression of genes related to the immune response in PAD, including lipocalin-2 (LCN2) or NGAL, which has been involved in atherogenesis in preclinical studies.

Methods: To study if the content of circulating EVs might reflect molecular changes locally, LCN2 levels were determined by RT-qPCR in femoral atherosclerotic plaques (n = 5) and in medium/large EVs released from femoral plaques into cell culture medium ex vivo (n = 5). EVs were isolated by centrifugation (2x20000xg) and characterized by NTA, TEM, and western blot. The study was approved by the Institutional Review Board of Complejo Hospitalario de Navarra (ref. 30/10), according to the Declaration of Helsinki on medical research. Written informed consent was obtained from all patients.

Results: LCN2 mRNA was detected in femoral plaques and in tissue derived EVs by RT-qPCR. Additional PCR experiments in plaque derived EVs showed the presence of the LCN2 full length transcript within EVs.

Summary/Conclusion: The transcriptional content of plasma EVs (liquid biopsy) might reflect in part the molecular changes within atherosclerotic tissue, and femoral plaque derived EVs.

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PT11.08

Cardioprotective effects of extracellular vesicles from immortalized parental stromal cells: a translational strategy

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Introduction: Myocardial is a significant cause of mortality and morbidity in developed countries and since current therapies are only palliative this pathology has arisen in the last decade. Mesenchymal stromal cell (MSC) derived extracellular vesicles (EVs) emerged as potential therapeutic element to reduce tissue damage after the ischaemic insult. EVs are known to recapitulate healing benefits exert by MSC transplantation in preclinical ischaemic models but EV based therapies face relevant difficulties such as high effective dose and manufacturing standardization. In this piece of work, we immortalized EV secreting MSCs to get high amount of EVs from a standizable and scalable source.
Methods: MSC were immortalized and secreted EVs (EVMSC-I) isolated by sequential ultracentrifugation. In order to evaluate the therapeutic potential of EVMSC-I we used both human and rat cardiomyocytes as well as cardiac microvascular cultures subjected to isquemia/reperfusion injury. We evaluated the capacity of EVMSC-I to reduce the consequences of the ischaemic insult measuring cell viability and functional parameters like contractile capacity in the cardiomyocytes and angiogenic properties in the cardiac microvascular cultures. EVMSC-I therapeutic effect was assessed in vivo in a rat model of cardiac infarction followed by histopathological studies.
Results: EVMSC-I significantly reduced cardiomyocyte death (22%±7.219%) and revert the beating rate reduction in a 45%±17.31% trigger by the ischaemic event. In this regard, EVMSC-I inhibit caspase 3 cleavage and reduce the appearance of ROS. In terms of post-ischaemic neoangiogenesis, we observed that EVMSC-I increased the capacity of vascular cells to form tubular structures. In addition, we observed that EVMSC-I reduce the consequences of cardiac infarction in vivo. Finally, intramuscular injection of EVMSC-I after permanent ligation of the descendent coronary reduced the infarct area in rats from 46% ±10.98% of the left ventricular area in control group to a 31.27%±8.619% in the group treated with EVMSC-I
Summary/Conclusion: Taking all together, our results show that EVMSC-I mitigate the consequences of the cardiac infarction by reducing tissue damage. More work needs to be done to understand the underlying biological processes.
Funding: ACIF/2017/318, RD160011/0004, PI19/00245, Co-funder by FEDER "Una manera de hacer Europa"

PT12: Biodistributio, Signalling, and ECM

Chair: Bong Hwan Sung – Department of Cell and Developmental Biology, Vanderbilt University

PT12.01

Acinetobacter baumannii transfers the efflux pump related substances via outer membrane vesicles

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Introduction: Clinically, the drug resistance situation of Acinetobacter baumannii is becoming increasingly serious, and its drug resistance has become a difficult problem for nosocomial infection and clinical treatment. In view of the relatively slow development of antibacterial drugs, exploring the resistance mechanism of Acinetobacter baumannii is of great significance to improve bacterial resistance and help clinical treatment. Studies have shown that outer membrane vesicles (OMVs) can transmit resistance genes to mediate the spread of drug resistance, and recent studies have confirmed that high expression of efflux pumps play an important role in the multidrug resistance of A. baumannii. In this study, we want to explore whether the outer membrane vesicles of Acinetobacter baumannii can transfer the efflux pump related substances.
Methods: First, ultracentrifugation and density gradient centrifugation were used to extract the OMVs of Acinetobacter baumannii antimicrobial-sensitive strains (ATCC19606) and antimicrobial-resistant strains. Then, nanoparticle tracking analysis (NTA) technology was used to analyse the particle size and distribution range of OMVs. Transmission electron microscopy (TEM) was used to identify their morphology and structure. Bradford method was used to determine the protein concentration of OMVs. Next, the OMVs of antimicrobial-resistant strains were incubated with the antimicrobial-sensitive strains and then the drug susceptibility test was done to determine whether OMVs of antimicrobial-resistant strains could transmit antimicrobial-resistance information to the antimicrobial-sensitive strains. Finally, PCR, qPCR and mass spectrometry were used to determine whether the efflux pump related genes were higher expression in OMVs of antimicrobial-resistant strains than those in antimicrobial-sensitive strains.
Results: Nanoparticle tracking analysis (NTA) detected the concentration and size distribution of OMVs of Acinetobacter baumannii strains. It showed that the extracted OMVs have a relatively uniform particle size and a size between 100–250 nm. TEM showed that OMVs had a typical vesicle structure. OMVs co-culture experiments showed that OMVs of the antimicrobial-resistant strains can indeed pass resistance to the antimicrobial-sensitive strains. And the efflux pump related genes were higher expression in OMVs of antimicrobial-resistant strains than those in antimicrobial-sensitive strains.
Summary/Conclusion: OMVs of the antimicrobial-resistant strains can indeed pass resistance to the antimicrobial-sensitive strains. The cause of acquiring antimicrobial resistance in sensitive strains may be caused by resistant strains passing efflux pump-related genes or proteins to sensitive strains.

PT12.02

Characterization of melanocytic extracellular vesicles during ageing of the choroid

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Introduction: The choroid is located at the backside of the light-sensitive retina and is highly vascularized. It contains pigmented melanocytes, and their melanin protects them against oxidative stress. Since ageing reduces the number of melanosomes in melanocytes and generates a stiffer extracellular environment, our hypothesis is that surrounding choroidal cells and the retinal pigment epithelium (RPE) are subject to more oxidative stress-related damages. This study aimed to characterize EVs released by human choroidal melanocytes in the context of intercellular cooperation during ocular ageing.

Methods: Melanocytic EVs were recovered from the conditioned culture medium of young/old melanocytes grown on hydrogels of varying stiffness (0.5–20 kPa) by differential centrifugation. The concentration and size distribution of melanocytic EVs were determined by high-sensitivity flow cytometry. Cryo-transmission electron microscopy combined with receptor-specific gold labelling were used to reveal their morphology, size and phenotype. The relative abundance of 8 surface markers was evaluated with the Exo-Check Exosome Antibody Array. The uptake of fluorescent melanocytic EVs by the RPE and choroidal endothelial cells was assessed by confocal microscopy.
Results: Choroidal melanocytes released EVs positive for Annexin-5 and the tetraspanin CD63. Young melanocytes produced more Annexin-5 positive EVs and EVs larger than 500 nm compared to older donors. The stromal stiffness impacted the concentration and size of melanocytic EVs. We confirmed the uptake of melanocytic EVs by endothelial and RPE cells.
Summary/Conclusion: EVs from choroidal melanocytes are internalized by surrounding endothelial cells and RPE. Age-related stressors modify the phenotype of melanocytic EVs. The identification of melanocytic factors that can protect retina/choroid cells from oxidative stress-induced cell death could lead to more efficient therapy for patients suffering from dry age-related macular degeneration.
Funding: Vision Health Research Network; Fonds de recherche du Québec – Santé (FRQS); Canada Foundation for Innovation

PT12.03

Extracellular vesicle heterogeneity and the impact of cellular phenotypic drift on the vesicular proteome

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Introduction: Owing to their proposed biocompatibility and ability to cross biological barriers, EVs represent an attractive therapeutic delivery platform. However, EVs are eminently heterogeneous. A better understanding of EV heterogeneity and its origins will allow for improved design of EV-based therapeutics. EV heterogeneity is mainly studied by focusing on distinct EV subpopulations. Other sources of heterogeneity, such as heterogeneity within EV secreting cells themselves, have been investigated in lesser detail. In this study, we assessed the phenotypic drift of cell derived EVs to explore the origins of EV heterogeneity and its potential impact.
Methods: Three independent samples of two MDA-MB-231 breast cancer cell sub-clones were cultured for six weeks. EVs were harvested weekly and analysed using the MACSplex Exosome Flow Cytometry kit. At two time points the proteome of EVs was analysed by LC-MS/MS mass spectrometry with subsequent Gene Ontology and REACTOME Pathway Analysis.
Results: The expression of over 600 proteins was de-regulated in EVs derived from the two different cell clones. Many de-regulated proteins were associated with biological processes predicted to affect potential EV toxicity (platelet activation, neutrophil degranulation, blood coagulation) and EV biological activity (antigen presentation, inflammation, TGF-beta/mTOR/WNT signalling). More surprisingly, within only two weeks, over 400 EV proteins, many associated with immune modulation, apoptosis, interleukins, cytokines and cell signalling pathways (including those affecting T-cell/B-cell receptors) were de-regulated between the two EV isolation time points.
Summary/Conclusion: Results suggest that temporal changes can be observed in the EV proteome (potentially by clonal drift, epigenetic changes or cellular genomic instability) over short time periods. These changes could cause significant differences in biological effects and delivery capabilities between EVs harvested from the same cells at different time points and conditions.

PT12.04

In vivo tracking and biodistribution analysis of mesenchymal stem cell-derived extracellular vesicles in a radiation injury murine model

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Introduction: Recent studies indicated that Extracellular vesicles (EVs) play key roles in intercellular communication and have great potential for clinical application. Understanding the biodistribution of EVs is therefore essential. Our previous works have shown the ability of mesenchymal stem cell (MSC)-derived EVs to protect haematopoietic cells from

radiation damage. In this study, we evaluated the biodistribution of MSC-EVs in a radiated mouse model.
Methods: Human MSC-EVs were harvested by ultracentrifugation and labelled with DiD lipid dye. The reliability of the labelling EVs was confirmed by sucrose gradient fractionation analysis. The distribution of EVs in radiation-exposed mice after EV intravenous administration were evaluated by Fluorescence Molecular Tomography and further confirmed by flow cytometry and confocal microscopy analysis.
Results: We observed that DiD labelled MSC-EVs appeared highest in liver and spleen, lower in bone marrow in tibias, femurs, and spine, and were undetectable in heart, kidney and lung. We found the significantly increased MSC-EV accumulation in spleen and bone marrow post-radiation appeared with an increase of uptake of MSC-EV by CD11b+ and F4/80 + cells, but not B220+ cells, compared to those organs from non-irradiated mice. However, there was a predominant EV accumulation in lung and less accumulation in spleen and liver; in mice infused with human lung fibroblast cell derived EVs (LFC-EVs) and there was no significant LFC-EVs accumulation change in the spleen or liver after radiation. We further found that increasing levels of irradiation caused a selective increase in vesicle homing to marrow and spleen. This accumulation of MSC-EVs at the site of injured bone marrow could be detected as early as 1 hour after MSC-EV injection and was not significantly different between 2 and 24 hrs. post-MSC-EV injection.
Summary/Conclusion: This study indicated the specific accumulation of MS-EVs at the site of injury of haematopoietic tissue in radiation injury mice.
Funding: This work was supported by the NIH grants 5UH2TR000880, 3UH3TR000880-03 S1, 5P20GM11 9943, and 5T32HL116249.

PT12.05

Linking fat to colorectal cancer: extracellular vesicle crosstalk

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Introduction: Colorectal cancer is the third most common cancer worldwide, and fourth leading cause of malignancy related mortality. Understanding the mechanisms of its growth and metastasis is key to elucidating new therapeutic targets and developing treatments in the clinical setting. Epidemiological evidence indicates an increased risk of cancer in obese patients, pointing to bidirectional communication between colon and adipose cells. Extracellular vesicles (EVs) are small membrane enclosed packages released by cells, capable of transporting bioactive cargo from donor to recipient cells and inducing phenotypic changes. Adipocytes are a key component of the tumour microenvironment and interactions between adipose tissue and tumour cells may be important in the growth and metastasis of cancer. In this study, we investigate the effects of colorectal cancer EVs on adipocytes in vitro, and potential induction of dedifferentiation to a more fibroblastic, pro-inflammatory phenotype.
Methods: EVs were isolated from SW480 and HT29 human colorectal cancer cell lines by differential ultracentrifugation and mature adipocytes generated by differentiation of the SGBS human pre-adipocyte cell line. Adipocytes were treated with EVs and their lipid content measured by oil red O to determine loss of lipids. Inflammatory cytokine profile was measured by ELISA to assess any increase in pro-inflammatory behaviour, and expression of late adipogenesis markers were determined by western blot.
Results: EV treatment was shown to reduce lipid accumulation in adipocytes, with up to 80% reduction in lipids observed at the 50 μg/mL dose. Treatment was also shown to reduce the expression of late adipogenesis markers, and increase secreted levels of pro-inflammatory cytokines IL-6 and IL-8 by over 3 fold and 10 fold respectively. These results provide evidence for colorectal cancer derived EV involvement in the dedifferentiation observed in cancer associated adipocytes in vivo, displaying an altered phenotype, releasing lipid energy stores to fuel tumour growth and increasing pro-inflammatory signalling.
Summary/Conclusion: Studies have shown colorectal cancer EVs may be involved in signalling which induces functional changes in cells within the tumour microenvironment. Our work indicates that EV mediated dedifferentiation of resident adipocytes may potentially contribute to a microenvironment favouring cancer cell growth and metastasis. Further work aims to elucidate the specific EV cargo which mediates these effects.

PT12.06

Regulation of cellular senescence by Interferon Induced Transmembrane Protein 3 contained in small Extracellular Vesicles

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Introduction: Ageing is a major risk factor for many human diseases. It is a complex process that progressively compromises most of the biological functions of the organisms, resulting in an increased susceptibility to disease and death. Senescence is a cellular phenotype characterized by a stable cell cycle arrest. Senescent cells are accumulated in the body during ageing. It contributes to develop age-related diseases and cancer. The alteration in intercellular communication with age has been demonstrated to be due to senescent cells developing a phenomenon denominated senescence-associated secretory phenotype (SASP). Exosomes are small extracellular vesicles (sEV) (30–120 nm) of endocytic origin whereas microvesicles are formed by shedding of the plasma membrane. They contain nucleic acids, proteins and lipid that generally reflect the status of the parental cell and can influence the behaviour of neighbouring cells.
Methods: In this study, we demonstrated that the small extracellular vesicles (sEV) contribute for transmitting paracrine senescence to proliferative cells Firstly, we evaluated the presence of exosome-like particles in the sEV from senescent cells by detection of exosome markers (Alix, Tsg101 and CD63), Transmission Electronic Microscopy (TEM) and Nanoparticle Tracking Analysis (NTA). To determine that sEV from senescent cells are mediators of the paracrine senescence, we performed functional assays using Cre-LoxP reporter system and high-throughput
Results: Besides, we confirmed at a single-cell level that the proliferative cells internalizing sEV from senescent cells activate senescence process using the Cre-reporter system. sEV protein analysis from senescent cells by mass spectrometry (MS) and validation of top candidates using a functional siRNA screen identify Interferon Induced Transmembrane Protein 3 (IFITM3), a component of non-canonical interferon (IFN) pathway, as partially responsible for transmitting senescence to proliferative cells.
Summary/Conclusion: In conclusion, we found that sEV are regulators of paracrine senescence and IFITM3 contained in senescent sEV has an important role in the intercellular communication mediated through sEV during cellular senescence1.
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PT12.07

Stiff matrix induces exosome secretion and promotes tumour progression

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Introduction: Extracellular matrix (ECM) remodelling and stiffening are associated with solid tumour progression. Stiff ECM promotes cell proliferation, epithelial-to-mesenchymal transition (EMT), metastasis and chemoresistance. Hepatocellular carcinoma (HCC) appears frequently in patients with liver cirrhosis or fibrosis while the mechanism remains unclear. Exosomes have been determined to serve as messengers to mediate intercellular communication and influence the extracellular. Tumour-derived exosomes have been shown to influence tumour progression, metastasis, drug resistance, angiogenesis and immune regulation. Thus, determining whether exosomes provide a mechanism by which stiff matrix modulates tumour microenvironment for tumour progression opens a new way to understand cirrhosis and oncogenesis. Here we identified the molecular mechanism of matrix stiffening induced exosome secretion and showed the different effect of exosomes induced by soft or stiff matrix on tumorigenesis.
Methods: Huh7 cells were cultured on acrylamide gels with the stiffness was modulated to 500 Pa (soft) or 10 k Pa (stiff). The exosomes in conditioned media were collected and analysed by nanoparticle trafficking analysis (NTA) and immunoblotting. Protein expression level in cells was screened by Reverse Phase Protein Array (RPPA). Inhibitor or shRNA were used to inhibit target proteins function. In vitro phosphorylation and GEF assay were used to verify Rabin8 phosphorylation and activation. Exosomes from cells on soft or stiff matrix were injected into mice to study their effect on tumour growth.
Results:

(1) Stiff matrix promoted exosomes secretion.
(2) Akt was activated by stiff matrix and was required for exosome secretion.
(3) Rab8 was activated by Akt and regulated exosome secretion.
(4) Rabin8 was a direct target of Akt.
(5) Stiff exosomes promote tumour growth.

Summary/Conclusion: Matrix stiffening promotes exosome secretion via Akt-Rabin8-Rab8 pathway, contributing to tumorigenesis.

PT12.08

Tridimensional fibroblast culture revealed a novel exososome-dependent extracellular matrix secretion mechanism

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Introduction: The extracellular matrix (ECM) is constituted of a variety of proteins and polysaccharides that are secreted locally and assembled into a thick 3D meshwork to provide biophysical and biochemical support to the surrounding cells, and regulate numerous cellular functions such as adhesion, migration and proliferation. Dysregulation of ECM components or aberrant ECM remodelling can lead to various pathologies, as well as to play important roles in wound healing. Although ECM secretion pathways are still largely unknown, the current paradigm is that ECM-associated proteins are synthesized in the endoplasmic reticulum and transported via the endosomes to the Golgi apparatus en route to the cell surface and released by exocytosis.
Methods: To study ECM secretion pathway, we used 3-dimensional (3D) cultured fibroblasts. This culture method technique has been used widely to generate tissue-engineered self-assembled stromal tissues, free of exogenous materials, and rely on long-term supplementation of sodium ascorbate into the culture medium. Non-cancerous fibroblasts, grown in conventional two-dimensional (2D) cellular cultures, are known to be a poor source of secreted exosomes when compared to cancerous fibroblasts.
Results: Here, we provide evidence that non-cancerous dermal fibroblasts can secrete high amounts of exosomes, containing different ECM proteins, when cultivated in a 3D fashion. We also demonstrated that dermal fibroblast-derived exosomes had the capacity to travel from one cell to another, induce cellular migration and promote wound healing.
Summary/Conclusion: Altogether, these findings reveal a novel exosome-dependent ECM deposition mechanism and suggest that the use of 3D-fibroblast cellular culture may emerge as an innovative approach in precision medicine to better study the role of patient-derived exosomes and ECM proteins in the establishment of cellular microenvironment in health and disease.

PT12.09

Redirected tropisms of extracellular vesicles and exomeres yield distinct biodistribution profiles

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Introduction: Bionanoparticles including extracellular vesicles and exomeres (collectively termed EVs), have been shown to play significant roles in diseases and therapeutic applications. However, their spatiotemporal dynamics in vivo have remained largely unresolved in detail due to the lack of a limited suitable method.
Methods: We developed a bioluminescence resonance energy transfer (BRET)-based reporter, PalmGRET, to enable pan-bionanoparticle labelling ranging from exomeres (< 50 nm) to small (< 200 nm) and medium and large (> 200 nm) EVs and larger EVs (> 50 nm).
Results: PalmGRET emits robust, sustained signals and allows the visualization, tracking and quantification of bionanoparticles from whole-animal to nanoscopic resolutions under different imaging modalities, including bioluminescence, BRET, and fluorescence. Using PalmGRET, we show that EVs released by lung metastatic hepatocellular carcinoma (HCC) exhibit lung tropism with varying distributions to other major organs in immunocompetent mice. EV proteomics identified HCC-EV lung tropic protein candidates associated with cancer progression, in which SLCO2A1 and CLIC1 expression on non-tropic EVs conferred lung-tropism, while CD13 gave spleen tropism. Our results further demonstrate that redirected lung tropism decreases EV distribution to the liver, whereas the spleen tropism significantly reduces over time delivery to most major organs distribution including the liver and kidney.
Summary/Conclusion: We established a multimodal and multi-resolution PalmBRET method to enable pan-bionanoparticle labelling and imaging and therefore quantification in live cells, whole animals, and

preserved tissues. The method can resolve the intricate spatiotemporal dynamics of EVs. PalmGRET revealed that EVs derived from lung metastatic HCC are lung tropic, and the tropism can be conferred to non-lung-tropic EV-293 T by decorating EVs with identified HCC-EV membrane proteins. Importantly, the enhanced EV delivery to tropic organs also significantly alters its distribution to other major organs. Our findings suggest that the dynamics of EV biodistribution and targeted design should be investigated at the organ systems level in EV biology and therapeutic developments, respectively.

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PT12.10

Tracking mesenchymal stem cell-derived extracellular vesicles (EVs) in a in vivo cancer model

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Introduction: Small Extracellular vesicles (sEVs) are nanoparticles (30–120mn) encircled by a phospholipid bilayer, derived from the endocytic pathway and released by all cells. sEVs have an inherent role in cell communication and deliver cargo to target cells. Mesenchymal stem cells (MSCs) and have a natural ability to home to tumours and metastases while avoiding the host immune response. It is hypothesised that MSC derived sEVs (MSC-sEVs) also possess tumour-homing and immune-evading capacities therefore could provide a novel targeted delivery vehicle for treatment of cancer. It is imperative to elucidate MSC-sEVs migratory itinerary in vivo to support translation to the clinical setting.

Methods: This study aimed to image the interaction of labelled MSC-sEVs with cancer cells in real time in vivo.

sEVs were isolated from wildtype MSCs and MSCs with stably expressing red fluorescent protein (RFP) (via lentivirus) by the combined techniques of differential centrifugation, microfiltration and ultra-centrifugation. Isolated sEVs were extensively characterised by Transmission electron Microscopy (TEM), Nanoparticle Tracking Analysis and Western Blot. NOD SCID Gamma (NSG) mice with dorsal skinfold window chamber (DSFWC) were injected with either MDA-MB-231 luciferase (Luc) expressing cells or HT-29-Luc cells. Bioluminescence imaging was performed to confirm tumour formation. A dose of 1x10^7 MSC-RFP-sEVs was directly added to the window chamber and RFP expression detected using a microscope with RFP filter attachments. 8x10^7 EVs were incubated with the radionuclide, Technetium-99m tagged Duramycin (99 mTc-Dur) for 30 minutes at room temperature. Excess radiolabel was removed using exosome spin column (Invitrogen™). The 99 mTc-Dur-sEVs were then added directly to the window chamber and charged particle imaging carried out.

Results: 18 hours post-administration; the RFP signal was localised at the tumour site. Radiolabelled sEV signal could be detected 10 minutes and 4 hours after administration. MSC-sEVs were successfully detected at the tumour site following direct administration using two different tagging and imaging approaches.

Summary/Conclusion: This promising preliminary data supports the potential of this approach for tracking MSC-sEV migration In Vivo. Future studies will investigate systemic tracking of MSC-sEV migration.

Funding: This work was funded by the Irish Association of Cancer Research (IACR) AOIFA mobility award. C.O.N. is supported by funding from the National Breast Cancer Research Institute (NBCRI).

PT12.11

Response To Irradiation Of Small Extracellular Vesicles Derived From Prostate Tumors

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Introduction: Tumor-derived small extracellular vesicles (sEVs) have emerged recently as mediators of tumorigenesis. However, the role of sEVs in response to irradiation, a widely used therapy in prostate cancer, is not fully understood.

Methods: Our study involved the TRAMP mouse model of prostate cancer. We used plasma sEVs isolated using differential ultra-centrifugation and further isolated using iodixanol gradient fractionation. We also

used Nanoparticle Tracking Analysis (NTA) to analyze sEVs. Mouse pelvises were irradiated using 10 Gy, for 5 consecutive days.

Results: We first observed that upon pelvic irradiation of TRAMP mice, the levels of the signaling oncogene c-Src are reduced in plasma-derived sEVs, while the average size of sEVs is increased from 50-100nms to 70-250nms. Furthermore, we show that the sEVs from irradiated cells lose the ability to stimulate anchorage independent growth and migration of recipient cancer cells. Additionally, sEVs from irradiated mice increase the amount of DNA damage in recipient cancer cells.

Summary/Conclusion: Overall, our data show that irradiation of TRAMP mice (and prostate cancer cells) significantly reduces the pro-metastatic and pro-anchorage-independent growth potential of sEVs when tested on human cells. Changes to the composition and behavior of a cancer cell sEV population via radiation therapy offers promise for future therapeutic approaches for prostate cancer.

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Please provide any keywords if applicable.: Small extracellular vesicles, TRAMP, prostate cancer, irradiation

PT13: Advances in EV Separation and Concentration

Chair: Navneet Dogra – Department of Genetics and Genomic Sciences, Department of Pathology, Icahn School of Medicine, Mount Sinai

PT13.01

Development and characterization of extracellular vesicles separation methods from human brain tissues and its application to Alzheimer's disease

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Introduction: There are emerging physiological and pathological functions of extracellular vesicles (EVs) in neurodegenerative diseases including Alzheimer's disease (AD). Brain derived-EVs contain pathogenic proteins, such as tau, amyloid beta (Aβ), which have been reported to contribute to cell-to-cell propagation in those diseases. Investigation of the brain-derived EV cargo, therefore, is important to further understand the mechanisms of progression in neurodegenerative diseases. We developed the EV separation method from unfixed frozen mouse and human brain tissues and assessed the protein composition.
Methods: To establish the EV separation method, we separated EVs from frozen mouse brain tissue using sucrose density gradient ultracentrifugation (SG-UC) or size exclusion chromatography to compare the results from the particle number, morphology and protein profiling by NTA, TEM and Mass spectrometry. EVs were then separated from cortical grey matter of AD (n = 20) and Control (n = 18) by SG-UC. Tau and Aβ in the EVs were measured by immunoassay. Differentially expressed EV proteins were observed by quantitative proteomics employing machine learning.
Results: The separated EVs were enriched in EV molecules and devoid of contaminant proteins by SG-UC, showing our method was successful. The levels of pS396 tau and Aβ1-42 were significantly increased in AD EVs. Annexin A5 (ANXA5), neurosecretory protein VGF, neuronal membrane glycoprotein M6-a (GPM6A), and Alpha-centractin (ACTZ) were differentially expressed in AD EVs. A combination of these 4 proteins were confirmed to predict AD with the 88% accuracy by machine learning.
Summary/Conclusion: These data suggest our method were suitable for the separation of brain-derived EVs and EV ANXA5, VGF, GPM6A and ACTZ can be potential biomarkers for monitoring the progression of AD.

PT13.02

EDTA stabilizes the concentrations of extracellular vesicles during blood collection

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Introduction: To establish reliable biorepositories for research on extracellular vesicles (EVs) as disease biomarkers, the release of EVs during blood collection and handling must be avoided. Currently, citrate is recommended as the anticoagulant for blood EV research, but citrate does not inhibit the release of EVs from activated platelets. The release of platelet-derived EVs excludes pneumatic tube transport and makes assays time dependent, thereby limiting clinical compatibility. Therefore, we aim to stabilize the release of platelet EV concentrations.
Methods: Blood samples were collected from healthy individuals and subjected to common circumstances known to induce platelet activation. Blood was (i) incubated with or without thrombin receptor-activating peptide 6 (TRAP; n = 7), a potent platelet activator, (ii) send to the lab by a routine blood transport

(pneumatic tube system; n = 3), and (iii) stored at room temperature or at 4°C for 6 hours (n = 3). The concentrations of EVs from platelets (CD61+), activated platelets (P-selectin+), erythrocytes (CD235a+), and leukocytes (CD45+) were determined by flow cytometry (Apogee A60-Micro).

Results: Following activation by TRAP, concentrations of platelet-derived and activated platelet-derived EVs increased 5.8-fold and 10.4-fold in citrate-anticoagulated blood, compared to 1.4-fold and 2.0-fold in EDTA-anticoagulated blood (EDTA vs citrate: $p = 0.018$ and $p = 0.043$, respectively). Preliminary data show that during pneumatic tube transport and routine sample handling, both platelet- and activated platelet-derived EVs were more stable in EDTA compared to citrate. The concentrations of EVs from erythrocytes and leukocytes were unaffected under all studied conditions.

Summary/Conclusion: To conclude, EDTA stabilizes platelet EV concentrations during and after blood collection, which would facilitate pneumatic tube transport, enhance reliability and thereby improves the establishment of reliable biorepositories for EV research.

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PT14: Microfluidic and Other Devices

Chair: Colin L. Hisey – Research Fellow, The University of Auckland

PT14.01

Circular Dichroism-based Exosome (CDEXO) detection microfluidic platform using their assemblies with chiral gold nanoparticles

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Introduction: Cancer-cell secreted extracellular vesicles, called exosomes, are an emerging biomarker for cancer liquid biopsy. Profiling of cancer-associated exosomes usually required lengthy, and multi-step procedures; therefore simple and easy-setup sensing methods are urgently needed for diagnosing cancer in a timely manner. Chirality, the foundational property of all biomolecules, including exosomal proteins, can be utilized for exosome detection and differentiation using recent advances in chiral nanostructures. We found that microfluidic sensors can be successfully implemented for successful detection of cancer-associated exosomes taking advantage of unusually high circular dichroism (CD) of chiral gold nanoparticles (AuNPs).
Methods: Circular Dichroism-based Exosome (CDEXO) detection utilizes chiroplasmonic enhancement of CD signatures of cancer-associated exosomes. We first synthesized donut-shaped AuNPs conjugated with L-cysteine and immobilized the AuNPs on a glass slide using a layer-by-layer assembly. The AuNPs on slide glass were surface functionalized by the standard biotin-avidin reaction after MUA treatment. Biotinylated annexin V marker, targeting phosphatidylserine (PS) expression on cancer-associated exosomes, was conjugated to the AuNP surface. 5 μl of exosome samples from cancer cells (A549 and H3255) or normal cells (MRC5) were injected into the PDMS microfluidic device and incubated for 30 minutes. The CD signal before and after exosome exposure was monitored, compared, and systematically analysed as a rapid technique for the detection of exosomes with high sensitivity.
Results: We showed that the CDEXO signals from cancer exosomes showed 26.6 folds absolute CD peak value change and 3.41 folds shift, respectively, compared to that of healthy exosomes. Importantly, the CDEXO sensing method takes less than 10 mins in terms of total scanning time and requires minimal sample volumes. From the preclinical studies using 7 blood samples from cancer patients and healthy donors, we found that cancer patients show stronger band shift and signal change comparing to that of healthy donors, implying our platform could be used for cancer diagnosis.
Summary/Conclusion: This new versatile and sensitive method based on chiroplasmonic exosome detection paves the way to profiling disease-associated exosomes in a timely manner for minimal volumes of liquid biopsies.

PT14.02

EV classification and fractionation strategy using surface charge labelling

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Introduction: The development of new classification technology is required based on the evaluation of physicochemical properties of exosome surfaces and the diversity of constituent molecules. In this presentation, we present the electric charge activated exosome sorting platform comprising microfluidic device technology and electric charge labelling technique.
Methods: The single nanoparticle analysis platform, which has been developed by our research group, images Rayleigh scattered light (elastically scattered light) obtained by irradiating nanoparticles with convergent laser light and provides information of individual particles by image processing. The method that utilizes electrokinetic phenomena, unlike the method using fluorescent labels, measures the properties of the particle surface without serious difficulty in principle even if the particle size is on the order of tens nanometres, and further enables to perform fractionation. Since the number of particles usually handled in exosome research or its envisioned application is enormous, it is not realistic to take an approach such as a cell sorter in which particles are sequentially

manipulated one by one following the measurement results of individual particles.

Results: Particles receive attraction or repulsion by an external field according to the charge density on the surface, so there is no need to control the external force, and it is possible to design a device that can autonomously fractionate particles according to the difference in zeta potential.

Summary/Conclusion: In conclusion, we have proposed and demonstrated the new concept of electric charge activated EV sorter.

Funding: This research was partially supported by the Center of Innovation Program (COI STREAM) from the Japan Science and Technology Agency.

PT14.03

High throughput exosome analysis by using reversible microfluidic electrochemical sensor system

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Introduction: Exosome is one of the important extracellular vesicles (EVs) released from parental cells and it contains various types of molecular cargos from its original cell including proteins, messenger RNA (mRNA), and micro RNA (miRNAs) [1]. The exosomes have recently emerged as biomarkers for early stage cancer detection because the number of exosomes originated from cancerous cells are significantly higher than those from normal cells [2]. Since many different types of exosomes exist in the whole blood, it is necessary to isolate and detect disease-specific exosomes. For this reason, the isolation and the detection of exosomes is an important research issue and has been studied by many groups. However, limitations such as low throughput and low recovery still make it difficult to use exosomes in diagnostics and therapeutics.

Methods: In this study, we developed an integrated microfluidic electrochemical biosensor to extract plasma from whole blood and subsequently detect cancer related exosomes in a continuous manner. This consists of two parts. The first part is a channel for extracting plasma containing exosomes from whole blood, and the second part is a channel combined with an electrochemical sensor for multiple detection of various exosomes in the extracted plasma. Previously, a Multi-Orifice Flow Fractionation (MOFF) channel that consists of a series of expansion and contraction structures has been developed in our group. In this channel, the blood cells are moved to sides of channels by hydrodynamic forces and then are eliminated to outlets. At this time, the plasma is moved to the electrochemical sensor part, the exosomes in the plasma are captured to the electrodes immobilized with the specific antibodies and are quantified the amount of cancer-related exosomes.

Results: Using this chip, blood cells were eliminated from the whole blood with over 90% of separation efficiency at 225 μl/min flow rate and exosomes were collected continuously with high recovery (~70%). In order to quantify various types of exosomes, a label-free electrochemical biosensor with Electrochemical Impedance Spectroscopy (EIS) was used for the continuous detection of exosomes. The limit of detection was 1x10^6 exosomes/ml.

Summary/Conclusion: The developed device is an integrated device capable of separating exosomes from whole blood with high purity and quantitating exosomes through the electrochemical sensor in a continuous manner.

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PT14.04

High-throughput multiparametric characterization and quantitation of extracellular vesicles by fluorescence-based microfluidic diffusion sizing

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Introduction: Progress in the field of extracellular vesicles (EVs) is hampered by the challenge to produce large amounts of EVs in a reproducible fashion (Paganini et al, Biotech. J., 1800528, 2019). The development of high-throughput techniques capable of simultaneously monitoring physical and biochemical properties of EVs would significantly simplify and accelerate the characterization process. In this context, microfluidic technology is emerging as an attractive platform. Here, we present a microfluidic device based on the combination of diffusion sizing and multi-wavelength fluorescence detection to simultaneously provide information on EV size, concentration and composition.

Methods: The diffusion of EVs in the microfluidic channel provides information on their size distribution, and four different staining protocols with high signal-to-noise ratios track different EV native molecules. EVs are separated from unbound fluorophores directly during the microfluidic analysis, therefore avoiding the need for sample pretreatments and allowing to operate the device as a single-step immunoassay.
Results: The microfluidic device coupled with complementary staining techniques allows to individually detect and size particle populations with different EV components such as lipids, primary amines and the EV marker CD63. We demonstrate that this approach can probe the abundance of EV-specific markers and impurities such as lipoproteins with high throughput and low sample consumption.
Summary/Conclusion: We present a microfluidic technique capable of characterizing and quantifying EVs at low costs, in a time-scale of minutes and requiring only up to 2 μL of non-pretreated sample. This method is an important complementary tool to the current array of biophysical methods for EV characterization, in particular for high-throughput screening applications.
Funding: H2020-EU.1.2.1-FET Open programme via the Grant agreement 801338.

PT14.05

Immunomagnetic isolation of specific subpopulations of exosomes for liquid biopsy via nano-architected porous materials

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Introduction: Exosomes offer the potential to reveal significant biological information in many areas of clinical importance by virtue of their RNA contents and protein surface markers. This abstract reports the fabrication of a device for high throughput targeted immunomagnetic capture of exosomes via the use of highly-ordered nano-architected porous metal lattice materials.
Methods: We have invented a fabrication technique to precisely make millions of nanoscale exosome sorting devices that can operate on unprocessed plasma. Each nanoscale device can precisely sort targeted exosomes from background vesicles but is too slow for practical use individually. However, the operation of millions of these devices in parallel preserves the precision of nanoscale sorting while also enabling high throughput and robust use on raw plasma samples. The metal lattice within which these devices are contained is assembled via metal electroplating onto a self-assembled polystyrene bead lattice with face-centred cubic (FCC) symmetry with 600 nanometre pores. The devices feature a conformally-coated layer of nickel-iron with gold passivation atop a base layer of nickel, resulting in a lattice of millions of nanoscale pores capable of magnetic sorting of exosomes tagged via surface-marker-based immunomagnetic labelling with magnetic nanoparticles.
Results: Compared to our previous work on immunomagnetic exosome capture via commercial track-etched membranes (TEMPO), this device offers superior capture due to increased surface pore density (>25x) and three-dimensional pore density (>1000x) alongside lower required sample volume due to decreased non-capturing volume in the device. Finite-element analysis simulations show that strong magnetophoretic traps emerge at the pore boundaries in this structure between higher-permeability metals such as nickel-iron permalloy and the lower-permeability sample fluid in the device. Preliminary experimental data shows that this device can isolate iron nanoparticles in solution with >100x enrichment from input and 49x capture efficacy versus TEMPO.
Summary/Conclusion: Current methods of exosome isolation such as ultracentrifugation and column chromatography all suffer from low throughput and limited yield. The application of inverse opal materials towards exosome capture offers the potential for isolation of specific exosome populations from very low clinical sample volumes or sparse biological signals.

PT14.06

Micropatterned growth surface topography affects extracellular vesicle production

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Introduction: Extracellular vesicles are micro and nanoscale packages released by all cells and play an important role in cell-to-cell communication by shuttling biomolecules to nearby and distant cells. However, producing enough EVs for many in vitro studies using conventional tissue culture techniques can be challenging, and despite the success of some bioreactors in increasing EV-production, it is still

unknown how many independent culture conditions like growth surface topography can alter the production and content of EVs.
Methods: Standard 150 mm petri dishes were patterned with 2 μm tall polystyrene microtracks spaced by 5, 10 and 20 μm across a 100 mm area using standard microfabrication techniques including photolithography, soft lithography and microtransfer printing. The micropatterns were characterized with SEM and profilometry, then activated with oxygen plasma and UV sterilized. MDAMB231 cells were seeded onto patterned and smooth (control) dishes and grown in serum-free media for the final 48 hours of culture. EVs were isolated using sequential ultracentrifugation of conditioned media and characterized using NTA, TEM and western blot. Cell morphology was imaged using immunocytochemistry and single cell migration was characterized using time-lapse microscopy and manual single cell tracking in FIJI.
Results: We demonstrate the simple and repeatable fabrication of microtracks across a large surface area in order to culture cells on topographically patterned growth surfaces. Furthermore, we show that the 5 μm spacing produced significantly more EVs than other patterns as well as the highest cell aspect ratio and average single cell migration speed ($p < .05$).
Summary/Conclusion: These findings have implications in both biomanufacturing of EVs and potentially in enhancing the biomimicry of EVs produced in vitro. However, further experimentation to assess the differences in cargo on patterned growth surface topographies compared to conventional methods is still required.
Funding: This project was funded by the Maurice and Phyllis Paykel Trust.

PT14.07

Using miscroscale thermophoresis and surface plasmon resonance to measure the interactions of extracellular vesicles

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Introduction: Microscale Thermophoresis (MST) is a recently developed biophysical technology for the analysis of molecular interactions. The motion of molecules in temperature gradients allows the quantification of biomolecule interactions by changes in conformation, charge and size of a molecule induced by a binding event. MST is a quick method, easy to handle, has a low sample consumption, has no limitation on molecule size, and enables measurements in solution, either in various buffers or complex biological liquids. These properties make MST an interesting tool for research of extracellular vesicles (EVs); therefore, our aim is to apply this method to EVs.
Methods: EVs were isolated from Jurkat cell line by differential centrifugation. Microscale Thermophoresis (MST) and Surface Plasmon Resonance (SPR) were used to analyse the interaction between antibody and EVs.
Results: We have demonstrated that interactions of EVs with antibodies could be analysed by MST. However, the tiny glass capillaries for sample mounting represent a challenge due to adhesion of EVs to their surface. We have tested commercial capillaries as well as prepared capillaries in house coated by liposomes or bovine serum albumin. The interactions between EVs and antibodies were confirmed by Surface Plasmon Resonance (SPR), which is an established method for studying the interactions of EVs.
Summary/Conclusion: Microscale Thermophoresis (MST) is a promising method for the analysis of EVs.
Funding: Financed by Slovenian research agency, grant No. P1-0391 and J4-9322.

PT14.08

Acoustofluidic separation of complex suspensions

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Introduction: The isolation of extracellular vesicles (EVs) from cell culture supernatants and complex body fluids, such as blood and urine, is of high importance for EV research as well as for future medical applications in diagnostics and therapy. Nevertheless, it is still challenging to reach the desired recovery, purity and specificity due to many manual and time intensive sample preparation steps. Conventional centrifugation for EV isolation or sample preparation prior to affinity-based separation methods can damage EVs and cells, leading to misinterpretation of results or inactive EVs. Alternative field flow fractionation methods employing acoustic fields are highly promising, but so far limited to laboratory usage, based on a complex (moulding) fabrication and/or hardly reproducible. Here, we present an innovative surface acoustic wave

(SAW)-based acoustofluidic device for gentle sorting of cells and particles.

Methods: Our device consists of interdigital transducers patterned on a piezoelectric substrate generating SAW propagating on the substrate surface. Upon interaction of SAW with our on-chip structured, fluid-loaden microchannels, an acoustic pressure field is developed across the fluid wherein particles are suspended. This pressure field can be employed to simply manipulate cells and particles based on their intrinsic properties, such as size, density and compressibility in continuous flow. The device is manufactured using precise and low-cost microtechnological methods and is suitable for reproducible mass fabrication.

Results: We demonstrated the separation of blood components, i.e. the sorting of erythrocytes and thrombocytes. Furthermore, we could also show results on thrombocyte activation indicating a gentle separation without damaging these shear-sensitive cells, as well as first results on plasma separation from whole blood samples and nanoparticle sorting.

Summary/Conclusion: Our unique acoustofluidic sorting technology for complex suspensions has the potential to overcome the need for time-effective, cheap and gentle separation of EVs.

Funding: This work was supported by EFRE InfraPro project "ChAMP: Chip-based acoustofluidic Medtech Platform".

PT14.09

Nanophotonic platform for cancer-associated exosomal microRNA detection

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Introduction: Exosomes have an important role in intercellular communication at physiological and pathological processes. Their cargo includes microRNAs (miRs), single-stranded non-coding RNAs, involved in alterations on recipient cells, such as development of tumourous phenotype and metastasis. More particularly, miR-21 excels due to its association with several cancers. Determining exosomal miRs as cancer indicators demands selective and accurate methods, which are not currently available or entail high costs. Colorimetric photonic-based assays are a promising label-free alternative, which dismisses complex apparatus for signal reading since biorecognition is detected by colour change. Moreover, the clinical and economic systems have also been demanding a decrease on the green footprint of biosensors, requirement fulfilled with naturally derived biomaterials.

Methods: Herein, the biosensor is constructed on a biopolymer matrix to meet the requirements of an eco-friendly disposable device, and it is based on a photonic structure obtained by imprinting a nanopattern on the polymer surface. Then, the surface is functionalized with the complementary oligonucleotide sequence of miR-21 as sensing probe. A label-free detection is thus envisioned and the sensor performance is evaluated by changes in the optical properties when the target is present.

Results: The combination of biological materials conducted to a biosensor support with great flexibility and low water permeability, allowing easy surface functionalization. The self-reporting ability of the photonic-based sensor enables high intensity colours detected by naked eye.

Summary/Conclusion: The alliance with the high selectivity of oligonucleotide hybridization is expected to offer great exosomal miR-21 recognition ability and an optimistic perspective for utilization in clinical setups.

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PT15 = OP1

Oral with Poster Session 1: Lungs

Chair: Uta Erdbrügger – University of Virginia

Chair: Peter Kurre, MD – Comprehensive Bone Marrow Failure Center, Children's Hospital of Philadelphia; Perelman School of Medicine, University of Pennsylvania

PT15.01 = OP1.01

Human urinary extracellular vesicles carry surface markers that are indicative of haematopoietic origin

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Introduction: Urinary extracellular vesicles (uEVs) are important intercellular communicators. By systems biology integration, uEVs prove to be relevant in genitourinary disease detection. However, it has recently been shown that labelled EVs administered to the circulation can be detected in the urinary system, as well. Thus, this pilot study aimed at phenotyping haematopoietic surface markers on uEVs to create enough plausibility for future non-invasive biomarker studies of circulation and immune disorders that may translate into urine but are not yet timely recognized.
Methods: Urine was obtained from healthy men signing a written informed consent (n = 31). Sampling was approved by the local ethics committee and in compliance with the Declaration of Helsinki. Cell-free urine was obtained by serial centrifugation and 10 ml, each, were utilized for the MACSPlex Exosome Kit, human (Miltenyi Biotec). The manufacturer's recommendations were followed to examine 37 distinct uEV surface markers of CD9+/CD63+/CD81+ vesicles in a multiplexed bead-based manner including respective controls. The Accuri C6 (BD) was utilized for data acquisition.

For further MISEV2018-compliant characterization, CD9+/CD63+/CD81+ uEVs were isolated by immunoaffinity and analysed by fluorescence nanoparticle tracking (f-NTA), transmission electron microscopy (TEM) and western blotting (WB).

Urinary creatinine (Ucrea) was determined to control for variances in urinary dilutions and used for data normalization.

Results: Except CD209, all other 36 surface markers could be identified. The most abundant markers were CD9 and CD63, which were detected in 93% of samples, followed by CD133/1 (84%), CD326 (81%), CD81 and CD24 (77%, each). CD3 (42%), CD9, CD45 (39%), CD49e (32%) and CD81 showed similar relative median fluorescent intensities (rMFI), while CD63 yielded significantly higher ($p = 0.009$) and all other markers significantly lower rMFI ($p < 0.011$).

TEM and f-NTA revealed cup-shaped vesicles (137 ± 22 nm) with 8.8 ± 7.0 E + 10 particles/g Ucrea. WB indicated uEV isolates that were positive for Alix, Syntenin, TSG101, CD9, CD63 and CD81 without any uromodulin or calnexin contamination.
Summary/Conclusion: Our results imply that considerable quantities of circulatory EVs are, indeed, filtered into urine and could serve as valuable non-invasive biomarkers for systemic dysfunctions.

PT15.02 = OP1.02

Cardiovascular risk markers are strongly related to numbers of circulating extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are small plasma membrane-derived vesicles released from various cells, which potentially affect many physiological and pathophysiological processes, and are emerging as a potential novel biomarker in cardiovascular diseases (CVDs). However, there is little information about the association of circulating EV levels with traditional cardiovascular risk markers and CVD risk score.
Methods: • Subjects (n = 40) aged 40–70 yrs with moderate risk of CVDs were recruited and assessed for body mass index (BMI), blood pressure (BP) and plasma lipid profile (triacylglycerol, total cholesterol and high-density lipoprotein).

• EVs were isolated from platelet-free plasma by size exclusion chromatography and analysed by both Nanoparticle Tracking Analysis (NTA) and flow cytometry (FCM). NTA was used to measure the concentration and size distribution of EVs population, and EVs were phenotyped by FCM via a 3-colour panel, which included Annexin V (for the majority of circulating EVs), CD41 (for platelet-derived EVs) and CD105 (for endothelial-derived EVs).

• The association between risk markers and EV numbers was examined by Pearson's correlation coefficient and stepwise multivariate regression model. Analysis of covariance (ANCOVA) was performed after adjustment for various variables to determine the correlation between the quartile range of EV numbers and 10-yr CVD risk detected by QRISK2.

Results: EV numbers, as determined by NTA, were strongly associated with BMI ($r = 0.602$, $p < 0.001$), blood pressure (systolic BP: $r = 0.359$, $p = 0.023$; diastolic BP: $r = 0.550$, $p < 0.001$) and plasma triacylglycerol levels ($r = 0.703$, $p < 0.001$). Plasma total cholesterol level was positively associated with platelet-derived EVs, determined by FCM ($r = 0.330$, $p = 0.038$). A multivariate regression model demonstrated that plasma triacylglycerol and diastolic BP independently predicted total EV numbers, with plasma triacylglycerol concentrations explaining 49.4% of the variance for total EV numbers. An additional 9.3% of the variance in total EV numbers was predicted by diastolic BP. ANCOVA of the 10-yr CVD risk score in the quartile range of total EV numbers were positively and independently associated.

Summary/Conclusion: BMI, blood pressure, plasma triacylglycerol and total cholesterol levels are strongly associated with EV numbers. Plasma triacylglycerol and diastolic BP independently predict circulating EV numbers. Elevated numbers of EVs are independently associated with 10-yr CVD risk.

Funding: This project is supported by Biotechnology and Biological Sciences Research Council (BBSRC)-Diet and Health Research Industry Club (DRINC) in the UK.

PT15.03 = OP1.03

Extracellular vesicles from cardiosphere-derived cells potentiate regulatory T cells

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Introduction: Extracellular vesicles from cardiosphere-derived cells (CDC-EVs) are known to be anti-inflammatory in various disease models. To further dissect the mechanism, we examined the effects of CDC-EVs on T lymphocytes.

Methods: Naïve CD4 + T cells were isolated from secondary lymphoid organs of Foxp3-RFP reporter mice, using magnetic-activated and fluorescence-activated cell sorting. Cells were subsequently polarized into effector subtypes (Th1, Th2, and Th17), as well as regulatory T cells (Tregs), and the effects of exposure to human-derived CDC-EVs on proliferation and cytokine production were assessed. CDC-EVs were isolated from serum-free, 15-day conditioned medium, using ultrafiltration by centrifugation.

Results: After polarization and culture for 5 days, CDC-EVs resulted in dose-dependent and cell-specific proliferative responses. Effector T cells (Th1, Th2, Th17) showed either no change in proliferation (Th1) or decrease in proliferation (Th2, Th17), compared to the vehicle control. In contrast, Tregs proliferated much more than control ($P < 0.0001$). Next, we sought to characterize the changes in cytokine production by each effector T cell and Tregs. Compared to the vehicle control, exposure of polarized effector T cells to CDC-EVs had little effect on the expression of characteristic cytokine genes, including Ifnγ and Tnfα (Th1), Il4 and Il13 (Th2), or Il17a and Il17 f (Th17). In contrast, exposure of Tregs to CDC-EVs resulted in ~1000-fold increase in expression of Il10, a key paracrine agent utilized by Tregs in suppression of inflammation. This response was specific to CDC-EVs insofar as it was not recapitulated with dermal fibroblast exosomes. Concentrations of IL-10 in the culture media of CDC-EV-conditioned Tregs mirrored the increases in gene expression.

Summary/Conclusion: CDC-EVs potentiate Tregs by increasing their proliferation and enhancing production of IL-10. This offers an attractive therapeutic approach to inflammatory diseases that relies on harnessing an endogenous mechanism of immunosuppression.

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PT15.04 = OP1.04

Prostanoids impair platelet reactivity, thrombus formation and platelet extracellular vesicle release in patients with pulmonary arterial hypertension

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Introduction: Prostanoids (epoprostenol, treprostinil and iloprost) induce vasodilation in advanced pulmonary arterial hypertension (PAH) but also inhibit platelet activation, thereby increasing the risk of bleeding. Therefore, the platelet function and extracellular vesicle (EV) concentrations were measured in PAH patients treated with prostanoids and compared to patients with PAH not receiving prostanoids.

Methods: Venous blood was collected from 42 patients treated with prostanoids (study group; n = 42, 50 ± 16 years, 70% female) and 38 patients not treated with prostanoids (control group; n = 38, 55 ± 19 years, 65% female). Platelet reactivity was analysed in whole blood by impedance aggregometry using arachidonic acid (AA; 0.5 mM), adenosine diphosphate (ADP; 6.5 μM) and thrombin receptor-activating peptide (TRAP; 32 μM) as agonists. In a subset of patients, concentrations of EVs from platelets (CD61+ and CD62p+; PEVs), leukocytes (CD45+, LEVs) and endothelial cells (CD146+, EEVs) were measured in platelet-depleted plasma by flow cytometry (A60-Micro). Platelet-rich thrombus formation was measured using a whole blood perfusion system.

Results: Compared to the control group, patients treated with prostanoids had lower platelet reactivity in response to AA and ADP ($p = 0.01$) and lower concentrations of PEVs and LEVs ($p \leq 0.05$). Furthermore, thrombus formation was delayed ($p \leq 0.003$) and thrombus size was decreased ($p = 0.008$) on prostanoids. Epoprostenol did not affect platelet reactivity in vitro, but decreased the concentrations of CD61+ PEVs ($p = 0.04$). In contrast, treprostinil and iloprost decreased both platelet reactivity in response to AA and ADP ($p \leq 0.05$) and the concentrations of PEVs ($p \leq 0.08$). All prostanoids delayed thrombus formation and decreased thrombus size ($p \leq 0.04$).

Summary/Conclusion: Patients with PAH treated with prostanoids have increased risk of bleeding both due to impaired platelet aggregation, EV release and thrombus formation, compared to patients not treated with prostanoids. Antiplatelet effect of prostanoids varies: whereas epoprostenol decreases the release of PEVs, treprostinil and iloprost impair platelet aggregation.

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Nanoflow cytometry identifies an imbalance of epithelium- and neutrophil-derived extracellular vesicles in the airway environment of paediatric cystic fibrosis patients

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Introduction: Progressive lung disease is the leading cause of mortality in cystic fibrosis (CF), a chronic condition characterized by recruitment of polymorphonuclear neutrophils (PMNs) into the airways. Newly arrived PMNs are exposed to extracellular vesicles (EVs) from the airway epithelium and PMNs recruited before them. In controlled experiments, these EVs were necessary and sufficient to induce pathological changes including reduced bacterial killing and immunosuppressive activities towards macrophages and T-cells. However, children with CF do not always show a high PMN presence in their airways, which suggests that the balance between PMN recruitment and the activity of other cells is still in flux in early stage disease.

Methods: We utilized spectral nanoflow cytometry to profile the single EV content of the bronchoalveolar lavage fluid (BALF) from 17 CF children (<6 years of age). For nanoflow cytometry, EVs were stained with Di-8-ANEPPs, and with EpCAM, CD66b and CD115 (to ascertain epithelial, PMN, and macrophage origins, respectively). Violet side scatter and/or fluorescence threshold triggering were used for EV detection.

Results: The ratio of neutrophil- to epithelial-derived EVs in CF BALF correlated positively with the percentage of PMNs that are present in the airways ($p = 0.003$, Spearman's rho = 0.689). This ratio also correlated with the PRAGMA disease score, which

quantifies airway damage by chest computed tomography (p = 0.001, rho = 0.857).

Summary/Conclusion: Using a method to quantify EVs from specific cell types in vivo, we demonstrated that the ratio of PMN- and epithelial cell-derived EVs tracks with airway damage and neutrophil influx, suggesting a critical interplay between these cells in early CF disease. This EV-focused method can be applied to other diseases in which sampling cells is difficult. Future experiments will use CF BALF biobanks to strengthen data presented here.

Funding: CF Foundation (TIROUV15A0), Emory Paediatrics Flow Core.

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The potential of crude extracellular vesicle microRNAs for the diagnosis of community-acquired pneumonia and for the detection of pneumonia-related sepsis as a severe secondary complication

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Introduction: Circulating cell-free microRNAs (miRNAs), often associated to extracellular vesicles (EVs), are essential for cell-cell communication in the pathogenesis of infectious pulmonary disorders. As early pneumonia diagnosis is often clinically challenging, advances in disease detection could improve outcomes. We characterized crude EV miRNAs as potential biomarkers for community-acquired pneumonia and sepsis as a severe secondary complication.

Methods: 142 individuals were enrolled into our study, subdivided into a training (volunteer n = 27, pneumonia n = 12, sepsis n = 28) and testing cohort (volunteer n = 20, pneumonia n = 18, sepsis n = 37). After precipitating crude EVs from sera (miRCURY Exosome Isolation Kit-Serum and Plasma) and extracting total RNA, small RNA sequencing was performed. miRNAs were selected as biomarker candidates by differential gene expression analysis (DESeq2) and sparse partial-least-squares discriminant analysis (mixOmics). Technical and biological validation was performed by reverse transcription quantitative real-time PCR. Group classification was predicted by partial-least-squares discriminant analysis. Gene targets and causal networks were identified by ingenuity pathway analysis.

Results: Differential gene expression analysis revealed 29 significantly regulated miRNAs in pneumonia compared to volunteers, and 25 miRNAs in pneumonia related to sepsis. Based on sparse-partial least discriminant analysis, group separation was achieved by 12 miRNAs as discriminators with high sensitivity and specificity (area under the curve of the receiver operated curve: volunteer: 0.982, pneumonia: 0.965, sepsis: 0.992). miR-193a-5p (log2FC = 1.86, padj = 1.49E-6) and miR-542-3p (log2FC = 1.67, padj = 3.29E-5) differentiated between pneumonia and volunteers and miR-1246 (log2FC = −2.41, padj = 1.78E-04) between pneumonia and sepsis. Expression levels of miR-193a-5p and miR-1246 were related to disease severity. miR-542-3p was higher expressed in pneumonia compared to volunteers and had equal expression in patient groups. Prediction of group classification in the testing cohort was 73.33%. Signalling networks were constructed for “cellular and humoral immune response”, “antimicrobial response” and “pathogen influenced signaling” involving the significantly regulated miRNAs.

Summary/Conclusion: Crude EV miRNAs are potentially novel biomarkers for community-acquired pneumonia and may help to identify patients at risk for progress to sepsis allowing early intervention and treatment.

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PT15.07 = OP1.07

microRNA exosome cargo from induced sputum: new tool for approaching asthma research

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Introduction: It remains unclear the specific mechanisms that lead to airways inflammation in asthma and the subsequent remodelling of the airways. Exosomes, small extracellular vesicles, has become in an important mechanism of cell-to-cell communication and participate in diverse biological processes including inflammation. In this study, we hypothesize that exosomes and their miRNA cargo

play an important role in the proinflammatory status of the upper airway of asthma patients, especially in those patients with severe asthma.

Methods: In a pilot study,3healthy subjects had induced sputum using standard methods. After several centrifugation steps, we were able to isolate exosomes from sputum supernatant by both precipitation and Size Exclusion Cromatography (SEC). Exosome size was observed with Transmission Electron Microscopy (TEM) and the protein markers CD63 and CD81 were analysed by Western Blot (WB). Then, total RNAs were isolated from sputum exosomes and 9 miRNAs (miR-103a-p, miR-191-5p, miR-320a, miR-200b-3p, miR-185-5p, miR-223-3p, miR-21-5p, miR-155-5p, let-7 g-5p), were evaluated by RT-qPCR. After the optimization of the methodology, 10 healthy adults subjects and 10 patients with persistent moderate-severe asthma, matched by age and sex were selected and induced sputum was collected.

Results: Exosomes isolated with both methodologies (precipitation and SEC) were observe under the TEM with a correct range of size. Furthermore, WB assay displayed a coherent protein profile for the exosome markers CD63 and CD81. However, SEC displayed low signal and the variability of between subjects was to higher. Using the optimized method of precipitation, we observed that after normalization, miRNA-320a showed a significant increased ($p = 0.02$) in asthma patients compared to control. This miRNA has been linked with an active proinflammatory status.

Summary/Conclusion: Our results confirm the presence of exosomes in induced sputum with promising applications in the field of asthma. The upregulation of exosomal miR-320a, which is related with inflammation, suggest that exosomes could play a crucial role in the chronic inflammation of airway described in asthma patients.

PT15.08 = OP1.08

Human nrf2-active multipotent stromal cell exosomes reverse pathologic delay in the healing of cutaneous diabetic wounds

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Introduction: Multipotent stromal cells (MSCs) have attracted much attention for their capacity to accelerate wound healing. Exosomes, nanosized extracellular vesicles, may be key to translating MSC therapy. We previously found that nuclear factor erythroid 2-related factor 2 (Nrf2) regulates MSC promotion of diabetic tissue repair. Here, we explore a novel role of Nrf2 in exosome biogenesis and investigate whether exosome treatment recapitulates the effects MSCs have on healing.

Methods: Exosomes were harvested by differential ultracentrifugation of conditioned bone marrow derived MSC media. For Nrf2-active exosomes, MSCs were incubated with potent Nrf2 activator, CDDO-Im. Exosomes and MSCs were vigorously characterized. Full-thickness humanized-stented wounds were created on adult Leprdb/db diabetic mice (db/db). Exosomes were injected intradermally and circumferentially to the wound margin.

Results: MSCs adopt an adherent fibroblast morphology, demonstrate robust osteogenic, chondrogenic, and adipogenic differentiation, express >95% positive MSC markers (CD44, CD73, CD90, and CD105) and <5% express negative markers (CD45, CD31, CD14, CD19, or HLA-DR). Immunoblotting of MSC exosomes shows enrichment for positive exosomal markers CD81, CD9 and TSG101. Nanoparticle tracking analysis (NTA) shows a nanoparticle population with mean diameter of 168.0 ± 6.5 nm. Transmission electron microscopy of exosomes reveals flattened cup-like spheres. NTA demonstrates that Nrf2-active human MSCs increase exosome secretion by 54%, compared to Nrf2-baseline MSCs ($p < 0.05$). Both Nrf2-baseline and Nrf2-active exosome treatment significantly reduced closure time to 15.5 and 14 days respectively, compared to 29.8 days for vehicle-treated wounds ($p < 0.05$). This reduction eliminated the delay in closure time compared to wounds of C57/B6 mice. Nrf2-active exosome treatment of db/db wounds reduced closure time by a further 2.6 days compared to untreated C57/B6 wounds. At day 10, exosome-treated db/db wounds have significant decreases in epithelial gap, expanded granulation tissue, and greater density of CD31+ vessels compared to vehicle-treated wounds.

Summary/Conclusion: Enhancing Nrf2 function in MSCs multiplies exosome yield. Our results demonstrate exosome-based therapies hold tremendous promise and warrant further investigation for rapid translation.

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PT15.09 = OP1.09

Extracellular vesicles from adipose tissue end endothelial cells of obese humans share miRNA cargos and increase prostate cancer aggressiveness in conjunction with Twist1

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Introduction: Obesity increases prostate cancer aggressiveness and adipose tissue (AT) is a rich source of extracellular vesicles (EV) that have been shown to contribute to pro-oncogenic effects in various malignancies. Twist1 is a key mediator of tumour cell metastasis. The goal of this study was to determine molecular and phenotypic changes of prostate cancer cells in response to EVs from obese human AT and the role of different levels of endogenous Twist1.

Methods: EV were harvested from human AT (ATEV) obtained from bariatric subjects or from AT endothelial cells treated with proinflammatory cytokines (PIC-EV) to mimic the obese AT environment. EVs were isolated by ultracentrifugation and characterized by electron microscopy, NTA and protein markers. We determined the effect of ATEV and PIC-EV on PC3-ML prostate cancer cells proliferation and invasion. EV miRNA cargo and transcriptome of PC3-ML cells treated with ATEV or PIC-EV were assessed using NanoString. To establish the contribution of Twist to the EV-related phenotypic and molecular changes in recipient cells, we used PC3-ML lines stably overexpressing or deficient in Twist1.

Results: ATEV from obese subjects and EV-PIC from AT endothelial cells both reduced invasion and increased proliferation in wild-type PC3-ML cells. A molecular signature showing decreased expression of genes mediating invasion, adhesion and metabolism supported these functional effects. Also ATEV and EV-PIC shared a subset of miRNA that target multiple MMPs, inhibit glycolytic genes and target cell cycle inhibitory genes. PC3-ML overexpressing Twist1 showed an increase in both proliferation and invasiveness and this phenotype was supported by the transcriptomic analysis following EV treatment.

Summary/Conclusion: EV produced by obese AT or by AT endothelial cells share a subset of miRNA that in conjunction with increased Twist1 expression contribute to tumorigenesis and metastasis of prostate cancer cells in vitro.

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PF01: EVS in Metabolism and Metabolic Disease

Chair: Michael Freeman – Cedars-Sinai Medical Centre

PF01.01

Release of larger extracellular vesicles post-acute exercise is associated with the exercise responder phenotype in youth living with obesity

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Introduction: Exercise is associated with various health benefits, including the prevention and management of obesity and cardiometabolic risk factors. However, a strong heterogeneity in the adaptive response to exercise training exists. Differential response to exercise training might be mediated by myokines (proteins, nucleic acids, metabolites) that can be released directly into the systemic circulation, or packaged within extracellular vesicles (EVs). The objective of this study was to evaluate if changes in EVs after acute aerobic exercise (AE) were associated with the responders phenotype following 6-week resistance exercise (RE) training.
Methods: This is a secondary analysis of plasma samples from the EXIT trial (clinical trial #02204670). Eleven sedentary obese youth (15.7 ± 0.5 years, BMI ≥ 95th percentile underwent an acute bout of AE (60% heart rate reserve, 45 min). Blood was collected before [time (AT) −15, 0 min], during [AT15, 30, 45 min], and after [15 min (AT60), 75 min (AT120)] exercise. Afterwards, youth participated in 6-week RE programme, and were categorized into responders or non-responders (NR) based on changes in insulin sensitivity (above or below 50 percentile). Primary outcome: EVs were isolated using size exclusion chromatography (Izon®) at baseline (AT0), immediately after AE (AT45) and after recovery (AT120). EV protein concentration, size, and zeta potential were analysed in a single-blind fashion.
Results: Responders had larger EVs (~141.1 nm) as opposed to NRs (~97.3 nm) at AT0 ($p < 0.05$) and this pattern was maintained at AT45 and AT120, though not significant ($p = 0.1$). NRs displayed differential EV size distribution (peaks at 100 nm or 300 nm), while EV distribution was highest at 250 nm in responders. No difference in average zeta potential or total EV protein yield was observed between groups. An increase in EV yield with exercise time and recovery was observed in both groups.
Summary/Conclusion: Our preliminary data suggest that EV size is significantly increased after an acute bout of AE in obese youth responders. Further research to delineate the role of EVs as predictors of exercise adaptation is warranted.
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PF01.02

Using dual-fluorescent reporter mice to track tissue-specific extracellular vesicles

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Introduction: Extracellular vesicles (EVs) from tissues like skeletal muscle (SkM) and adipose tissue (AT) have been implicated in human disease but are understudied. SkM is likely a major player in EV biology as it accounts for ~35% of total body mass. Tools to define cellular EV origin are needed because tissues like SkM are comprised of a variety of cell types. Here, we describe our ongoing efforts using the dual fluorescent mG/mT mouse as a tool to analyse SkM-myocyte derived EVs.
Methods: Wild-type (WT) and mG/mT mice were used for these studies. mG/mT mouse cells express membrane-tagged red (mT) or green (mG) fluorescent protein in the absence or presence of Cre, respectively. We made SkM myocyte mG expressing mice using a mouse expressing Cre on the human skeletal actin promoter. Blood was collected via cardiac puncture and platelet-free plasma was obtained via centrifugation. Plasma EVs were isolated using Exoquick, Exoquick-TC or size exclusion chromatography. SkM and AT were dissected into ~5mm chunks, placed in serum-free DMEM and incubated for 24 hours. Tissue-derived EVs were isolated using Exoquick-TC. EV abundance was determined with a Horiba ViewSizer.

Individual EVs were analysed with a Cytek Aurora spectral flow cytometer. Settings were optimized using polystyrene beads and spectral unmixing was performed to allow detection of mG and mT.

Results: In WT mice, SkM releases >100 times more EVs than adipose tissue per unit of mass ($p < 0.01$ using paired Student's t-test). Since SkM is also a major component of total body mass, these data further emphasize the importance of SkM-derived EVs. SkM-derived EVs from WT mice were not fluorescent (<0.1% of events). EVs from mG/mT mouse SkM overwhelmingly expressed mG (>95% of events) with negligible (< 1%) expression of mT. AT-derived EVs robustly expressed mT but lacked mG.

Summary/Conclusion: These data provide "proof-of-principle" that mG and mT are readily incorporated into EVs secreted ex vivo. Surprisingly however, plasma EVs from mG/mT mice expressed very little mG (~3%) or mT (~ 4%). This observation was confirmed with three separate isolation techniques. We are currently exploring possibilities to explain this finding, including: 1) modification of EVs post-secretion, 2) clearance of fluorescent EVs by the liver or 3) that EVs secreted from tissues remain predominantly in the interstitial space.

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PF01.03

Endothelial CD36 delivery of FA loaded extracellular vesicles is critical for thermogenesis.

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Introduction: Membrane CD36 facilitates tissue fatty acid (FA) uptake. We recently found that endothelial cell (EC) CD36 controls muscle and adipose tissue FA uptake, and influences the tissue's metabolic phenotype. The mechanism for CD36-facilitated FA uptake is unknown. Here we examined the role of EC CD36 in thermogenesis and in FA delivery to brown fat tissue.

Methods: Adult male mice were housed individually, restricted from food during acute (4 hr) cold exposure (4°C) with core temperature monitored every 30 minutes. After 4 hours, animals were sacrificed and samples collected for analysis. For cellular studies, human microvascular (Lonza) or primary murine microvascular EC were used. For primary cells, crude cell pellets from lung homogenates were purified using mouse-CD31 magnetic beads (Miltenyi). For microscopy studies, alkyne FA (Cayman) was added to cells and to enable visualization of internalized FAs, click chemistry (Invitrogen) used to label alkyne-FA with Alexa 568. For radioactive studies, primary lung EC were serum starved for 5 hrs and incubated overnight with 3H-oleic acid bound to FA-free BSA (2:1 ratio). Media was collected, clarified by centrifugation to remove microvesicles and debris. Small extracellular vesicles (sEVs) were isolated from clarified media using Total Exosome Isolation reagent (Invitrogen) and counted for radioactivity.

Results: Basal core body temperatures are similar in mice lacking EC CD36 (ecCD36-/-) compared to controls (CD36fl/fl). However, during cold exposure at 4° C, ecCD36-/- are unable to maintain body temperature ($p < 0.001$). Plasma free FA are higher in cold exposed ecCD36-/- indicating FA clearance by brown fat is impaired. Mitochondrial function and expression of thermogenic and mitochondrial genes in brown fat from ecCD36-/- and CD36fl/fl mice were similar. These data suggested that endothelial delivery of FAs is necessary for thermogenic maintenance of body temperature.

To examine FA handling by ECs we used alkyne FAs to visualize the process. We found that FAs are transferred by microvascular EC through caveolae-mediated transcytosis involving Src signalling and Cav-1 phosphorylation. The internalized Cav-1 and CD36 positive vesicles containing FAs are released as sEVs. To determine the dependence of CD36 on this process, we treated primary microvascular EC with radiolabeled FA and found that sEVs secreted by CD36-/- cells contain less labelled-FA ($p = 0.05$).

Summary/Conclusion: Endothelial delivery of FA is critical for thermogenesis. Our working model for the mechanism of FA uptake by brown adipose tissue is the following: Endothelial cells transfer the FA through caveolae-mediated transcytosis and secrete small extracellular vesicles (sEVs) that help deliver FAs to brown adipocytes.

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PF01.04

Nanovesicles from orange juice restore the intestinal functions altered during diet-induced obesity in mice.

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Introduction: Diet-induced obesity modifies intestinal permeability leading to bacteria infiltration and to a decrease in the number of immune cells protecting mucosa. As orange consumption is beneficial for human health and used in preventive medicine, we determined whether orange juice-derived nanovesicles (ONV) might be recommended as nutritional strategies for the treatment of intestinal complications associated with obesity.

Methods: ONV isolated from fresh orange juices were characterized by lipidomic, metabolomic, microscopy, NTA and for their stability during digestion. Intestinal barrier (IB = Caco-2 cells+HT-29 cells differentiated with oleic acid) were treated with ONV and co-cultured with adipocytes to monitor IB fat absorption and release. Obesity was induced in mice fed for 12 weeks with a high-fat high-sucrose diet (HFHS mice vs standart chow diet mice). Then half of the HFHS mice were gavaged with 150 micrograms/day for 4 weeks.

Results: ONV did not modify high-fat high-sucrose diet-induced obesity and insulin resistance but reversed diet-induced gut modifications. Six hours post-gavage, ONV accumulated preferentially in jejunum involved in lipid absorption. In jejunum, and no other intestinal region, ONV increased villi size, restored immune response and decreased barrier permeability in HFHSD mice. In addition, ONV-treated mice had increased expressions of ACAT2, ANGPTL4 and DGAT1, but a decreased expression of FABP2, FATP4, MTP vs HFHSD animals, which indicated that fat absorption, TG synthesis and chylomicron release were strongly reduced. Similarly to other plant-derived nanovesicles, these results were likely associated with ONV lipid and metabolite compositions (strong enrichment in bioactive phospholipids: PE, PA, PC, PI and leucine) as ONV did not resist to harsh digestive conditions in vitro and were poorly incorporated in enterocytes. As the effects of ONV on the decrease in TG content and epithelial cell growth were also observed in vitro, gut microbiota unlikely participate to these effects.

Summary/Conclusion: ONV are important bioactive compounds of orange juice and for the first time we demonstrated that they can modulate lipid metabolism in the intestinal barrier associated with morphological changes. Interestingly ONV treatment targets MTP and ANGPTL4 mRNAs, 2 therapeutic intestinal targets to reduce plasma lipids and for attenuating inflammation in gastrointestinal diseases. Therefore, ONV might be used to reduce the development of dyslipidemia-associated diseases and to restore intestinal functions in obese patients.

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PF01.05

Association, structure, and function of fibronectin in extracellular vesicles from hepatocytes

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Introduction: We have shown that extracellular vesicles from normal hepatocytes have anti-fibrogenic activity and that they preferentially bind to hepatic stellate cells (HSCs, the principal fibrosis-causing cell in the liver) and hepatocytes. In this study, our goal was to determine the molecular nature of the EV components involved in cell binding. Fibronectin (FN1) is a key component of extracellular matrix, functioning in processes including cell adhesion, differentiation, and wound healing. Two types of FN1 are present in vertebrates, of which the soluble plasma FN1 is derived principally from hepatocytes, while cell-associated FN1 is produced by numerous cell types. Here we describe a novel function of plasma FN1 in facilitating binding of hepatocyte EVs to target cells.

Methods: Differential ultracentrifugation was used to collect EVs released by parental mouse AML12 hepatocytes, FN1 KO AML12 cells in which FN1 was ablated using CRISPR-cas9, primary human or mouse hepatocytes, or human HepG2 cells, or from human or mouse serum. EVs were characterized by nanosight tracking analysis (NTA), Western blot, iodixanol gradient ultracentrifugation, and mass spectrometry. The binding efficiency of PKH26-labelled EVs from parental (EV-Hep) or FN1 KO (EV-HepFN1 KO) AML12 cells was analysed in hepatocytes or HSCs. Swiss Webster mice were injected with CCl4 for five weeks to induce liver fibrosis, with some mice also receiving i.

p. administration of EV-Hep or EV-HepFN1 KO over the last two weeks, followed by determination of hepatic fibrogenic genes by qRT-PCR.
Results: EV-Hep or EV-HepFN1 KO were 50–200 nm in diameter and positive for common EV markers (CD63, CD9, flotillin-1). Mass spectrometry showed that FN1 was the most abundant protein in EV-Hep and comprised principally the plasma form. The abundant presence of EV FN1 was verified by Western blot and co-immunoprecipitation with anti-CD9 or anti-flotillin-1. Western blot showed that FN1 was also abundant in EVs from primary human or mouse hepatocytes, HepG2 cells, and human or mouse serum. FN1 and EV-Hep co-sedimented at a density of ~1.15 g/ml. EV-HepFN1 KO yield and size-range were similar to those of EV-Hep, suggesting that EV biogenesis is FN1-independent. As compared to EV-Hep, the binding of EV-HepFN1 KO to target cells was highly reduced whereas EV binding was independent of FN1 expression by the target cells themselves. Both EV-HepFN1 KO and EV-Hep were anti-fibrogenic in vivo but only EV-Hep attenuated collagen 1□1 expression in mouse HSCs in vitro.
Summary/Conclusion: FN1 is abundantly associated with hepatocyte EVs and facilitates EV binding to target hepatocytes or HSCs. Additional studies are needed to clarify the functional role of FN1 in mediating EV-Hep anti-fibrogenic actions in vitro or in vivo.

PF01.06

Elevated glucose increases soluble and aggregated forms of human islet amyloid polypeptide in islet-derived extracellular vesicles – Implications in type 2 diabetes and islet transplantation

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Introduction: Type 2 diabetes (T2D) is characterized by reduced beta cell mass and function. Islet amyloid, formed by aggregation of human islet amyloid polypeptide (hIAPP), contributes to progressive beta cell loss in T2D. Amyloid also forms in human islets during pre-transplant culture and following transplantation in patients with type 1 diabetes (T1D) which is associated with graft failure. The cellular mechanisms underlying islet amyloid formation are still unclear. In this study, we examined the potential role of islet-derived extracellular vesicles (EV) in the clearance of soluble and aggregated (pro)IAPP species from beta cells and amyloid formation.
Methods: Human islets isolated from cadaveric pancreatic donors (n = 4 donors) and wild-type or hIAPP-expressing (hIAPP+) transgenic mouse islets (n = 4/group) were cultured in normal (5.5 mM) or elevated (11.1 mM) glucose to form amyloid. EV (exosomes) were isolated from culture medium using classical centrifugation and ultracentrifugation. Purified EV were analysed by nanoparticle tracking analysis. Western blot analysis and double immunogold transmission electron microscopy were performed to verify the presence of EV markers as well as (pro)hIAPP species and oligomers (aggregates).
Results: Human islets formed amyloid during culture with elevated glucose which was associated with progressive beta cell apoptosis. (Pro)IAPP species were detectable in EV released from human islets cultured in normal and elevated glucose. The latter markedly increased (pro)IAPP content in islet-derived EV. Interestingly, hIAPP aggregates (oligomers) were present in the majority of EV released from human islets cultured in elevated glucose but were not detectable in islets cultured with normal glucose. Similarly, EV released from hIAPP+ mouse islets which formed amyloid during culture had higher (pro)IAPP content compared to wild-type islet-derived EV. Moreover, hIAPP oligomers were present in EV derived from hIAPP+ islets but not WT islets.
Summary/Conclusion: In summary, our data show that (pro)IAPP species are present in islet-derived EV and that elevated glucose increases (pro)hIAPP and its aggregates in EV released from islets. Islet-derived EV may play a key role in the process of amyloid formation in T2D and human islet grafts.
Funding: University of Manitoba Research Grants Program (URGP).
on.

PF01.07

Contraction, but not glycolysis, regulates the size of skeletal muscle EVs secreted ex vivo.

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Introduction: Skeletal muscle (SkM) is a metabolically active tissue and accounts for ~35% of total human body mass. Acute exercise increases secretion of extracellular vesicles (EVs), but the mechanisms responsible

are unknown. Muscle contraction increases the demand for ATP which requires intercellular communication in order to adapt. We hypothesized that this "metabolic stress" during contraction increases SkM EV secretion.

Methods: We tested our hypothesis using an ex vivo EV secretion assay. All studies were approved by the Colorado State University Institutional Animal Care and Use Committee. Vastus medialis muscle (SkM) from male C57Bl/6 J mice (n = 6) or female mT/mG mice (n = 6) was cut into ~5 mg pieces and added to 12 well plates (~50 mg/well) filled with 1 ml of serum-free DMEM and placed in a cell culture incubator at 37 C for 24 hours. SkM from male mice was treated with 2-deoxyglucose (2-DG) (0.1 nM – 100 mM) to induce metabolic stress via inhibition of glycolysis. SkM from female mice was treated with 10uM of blebbistatin (BLEB), a contraction inhibitor. After incubation, SkM mass was measured and conditioned media was centrifuged (3,000 x g for 15 min) to remove cell debris. EVs were isolated using ExoQuick-TC. NTA was performed on isolated EVs using a Horiba Viewsizer 3000. EV secretion was normalized to tissue mass and culture media volume then reported as ([Particle]/mL/mg tissue). Statistical comparisons for 2-DG experiments were made using a repeated measures 1-way ANOVA. BLEB experiments were analysed using a paired Student's t-test.

Results: There was a trend towards greater EV abundance ($p = 0.07$) as a function of 2-DG treatment, but no effect on EV diameter ($p = 0.37$). BLEB treatment did not alter EV abundance ($p = 0.69$), but significantly reduced EV mean diameter ($p = 0.007$; 11% decrease; DMSO: 114.9 ± 5.1 vs. BLEB: 103.9 ± 4.2).

Summary/Conclusion: Contrary to our hypothesis, inhibition of glycolysis with 2-DG did not stimulate SkM EV secretion. However, BLEB did appear to promote the release of small EVs and/or inhibit secretion of larger EVs. Ongoing efforts are focused on testing other metabolic stressors and defining how blebbistatin promotes small EV secretion.

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PF02: EVs as delivery vehicles

Chair: Karen Bussard – Thomas Jefferson University

PF02.01

Cell-penetrating sC18 peptide-modified extracellular vesicles for intracellular delivery

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Introduction: Extracellular vesicles (EVs, exosomes) are nanovesicles (30–200 nm) secreted from various types of cells. Because of vesicular encapsulation of miRNAs and enzymes, the EVs play crucial roles in cell-to-cell communication by delivering these functional molecules to other cells [1]. On the other hand, the EVs are highly expected as next generation therapeutic tools due to pharmaceutical advantages such as controlled immunogenicity, effective usage of cell-to-cell communication routes, artificial modification and encapsulation of functional molecules. However, cellular targeting and uptake efficacy of the EVs are insufficient to be utilized as therapeutic tools [2, 3]. In this study, we newly developed EVs decorated with cell-penetrating sC18 or (sC18)2 peptides, which are derived from the C-terminal domain of the cationic antimicrobial protein, CAP18, because the peptides can be efficiently internalized by breast cancer cells. [4, 5].

Methods: All peptides were prepared by Fmoc-solid phase synthesis. Secreted EVs from CD63-GFP stably expressing HeLa cells were isolated by ultracentrifugation. Cellular uptake of EVs was analysed using a flow cytometer and a confocal laser microscope. Encapsulation of saponin in the EV was conducted by electroporation.

Results: sC18 peptide is known as one of cell-penetrating peptides, and branched structure of sC18 peptides, (sC18)2, further enhances the cellular uptake [5]. In this research, we examined the effects of the peptide modification on cellular EV uptake, and modification of the sC18 or (sC18)2 peptides on EV membranes was conducted via stearyl moiety. As our results, increased macropinocytotic cellular uptake by modification of the peptides was successfully attained. Especially, the modification of (sC18)2 peptides showed higher cellular uptake and macropinocytosis induction efficacy than that of sC18 peptides. In addition, anticancer protein, saporin toxin-encapsulated EVs modified with the (sC18)2 peptides significantly enhanced their biological activity with dependency of glycosaminoglycan expression on targeted cells.

Summary/Conclusion: The cell-penetrating (sC18)2 peptide-modified EVs shows high abilities to be effectively internalized by cells and are applicable for intracellular delivery of therapeutic molecules. This study is expected to contribute to development of intracellular delivery techniques based on EVs.

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PF02.02

Natural or synthetic: a comparison of the efficiency of extracellular vesicles vs synthetic carriers for RNA delivery

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Introduction: RNA therapeutics possess high potential which is yet to be realised, largely due to difficulties involved in delivery to the cytoplasm of target cells. Extracellular vesicles (EVs) possess numerous features that may help overcome this hurdle and have emerged as a promising RNA delivery vehicle candidate. Despite extensive research into the engineering of EVs for RNA delivery, little is known about how their intrinsic RNA delivery efficiency compares to current synthetic RNA delivery systems. Using a novel CRISPR/Cas9 based RNA transfer fluorescent stoplight reporter system,

we here compared the delivery efficiency of EVs to state-of-the-art DLin-MC3-DMA lipid nanoparticles (LNPs).

Methods: EVs were isolated from MDA-MB-231 cells expressing either a targeting or non-targeting control sgRNA and applied to HEK293 T stoplight+ reporter cells. LNPs containing targeting sgRNA were titrated onto HEK293 T stoplight+ reporter cells to determine the minimum effective dose. LNP and EV particles were characterized using nanoparticle tracking analysis, dynamic light scattering and zeta potential analysis. sgRNA copy number was determined using RT-qPCR.

Results: EVs were 140 ± 7 nm in diameter as measured by DLS and possessed a negative surface charge of −25.3 ± 3.3 mV. RT-qPCR and NTA analysis indicated that sgRNA EV loading was low, with only 1 in 3.8e05 ± 3.5e05 EVs containing a single sgRNA copy. Nevertheless, EVs containing targeting sgRNA induced significant reporter activation while EVs containing non-targeting sgRNA did not. LNPs were 51 ± 0.6 nm in diameter and possessed a neutral charge. These particles also induced significant reporter activation when loaded with targeting sgRNA. When delivered via EVs, only between 4 to 54 sgRNA copies per cell were required to induce statistically significant reporter activation. In contrast, the minimal effective sgRNA dose when delivered by LNPs was considerably higher at approximately 9e03 copies per cell.

Summary/Conclusion: MDA-MB-231 EVs deliver RNA in a highly efficient manner and are functional at sgRNA concentrations several orders of magnitude lower than those required for LNP mediated delivery. This underlines the potential of EVs as RNA delivery vehicles and highlights the need to study the mechanisms by which EVs achieve their efficiency in order for improved development of RNA therapeutics.

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PF02.03

The role of circulating extracellular vesicles in patients with chronic chagas disease

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Introduction: Chagas Disease is a neglected tropical disease (NTD) caused by the flagellated protozoan Trypanosoma cruzi. It is a major public health problem in Latin America, and it is now expanding over the globe through immigration of infected individuals. Eukaryotic cells release extracellular vesicles (EVs) that circulate in body fluids and have an important roles in intercellular communication, both in physiological and pathological conditions. Objectives. Our study proposes to characterize and to compare the circulating EVs isolated from plasma of the Chronic Chagas Disease (CCD) patients with healthy individuals (controls).

Methods: Peripheral blood was collected from patients and controls in the presence of EDTA and EVs enriched from plasma by differential ultracentrifugation. The obtained EVs were characterized and quantified by Nanoparticle Tracking Analysis (NTA) and added to human THP-1 cells. After 48 h, the cell supernatants were analysed by ELISA for the presence of cytokines.

Results: Lower amounts of EVs were obtained from CCD patients in comparison with control individuals. However, the same amount of EVs of CCD were more capable of inducing cytokines such as IFN-gamma and IL-17 in relation to controls.

Summary/Conclusion: Although less EVs are present in the blood of CCD, these EVs induce high inflammatory reactions on macrophages suggesting a possible role of these EVs in the establishment of chronic disease.

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PF02.04

Extracellular Vesicles – a Trojan horse for therapeutic agent delivery

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Introduction: Extracellular vesicles (EVs) may prove to be one of the optimal payload carriers for therapeutic agents. While they travel through the extracellular space, the EV's lipid membrane layer shields their luminal cargo from deleterious external factors. When autologous EVs are used to protect this therapeutic cargo, little immunogenic effects are expected compared to viral

vectors and artificial structures, such as liposomes. Their usage is potentially manifold, and they are ubiquitously present in all body tissues and fluids. The key is to develop a manageable EV loading agent for adoptive transfer therapies.

Methods: To exploit the unique properties of EVs, highly positively charged proteins were used to load them with multiple biomolecules, such as a Cas9 protein or Dicer substrate dsRNA as a functional payload and to improve their apparently inadequate natural ability to deposit cargo into the cytoplasm of recipient cells.

Results: Highly positively charged proteins can associate with and/or diffuse through a phospholipid bilayer (Thompson et al. 2012). When these kinds of charged proteins are mixed with isolated EVs in vitro, they are loaded into the EVs. The positive charge of the protein has the advantage that it can associate with negatively charged agents, such as RNA species, and aids the associated molecule to also incorporate into the EV. Moreover, the positive charge of the protein helps with cargo delivery, and thus overcoming the bottleneck of the EV's cargo to escape the endosome post-uptake in a recipient cell. Self-quenching fluorescent lipid dyes demonstrated that discharge of the highly positive EV cargo into the cytoplasm is concomitant with lipid mixing between the membrane of EVs and the membrane of the recipient cell. When eGFP-expressing microglia were exposed to EVs loaded with a Dicer substrate dsRNA able to silence eGFP via the positively charged protein, the uptake of Dicer substrate dsRNA was concomitant with a decrease in eGFP expression in the microglia. A similar result was achieved when EVs were loaded with Cas9 protein conjugated to the highly positively charged protein. Post-uptake of these Cas9-loaded EVs, microglia expressing anti-eGFP sgRNA (single guide RNA) lead to decreased eGFP expression.

Summary/Conclusion: Our EV delivery technology has the capability of delivering multiple biomolecules, such as protein and RNA cargo and demonstrates post-uptake of the EV functionality of the EV delivered cargo in the recipient cell.

PF02.05

Hybrid extracellular vesicles – biomimetic tool for drug delivery to repair endothelial cell dysfunction

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Introduction: Traditional drug delivery systems (DDS) are usually based on liposomes, micelles or dendrimers. Unfortunately, many DDS cause side effects including organ toxicity and/or unexpected immune response. In living organisms, extracellular vesicles (EVs) are responsible for delivering biologically active molecules to distant cells. In vitro loading of therapeutic compounds into EVs is still not effective and needs developing new strategies. For these reasons we aimed to design hybrid extracellular vesicles (hEVs) with high loading capacity for DDS.

Methods: For hEV synthesis, we used human endothelial derived EVs. Using freeze/thawing method we fused them with liposomes composed of cholesterol and one of the three lipids: DOPC, sphingomyelin or phosphatidylserine. To confirm membrane fusion, we applied a spectroscopy ruler – FRET (Förster Resonance Energy Transfer) and CryoTEM imaging technique. We characterized hEVs using NTA (for size distribution evaluation), DLS (zeta potential) and Western blot (for detection of EVs markers). We evaluated loading efficiency using calcein as a model drug. Additionally, we performed cytotoxicity tests.

Results: In the CryoTEM imaging, pure and homogenous hEV population with a diameter of 65 ± 15 nm was detected. Additionally, we observed changes in zeta potential and in size distribution after fusion. FRET measurements showed increased fusion efficiency with the increasing number of freeze/thawing cycles and dependence on a lipid-to-protein ratio in EVs. Additionally, hEV had higher loading efficiency than liposomes and sole EVs and that their internalization by endothelial cells did not cause a cytotoxic effect.

Summary/Conclusion: Based on cryo-TEM and FRET, we confirmed that our fusion method of hybrid EVs is effective and can be applied as a delivery platform for DDS to endothelial cells.

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PF03: EVs of Non-mammalian Organisms

Chair: Yong Song Gho – Department of Life Sciences, Pohang University of Science and Technology

Chair: Franklin W.N. Chow – Postdoc, Institute of Immunology and Infection Research, Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh

PF03.01

Investigating the effects of oxidative stress on extracellular vesicle function and cargo loading

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Introduction: Extracellular vesicles (EVs) have been demonstrated to be important mediators of intercellular communication in various contexts, including in response to a range of stressors. The functional activity of these EVs in recipient cells may, in part, be driven by changes to their biological cargoes. However, the molecular details of the underlying EV biogenesis and loading processes, and how this may vary in different conditions, is poorly understood.

Methods: We first studied the effect of oxidative stress on the functional activity of EVs in recipient cells using cell viability and mitochondrial membrane potential assays in Drosophila S2R+ cells. We then carried out total RNA sequencing of EV and cellular RNA under three stress conditions and compared results to existing data in mouse cells. Further to this we have used a bioinformatic pipeline to identify sequence motifs enriched in EVs under stress.

Results: Functional assays indicated changes to cell viability and mitochondrial membrane potential in recipient cells, which were donor cell-stress dependent. Subsequent characterisation of RNA showed an enrichment of ribosomal RNA in EVs relative to cells, but no significant changes to other biotypes. Comparative analysis has also uncovered a set of genes enriched in EVs under oxidative stress, and a further subset whose enrichment may be evolutionarily conserved in mouse. We also identified potential EV-loading motifs which may assist in RNA loading specifically under stress.

Summary/Conclusion: We have shown that EVs derived from oxidatively stressed cells show dose-dependent differences in RNA cargo and identified potential sequence motifs that may have a role in its loading. We are now validating the biological significance of these findings by combining different in vivo approaches in Drosophila. This will enable us to gain insights into the basic mechanisms which govern EV loading in different contexts, and ultimately the molecular mechanisms underlying EV-mediated intercellular communication.

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PF03.02

Mutant p53 governs tumour microenvironment dynamics via exosomes and outer membrane vesicles

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Introduction: Mutations in TP53 are considered one of the most frequent genetic alterations in human cancer. Besides the abrogation of the wild-type (WT) p53-mediated tumour suppression, a distinct set of missense mutations was reported to endow mutant p53 proteins with novel activities termed gain-of-function (GOF). Even though mutations in TP53 are typically thought to arise in the tumour cells rather than in the stroma, the non-cell-autonomous effects of these mutants over the tumour microenvironment are poorly understood.

In the presented studies, focusing on colon cancer as well as on lung cancer microbiome, we investigated intercellular interactions mediated by exosomes and

outer membrane vesicles (OMVs) in the context of cancers harbouring mutant p53.
Methods: p
Results: In the colon, tumour cells harbouring mutp53 were found to exert a non-cell-autonomous effect over macrophages. When exposed to tumour cells harbouring mutp53, monocytes became polarized towards a distinguished subset of macrophages characterized by TAMs-related markers. The mutant p53 affected TAM were characterized as TNF-αlow/IL-10 high, over expressing CD-206 and CD163, with decreased phagocytic ability and increased invasion and matrix degradation potency. Investigating the exosomal transfer from mutp53 tumour cells to macrophages, revealed a mutp53-specific miRs signature led by miR-1246 promoting the TAM phenotype and creating an invasive front together with tumour cells. MiR-1246 was also found to be the top mutp53-associated miR in a cohort of 57 human colorectal resected tumours.

Separately, in two lung cancer cohorts, we identified a signature of microbiome members associated with p53 mutations. Acidovorax Temperans, a Gram negative bacterium, was found to be abundant in tumours of patients with mutant p53. We found a significant increase in tumour volume in animals inoculated with Acidovorax temperans as compared to Sham treated animals, and increased lung weight as a percent of total body weight. These preliminary data indicate that Acidovorax temperans contributes to lung tumorigenesis in the presence of activated K-Ras and mutant p53. OMVs shed by Acidovorax temperans promoted inflammatory signalling in lung carcinoma cells and elevated CD47 expression on tumour cells and SIRPα levels on macrophages.
Summary/Conclusion: Altogether, these findings are consistent with a microenvironmental role for specific "hot-spot" GOF p53 mutants tightening the interaction between the tumour cell and the immune compartment in colon cancer. In both colon and lung cancer, mutant p53 facilitates cellular interactions within the tumour microenvironmets mediated by vesicles.
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PF03.03

S. oneidensis vesicle loading is driven by BAR domain protein and outer membrane cytochrome interplay

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Introduction: The metal respiring bacterium S. oneidensis creates outer membrane extensions and outer membrane vesicles that are sculpted by the novel BAR domain protein BdpA. These vesicles and extensions incorporate mutliheme cytochromes involved in extracellular electron transfer to metals and electrodes. However, the physiological relevance of incorporating these cytochromes into the higher order 3D architecture of a vesicle or extension is unknown. Given that BAR domains serve as a protein sorting mechanism in Eukaryotes, we investigated the pathway crosstalk between BdpA and outer membrane multiheme cytochromes as means to understand the physiological significance of membrane architecture.
Methods: o this end, vesicle morphology and content was measured using dry weights, dynamic light scattering, fluorescence microscopy and comparative proteomics from wild type S. oneidensis and deletion strains.
Results: Cells lacking BdpA make large amorphous vesicles that are dense with protein. In contrast, a strain lacking outer membrane cytochromes recruits less total protein into smaller vesicles. Proteomics to show that both BdpA and multiheme cytochromes are involved in recruiting other proteins to outer membrane vesicles and have a reciprocal relationship.
Summary/Conclusion: in the absence of BdpA, protein crowding has to become the main driving force of vesiculation and BdpA is essential for efficient incorporation of cytochromes. However, multiheme cytochromes are not only vesicular cargo, but are also important for shaping and loading vesicles. Both of these situations make it clear that vesicles play a role in increasing the respiratory surface area of S. oneidensis cells. Moving forward, we hope to be able to control Bdpa and cytochrome levels for selective recruitment of technologically relevant payloads.

PF03.04

Identification and evaluation of potential new biomarkers of trematode infections in Extracellular Vesicles

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Introduction: Fascioliasis caused by Fasciola hepatica represents a major economic loss and clinical burden in cattle farming worldwide. Extracellular vesicles (EVs) contain pathogen-derived molecules that represent novel biomarkers of disease. In the present study, we have identified potential new biomarkers of F. hepatica infection in EVs present in sera of infected cattle.

Methods: Parasites and sera were obtained from local abattoirs (Valencia, Spain, and Medellin, Colombia, respectively). 38 sera from infected and 32 from healthy animals.

Parasites were cultured, and EVs obtained by size-exclusion chromatography (SEC) and characterized by NTA, TEM and proteomic profiling.

Recombinant proteins from F. hepatica EVs (enolase and Fh16.5 tegumentary protein) were produced, and coupled to magnetic beads. Measurement of bovine IgG antibodies was performed using Luminex bead array technology.

Results: A total of 239 proteins were identified associated with EVs as shown by the presence of typical EV-markers (Tsg101, Alix, CD63). Two parasite proteins, enolase and the Fh16.5 tegumentary protein were produced as recombinant proteins and used for detection of cattle IgG employing Luminex bead array technology. Interestingly, significant differences were found in the fluorescence values of both recombinant proteins allowing discrimination between sera from infected and non-infected cattle. The use of the Fh16.5 protein generated a highly significant difference between the two groups (p value = 0.003614); as did enolase (p value was 0.02294).

Summary/Conclusion: This study demonstrates the usefulness of EV proteins as new biomarkers for early diagnosis of helminth infections using Multiplex assays, a technology that may also be applied to other parasite EV molecules.

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PF03.05

Life stage-specific glycosylation of schistosome-derived extracellular vesicles

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Introduction: Glycans play an essential role in pathogen-host interactions. Larvae and adult worms from Schistosoma mansoni release distinct subsets of glycoconjugates as excretory/secretory (ES) products. Extracellular vesicles (EVs) are also among the ES products. We recently found that schistosomula-derived EVs are glycosylated and bind human dendritic cells via C-type lectin receptor (CLR) DC-SIGN, leading to increased IL-10 and IL-12 release. Here we investigated the glycosylation profile of EVs released by S. mansoni adult worms, compared this to schistosomula EVs, and addressed how this may affect parasite-host interactions via CLRs.

Methods: EVs from cultured S. mansoni parasites were obtained by ultracentrifugation and purified with iodixanol density gradients. Isolated EVs were analysed by NTA and cryo EM. N-glycan and lipid glycan content was determined by mass spectrometry. Density gradient fractions with EVs were loaded onto SDS-page gels followed by Western Blot (WB) analysis using anti-glycan monoclonal antibodies (mAbs).

Results: Cryo EM showed that adult worm EVs lacked the long thin filaments that are characteristic for schistosomula EVs. Additionally, in contrast to schistosomula EVs, glycolipids could not be detected in the adult worm EVs. Mass spectrometry analysis showed that the most abundant N-glycans in the adult worm EVs contained GalNAcβ1–4GlcNAc (LacDiNAc, LDN) motifs, which correspond to previously published overall glycan profiles of this specific life stage. Other differences in EV glycosylation between the two life stages were observed by WB using anti-glycan mAbs: adult worm EVs showed a paucimannosidic glycan motif whereas in the schistosomula EVs Galβ1–4(Fucα1–3) GlcNAc (Lewis X) was detected in line with previous MS analysis.

Summary/Conclusion: The adult worm-derived EVs contain life stage-specific N-glycans and show a distinct glycosylation profile compared to the schistosomula EVs. The LDN motifs suggest that adult worm EVs may interact with the macrophage galactose-type lectin (MGL) on host cells and most likely have other immunological consequences than the schistosomula EV-DC-SIGN interaction.

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PF03.06

Isolation and characterization of Extracellular Vesicles from phloem sap reveals their possible role in defence against phloem-feeding insects

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Introduction: Phloem plays a central role in plant function, as it is the responsible for the translocation of photoassimilates from source- to sink-organs, and a long-distance route for signals distribution. Due to the sap high nutrient content, sieve elements are primary target for plant pathogens and pests.

In this work we aimed to isolate and characterize Extracellular Vesicles (EVs) from Cucumis melo phloem sap, derived from plant either exposed or not to the melon aphid, Aphis gossypii (Hemiptera: Aphididae).

Methods: Phloem exudates from 5-week-old melon plants, either uninfested or infested with adults of A. gossypii (n = 15, 4 replicates each), were collected by cutting the stem with a sterile razor blade between first and second expanded leaf from the top. EVs were isolated by Size Exclusion Chromatography, and analysed by Nanoparticle Tracking Analysis (NTA) and Transmission Electron Microscopy. EVs proteome was determined by quantitative mass spectrometry.

Results: EVs from phloem sap were successfully isolated in every condition. No significant differences were detected among distinct samples, neither in particle concentration and size by NTA, nor in protein concentration.

Most importantly, a total of 381 different proteins were identified in phloem sap EVs, including 152 present in exosome databases (ExoCarta). On top of that, 44 differentially expressed proteins were identified in EVs derived from aphid infested or uninfested plants (p value < 0.05).

Summary/Conclusion: Understanding how plants trigger their defences against pests and pathogens is important to develop new control measures. The characterization of several proteins in EVs from the phloem sap provide valuable information on long distance signalling in plants.

Moreover, as plants lack an immune system comparable to animals, the different protein content in phloem sap EVs after exposure to aphids could indicate their important role in delivering inducible defences against invading pests and pathogens.

PF03.07

Extracellular vesicles from nematode species Heligmosomoides bakeri and Trichuris muris contain distinct small RNAs that could enable niche specificity in the host

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Introduction: Gastrointestinal nematodes are extremely prevalent parasites that infect most animals and ~24% of human population. Their success as parasites is attributed to their ability to secrete diverse molecules that modulate the host immune system. Extracellular vesicles (EVs) are one of the immune modulatory compounds they release that directly modulate host cells. Our goal is to understand how the small RNA (sRNA) cargo underpins EV function, using a comparative analysis of EV cargo from diverse nematode species.

Methods: We first compared how different EV isolation methodologies (Ultracentrifuge (UC), size fractionation, sucrose gradient floatation) effect the small RNAs detected in H. bakeri EVs using different library preparation kits (CleanTag, TruSeq), with or without polyphosphatase treatment. We then compared this to small RNA libraries from T. muris EVs using comparable methods, UC EV purification, with or without polyphosphatase treatment and using the CleanTag library preparation kit.

Results: EVs from both species contained miRNAs, however the miRNA gene familes in H. bakeri and T. muris EVs are distinct. The miRNA content detected in EV samples collected by different purification protocols is robust. The largest difference in detected miRNAs was found when comparing different library preparation kits. Although both H. bakeri EVs and T. muris EVs were dominated by sRNAs derived from intergenic or repetitive elements in the parasite genomes, only in H. bakeri EVs were these secondary siRNAs.

Summary/Conclusion: H. bakeri and T. muris EVs contain distinct small RNA cargos, which may underpin their ability to colonise different host niches, and/or modulate the host immune system differently. T. muris EVs do not contain secondary siRNAs, in contrast to H. bakeri, however they are dominated by sRNAs derived from intergenic or repetitive regions. Comparative analysis of helminth EVs could help pinpoint the sRNAs involved in cross-species communication.

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PF04: EV Nucleic Acid Biomarkers

Chair: Robert Raffai – University of California & VA Medical Center, San Francisco

Chair: Phil W. Askenase – Yale University

PF04.01

Circulating non-coding RNA biomarkers of cancers in extracellular vesicles and serum

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Introduction: Extracellular vesicles (EVs) are secreted from various cells including cancer cells and known to contain protein and small RNAs including miRNA isoforms (isomiRs). Therefore we also focused on isomiRs including other small non-coding RNAs for biomarker discovery. Although liquid biopsies using small RNAs are promising biomarkers for early detection of cancer, current approaches to detecting and analysing miRNAs in the blood are still inadequate. Artificial intelligence (AI) data analysis may provide better algorisms for diagnosing cancer.
Methods: Small RNAs were isolated from serum or purified EVs using a miRNeasy Mini Kit (Qiagen) and quantified by using the Ion S5™ next-generation sequencing system. (Thermo Fisher Scientific). EVs were purified using total exosome isolation Reagent (Invitrogen™). AI data analysis was performed using JMP® Genomics and DataRobot enterprise AI platform.
Results: Three small RNAs, isomiR of miR-21-5p, miR-23a-3p, and tRF-Lys (TTT) were significantly upregulated in breast cancer patients compared with the healthy cancer-free individual. The combination algorithm using these three small RNAs allows for a more accurate diagnosis of the area under the curve (AUC) 0.92. To test the possibilities that these small RNAs are derived from cancer cells, we isolated EVs from the serum and performed NGS analysis to profiled serum small RNAs in EVs. Interestingly we found that two small RNAs, miR-21-5p and miR-23a-3p, also high in breast cancer EVs, indicating that these small RNAs were expected to be derived from cancer cells. In oesophagus cancer, we also performed NGS analysis and identified twenty-four miR/isomiRs candidates for diagnostic biomarkers. A multiple regression model selected miR-30a-5p and two isomiRs (miR-574-3p and miR-205-5p). The AUC of the panel index was 0.95. We also performed AI data analysis and discovered the novel algorisms that can diagnose breast and oesophagus cancer more accurately.
Summary/Conclusion: We demonstrated combinations of circulating non-coding RNAs containing EVs potentially useful for the detection of early-stage breast and oesophagus cancers. In addition, The DataRobot enterprise AI platform enables us to the more accurate diagnosis of cancers at the early stage.

PF04.02

Identification of novel EV-associated miRNAs as toxic biomarkers in mouse

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Introduction: Recent findings reveal that extracellular vesicles (EVs), secreted from cells, are circulating in the blood. EVs are classified into exosomes (40–120 nm), microvesicles (50–1,000 nm) and apoptotic bodies (500–2,000 nm). EVs contain mRNAs, microRNAs, and DNAs and have the ability to transfer them from

cell to cell. Recently, especially in humans, the diagnostic accuracy of tumour cell type-specific EVs as biomarkers is more than 90%. In addition, microRNAs contained in the EVs are being identified as specific biomarkers in blood for chemical-induced inflammation and organ damage.

Therefore, microRNAs contained in the EVs released into the blood from tissues and organs in response to adverse events such as chemical substances and medicine are expected to be useful as novel biomarkers for toxicity assessment. In this study, we aimed to identify target organs by comprehensive analysis of EV RNAs in the blood of mice after chemical exposure to establish a highly sensitive "Next Generation type" toxicity test for chemical substances and medicine using EV RNA in blood as a biomarker.
Methods: All animal studies were conducted in accordance with the Helsinki Declaration and the guidelines approved by the animal care committee of the National Institute of Health Sciences. C57BL/6 J male mice (12 weeks) were orally dosed with CCl4 (vehicle, 7, 70 mg/Kg). Serum were separated from blood after 2, 4, 8 and 24 hours after CCl4 administration. The serum was centrifuged at 10,000 x g to remove cellular debris and subsequently ultracentrifuged 100,000 x g. The pellet is resuspended in PBS and ultracentrifuged 100,000 x g again. The comprehensive small RNA-seq of collected EVs were performed according to the manufacture's protocols.
Results: We succeeded in isolating more than 10 novel small RNAs, which could be used as novel highly sensitive biomarkers for hepatotoxicity due to carbon tetrachloride (CCl4: 7 mg/Kg & 70 mg/Kg). Well known hepatotoxicity biomarkers, miRNA-122 and miRNA-192 were upregulated more than 1000-fold in the administration of 70 mg/Kg CCl4, but not responded in the administration of 7 mg/Kg CCl4.
Summary/Conclusion: These results suggest that mir-122 and mir-192 are mainly released from liver to blood directly only in the administration of 70 mg/Kg CCl4, while novel more sensitive hepatotoxicity biomarkers which responded in the administration of both 7 mg/Kg and 70 mg/Kg CCl4 should be included in the EV. Our novel biomarkers will accelerate a rapid evaluation of chemical substances and medicine in Nonclinical Safety Evaluation.
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PF04.03

Cancer-specific genomic alterations in plasma large EVs from patients with advanced cancer

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Introduction: Advancements in sequencing technologies have allowed analysis of the genomic landscape of cancer using circulating cell-free(cf) DNA. However, cfDNA does not originate only from tumour cells. We recently demonstrated that most of the DNA circulating in plasma of cancer patients is associated with large EVs (L-EVs), and that L-EV-associated DNA reflects genomic aberrations of the cells from which L-EVs arise. Since L-EVs are specifically released by tumour cells, we explore their potential to report cancer-specific genomic alterations in patient plasma and compare it to cfDNA.
Methods: Differential ultracentrifugation, tunable resistive pulse sensing, Qubit dsDNA High Sensitivity assay, capillary electrophoresis, whole exome sequencing (1500–2000x), targeted sequencing (QIAseqTM), flow cytometry.
Results: We show here that L-EVs in the size range of >1 micrometre are present exclusively in plasma obtained from cancer patients and absent in plasma from healthy donors. In agreement with this finding, double-stranded(ds) DNA is detected only in L-EV fractions of patient plasma and not in those obtained from healthy donor plasma using the same protocol. We also demonstrate that the fragments of dsDNA associated with circulating L-EV are larger in comparison with cfDNA (>10,000 bp versus ~170 bp). A large-scale analysis of L-EV DNA obtained from plasma of patients with metastatic castration-resistant prostate cancer (mCRPC) as well as with non-small cell lung cancer (NSCLC) demonstrates that DNA associated with circulating L-EVs reports cancer-specific genomic alterations in both types of cancer. We further investigate if L-EV-associated DNA is intra- or extravesicular and demonstrate that it is present in both forms. We finally compare the purity of the tumour signal in intravesicular L-EV DNA, total L-EV DNA, and cfDNA obtained from patient plasma.
Summary/Conclusion: Our results demonstrate that circulating L-EVs contain high quality, large molecular weight DNA that contains cancer-specific genomic

alterations, supporting the use of L-EVs as a source of tumour-derived DNA in plasma.

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PF04.04

Detection of EGFR mutations in exosomal RNA and protein mirrors disease status in metastatic lung cancer patients

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Introduction: Epidermal growth factor receptor (EGFR) mutation driven lung adenocarcinoma (AC) represents a unique subgroup that lends itself to treatment with oral EGFR tyrosine kinase inhibitors. Current methods that are used to detect these mutations (e.g. L858 R or the resistance mutation T790 M) involve invasive tumour biopsies or blood circulating tumour DNA (ctDNA) and cell free DNA (cfDNA). The sensitivity of blood ctDNA and cfDNA is limited by the frequency of genomic alterations in the EGFR gene; additionally, ctDNA does not reflect changes in the EGFR protein, against which novel therapies are in development. There remains a need to develop blood-based biomarkers that can circumvent these disadvantages and replace the more standard, invasive tumour biopsies. We propose the study of exosomes for treatment monitoring as well as to identify EGFR resistance related genomic and proteomic changes.

Methods: We enrolled 10 patients with metastatic lung AC: 8 with EGFR mutations and 2 without (control). From the patients with EGFR mutant lung AC, we processed 25 blood samples through the patients' treatment course, using ultracentrifugation to isolate exosomes. We then used both droplet digital PCR (ddPCR) to test exosomal RNA (exoRNA) for the mutation of interest and western blots to test protein resulting from exon 19 deletion or L858 R mutations.

Results: From 6 patients with EGFR exon 19 deletion mutations, we detected identical mutations in exoRNA from 15/19 samples. ExoRNA based mutational load increased and mirrored clinical progression in 2 patients. Three patients whose cancer remained stable demonstrated a decrease in their exoRNA. One patient had blood drawn only at 2 points and was therefore not plotted. ExoRNA from 2 patients with L858 R and T790 M mutations demonstrated the corresponding mutations; however, exoRNA did not mirror their disease course. We also demonstrated mutant EGFR protein presence in exosomes from 3 patients. Finally, we tested cfDNA for EGFR mutations from four matched samples using ddPCR. We detected matched mutations in exosomes in all four, while cfDNA mutations were only detected in 2/4 patients.

Summary/Conclusion: In summary, we detected EGFR mutations in 19/23 exosome samples isolated from metastatic lung AC. Our results set the stage for optimization of exoRNA methods and inform future experiments relating to exosomal cargo in patients with EGFR mutant lung AC.

PF04.05

Identification of plasma-derived, EV-based biomarkers for glioblastoma

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Introduction: Glioblastoma multiforme (GBM) is the most malignant and aggressive primary brain cancer in adults, with an incidence of 3.2 per 100,000 people. Currently, diagnosis is only performed via histopathological investigation of a tissue sample from a GBM lesion, complemented with molecular diagnostics for identification of select biomarkers. MRI is the standard of care for follow-up and monitoring of treatment response. Therefore, development of a "liquid biopsy" to obtain disease-relevant information from patient's body fluids is highly desirable.

Methods: We present the results from a clinical study in which extracellular vesicle (EV)-derived mRNAs and long non-coding RNAs were profiled from the plasma of GBM patients and control individuals. We obtained plasma from 8 patients at the time of initial diagnosis, and 8 matched controls by sex and age. EV-associated RNA was isolated from 1–2 mL plasma and RNA-seq was performed using our proprietary pipeline. Sequencing data was analysed for differential gene expression.

Results: We observed 95 mRNAs as differentially abundant between GBM and control samples, with 82 mRNAs enriched in GBM samples and 13 mRNAs enriched in control samples ($p < 0.05$). Correlation based on differentially abundant mRNAs separated GBM and control samples into two unique populations. Eight differentially expressed mRNAs were

previously identified as part of the mesenchymal GBM subtype. These data, while preliminary, provide a potential basis for the further development of a non-invasive GBM gene panel test.

Summary/Conclusion: We have identified a novel RNA signature for GBM from plasma derived EVs, which differs from previously identified biomarkers isolated from tissue. Further work will refine this signature to enable detection, characterization, and patient monitoring for GBM with minimally invasive techniques.

Funding: Bio-Techne Corporation

PF04.06

Identification of a saliva exosomal RNA signature for Sjogren's syndrome

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Introduction: Sjogren's syndrome (SS) is a systemic autoimmune disease in which inflammation progressively damages the moisture producing glands of the afflicted. 4 million Americans are estimated to be suffering from the disease, 90% of which are women with an average age of 40. Overlapping symptoms with other health conditions and co-morbidities make SS particularly difficult to diagnose, with average time to diagnosis of 3 years. Saliva exosomal RNA profiling has been primarily focused on small RNAs and has been limited thus far due to the large contribution of sequencing reads from the oral microbiome. A non-invasive saliva exosomal RNA (exoRNA) based test capable of diagnosis would be highly desirable.

Methods: We began by first developing a novel long RNA-Seq workflow to selectively enrich and profile human exosomal mRNAs and long non-coding RNAs (lncRNAs) from saliva. We then profiled salivary exoRNA obtained from 7 SS patients and 7 healthy matched controls. Finally, we performed differential gene expression analysis to obtain an exoRNA signature for SS.

Results: RNA-Seq data analysis demonstrated highly efficient enrichment of human transcriptome, with over 80% of reads mapping to the transcriptome. Further RNA biotype analysis showed over 75% of transcriptome reads mapped to protein coding genes and lncRNAs. We detected over 10,000 mRNAs and approx.1000 lncRNAs. Differential expression analysis (DEx) of SS vs. healthy control exoRNA identified 455 upregulated genes, including 415 mRNAs and 37 lncRNAs ($p < 0.05$). 120 genes were found to be downregulated in SS, including 114 mRNAs and 6 lncRNAs. Gene ontology analysis of DEx genes revealed enrichment of genes involved in various immune system related pathways. Most importantly, principal component analysis (PCA) resulted in clear separation of SS patients from healthy controls.

Summary/Conclusion: Our optimized RNA-Seq workflow enables saliva-based liquid biopsy for biomarker discovery. The gene signature identified in this ongoing study could potentially provide a non-invasive molecular means of diagnosing Sjogren's syndrome.

Funding: Bio-Techne Corporation

PF04.07

Endometrial fluid derived EVs as low invasive diagnostic biomarkers of implantative endometrium

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Introduction: Increasing embryo implantation rates has become one of the greatest challenges in assisted reproduction techniques. Usually an endometrial biopsy is done to identify a receptive endometrium, which prevents embryo transfer in the same cycle, as it is detrimental for the implantation. The implantation is a complex process, which requires a synchrony between the development of the embryo and the endometrium, but also, an adequate embryo-endometrial cross talk. The presence of extracellular vesicles (EVs) as mediators of this communication has been describe in the endometrial fluid. Therefore, we hypothesize that the molecular analysis of the content of the EVs and companion molecules

from endometrial fluid could be a non-invasive method to recognize an implantative endometrium and consequently improve the implantation rates.

Methods: The objective is to define a simple, sensitive and reproducible non-invasive EV-based method that allow the quick identification of an implantative endometrium by means of miRNA analysis. For the establishment of a robust methodology for analysing EVs from endometrial fluid in clinical settings, where the sample is limited and no sophisticated equipment is available, five different methodologies were compared in triplicate. Two of them consisted in the direct extraction of RNA while in the other three, before the RNA extraction an enrichment of EVs was done. SmallRNAseq was performed to determine the most efficient method. Once the best method was selected, it was applied in a set of real samples with different implantation outcome. The content of miRNAs (mainly associated with EVs) of endometrial fluid samples from women in whom the implantation was successful (n = 15) and unsuccessful (n = 15) were analysed.

Results: Our results show that the protocols with a previous enrichment step of EVs obtained a higher miRNA expression. The results obtained from the differential analysis of the set of samples with different implantation outcome are being analysed and it is expected that the results will be available by the time this communication is presented.

Summary/Conclusion: This work demonstrates that it is possible to obtain and analyse EVs and EVs-associated miRNAs from a small volume of endometrial fluid samples, which allows the use of EV-miRNAs as a low-invasive biomarkers for the detection of an implantative endometrium.

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PF04.08

Small RNA cargo of EVs is affected by hormone treatment in prostate cancer

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Introduction: Small RNAs are recently reported as a regulator for prostate cancer progression to castration-resistant disease. Our previous work has shown that EVs protein cargo is affected by male steroid hormone, dihydrotestosterone (DHT). In this study, we assess the small RNA cargo of EVs in response to androgen manipulations.

Methods: Androgen receptor-positive LNCaPs are grown in CSS medium to deplete the androgens. Media were then replaced with vesicle-depleted CSS medium ± 10 nM dihydrotestosterone (DHT) ± 10 μM Enzalutamide (ENZ) for 48 h. EVs were isolated using sequential ultracentrifugation (2000 g for 20 min, 10,000 g for 30 min, 100,000 g for 2 h), washed once in PBS. Protein and RNA were collected from both parent cells and conditioned medium to allow direct comparison between S-EVs cargo and cells. Small RNA NGS libraries were prepared using the Illumina's TruSeq Small RNA Library Prep kit and single-end sequenced at a read length of 50 nucleotides (nt). Fastq library files were processed using a custom-designed pipeline. Adapters were removed using the Cutadapt tool, trimmed reads were mapped with high stringency against ribosomal sequences using Bowtie2. SnoRNA and tRNA fragments were identified using the FlaiMapper software. Remaining reads were mapped against the human genome hg38 using Bowtie2.

Results: We found that the presence or absence of androgens does not significantly change the amount of total RNA in small EVs (S-EVs). However, hormone stimulation altered the small RNA content of S-EVs, in parallel with our previous published data on EV protein cargo. DHT increased the abundance of snoRNA in cells, while a reduction of snoRNAs was observed in the S-EVs fraction. Interestingly, DHT induced the formation of cell filopodia that are not inhibited by androgen inhibitor Enzalutamide. Pathway analysis indicates the p53 mediated regulation driven by miRNAs found in S-EVs upon exposure to DHT. The expression profile of snoRNA and tRNA fragments in DHT treated cells resembles results from clinical prostate cancer specimens.

Summary/Conclusion: Our findings show that androgen manipulation alters both S-EV derived protein and RNA cargo. Changes in the S-EV RNA profile due to treatment with androgens are not identical to small RNA profiles in parental cells, indicating a specific sorting mechanism of S-EV small RNA upon androgen manipulation. Further, DHT induces the formation of cell filopodia irrespective of Enzalutamide, suggesting cargo selection of S-EVs. We conclude that small RNA EV cargo can be utilised to as prostate cancer biomarkers in androgen targeted treatments.

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PF05: EV Protein Biomarkers

Chair: Jennifer Jones – Laboratory of Pathology, National Cancer Institute, National Institutes of Health

Chair: Patricia Midori Murobushi Ozawa – Department of Cell and Developmental Biology, Vanderbilt University School of Medicine

PF05.01

Detection of PD-L1 in circulating extracellular vesicles

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Introduction: Cancer immunotherapy, such as PD-L1 blockade, is a method to eliminate cancer cells. Ectopic expression of PD-L1, on the surface of tumour cells, has been associated with tumour persistence and as an important predictor of therapy response. A test that, specifically and accurately, detects PD-L1 is critically important in order to identify patients that would benefit from these treatments. Emerging evidence has shown that extracellular vesicles (EV) can carry immune checkpoint molecules, such as PD-L1, and whose expression have been correlated with tumour immunity response. With a multitude of commercially available antibodies identifying appropriate clones and associated assay is important in order to standardize the diagnostic modality used.
Methods: PD-L1 expressing cancer cell lines were used to generate EVs. PD-L1-MYC vector was transfected to generate an overexpression system. ExoView® sensors containing different anti-PD-L1 clones were generated. Samples (cell derived and plasma) were incubated on chips to allow the antibody to bind the antigen on the EV. After incubation, chips were immunolabeled with fluorescently labelled antibodies against PDL-1 or EV associated markers. ExoView R100 reader was used to enumerate the EVs captured on the sensor surface and analyse the expression of PDL-1 on single vesicle through fluorescence imaging. Immunoprecipitation and mass spectrometry (IP/MS) were employed as an orthogonal method to verify the specificity of the assay.
Results: To study the detection efficiency of the antibodies, engineered PD-L1-MYC EVs were used. Under these circumstances, all the tested antibodies were able to capture EVs. When testing endogenous PD-L1 positive EVs from different cancer cell lines, only 28.8 and 73 10 clones consistently bound to EVs. In addition, EVs derived from plasma demonstrated to be positive for PD-L1, however, only clone 28.8 was able to immobilize these EVs. The results suggested that clone 28.8 could be a potential PD-L1 antibody to detect PD-L1 positive EVs originating from various sources. To confirm these results, and assure the specificity of the antibody targeted IP/MS was employed.
Summary/Conclusion: In combination with the ExoView platform, anti-PD-L1 antibodies can be screened and potentially used to generate a non-invasive EV-specific assay that could detect this protein in patients.

PF05.02

Differences in extracellular vesicle protein cargo is dependent on head and neck squamous cell carcinoma cell of origin

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Introduction: Head and neck squamous cell carcinoma (HNSCC) is the sixth most common, eighth most fatal cancer worldwide and includes cancers of the oropharynx, larynx, hypopharynx, and oral cavity. In 2017, there were over 49,000 new cases and 9,700 deaths estimated in the USA alone. Despite recent advances in treatment, including radiation, chemotherapy, surgery, concurrent chemoradiation, and immunotherapy, many tumours develop resistance and progress. Patients develop metastases or tumours recur locally or regionally; the 5-year overall survival rate for HNSCC is only 40–50%. Factors that contribute to poor survival for patients with HNSCC include late stage diagnosis, lack of reliable markers for early stage detection, high level of biologic heterogeneity, and local recurrence and distant metastases after treatment.
Methods: This study used 8 representative HPV-positive and HPV-negative HNSCC cell lines, one HPV-transformed cell line. and two non-cancer oral keratinocyte cell lines. EVs were isolated using differential

ultracentrifugation and PEG precipitation/ultracentrifugation. EVs were characterized by TEM, NTA, and Wes protein analysis for reported EV markers. EV and whole cell lysates were assessed by LC-MS/MS analysis using the Tandem Mass Tag-10plex kit. Cluster analysis was performed on the fold-change peptide spectrum matches (PSM) for the EVs from the HNSCC lines compared to the EVs from the normal keratinocyte line (NOKsi). Protein was measured using a capillary-based electrophoresis instrument.

Results: CD9 and AnnexinV were detected in all of the EV lysates tested, while Calnexin was detected in all of the whole cell lysates and none of the EV samples tested. Selected proteins STAT3, HLA-A, Tenascin, E-Cadherin, β Catenin, Cytokeratin 19, EPHA2, and CD59, and HPV-related markers p16, p53, RB, Cyclin D1, and EGFR were tested using the WES platform. EVs from HPV-positive cell lines showed higher protein levels compared to EVs from HPV-negative cell lines in STAT3, HLA-A, and Tenascin. Only KERT19 demonstrated lower protein levels in EVs from HPV-negative cell lines. Of the common HPV-associated HNSCC markers: EGFR, p53, RB, Cyclin D1 and p16, only EGFR was positive in any the EVs tested. The remaining proteins queried, E-Cadherin, β Catenin, EPHA2 and CD59 showed varying protein levels in EVs from both HPV positive and HPV-negative cell lines.

Summary/Conclusion: Our findings suggest that these proteins may be potential HNSCC EV markers that may be 1) selectively included in EV cargo for export from the cell as a strategy for metastasis, tumour cell survival, or modification of tumour microenvironment, or 2) representative of originating cell composition, which may be developed for diagnostic or prognostic use in clinical liquid biopsy applications.

PF05.03

Validation of antibodies on western blot for extracellular vesicles from biological human samples and cancer cell conditioned media

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Introduction: One of the major challenges in Extracellular vesicles (EVs) research is to prove the particles that are isolated are true EVs, rather than other co-isolated contaminants, like lipoproteins. ISEV recommends using multiple assays to characterize EVs. This study aims to validate the positive and negative protein markers for extracellular vesicles from plasma, urine and prostate cancer cell conditioned media (CCM).

Methods: Membrane and cytosolic fractions of MCF7 cells served as positive and negative controls for all antibodies validated. EVs were isolated from plasma of healthy volunteers, urine of healthy volunteers and CCM of PC-3 cells using differential ultracentrifugation. Eight protein markers were assessed: positive markers CD63, CD81, CD9, Flotillin1 (Flot1), Alix and Tumour susceptibility gene 101 (TSG101), negative marker calnexin (CANX), and contaminant markers Apo-A1 for plasma and THP for urine. Tetraspanins are small transmembrane proteins expressed in EVs. Flot1 is membrane protein that forms microdomains in the plasma. Alix and TSG101, an accessory protein of the endosomal sorting complex required for transport, are involved in the biogenesis of EVs. They are positive markers for EVs. CANX is in the membrane of the endoplasmic reticulum. Apolipoprotein-A1 (Apo-A1) is the protein components of lipoproteins, therefore it is marker of contamination for Plasma EV. Tamm-Horsfall protein (THP) is contamination marker for Urine EV, because it is most abundant protein in human urine.

Results: All antibodies were validated in the correct positive and negative control, thus confirmed as usable and reliable antibodies for Western Blot. In Plasma EV, CD63, CD9, CD81 and Flot1 were positive and CANX and Apo-A1 were negative. In Urine EVs, CD9, CD63, Flot-1, Alix and TSG101 were positive and CANX and THP were negative. In CCM EVs, CD9, CD63, Flot1, Alix and TSG101 were positive and CANX was negative.

Summary/Conclusion: We confirmed a high degree of EV purity from 3 sample types: urine, plasma, and CCM. Of particular importance, we confirmed that EVs isolated from biologic patient samples, plasma and urine, had low contamination. Future work will use these methods to confirm purity of EV samples prior to addition analysis, such as examining EV cargo and biologic significance.

PF05.04

Proteomic study of mesenchymal stem cells derived exosomes modified using miR.

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Introduction: The project we are working on is to modify the immunogenic profile of human CMMs from the umbilical cord stroma through its stable transfection with anti-miR-21-5p, and therefore of the exosomes that these cells generate, for use in free-cell therapy to treat inflammatory process.
Methods: EVs released from a primary culture of human umbilical cord mesenchymal stem cells and from primary culture of human umbilical cord mesenchymal stem cells miR21-/- modified through stable lentiviral transfection were isolated by ultracentrifugation processes, characterized by transmission electron microscopy (TEM) and measured by nanoparticles tracking analysis (NTA). Protein extraction from EVs was made using RIPA buffer and after checking protein integrity the total EV proteins. We performed a shotgun proteomic study using a TMT (10-plex) label of the total miR21-/- exosomes protein comparing it with normal exosomes. After labelling the LTQ-Orbitrap platform of ProteoRed was needed for fraction injections and data acquisition. Proteome Discoverer 2.2 (Thermo) was used for protein processing and quantification.
Results: A total of 1.861 proteins were identified at least with a unique peptide and we have able to establish the proteomic profile of miR21-/- exosomes against normal exosomes. We found out several protein modulated by miR21 and related to inflammation.
Summary/Conclusion: We have able to establish the proteomic profile of miR21-/- exosomes against normal exosomes focusing on proteins involving inflammation process. All those results seem indicate that exosomes could be modified, which could be used as an anti-inflammatory free-cell therapy.
Funding: ProteoRed Concept Test Project Grant.

PF05.05

A novel extracellular vesicle isolation method used to discover urine liver disease biomarkers

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Introduction: Hepatocellular carcinoma (HCC) is the 6th most common cancer worldwide and the 3rd most common cause of cancer death; additionally, its incidence is increasing. While outcomes for early HCC are superior to those for late stage disease, early detection of HCC remains a challenge. Current guidelines have suboptimal sensitivity and specificity. In this pilot study, we hypothesize that urine extracellular vesicles (EVs) may identify candidate biomarkers towards the development of an inexpensive, widely accessible screening assay for the early detection of HCC.
Methods: Urine samples from 20 healthy subjects, 19 subjects with cirrhosis, and 19 subjects with cirrhosis plus HCC were collected and processed using YMATRIX columns to isolate EV-associated protein and miRNA. Protein was analysed using a tandem mass tag method on a Thermo Scientific Orbitrap Fusion mass spectrometer with Comet/PAWS and EdgeR processing. miRNA was analysed using a targeted Firefly microarray from Abcam. Differential expression and predictive modelling for the presence of HCC and cirrhosis was performed to identify candidate miRNA and protein biomarkers.
Results: For miRNA, 39 samples were eligible for analysis after low expression filtering. We used pair-wise ratios of 34 cancer-associated miRNAs by gradient boosting of decision trees to develop a predictive model for HCC. Our best model had a sensitivity and specificity of 0.93 and 0.92 respectively using 6 miRNAs to distinguish HCC from cirrhosis. All samples were eligible for protein analysis. Based on differential expression and biologic relevance, we identified 10 protein candidate biomarkers. Interestingly, we found liver-selective proteins and known HCC/cirrhosis plasma/tissue markers, demonstrating proof-of-concept for the method.
Summary/Conclusion: Urine extracellular vesicles contain liver-selective proteins and known liver disease serum biomarkers as well as novel miRNA and protein biomarkers that are significantly up-regulated in disease samples. The described candidate biomolecules may be easily accessible biomarkers with which to develop a sensitive and specific universal screening diagnostic for the early detection of cirrhosis and HCC.
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PF05.06

Evaluation of plasma extracellular vesicles in Rheumatoid Arthritis and Systemic Lupus Erythematosus

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Introduction: Circulating extracellular vesicles (EVs) are known to carry biologically active molecules which contribute to homoeostatic and pathologic functions. EVs and their cargo have been associated with the development and perpetuation of autoimmune (AI) diseases such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE). Understanding the content of EVs and how they correlate with disease state, activity, and response to therapy could provide a valuable tool for patient tailoring in AI diseases.
Methods: Samples were collected after independent review board approval and patient informed consent was obtained. EVs were isolated from EDTA plasma from patients with RA (n = 20) or SLE (n = 20) and from age- and sex-matched healthy controls (n = 20). EV size and concentration were assessed by Nanoparticle Tracking Analysis (NTA), miRNA content by NanoString, and protein content by Luminex 41-plex Immunoassay.
Results: NTA revealed a higher concentration of EVs in RA ($p = 0.042$), but not SLE ($p = 0.278$) plasma compared to healthy controls. Additionally, the size of EVs from RA and SLE samples compared to those of healthy controls were significantly larger ($p = 0.0074$ and $p = 0.0004$, respectively). Analysis of miRNA identified differential expression of 8 and 11 miRNA in RA and SLE EVs, respectively, compared to healthy controls. All 8 species that changed in RA EVs were also differentially expressed in SLE EVs. Furthermore, when we analysed the protein content, we found that 2 proteins in RA EVs and 4 proteins in SLE EVs were differentially expressed compared to healthy control EVs. Of these analytes, MDC was downregulated in EVs from both RA and SLE samples compared to those of healthy controls ($p = 0.04$ and $p = 0.0004$, respectively).
Summary/Conclusion: Plasma EVs from patients with RA and SLE displayed altered size, miRNA and protein content compared to EVs from healthy controls. Additional analyses, correlating findings with disease metrics, will be performed and may reveal biomarkers with relevance to clinical outcomes. Identification and analysis of EVs circulating in patients with AI diseases may present a new paradigm for future biomarker strategies.

PF05.07

Using extracellular vesicles as a template for autoimmune diagnostic screening

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Introduction: The peptidergic G-protein coupled receptors (GPCRs) are cell-signalling transmembrane proteins, which in their native form comprise of seven segments embedded in the cell membrane. This structural advancement is believed to be maintained in extracellular vesicles (EVs). In autoimmune diseases, the presence of autoantibodies towards GPCRs is not uncommon, and to detect plasma autoantibodies, EVs carrying GPCR will be used as template in a novel microarray screening tool.
Methods: Purified EVs from HEK293 cells were printed on different types of surfaces; polymer coated glass slides and hydrophilic and hydrophobic plastic 96 well plates. Five different print buffers were tested in a multiplex assays. Spots containing EVs were stained with biotinylated antibodies (CD9, CD63, CD81, ADRβ2, Hsp70, EPCAM and Flotilin-1) followed by binding of Cy5-labelled streptavidin and visualized microarray scanner.
Results: The outcome of these experiments was promising, as some of the chosen printing buffers showed increased tendencies to bind EVs. The EV presence was verified with a panel of markers known to be present on small EVs. In addition, the EV content of the adrenergic beta-2 receptor (ADRβ2-receptor), which is a GPCR of interest in autoimmune diseases, was verified in some of the experimental setups.
Summary/Conclusion: The approach of using EVs as template in a screening tool possesses the potential to easily screen for autoimmune illness markers in diagnostic purposes. Using the microarray technology allows the screening to be multivariate, specific and highly sensitive.

PF05.08

Circadian variation of extracellular vesicles secreted in urine: Analysis of time point collection and normalization strategy.

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Introduction: Urinary extracellular vesicles (uEVs) are an ideal source of biomarkers for kidney and urogenital diseases. Despite the great deal of interest generated by

uEVs, little is known about its collection time and normalization approach. The majority of the studies on uEVs focus on spot urine collection based on the assumption that it accurately reflects the renal function, although time point of collection is not standardized. Therefore the practice to collect spot urine does not allow for calculating and standardizing accurately the uEV excretion rate which may vary during the day. In addition, no research has been carried out yet to show the quantitative and qualitative difference of uEVs between spot urine and 24 h collections.The aim of this study is to compare uEVs excreted in all single voids during a 24 hour collection period and compare it with 24 hour collection performed.

Methods: uEVs were enriched by differential centrifugation and electron microscopy, western blot, nanoparticle tracking analysis, tuneable resistive pulse sensing and imaging flow cytometry were used to quantify uEVs and associated markers variation during the 24 hour. Creatinine, urine osmolality and particle concentration were used to normalize the assessed analytes.

Results: Electron microscopy showed a heterogeneous population of EVs and western blot confirmed the presence of EV markers (TSG101, ALIX and CD9). RNA was extracted by a column-based method (miRNA extraction kit Qiagen) and cel-39 miRNA was spiked in each sample. A multiparametric detection of nephron markers podocalyxin, aquaporin-2 and uEVs pan tetraspanins (CD9 + DC63 + CD81) was performed utilizing imaging flow cytometry. Whereas the uEV composition did not change across the 24 hours analysis, the quantity of uEVs and related markers fluctuated during the day depending on the hydration and excretion rate.The results of a 24 hour urine collection reflected the average results of all single voids over a 24 hr period. Creatinine and particle count normalization failed to normalize "outliers".

Summary/Conclusion: This study represents the very first report which compares single void urine versus 24 hour uEV analysis. We concluded that the 24 hour collection is the preferred choice for a robust and rigorous assessment of uEVs and its associated markers.

PF05.09

Porcine body fluids differ in small extracellular vesicle counts: comparison of blood plasma, seminal plasma and cerebrospinal fluid as vesicle sources for proteomic analyses

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Introduction: Extracellular vesicles (EVs) released from cells to body fluids are extensively studied as potential carriers of nucleic acid and protein biomarkers, particularly in cancer research. Our aim was to compare small EV content in body fluids of the biomedical porcine model to assess their suitability for subsequent proteomic analyses.

Methods: Small extracellular vesicles were enriched from freshly collected porcine blood (plasma), cerebrospinal fluid (CSF) and semen by ultracentrifugation. Size, quantity and quality of isolated particles were characterized by transmission electron microscopy, flow cytometry and western blots. Protein composition of seminal plasma-derived vesicles was analysed using LC-MS/MS (tripleTOF with SWATH quantification).

Results: Seminal plasma yielded twice the number of small EVs than the blood plasma. Approximately 13times less EVs were obtained from CSF compared to seminal plasma. Proteomic analysis of seminal plasma EVs resulted in approx. 1500 identified proteins including proteins involved in exosome biogenesis and transport (74 of the 100 most frequently identified proteins in EVs according to Exocarta database).

Summary/Conclusion: Porcine seminal and blood plasma (single ml volumes) are reasonable sources of EVs for proteomic analyses. In contrast, EV counts in CSF are very low. Techniques for the EV enrichment and proteomic analysis implemented in this study may be applied to biomarker discovery in porcine model of diseases as well as adopted to other species, including human.

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PF05.10

Identification of potential pancreatic cancer therapeutic targets in the cargo of extracellular vesicles released by human macrophages

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Introduction: Macrophages contribute to therapy inefficiency in pancreatic ductal adenocarcinoma (PDAC), possible through secretion of extracellular vesicles (EVs). We aimed to understand the impact of EVs released by macrophages on PDAC cellular response to gemcitabine (GEM) and to identify novel targets for therapeutic intervention.

Methods: EVs released by polarized pro-inflammatory or anti-inflammatory macrophages, derived from human healthy blood donors, were isolated by differential centrifugation. EVs were characterized by Nanoparticle Tracking Analysis, Transmission Electron Microscopy and Western Blot. Response to GEM was analysed in PDAC cells with or without prior co-culture with EVs, using SRB assay. Protein content of EV's cargo was assessed by proteomic analysis. Potential candidates interfering with GEM response were identified and confirmed using human recombinant proteins (rh) or specific pharmacological inhibitors. In addition, tissue samples from 3 metastatic PDAC and from healthy non-pathological tissues were immunohistochemically stained for the identified proteins, as well as for CD68, a macrophage lineage marker. Using The Cancer Genome Atlas (TCGA) database, we assessed the association of the identified candidates with the overall survival of 176 PDAC patients.

Results: Our results showed that large EVs (10 k-centrifugation pellet) shed by the distinctly polarized macrophages decreased BxPC3 PDAC cellular sensitivity to GEM, in an EVs-concentration dependent manner. Proteomic analysis of those EVs identified Chitinase 3-like 1 (CHI3L1) and Fibronectin (FN) as one of the most abundant proteins. Both EVs and rhCHI3L1 or rhFN induced GEM resistance, involving the ERK signalling pathway. Moreover, Pentoxyfilline and Pirferidone, two FDA-approved drugs inhibitors of CHI3L1 and FN, respectively, increased PDAC cellular sensitivity to GEM. Immunohistochemistry data confirmed that CHI3L1 and FN are expressed in the stroma of human PDAC tumour samples, associated to the presence of macrophages, and have no relevant expression in healthy pancreas. Using TCGA, we also found an association between CHI3L1 and FN gene expression with PDAC patients' overall survival, response to GEM and macrophage infiltration.

Summary/Conclusion: This work highlights the relevance of EVs shed by human macrophages to GEM response and identified CHI3L1 and FN as potential therapeutic targets in PDAC.

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PF06: EVs in Reproduction and Pregnancy

Chair: Carlos Salomon, MSc, DMedSc, PhD – Exosome Biology Laboratory, Centre for Clinical Diagnostics, University of Queensland Centre for Clinical Research, Royal Brisbane and Women's Hospital, The University of Queensland

PF06.01

In vivo derived bovine blastocyst secrete different population of extracellular vesicles compared to vitro produced counterparts

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Introduction: In vitro produced (IVP) bovine embryos secrete extracellular vesicles (EVs) that participate in cell communication. Cargo and characteristics of EVs (size mean: SM, size mode; SMo and concentration: C) vary during preimplantation development according to the origin and competence of embryos. In vivo produced (IVV) embryos have better developmental potential than IVP. Here we aim to evaluate if IVV embryos secrete different population of EVs compared to IVP counterparts.
Methods: For IVV embryos, heifers were superovulated and inseminated with commercial semen. Embryos were recovered at morula stage and cultured individually in EVs depleted media (DOFd) until day7. IVP embryos were produced by in vitro fertilization and cultured in groups (25 zygote per well) until morula stage, at this point were cultured further individually in SOFd. At day 7, all embryos were classified according to developmental stage and culture media (CM) were collected from embryos that reached the blastocyst stage. Blastocysts were transferred to fresh media until day 11 to assess their post-hatching competence. CM from competent embryos, (diameter > 270 μm) were used for EVs analysis (IVV: n = 26; IVP: n = 30). EVs were analysed using NTA. Data were analysed by Wilcoxon test or Pearson correlation. Statistic signification for $p < 0.05$.
Results: At day 7 IVV embryos had a greater diameter than IVP (IVV: 190.7 μm; IVP: 165.7 μm; $p < 0.05$). However, IVP embryos reached a bigger diameter at day 11 (IVV: 289.9 μm; IVP: 381.9 μm; $p < 0.05$). Isolated particles from CM of both groups were classified as EVs by their morphology using TEM and by the expression of surface markers CD9, CD63 and CD81. EVs parameters for IVV were: SM = 149.6 nm; SMo = 116.4, C = 1.2 x108xml while for IVV: SM = 124.7 nm; SMo = 93.5 and C = 1.5x108xml. Both SM and SMo were statistically different between groups. Also SM and SMo positively correlate with embryo diameter at day 7.
Summary/Conclusion: IVV embryos secrete bigger EVs than IVP, what may point to a higher competence of in vivo derived embryos. This coincides with our previous finding that competent IVP embryos secret bigger EVs compare to non-competent IVP embryos (Mellisho et al., 2019).
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PF06.02

Extracellular vesicles as messengers in early embryo-maternal communication

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Introduction: Behind the beauty of each pregnancy, stands an incredible complexity. The most critical stage of the pregnancy, unfortunately marked by a high rate of pregnancy loss, is the embryo implantation. Its success depends on a proper embryo development synchronised with timely acquired uterine receptivity. Extracellular vesicles (EVs) are considered now as important players in embryo-maternal communication. Despite the ongoing effort, still little is known about precise role of EVs in modulation of embryo-maternal milieu. Thus, the overarching aim of this study was to determine the effect of the delivery of uterine EVs to primary porcine trophoblast (pTr) cells in vitro.
Methods: Several methods (e.g., Western blot, NTA) were used to characterize EVs isolated from porcine uterine lumen during early pregnancy (day [D] 12 and D16). TaqMan Array Cards were applied to profile miRNAs carried by uterine EVs. Ingenuity Pathway

Analysis tools were used to identify in silico biological pathways and functions governed by detected miRNAs. Expression of putative targets of selected miRNAs was tested using qPCR after in vitro delivery of uterine EVs to pTr cells.

Results: Careful characterisation confirmed that uterine lumen is enriched with a diverse population of EVs caring miRNAs. Interestingly, 36 out of 79 detected miRNAs showed difference in abundance between tested days of pregnancy and half of them was exclusively detected on D16. Identified miRNAs were characterized as potent regulators of cellular development, growth, proliferation, and movement, in addition to their involvement in organismal and embryonic development. The expression of 20 genes identified as a possible miRNA targets was tested after EVs delivery to pTr cells in vitro. Both down- (e.g., PTGER4) and up-regulated (e.g., LIFR) genes were found ($p < 0.05$); involved in the same molecular and cellular functions enriched by detected miRNAs.

Summary/Conclusion: Uterine lumen is enriched with EVs transporting miRNAs, which may have an impact on proper embryo development and implantation. In addition, delivery of uterine EVs to the trophoblast cells affect the expression of miRNA targets involved in embryo-maternal communication. Altogether, these results show an important role of EVs and their cargo during early pregnancy in pigs.

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PF06.03

Arrdc4-dependent extracellular vesicle biogenesis is required for sperm maturation

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Introduction: Extracellular vesicles (EVs) are emerging as important players in cell to cell communication in reproductive systems. Notably, EVs have been found and characterized in the male reproductive tract, however direct functional evidence for their importance in mediating sperm function is lacking. We have previously demonstrated that Arrdc4, a member of the alpha-arrestin protein family, is involved in EV biogenesis and release. Here we show that Arrdc4-mediated EV biogenesis is required for proper sperm function.

Methods: EVs were harvested from wild type and Arrdc4-/- epididymal cells using differential ultracentrifugation, then characterised using Nanoparticle Tracking Analysis and transmission electron microscopy. Sperm motility was measured using Computer assisted sperm analysis and ImageJ. Fertilisation capacity was measured using the following assays: Capacitation-associated tyrosine phosphorylation, calcium ionophore induced acrosome reaction, Zona pellucida binding assay and In vitro fertilization with time-lapse imaging of embryo development. Immunohistochemistry was also used to visualise two pronuclei formation and blastocyst morphology. Arrdc4-/- sperm was supplemented with wild type EVs in the above assays to assess whether they could restore function.

Results: Sperm from Arrdc4-/- mice develop normally through the testis but fail to acquire adequate motility and fertilization capabilities through the epididymis, as evidenced by reduced motility, premature acrosome reaction, reduction in zona pellucida binding and production of two-cell embryos. We observed a significant reduction in EV production by Arrdc4-/- epididymal epithelial cells, and addition of wild type EVs to Arrdc4-/- sperm dampens the acrosome reaction and restores zona pellucida binding.

Summary/Conclusion: These results indicate that Arrdc4 is important for proper sperm maturation through the control of extracellular vesicle biogenesis.

Funding: NHMRC

PF06.04

Maternal EV-derived myomiRs are associated with the development of large-for-gestational age babies in pregnancies complicated by gestational diabetes

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Introduction: Gestational diabetes (GDM) is among the most common pregnancy complications. Despite treatment, up to 25% of pregnancies complicated by GDM result in infants being born large-for-gestational-age (LGA). This not only causes problems at birth but predisposes offspring to developing cardio-metabolic disease in adulthood. There are no treatments for LGA as the cause is unclear, although it is associated with altered placental vascular development. microRNAs (miRNAs) regulate placental development; they are produced within cells but can be released into the circulation inside EVs, which in turn can be transported into target cells and tissues to influence cellular processes.

We aimed to characterise circulating EVs in pregnancies complicated by GDM-LGA and determine if EV-derived miRNAs have the potential to influence placental development.

Methods: Maternal serum and plasma samples were collected from women with pregnancies complicated by GDM at 24–32 weeks gestation; placental tissue was collected at delivery and birth outcomes recorded. Serum and plasma EVs were isolated and characterised by electron microscopy (shape), nanoparticle tracking analysis (NTA; size/concentration), and Western blotting (EV-enriched proteins). miRNA QPCR arrays were performed on EVs. miRNAs were quantified in placental tissue via QPCR.

Results: EM and Western blotting confirmed isolation of EVs and NTA revealed no significant difference in size/concentration in GDM-LGA pregnancies (n = 7) compared to GDM-AGA (n = 13; $p > 0.05$). Several EV miRNAs were altered in maternal circulation in GDM-LGA compared to GDM-AGA (n = 7/group; >twofold-change; $p < 0.05$), including four skeletal muscle-specific "myomiRs": miR-1-3p, miR-133a-3p, miR-133b, and miR-499a-3p (all increased). All four myomiRs were present in placenta but only miR-1-3p was significantly altered in GDM-LGA compared to GDM-AGA (n = 12–14/group; $p < 0.05$).

Summary/Conclusion: EV-bound myomiRs could have predictive value for aberrant foetal growth in cases of GDM. miR-1-3p regulates vascular development in other systems, so we propose that miR-1-3p contributes to LGA by influencing placental vascular development, however further work is required to establish this.

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PF06.05

A new type of nanovesicles in seminal plasma: the myelinosomes

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Introduction: Seminal plasma is particularly rich in extra cellular vesicles. Myelinosomes are membranous organelles described throughout the seminiferous epithelium of the testis but never reported in semen. The aim of this study was to look for the presence of myelinosome vesicles in human seminal plasma.

Methods: Because of the viscosity of seminal gel and its water-holding capacity, classical transmission electron microscopy does not seem to be an optimal technique to reveal the presence of myelinosomes in this fluid. Cryo-electron microscopy is a technique that allows visualization of nanosized structures without prior fixation or addition of heavy metals for contrast. The sample is therefore visualized as close to its native state as possible. Using standard myelinosome preparation from TM4 Sertoli cells, we first analysed the appearance of "standard" native myelinosomes by cryo EM and then compared it with the vesicles from human seminal plasma samples.

Results: We have specified by cry-EM the morphological aspect of "standard" myelinosomes isolated from the culture media of TM4 Sertoli cells. The vesicles with the same morphological appearance were revealed in human seminal plasma specimens.

Summary/Conclusion: Myelinosomes are membranous organelles found in the seminiferous epithelium of the testis and secreted by the somatic Sertoli cells in the lumen of the seminiferous tubules.The preparations from human seminal plasma contains a population of large EV (average diameter 200 nm) whose morphological appearance resemble those of myelinosomes. Defining the specific biomarkers and functionalities of myelinosomes in human seminal plasma are the concerns to be addressed in our further research.

Funding: CHU de Rennes

Univ Rennes Inserm, Irset (Institut de recherche en santé, environnement et travail) – UMR_S 1085,

PF07: EVs in Kidney, Urinary Tract, and Related Diseases

Chair: Luca Musante – University of Virginia School of Medicine

Chair: Dylan Burger, PhD – Chronic Disease Program, Ottawa Hospital Research Institute

PF07.01

Tuberous sclerosis complex axis controls extracellular vesicles production and protein content

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Introduction: More than one million patients worldwide suffer from tuberous sclerosis complex (TSC) and have mutations in either TSC1 or TSC2 genes. Together, the TSC proteins regulate mTORC1 activity. All TSC patient post-mortem samples exhibit renal disease and 40% of patients with TSC experience a premature loss of renal function. Mouse and human studies are incongruity with the second somatic hit mechanism of disease, because of the low percentage of cystic cells exhibiting loss of TSC expression. We posited that the loss of a TSC protein expression may alter extracellular vesicle (EV) biology and contribute to disease.
Methods: We used CRISPR/CAS9 to disrupt the Tsc2 gene in mouse inner medullary collecting duct (mIMCD) cells, and isolated EVs using gel filtration from the isogenic cell lines. We characterized the EVs using tunable resistive pulse sensing (TRPS), dynamic light scattering (DLS), transition electron microscopy (TEM), and wester blot analysis. We further performed mass spectroscopy on the EV proteins.
Results: Loss of the Tsc2 gene in mIMCD cells induced a greater than three-fold increase in EV production compared to the same cells having an intact Tsc axis. Electron microscopy confirmed the purity and spherical shape of EVs. Both TRPS and DLS demonstrated that the isolated EVs possessed a heterogenous size distribution. Approximately 90% of the EVs were in the 100–250 nm size range. Western blot analysis using proteins isolated from the EVs revealed the cellular proteins Alix and TSG101, the transmembrane proteins CD63, CD81 and CD9, and the primary cilia-related Hedgehog signalling-related proteins Arl13b. Proteomic analysis of EVs identified a significant difference between the Tsc2-intact and Tsc2-deleted cells that correlated well with the increased production.
Summary/Conclusion: EVs may be involved in tissue homoeostasis and cause disease by overproduction and altered protein content. The EVs released by renal cyst epithelia in TSC complex may serve as a tool to discover the mechanism of TSC cystogenesis and in developing potential therapeutic strategies.
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PF07.02

Extracellular vesicles derived from human amniotic fluid stem cells: characterization and therapeutic effect in a model of chronic kidney disease

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Introduction: We have shown that EVs derived from amniotic fluid stem cells (AFSC) of mouse origin present therapeutic effect in an animal model of chronic kidney disease, Alport Syndrome (AS). In light of clinical translation, we isolated AFSC-EVs of human origin, characterized their cargo and evaluated thier therapeutic effect in vivo.
Methods: Human clonal AFSC were derived from amniotic fluid collected after volunteer donors provided consent. EVs were obtained from AFSC and identity and purity were assessed by RNA-seq and proteomics. Potency of hAFSC-EVs was evaluated by performing in vivo studies. EV biodistribution was evaluated by MRI and therapeutic effect by measuring renal function and mice life-span. Bulk RNA-seq was performed on glomeruli obtained from injected and non-injected mice to identify potential EV regulating targets.
Results: Proteomic profiling identified 675 intact proteins and RNA-seq data identified 2,535 miRs in hAFSC-EVs. hAFSC-EV "fingerprint" was assessed by performing GO analysis on the 100 most highly expressed proteins and miRs. The results identified pathways involved in tissue homoeostasis such as mTOR pathway, TGFβ and VEGF pathways. When

injected in vivo into AS mice, biodistribution studies showed that hAFSC-EVs localized in the kidney, corrected proteinuria. No side effects (including teratoma) were noted in the treated mice. RNA-seq of glomeruli obtained from treated AS mice showed similar gene expression patterns to wilt type mice, by cluster analysis. Our data indicated that hEVs highly modulated pathways involved in collagen and matrix deposition remodelling, in addition to downstream targets of VEGF, FGF, TNF, angiotensin and preserved glomerular cells structure and function.

Summary/Conclusion: Our protocol for hEVs derivation is reproducible and allows derivation of EV lots with the same identity (specific cargo of proteins and miRs) and potency (present therapeutic effect in AS). hAFSC-EVs modulated signalling pathways that are central to maintaining glomerular homoeostasis and preserved glomeruli structure with improved kidney function. This suggests the possibility of using hAFSC-EVs as a new therapeutic option for treating renal failure in humans.

Funding: -Intramural CHLA funding
-Pilot Imaging CHLA Core Funding

PF07.03

A millifluidic in vitro model of glomerular filtration to test the regenerative effect of extracellular vesicles under dynamic conditions

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Introduction: Recent studies have shown that stem cell-derived extracellular vesicles (MSC-EV) therapy improves renal outcomes in models of acute ad chronic renal disease. However, to better investigate the molecular mechanisms of EV-induced regeneration, and to define new EV sources, devices that mimic 3D organ architecture and flow conditions are needed. The aim of our work is to evaluate the regenerative potential of naïve and engineered EV in a millifluidic in vitro 3D model of glomerular damage in continuous perfusion.

Methods: Methods: we set a millifluidic in vitro 3D model of glomerular filtration, a three-layers structure composed by human podocytes and glomerular endothelial cells, and, in between, of a basement membrane of Collagen type IV. The barrier thus formed is set up inside a bioreactor, in a closed milli-fluidic circuit in which fluid flows continuously at a certain flow rate. We reproduced different pathological conditions and tested the localization and effect of EVs in a dynamic system.

Results: Results: we obtained a standardized protocol and an adequate configuration of the milli-fluidic circuit subject to continuous reperfusion. Renal damage was induced by doxorubicin or by hypoxia-reperfusion injury. We evaluated uptake, cargo transfer and effect of naïve and miRNA engineered MSC-EVs or of Klotho engineered ineffective EVs administered into the dynamic co-culture system. EVs were able to pass through the system and to deliver to podocytes pro-regenerative factors, promoting survival and limiting permeability.

Summary/Conclusion: In conclusion, we highlighted the effects of MSC naive EVs as well as of engineered EVs in a model of glomerular damage under EV continuous perfusion.

Funding: This study was supported by Regione Piemonte POR FESR 2014/2020 – Bando Piattaforma Tecnologica Salute e Benessere – Project "Terapie Avanzate per Processi Fibrotici Cronici (EVER).

PF07.04

Extracellular vesicles from kidney cancer tissue and comparison of two quantitative methods

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Introduction: Worldwide, renal cell carcinoma (RCC) is 8th most common cancer in men and 10th most common in women. New biomarkers are needed to aid RCC-diagnosis, provide prognostic information, and to predict response to modern targeted therapies. Extracellular vesicles (EVs) are an emerging source of cancer biomarkers because all cells, including cancer cells, secrete EVs into biofluids as blood and urine. However, benign cells contribute to EV populations isolated from blood and urine reducing the disease-specificity. We have developed a protocol for EV isolation directly from human RCC tissue that can increase tumour-specificity of biomarkers.

Methods: We obtained technical and biological replicates from normal kidney tissue and clear cell RCC tissue. Serum-free media was incubated with the specimens. A combination of differential centrifugation, filtration, and ultracentrifugation was used for EV isolation. EVs were quantitated using two methods, allowing for comparison between NanoSight NS300 and NanoFCM. TEM was used to determine presence of intact vesicles in the EV samples. Presence of EV

protein markers (CD81, CD63, flotillin-1), and absence of cellular debris (calnexin), were assessed using Western Blot.

Results: Particle concentrations for the technical replicates of normal kidney EVs were 1.44 × 1010 ± 2.51 x 109 p/mL (mean ± SD) measured by NanoSight, and 1.67 × 1010 ± 6.89 x 109 p/mL (mean ± SD) measured by NanoFCM. Vesicle concentrations for replicates RCC EVs were 1.80 × 1010 ± 3.59 x 109 p/mL (mean ± SD) measured by NanoSight and 1.68 × 1010 ± 4.18 x 109 p/mL (mean ± SD) measured by NanoFCM. Among different patients, we observed an acceptable biological variance in EV counts. A head-to-head comparison of NanoSight and NanoFCM demonstrated differences in total particle counts of less than fivefold. NanoFCM performed better in measuring particle size distribution. Small EVs were visible on TEM images in all technical and biological replicates. EV markers were positive in all samples and calnexin was negative.

Summary/Conclusion: We optimized EV isolation from RCC tissue and normal kidney. The product of our protocol contains small EVs in high abundance. The protocol can contribute to study tumour microenvironment and biomarker discovery in kidney cancer.

Funding: This work was supported by Cure for Cancer foundation (Amsterdam, The Netherlands), the Prostate Cancer Foundation, the Patrick C. Walsh Prostate Cancer Research Fund, the William and Carolyn Stutt Research Fund, and the National Cancer Institute.

PF07.05

Tetraspanins on serum derived-EVs enable detection of renal cell carcinoma

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Introduction: Renal cell carcinoma (RCC) is a lethal urological cancer with an incidence rate which accounts for about 3% of all human cancers. Nearly 40% of RCC patients are diagnosed with either locally invasive or metastatic disease. Added to this, there is a dearth of reliable biomarkers for early diagnosis. These facts highlight the urgent need for novel non-invasive diagnostic tools. Extracellular vesicles (EVs) are considered a promising biomarker target for diagnosis of various malignancies. However, investigation of EVs typically demands isolation of them from body fluids, which is difficult and time-consuming process. The aim of our study was to develop EV-based assay for the early and non-invasive detection of RCC using a highly sensitive nanoparticle-aided time-resolved fluorescence immunoassay (TRFIA).

Methods: EVs from the serum of renal cell carcinoma (RCC), bladder cancer (BlCa), benign and healthy samples were captured with biotinylated anti-tetraspanin-antibodies (CD63 or CD81) immobilized on streptavidin coated microtitration wells. The captured EVs were detected using 95 nm europium dyed nanoparticles (polysterene beads packed with ~30,000 Eu3+ chelates) conjugated with anti-tetraspanin-antibodies (CD63 or CD81). Isolated EVs-derived from four-cancer cell lines was taken to validate this TRFIA. Serum samples from RCC (n = 14), BlCa (n = 14), benign prostate (n = 14), and healthy (n = 10) controls were analysed. This study was conducted following the guidelines of Helsinki Declaration. Participants had given written informed consent.

Results: This TRFIA can measure purified-EVs with high sensitivity. The CD63-CD63 assay enabled significant discrimination of RCC patients from BlCa (4.3-fold, p = 0.001), benign (fivefold, p = 0.0007) and healthy (2.8-fold, p = 0.010) controls. Similarly, the CD81-CD81 assay also showed a discrimination of RCC patients from BlCa (6.3-fold, p = 0.001), benign (3.7-fold, p = 0.020) and healthy (1.8-fold, p = 0.003) samples.

Summary/Conclusion: The results obtained from this study suggest that the EVs derived from the serum of patients with pathological conditions display varying amounts of tetraspanins. Detection of such varied expression of tetraspanins using non-invasive techniques may play a major role in the early detection of RCC. Further validation with large cohort of samples is required.

Funding: DPMLS-Graduate school, University of Turku, Finland.

PF07.06

Extracellular vesicles as a source of cancer biomarkers in the urine of urothelial carcinoma patients: characterization of a two-step protocol for the isolation of urine-derived EVs

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Introduction: Urothelial carcinoma (UC) is a malignant cancer that affects the urothelial cells, representing 90% of all bladder tumours. At diagnosis 75% of bladder cancers are non-muscle invasive tumours. Importantly, upon transurethral resection of the bladder tumour, nearly 35–80% of these patients will experience disease relapse and 10–20% will progress to muscle invasive tumour, requiring thereby, a rigorous and expensive follow-up. Currently, this is performed through the frequent use of highly invasive cystoscopy and the low sensitivity urine cytology. Thus, innovative liquid biopsy-based biomarkers that circumvent these drawbacks are highly desirable for improved UC clinical management. Here, we AIM to implement a protocol for the isolation and characterization of extracellular vesicles (EVs) from UC patients' urine samples.

Methods: A two-step protocol involving ultracentrifugation (UCt) and by size-exclusion chromatography (SEC) was optimized for urine samples. The isolated urine-derived EVs from 9 UC patients were then characterized according to their size, concentration (NTA), morphology (TEM), protein amount (Lowry method), presence of EV-associated and disease-associated protein markers (Western blot).

Results: Isolated urinary EVs from UC patients had a size ranging from 50nm to 400 nm with characteristic EV morphology, express EV-associated markers as CD63 and HSP70 and were negative for cell debris markers. The recovery yield and purity of isolated EVs following each isolation technique was characterized. Upon UCt, SEC was required to deplete most of the EV-associated THP and albumin protein contaminants. Some disease-associated protein markers were highly enriched in isolated urinary EVs compared to crude urine.

Summary/Conclusion: Taken together, these results indicate that a two-step EV isolation protocol was properly implemented and validated in UC patients' urine samples. Notably, several EV-associated disease biomarkers were detected in the urine of UC patients. This EV-based liquid biopsy might provide the means for real-time monitoring of residual disease and relapse in UC patients.

PF08: EVs in Cancer Pathogenesis

Chair: Jeffrey Franklin – Vanderbilt University Medical Center

Chair: Aurelio Lorico – Roseman University of Health Sciences

PF08.01

Extracellular vesicles are involved in the expansion of the mesenchymal signature in glioblastoma tumours

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Introduction: Glioblastoma multiforme (GBM) is a very aggressive type of brain tumour. Different GBM molecular subtypes (proneural, mesenchymal and classical) often co-coexist within the same tumour, with the mesenchymal subtype driving the tumour progression. Recently, our lab demonstrated that the cargo of extracellular vesicles (EVs) could mirror the molecular background of the GBM cells from which they were derived. Altogether, we believe that GBM cell-derived EVs can be directly involved in the expansion of the mesenchymal signature in tumours, thus supporting GBM aggressiveness.
Methods: Non-mesenchymal (T98 & U138) GBM cells were "primed" using EVs derived from mesenchymal-like (U87 & LN18) GBM cells. EV-primed GBM cells were then co-cultured with their non-primed counterparts to determine whether the mesenchymal signature can "spread" from cell to cell via EVs. Effect on cell proliferation, migration and invasion (in hyaluronic acid hydrogels) was assessed following EV treatment and co-culture. The expression of mesenchymal GBM markers was measured by western blotting. Further mass spectrometry analysis of cell and EV content was undertaken to describe potential underlying mechanisms.
Results: Co-culture with EV-primed GBM cells significantly increased proliferation and hydrogel invasiveness of non-mesenchymal cells. Interestingly, the stimulating effect of co-culture was even stronger on the proliferation of EV-primed GBM cells. Moreover, further proteomic analysis revealed that expression of mesenchymal GBM markers such as CD44 was increased in non-mesenchymal cells following co-culture.
Summary/Conclusion: Our data suggest that EVs from mesenchymal GBM cells can be uptaken by GBM cells from different subtypes, thus stimulating tumour progression. Overall, we think the present study provides with new insights for the understanding of GBM recurrence and the development of potential therapeutic strategies.
Funding: This work is supported by Action Against Cancer, The Colin McDavid Family Trust, The Rothschild Foundation, The Bernard Sunley Foundation, The Searle Memorial Charitable Trust, Mr Alessandro Dusi and Mr Milan Markovic.

PF08.02

Inhibition of extracellular vesicle release in triple negative breast cancer

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Introduction: Triple-negative breast cancer (TNBC) is the most aggressive form of breast cancer. Previously we reported that the heterogenous population of EVs released from TNBC cells promotes the growth and aggression of recipient cells. Here we investigated if, by using compounds proposed to inhibit EV release i.e. calpeptin and Y27632 (to block those budding at cell membrane) and GW4869 and manumycin A (to block EVs from MVBs), we could reduce the associated transmission of aggressive phenotype.
Methods: EVs were separated from medium conditioned by TNBC cell line Hs578Ts(i)8, using a discontinuous optiprep density gradient, after the cells were treatment for 48 hrs with the compounds listed above. EVs (pooled fractions 3–9 with a density range of 1.03–1.16 g/mL) were characterised by NTA, BCA, lipid assay, immunoblot, TEM and flow cytometry. To investigate the functional effects of the EVs released, proliferation and migration assays were performed on Hs578T and MDA-MB-468 cells using the EV to cell ratios of 1 × 105 EVs/3x103 cells, 1 × 106 EVs/3x103 cells, 1 × 107 EVs/3x103 cells to evaluate dose-response. EV-TRACK ID EV190109 (Score of 88%).

Results: GW4869 significantly (p = 0.035) decreased EV release from Hs578 Ts(i)8 cells. Manumycin A and a combination of calpeptin and Y27632 (Combo) decreased EV release, but significance was not reached. Conversely, calpeptin and Y27632 actually increased EV release; but not significantly. Of the reduced numbers of EVs released following GW4869 treatment, HLA-DR+ EVs were significantly (p = 0.032) enriched. None of the EVs analysed significantly changed Hs578 T or MDA-MB-468 growth rates. However, EVs from cells treated with calpeptin (p = 0.007), GW4869 (p = 0.025), manumycin A (p = 0.01) and Combo (p = 0.001) caused significant reduction in MDA-MB-468 migration compared to the effects of EVs from untreated cells. Similarly, EV from cells treated with GW4869 (p = 0.011), and Combo (p = 0.018) caused significant reduction in Hs578 T migration.

Summary/Conclusion: While GW4869 was the only compound that caused a significant decrease in quantities of EV released, the EVs that continued to be released following treatment with GW4869 or calpeptin and Y27632 significantly reduced migration of both recipient cell lines.

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PF08.03

Extracellular vesicles from highly metastatic lung cancer cells induce barrier impairment, permeability, and epithelial-to-mesenchymal plasticity in a 16-day mature bronchial epithelium

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Introduction: Epithelial-to-mesenchymal (EMT) transition plays an integral role in cancer metastasis, which is responsible for as much as 90% of cancer mortality. Cancer exosomes induce EMT in bronchial epithelial cells, however, the epithelial cells inhibit EMT when allowed to form a mature epithelial barrier with apical-basal polarity. It is not known if cancer-derived extracellular vesicles (EVs) can induce EMT and more importantly, barrier disruption in a mature epithelium. Here, we show that EVs from a highly metastatic lung cancer cell line (Calu6) are) are not only sufficient to induce EMT in non-tumorigenic bronchail epithelial cells (BEAS-2B), but are also capable of disrupting a 16-day mature bronchial epithelial barrier by significantly reducing TEER, inducing sixfold increase in permeability and complete loss of E-cadherin at cell-cell tight junctions.

Methods: BEAS-2B and Calu6 EVs were characterized using electron microscopy, NanoSight and western blotting for exosome-specific features. For permeability studies, BEAS-2B cells were cultured in transwell for 16 days to establish an intact epithelium – confirmed by measuring TEER (trans-epithelial electrical resistance). Intact BEAS-2B monolayers were treated with Calu6 EVs at 1, 10 and 20 μg/ml for 24 hrs, and barrier intactness and permeability were evaluated by measuring TEER, apical-basolateral translocation of dextran beads and confocal imaging of tight junctions (E-cadherin). For EMT experiments, BEAS-2B cells treated with Calu6 EVs at 1 and 10 μg/ml were evaluated for E-cadherin and Vimentin levels by qRT-PCR and western blot after 48 hrs.

Results: BEAS-2B and Calu6 EVs were enriched in 50–200 nm size range, and CD9 and CD81 were enriched in the EV fraction in contrast to the cell lysate and vice versa for GP96. Calu6 EVs significantly impaired 16-day mature BEAS-2B monolayer's barrier properties, which at the highest dose caused 33% reduction in TEER from 27.0 ± 2.5 to 18.2 ± 3.2 Ω.cm2 (n = 4). This was further confirmed by ~sixfold increase in dextran beads' apical-basolateral translocation in 30 min (14.8 ± 7 ng/ml in control vs 87.3 ± 51 ng/ml in treated) (n = 3) and complete loss of E-cadherin expression at cell-cell tight junctions (n = 3). At the transcript level, Calu6 EVs induced significant downregulation of E-cadherin by 36% and upregulation of Vimentin (mesenchymal marker) twofold (n = 3) in BEAS-2B cells, indicating transition into mesenchymal phenotype.

Summary/Conclusion: We demonstrated the involvement of EVs derived from highly metastatic lung cancer cells in inducing EMT in bronchial epithelial cells and epithelial barrier disruption – the initial stage of the intravasation process.

PF08.04

Grp78 plays a crucial role in the extracellular vesicle-promoted radioresistance of irradiated head and neck cancer cells

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Introduction: Small EVs released from irradiated head and neck squamous cell carcinoma (HNSCC) cells increase resistance of recipient HNSCC cells to radiation in vitro. We have identified the Glucose-regulated protein 78 (Grp78), a chaperone protein of the HSP70

family which is involved in cellular stress responses and associated with worse survival in head and neck cancer patients, as an essential component of the EV-mediated radioresistance.

Methods: Small EVs were isolated from conditioned medium from irradiated and non-irradiated BHY HNSCC cells by combined microfiltration (0.22 μm) and differential ultracentrifugation. Grp78 surface expression was measured by proteomic analysis, immunoblotting and bead-FACS. Radiation resistance of BHY cells was determined by a clonogenic survival assay.

Results: Increased Grp78 was identified on the surface of EVs from irradiated cells. The increase in EV Grp78 correlated with increased Grp78 expression at the donor cell surface. The Grp78 content of recipient cells also increased upon transfer of EVs from irradiated, but not non-irradiated cells, ultimately leading to enhanced cell survival. To check a potential role of elevated Grp78 in radiation resistance we overexpressed Grp78. Here the modest (3x) overexpression of Grp78 was sufficient to confer an enhanced radioresistant phenotype to the BHY cells. A correlation between Grp78-dependent increase of radioresistance and activation of the Akt pathway is yet to be determined.

Summary/Conclusion: Our results suggest a pivotal role for EV-transferred Grp78 in modulating the radiation response of recipient HNSCC cells. Radiation directly increases the cellular and vesicular Grp78 levels, and subsequent EV-mediated transfer leads to enhanced Grp78 levels and radioresistance in recipient cells. This study provides new mechanistic insights into the effects of EVs in radiation response and elucidates an interesting target protein and novel strategies for the improvement of radiotherapy.

PF08.05

3D modelling of EV release in progressing prostate cancer

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Introduction: The modelling of cancer progression should be capable to translate acquired knowledge of cell behaviour to the real human body conditions. However, the extracellular vesicles (EVs) isolated from 2D cell models are commonly exploited in research. Taking into account the specificity of the prostate cancer (PC) environment, and a strong need of early diagnosis of castrate-resistance by prostate cancer (CRPC) patients, we suggest in-depth profiling of different EV subtypes isolated from 3D culture as a new tool to model the progressing PC.

Methods: Cells from hormone-resistant prostate carcinoma 22-RV1 line were cultured in 2D and 3D conditions, using 3D CoSeedisTM. ACD plasma controlled for haemolysis and remaining platelets was taken from patients with PC and CRPC. The 40 fractions of 4 EV subtypes from cell culture and plasma were obtained by differential centrifugation (DC) followed by iodixanol density gradient purification. Each of the fractions was measured by Nanoparticle Tracking Analysis (NTA), Tunable Resistive Pulse Sensing (TRPS) followed by ELISA. For that, CD63 and CD9 were used as EV markers, ApoB and ApoA1 for lipoprotein contaminants control, and CD3, CD41 and PSMA as tissue-specific biomarkers for determination of fractions containing EVs of different origin. EV-contained fractions were subjected to Next Generation Sequencing (NGS).

Results: In 3D conditions, the 22-RV1 cells produce up to 1000-times higher EV number than in 2D. Size and density distribution of EVs derived from 3D cultures but not of 2D resembled plasma EVs. Size distribution and biomarker expression among different EV subtypes allowed distinguishing between PC and CPRC-derived samples, indicating a potential to translate these results into clinics for early CPRC detection.

Summary/Conclusion: This work demonstrates a new approach to study the secretome of a progressing PC under 3D conditions. The profiles of EV subtypes produced by cancer cells growing in a 3D spatial architecture resemble the profiles of plasma EVs and can serve a useful tool for the establishment of new biomarkers.

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PF08.06

Multiplex analysis of renal cell carcinoma cell extracellular vesicles to identify potential clinically relevant markers

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Introduction: Renal cell carcinoma (RCC) is the most common primary renal neoplasm, with over 80,000 cases in the US alone each year. Early detection of RCC leads to consistently better patient outcomes, and extracellular vesicles (EVs) isolated from patient samples may prove to be a valuable clinical tool in the future. EVs are abundant in blood and urine and show a large amount of heterogeneity but are difficult to analyse due to their small size and difficulty in isolation. Here, we employ a multiparametric analysis of EV surface markers to identify a set of markers that may prove clinically relevant in future studies.

Methods: RCC cell lines VOK111, VOK130, and VOK151 were cultured in flasks containing 500 mL of EV-depleted media (10% FBS, centrifuged 18 hr x 100,000 g). When cells reached ~75% confluency, the conditioned media was collected and spun at 2,500 g for 10 mins two times to deplete any remaining debris, leaving ~ 450 mL of media. This media was concentrated to a final volume of ~ 7 mL using a PALL jumbosep 100 kDa MWCO filter. This concentrate was purified from protein by using an Izon qEV-10 column, collecting 5 mL fractions. Protein content of each fraction was analysed using A280 absorbance while concentration and diameter distribution were determined through nanoparticle tracking analysis (NTA). Pooled samples made of the three most concentrated fractions were concentrated to a final volume of ~190 μL using the PALL Microsep 100 kDa filter and then used for analysis in the Miltenyi MACSPlex exosome kit. Flow cytometric data were generated by the CytoFLEX S and analysed using FlowJo and MPAPASS software. These positive signals were verified through bead-only controls and titrations.

Results: The MPAPASS software allowed for heatmap generation, data reduction, clustering and visualization of expression patterns. Of the 11 detection antibodies used across 39 capture beads, CD276, CD26, CD82, Beta-2 Microglobulin, and CD151 were found to be prevalent in these RCC EVs. These markers were found to be co-expressed particularly with CD63, CD81, and CD29.

Summary/Conclusion: The use of multiplex analysis allowed for detection of five distinctive surface markers found to be prevalent in EVs collected from RCC cell lines. These results demonstrate the utility of multiplex analysis and MPAPASS software for identifying potential markers of interest and provide proteins that are worth exploring further. The next steps to this work will be developing custom multiplex arrays that tailor capture and detection of EVs specifically for RCC pathology.

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PF08.07

Low molecular weight protein tyrosine phosphatase (LMWPTP) carried by colorectal cancer cells-derived extracellular vesicles as a player in tumour-educated human fibroblast

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Introduction: Extracellular vesicles (EVs) are double-membrane-bound nanovesicles released by cells playing a key role as mediators of intercellular communication. Low Molecular Weight Protein Tyrosine Phosphatase (LMWPTP) is upregulated in several cancers type, including colorectal cancer (CRC), and it has been correlated with aggressiveness, chemoresistance and poor prognostic.

Methods: The aim of this study was to determine whether CRC cells release LMWPTP-enriched-EVs and influence tumour microenvironment-associated cells as a representative tumour education. CRC cells, HCT116 and HT29, were cultured in serum-free medium for 24 hours. Conditioned medium was concentrated by ultrafiltration (MWCO 10 kDa) and EVs were isolated by Total Exosome Isolation Reagent (Invitrogen). EVs were characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and western blotting (WB). LMWPTP levels were analysed by WB and sandwich-ELISA. To evaluate tumour education, HFF-1 fibroblasts were used as recipient cells. The uptake of EVs (PKH26 fluorescently labelled EVs), proliferation (viability) and migration (wound healing assay) were analysed in a co-culture model of CRC-derived EVs and HFF-1.

Results: NTA showed a higher concentration of EVs released by HT29. HCT116 and HT29 EVs displayed a mean diameter around 140 nm and a cup-shaped morphology. Isolated EVs were positive for EVs-markers CD81 and TSG101 and negative for GM130 a non-EVs marker. HT29 lineage as well as derived-EVs are LMWPTP-enriched in comparison to HCT116 cells and EVs. Upon incubation, fluorescently HCT116 and HT29 derived EVs were internalized into HFF-1 cells in a perinuclear region. EVs derived from both cells increased the viability and proliferation of HFF-1 cells. Intriguingly, EVs derived from HT29 promoted cell migration.
Summary/Conclusion: In conclusion, for the first time, we showed that LMWPTP can be carried by EVs derived from CRC cells and LMWPTP-enriched-EVs can modulate biological aspects of HFF-1 fibroblast. Overall, our findings point LMWPTP out as important player in tumour-educated fibroblast.
Funding: This study was supported by São Paulo Research Foundation (FAPESP) – Grants: 2018/03593-6 and 2015/20412-7.

PF08.08

Exosomal miR-181a Inhibition by Vincristine and Prednisone in Paediatric Acute Lymphoblastic Leukaemia.

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Introduction: Vincristine and prednisone are standard agents in treatment of paediatric acute lymphocytic leukaemia (P-ALL). Mechanistically, vincristine induces apoptosis by blocking microtubules formation, while prednisone binds to cytoplasmic receptors and inhibits DNA synthesis, both of which lead to apoptosis. The effect of these agents on exosomal micro-RNA expression and its functional regulation is not yet investigated. Elevated levels of miR-181a in circulating exosomes (nanoparticles) has been shown to lead to progression in several cancers, including ALL. We have previously shown that leukaemia-derived exosomes induce leukaemia cell proliferation via up-regulating of miR-181a expression and silencing of exosomal miR-181a reverses this exosome-induced cell proliferating effect. The objective is to investigate the effect of vincristine and prednisone on exosomal mi-R181a expression in ALL.
Methods: JM1, SUP-B15, and NALM-6 leukaemic cell lines were treated in vitro with vincristine (0.1 to 4.0 μM) and prednisone (0.1 to 12.0 μM) in exo-free medium and apoptosis was measured by MTS assay. Total RNA of exposed cell lines was isolated and cDNA was prepared for miR-181a analysis. Expression of miR-181a was analysed by q-PCR.

Exosomes from conditioned medium of exposed cell lines were isolated by ultracentrifugation method. Purity and particle size of exosomes were confirmed by western blot and nanoparticle tracking analysis (NTA) assay respectively. Total exosomal RNA was isolated from exosomes (Exo-RNA) by Trizol method. Synthesis of cDNA was carried out with the miScript II RT kit (Qiagen).
Results: Vincristine and prednisone promote apoptosis in leukaemia cell lines (JM1 and SUP-B15) in a dose-dependent manner. Both cellular and exosomal miR-181a expression was down-regulated by vincristine and prednisone exposure in all three leukaemia cell lines (JM1, SUP-B15, and NALM-6). These observations demonstrate that cellular miR-181a down regulation in the parental cells is stable and can be transferred to exosomes, confirming the concept that exosomes are the fingerprint of parent cells.
Summary/Conclusion: Our data suggest that the vincristine and prednisone anti-proliferative effect in P-ALL maybe induced by another yet unexplored pathway, that suppresses miR-181a at a cellular and exosomal level in P-ALL, resulting in apoptosis.
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PF08.09

Secreted extracellular vesicles from renal cell carcinoma cells

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Introduction: Clear cell Renal Cell Carcinoma (ccRCC) represents the most common form of kidney cancer and is among the most lethal of all genitourinary cancers. Despite surgery and medication therapy, most patients with metastatic ccRCC have a poor prognosis. Intratumoural hypoxia is a key factor involved in renal cancer progression and it is known to promote secretion of EVs by many types of tumour cells.
Methods: RCC-derived Renca cells, embryonic kidney derived UB cells, and primary mouse hepatocytes were used in the study. EVs were purified from cell culture media by gradient ultracentrifugation, sequential ultracentrifugation and Exo-spin™ columns. Before EV isolation cells were kept for 24 h either under normoxia or hypoxia (1% oxygen). EVs were analysed by transmission electron microscopy with negative staining and immunolabeling, by nanoparticle tracking analysis (NTA) and

Western blotting. Cells proliferation and viability were assayed by live cell imaging using IncuCyte ZOOM (Essen BioScience), cell metabolic activity by Seahorse XF Analyser (Agilent), RNA expression by qPCR and ddPCR. Proteins were identified by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). RNA libraries were made using NEBNext small RNA library prep kit, and sequenced on NextSeq550 (Illumina).

Results: We showed that hypoxia induced production of EVs by RCC cells, and characterized differences in protein and RNA content of EVs generated by Renca cells cultured under normoxic and hypoxic conditions. We also showed that RCC-produced vesicles modify key features of tumorigenesis (gene expression, metabolic activity, motility, and growth) of target cells. These data were obtained by using two target cell types: model mouse kidney cells and primary mouse hepatocytes, which represent typical site of RCC metastasis with an exceptionally poor prognosis. We proposed that a possible mechanism of EV action in RCC is related to changes in caveolin-1 function. We also tracked Renca-derived EVs in a chick embryo model and in a novel kidney organoid co-culture assay developed by our group (Xu et al., 2017).

Summary/Conclusion: Hypoxia may influence tumorigenic properties of RCC by changing rates of production and composition of EVs.

Funding: The study was supported by Finnish Cancer Foundation grants.

PF08.10

Exosomes synthesizing HER2 miRNA and engineered to adhere to HER2 on tumour cells surface exhibit enhanced anti-tumour activity

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Introduction: Exosomes are small extracellular vesicles averaging 100–150 nm in diameter. They serve as a means of intercellular communication. Typically they consist of structural proteins as well as selected proteins, miRNAs, mRNAs, and long noncoding RNAs. Thus in an earlier report this laboratory designed a miRNA targeting a major herpes simplex virus regulatory protein. As predicted by the nucleotide packaging signal the miRNAs were packed in exosomes and on exposure to infected cells significantly reduced virus yields. HER2 (human epidermal growth factor receptor 2) plays an important role in the neoplasia of some breast cancers. The protein is exhibited on the cell surface and is the target of therapeutic antibodies.

Methods: Firstly, we report on the construction of a miRNA targeting the synthesis of HER2 both in cells constitutively expressing HER2 and in cells transfected with a plasmid encoding HER2. Secondly, we report that the miRNA targeting the synthesis of HER2 reduced the viability of HER2 positive cancer cells both in cell culture and in implanted tumours. Lastly, we enhanced the anti-tumour activity of the exosomes by binding to the exosome surface a ligand with affinity for the HER2 on the surface of tumour cells.

Results: The 293-miR-HER2 exosomes package with miRNA designed to block HER2 synthesis and deliver to cells. These exosomes kill cancer cells dependent on HER2 for survival but have no effect on cells lacking HER2 or which were engineered to have HER2 but do not depend on it for survival. The 293-miR-XS-HER2 exosomes carry in addition a peptide which enables the exosome to adhere HER2 on the surface of the cancer cells. In consequence, these exosomes preferentially enter and kill cells exhibiting HER2 on their surface. The exosomes with 293-miR-XS-HER2 are significantly more effective in shrinking the size of HER2-positive tumours implanted in mice than the 293-miR-HER2 exosomes.

Summary/Conclusion: Our studies indicate that exosomes carrying miRNA against HER2 have no effect on HER2 negative cells it was nevertheless desirable to increase the uptake of exosomes carrying the HER2 miRNAs by HER2-positive tumour cells. To this end we modified the exosomes to exhibit on their surface a peptide that bound the exosomes to the HER2 on the surface of cancer cells. In consequence, we significantly enhanced the uptake of exosomes carrying the miRNAs directed against HER2 by HER2 positive cells.

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PF08.11

Systematic characterization of ovarian cancer-derived exosomes unveil miRNAs interfering with CD8 + T cell activation

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Introduction: CD8+ tumour-infiltrating lymphocytes (TIL) have been widely reported to correlate with cancer patient survival, including ovarian cancer. Even with the presence of TILs, immunotherapy has limited success in ovarian cancer. Understanding the interaction between CD8+ TIL and tumour cells is thus important. Our hypothesis is that tumour-derived exosomes are released and taken up by CD8+ TIL such that specific miRNAs contained within modulate physiological processes that inhibit CD8 + T cell activation. We aim to identify miRNAs carried in tumour-derived exosomes that inhibit CD8 + T cell activation in ovarian cancer.
Methods: We purified exosomes from nine ovarian cancer cell lines and stocked in high concentration. Interferon-gamma (IFN-gamma expression screening was performed after 3 days of co-incubation of tumour derived exosomes, CD8 + T cells, and activators in conditioned medium. Cell counts and viability were tested by trypan blue staining at day 0 and day 3. RNA-seq for exosomes were generated to identify miRNAs critical in differentiation effects on CD8 + T cell activations. MicroRNA target matching uncovered target mRNAs while enriched pathway analysis predicted potential signalling pathways involved.
Results: Our IFN-gamma screening results indicated the exosomes exhibit different behaviours in interfering CD8 + T cell activation owing to different donors. Exosomes derived from PEO.1 and OVCA432 cells have consistent polarized results in IFN-gamma expression. Exosomes derived from PEO.1 remained a low IFN-gamma expression and from OVCA432 stayed at relatively high level. Small RNAs profiling analysis between the two cell lines identified 56 miRNAs ($p < 0.05$), and 13 miRNAs have been reported with validated targeting information, and 10 out of 13 have targets involved in immune signalling. 210 mRNA targets were uncovered by target matching. CMap search identified complex connections among mRNAs with the top 20 enriched pathways actively involved in cell cycle and immune related behaviours.
Summary/Conclusion: Our IFN-gamma screening identified crucial miRNAs in ovarian cancer exosomes interfering CD8 + T cell activation. Computational modelling on both experimental and public multi-omics datasets predicted promising signalling pathways of tumour-immune crosstalk for functional validation.
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PF08.12

Irradiation of breast cancer cells alters the quality of DNA cargo in the exosomes that they produce

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Introduction: Irradiation of breast cancer cells with an immunogenic dose (8GyX3) leads to accumulation of cytosolic DNA that is sensed by cGAS leading to interferon type I (IFN-I) signalling via cGAS/STING pathway [1–3]. We previously showed that tumour-derived exosomes (TEX) secreted by irradiated (8GyX3) (RT-TEX) but not untreated (UT-TEX) TSA carcinoma cells carry DNA that stimulates the production of IFN-I in recipient dendritic cells (DC) via the cGAS/STING pathway [4]. Moreover, mice vaccination using RT-TEX, but not UT-TEX, elicited anti-tumour immune response inhibiting tumour growth [4].

Here, we hypothesized that the differential ability of RT-TEX and UT-TEX to activate IFN-I in recipient DCs is due to qualitative differences in DNA cargo of RT-TEX compare to UT-TEX.
Methods: The length of DNA purified from TEX and from the cytosolic fraction of TSA cells was measured by Agilent Bioanalyzer. The DNA cargo of TEX was analysed by whole-genome sequencing (WGS) and whole-genome bisulphite sequencing. The percentage of methylation of total DNA in TSA cells was quantified by 5-methyl cytosine DNA Elisa kit.
Results: DNA fragments with size between 60 and 250 bp were enriched in RT-TEX compared to UT-TEX, as well as in the cytosolic fraction of irradiated compared to mock-treated TSA cells. WGS revealed that the entire genome was represented in TEX DNA cargo, regardless of RT. More than 99% of TEX DNA was of nuclear origin, but mitochondrial DNA was increased in RT-TEX. Interestingly, we found that RT decreases the level of methylation in both exosomal and total DNA in TSA cells compared to the controls.
Summary/Conclusion: These data support the hypothesis that immunogenic RT alters some characteristics of the exosomal DNA cargo, mirroring molecular changes occurring in parent irradiated breast cancer cells. The enrichment in DNA fragments of 60–250 bp in RT-TEX is intriguing considering that cGAS is optimally activated by DNA in this length range [5]. We are currently investigating which features of the cargo DNA that differ between UT-TEX and RT-TEX may explain the differential ability to induce IFN-I pathway

activation in recipient DCs. The identification of a DNA signature associated with the ability of TEX to activate the cGAS/STING pathway could provide a circulating biomarker of the RT-driven immunogenic tumour response.

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PF08.13

IRE1 inhibition modulates immune phenotype of triple negative breast cancer cells and cancer-derived extracellular vesicles

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Introduction: Triple negative breast cancer (TNBC) is among the most difficult cancer subtypes to treat and continues to cause a high number of cancer-related deaths annually. Extracellular vesicles (EVs) transfer cell type-specific cargo and have important implications in disease initiation, therapy and outcome. Upon treatment of cancer cells with low-dose chemotherapy, released EVs are able to transfer phenotypic traits to other cancer cells. New treatment strategies for TNBC, like inhibitors of the ER stress pathway (IRE1) might impact on EV biogenesis, cargo delivery and response of cells in the cancer microenvironment. Our aim is to identify immune modulatory alterations in breast cancer cells and cancer derived EVs upon treatment with inhibitors of the ER stress pathway.

Methods: Human TNBC cell lines were treated with IRE1 inhibitor MKC8866 and cells were analysed for immune modulatory surface markers, like HLA-I, B7-H molecules and different integrins. Mitochondrial and lysosomal activities were investigated by the use of a Mito- and Lysotracker and analysed by ImageStream (ISX) technology. Extracellular vesicles were isolated from cell culture supernatants by sequential centrifugation, quantified by Nanoparticle tracking (NTA) and characterized by Exosome bead array. Single EV analysis of total cell free supernatants and of isolated EVs was performed by ISX and marker positive EVs were quantified for absolute fluorescence signals and total amount by objectives/ml. EV uptake into T cells was investigated by the use of different EV labelling strategies.

Results: Several immune relevant surface markers (HLA-I and CD54) are downmodulated by IRE1 inhibition across different cell lines. Cell surface expressed CD63 and B7-H3 show cell line specific downmodulation profiles upon IRE1 inhibitor treatment. Other immunomodulatory marker such as B7-H1 and B7-H4, integrin CD29, cell adhesion-promoting CD146 and stemness/metastasis marker (CD44 and SSEA) are unaltered on IRE1 treated breast cancer cells. Cancer cell derived EVs were tetraspanin positive (CD9, CD63, CD81), similar in number and showed differential expression of immune markers upon IRE1 treatment. Mitochondrial and lysosomal activities were unaltered under IRE1 inhibition, whereas cell proliferation was diminished. No breast cancer-derived EV uptake of externally labelled EVs into healthy T cells could be detected.

Summary/Conclusion: Ongoing analyses focus on the multicolour analysis of multiple markers on single EVs by imaging flow cytometry and on the functional impact of cancer derived EVs on T cells delivered by EV receptor binding.

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PF09: EVs in Blood Disorders

Chair: Uta Erdbrügger – University of Virginia

Chair: Larry Harshyne – Thomas Jefferson University

PF09.01

Comparison of three isolation protocols to search extracellular vesicles signature in sickle cell disease patients

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Introduction: Sickle cell disease (SCD) is an inherited disorder characterized by chronic haemolysis and continuous activation of different cell types. Extracellular Vesicles (EVs) were described to be at increased levels in SCD patient's plasma compared to healthy subjects and were associated with several clinical manifestations such as leg ulcers and stroke. SCD patient's plasma has increased concentrations of haem, free-Hb and other proteins and lipoproteins as chronic haemolysis consequence. Here, we report the comparison of three mostly used isolation protocols to search EV signature in SCD patient's plasma by flow cytometry.
Methods: Blood samples were obtained from SCD patients (n = 3) following Wisgrill et al., (2016) protocol. Three different EV isolation protocols were used: differential centrifugation (DC), ultracentrifugation (UC) and size-exclusion chromatography (SEC). Lactadherin and calcein-AM were used to detect phosphatidylserine (PS)+ vesicles and membrane integrity, respectively. Platelet-derived EVs (PEVs), endothelial-derived EVs (EEVs), leucocyte-derived EVs (LEVs) and monocyte-derived EVs (MEVs) were quantified. Silica beads were used to define EVs gate and samples were acquired in the CytoFLEX cytometer platform.
Results: The quantification of PEVs in UC, DC and SEC samples was, respectively, 31x106, 8,5x106 and 9,7x106 events/mL mean, EEVs was 6,4x106, 1 × 106 and 4,3x106 events/mL mean, LEVs was 2x106, 6 × 105 and 1,3x106events/mL mean and MEVs 5,7x105, 6,5x105 and 3,7x105 events/mL mean. UC samples demonstrated a higher concentration of EVs, which could be more useful to functional studies than DC and SEC, however, it took more time to separate than DC. DC was the fastest method to separate EVs from plasma, being useful to study large patients cohorts, but showed the smallest overall number of EVs. SEC also demonstrated high capability to detect EVs in plasma and the possibility of obtaining a purer sample, although it is the most expensive and time-consuming method among all tested. All EVs populations were detected in the three protocols tested.
Summary/Conclusion: In summary, all protocols tested were efficiently to detect EVs in SCD patient's plasma and the definition of the best protocol may vary based on the research aim and time and budget available.
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PF09.02

Extracellular vesicles from patients with sickle cell disease disrupt gap junctions

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Introduction: Aberrant cell-cell interactions involving the endothelium are central to the pathophysiology of sickle cell disease (SCD), including acute chest syndrome (ACS), a deadly and unpredictable complication. We previously demonstrated that the plasma of SCD patients contains increased circulating small extracellular vesicles (EVs) compared to controls and that those vesicles can disrupt endothelial integrity in vitro by affecting adherens junctions and VE-cadherin. The current study was designed to examine the effects of those EVs on other cellular junctions including tight (zonula occludens 1, ZO-1) and gap junctions (connexin43, Cx43) and to test the hypothesis that the junctions would be more severely affected by EVs isolated from patients during an episode of ACS than by ones isolated from the same patient at baseline.
Methods: We identified subjects with SCD in our biobank who had plasma isolated at baseline and at the beginning of an admission for ACS. EVs were isolated from platelet free plasma using established methodologies. To determine the effects on endothelium, cultures of human microvascular endothelial cells were treated with EVs for 48 h and studied by immunofluorescence, immunoblotting and RT-qPCR. Gap junction-mediated

intercellular communication was assessed following microinjection of Lucifer yellow and neurobiotin.
Results: The distribution and abundance of ZO-1 at the plasma membrane were minimally affected by SCD EVs. While baseline EVs did not affect the distribution of Cx43, EVs isolated during an episode of ACS caused loss of Cx43 from the plasma membrane. The integrated intensity of Cx43 membrane staining was decreased by ~20% following treatment with ACS EVs. Cx43 protein decreased on average by 32%, Cx43 mRNA levels by 21% and neurobiotin transfer by 67–94% in cells treated with ACS EVs, compared to baseline EVs.
Summary/Conclusion: Circulating EVs in SCD affect multiple components of endothelial junctions. Gap junctions composed of Cx43 are the most sensitive of the cell-cell junctions, since their abundance and function are reduced by ACS EVs even when the endothelial monolayer appears intact. Cx43-mediated intercellular communication may be an early and sensitive event in the endothelial disturbance caused by EVs in SCD patients.
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PF09.03

The effects of platelet concentrate storage time on extracellular vesicle interactions associated with fibrin clot formation in-vitro

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Introduction: Platelet concentrates (PCs) have been utilised for decades to prevent bleeding in thrombocytopenic patients and to stop active bleeding. The storage of PCs however is a logistical challenge due to the limited 7 day shelf life under standard conditions. During storage, platelets undergo a number of mechanical and biochemical changes contributing to the short shelf life of a PC. These changes are collectively known as the platelet storage lesion. Platelet extracellular Vesicles (PEVs) are known to increase throughout PC storage, due to an increase in platelet activation. As PEVs have previously been shown to be pro-coagulant and increase in Annexin V binding over PC storage. The aim was to investigate the effect of PC storage time on extracellular vesicle interactions on fibrin clot formation.
Methods: PCs were sampled on alternate days up to 10 days of storage and centrifuged to achieve acellular plasma. The plasma was subjected to ultracentrifugation (100,000xg) to pellet EVs. The size and concentration of EVs was assessed using Nanoparticle tracking analysis software, followed by a western blot to confirm EVs were of platelet origin. The PEVs were added at a fixed number to a control pooled plasma sample with added thrombin and tissue plasminogen activator. The time to clot and 50% lysis time were recorded by using the turbidometry of the plasma over time.
Results: EVs isolated from the PC were confirmed to be of platelet origin by western blot using CD41 as a marker of platelet origin and CD9 as an EV marker. PEVs caused a significant increase effect on the fibrin clot formation ($P < 0.001$) when compared to the control plasma. PEVs also had a significant effect ($P < 0.01$) on the fibrinolysis time, extending the time taken to lyse the clot. The time point during storage of PEV isolation had no significance ($P > 0.05$) on the fibrin clot.
Summary/Conclusion: PEVs from PCs significantly enhance fibrin clot formation and extend clot lysis time, irrespective of PC storage time. Trauma patients could benefit from a PC unit with high PEV numbers; however, more research into the potential negative effects of high PEV numbers, including surplus platelet activation in oncology patient groups, is required.
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PF09.04

Characterization of miRNA from serum derived exosomes in a mouse tibia fracture model of Complex Regional Pain Syndrome

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Introduction: Complex regional pain syndrome (CRPS) is a debilitating chronic disease that occurs after trauma to the periphery and is intimately associated with nerve injury. Its presentation is often described as an injury that is disproportional to the inciting event and manifests neuropathic pain, systemic inflammation, and immune dysregulation. Owing in part to our poor understanding of disease aetiology, current treatments for CRPS are insufficient and as a

disease of exclusion there is a lack of quantitative diagnostic markers. Exosomes are small extracellular vesicles (sEVs) 30–100 nM in size which provide a means of cellular communication through their cargo molecules (protein, miRNA, mRNA, lipids), and have demonstrated promise in uncovering mechanisms of disease manifestation and identifying potential diagnostic markers. We have shown previously that CRPS patients have differential expression of several miRNAs in serum derived sEVs as compared to healthy controls, but little is known on how this compares to the established mouse tibia fracture model of CRPS.

Methods: Mice undergoing fracture were anesthetized and subjected to a unilateral tibia fracture followed by casting of the injured limb. After confirming the establishment of pain hypersensitivity, serum samples were collected from fracture model and control mice three weeks post-injury. sEVs were isolated by differential centrifugation and characterized using nanoparticle tracking analysis, transmission electron microscopy and western blotting. RNA-seq analysis is being performed to identify differentially expressed miRNAs.

Results: Nanoparticle tracking analysis showed no significant difference in the number or size of sEVs present in the serum from the fracture model and control mice. RNA-seq is ongoing and differential miRNA expression in sEVs from fracture model will be compared to control samples. Comparative studies identifying miRNAs that are common between CRPS patients and the rodent model will facilitate the development of correlational outcomes between preclinical and human studies.

Summary/Conclusion: Identification of similarities and differences between CRPS patients and animal models will aid in directing future studies at clinically relevant aspects of CRPS aetiology and identifying potential diagnostic markers for CRPS patients.

PF09.05

Extracellular Vesicle-based liquid biopsy in Acute Myeloid Leukaemia: a reliable source of residual disease biomarkers?

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Introduction: Acute myeloid leukaemia (AML) is an haematopoietic stem cell disorder with a poor 5-year survival rate. Monitoring of Measurable Residual Disease (MRD) in AML patients receiving chemotherapeutic treatment is useful to assess therapy response and predict relapse. Indeed, many different leukaemia associated immunophenotypic protein markers (LAIPs) are presently useful to detect MRD. Nevertheless, their analysis currently requires invasive bone marrow aspirates, thus severely hindering real-time monitoring of the disease. Therefore, alternative peripheral blood-based methods are highly desirable for an easy, real-time and cost-effective monitoring of AML progression.

This work AIMs was to assess the feasibility of a peripheral blood EV-based liquid biopsy method for AML disease monitoring, based on the detection of LAIPs with a known negative impact on the prognosis of AML.

Methods: The profile of EVs isolated from 12 paired samples from AML patients' blood plasma collected at diagnosis, complete remission (and some at relapse) was compared and correlated with clinical data. For that, a size-exclusion chromatography (SEC) method was optimized to isolate the circulating EVs from the blood plasma. The EVs of the 12 paired AML patients' blood samples were then characterized according to their size (DLS/NTA), morphology (TEM), protein-to-lipid ratio (Lowry/Sulpho phosphovanillin assay), surface charge (Zeta-Sizer) and protein cargo (Western blot).

Results: SEC allowed the isolation of size-resolved plasma-derived EVs from the peripheral blood of AML patients. Isolated EVs had a size ranging from 30 nm to 300 nm with an intact morphology, expressing EV-associated markers such as HSP70, CD63, CD81 and CD9. Size-resolved EVs also had a differential expression of Mitofilin, Actinin-4, Syntenin-1 and Annexin-XI proteins. Several LAIPs were detected in the isolated EVs and their relative abundance changed throughout the stage of the disease.

Summary/Conclusion: Our preliminary data shows that AML patients' circulating EVs carry relevant immunophenotypic protein markers, which might predict AML clinical outcome.

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PF10: Cell-EV Interaction, Uptake, and Fusion

Chair: Terri F. Bruce, MD, PhD – Clemson University

PF10.01

Profiling mRNAs of parental prostate cancer cells with different phenotypes and their daughter extracellular vesicles using the NanoString low RNA input nCounter assay

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Introduction: Cell plasticity regulated by the balance between the epithelial-to-mesenchymal transition (EMT) and MET is critical in the metastatic cascade. Extracellular vesicles (EVs) may play an important role in this balance by shuttling molecular cargos into recipient cells. This study aims to evaluate the feasibility of profiling mRNAs of parental prostate cancer (PCa) cells with different phenotypes and their daughter EVs using the NanoString low RNA input nCounter assay.
Methods: PC3-Epi and PC3-EMT cell lines representing epithelial and mesenchymal phenotype, respectively, were generated from original PC3 cell line. The cell culture supernatant was first pre-cleared for any dead cells and debris by centrifugation at 1000 × g for 20 min. Without disturbing the pellet, the supernatant was then transferred to a fresh ultracentrifuge tube and centrifuged at 10,000 × g for 20 min at 4°C. The remaining supernatant was then centrifuged to isolate the EVs at 100,000 × g for 120 min at 4°C. The EVs pellet was further washed in 1× PBS followed by a second centrifugation at 100,000 × g for 120 min at 4°C. The final EVs pellet was resuspended in 1× PBS for subsequent characterization (transmission electron microscopy, nanoparticle tracking analysis and Western blot) and nCounter assays. The total RNA of cells and their daughter EVs were assayed by the nCounter PanCancer Progression Panel to determine expression of 770 selected mRNAs. The NanoString nCounter Low RNA Input Kit with the multiplex 770-gene primer pool was used for the pre-amplification of mRNA and overnight hybridization with the PanCancer Progression panel. Each sample type was submitted to the assay in biological triplicate.
Results: When comparing all 12 samples, Eisen Cluster analysis separated all the cells and all EVs into two groups, regardless of their phenotypes. In subgroup analysis, the expression patterns between PC3-Epi and PC3-EMT cells were significantly different. CLEC2B, KDR, CRIP2, IL13RA2, CC2D1B were significantly upregulated in PC3-EMT cells, while CXCL8, EPCAM, ESRP1, TGFB2, CDH1, S100A14, OVOL2 were significantly downregulated in PC3-EMT cells. The expression patterns between PC3-Epi and PC3-EMT EVs were also significantly different. TBX1, CAV1, COL4A1, SLC35A3, MYC, ITGB2, TIMP4, CAMK2B, PTGDS, P3H2, ITGB6, VIM, STAT3 were all significantly downregulated in PC3-EMT cell derived EVs.
Summary/Conclusion: The NanoString low RNA input nCounter assay can provide reliable mRNA expression profiling of EVs. The mRNA expression patterns are very different between cells and their daughter EVs. Both cells and EVs with different phenotypes have different gene expressions.

PF10.02

Cancer cell-derived EVs containing alphaV beta6 Integrin regulate CD163, IL-6 and IL-10 levels in peripheral blood mononuclear cells

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Introduction: Extracellular vesicles (EVs) mediate communication in the tumour microenvironment and play an important role in cancer progression. Previously, we have shown the enrichment of alphaV beta6 integrin in small extracellular vesicles (sEVs) isolated by differential ultracentrifugation and iodixanol density gradient from PC3 prostate cancer cells. We have also shown in the past that alphaV beta6-positive sEVs induce peripheral blood mononuclear cell (PBMC) polarization by increasing the expression of pro-tumorigenic M2 markers, such as CD163 and CD204. Finally, we have demonstrated that down-regulation of alphaV beta6 integrin up-regulates the STAT1-Interferon Stimulated Genes (ISGs) pathway in cancer cells and in sEVs released by them.
Methods: In order to investigate whether prostate cancer cell-derived vesicular STAT1 has a causal effect in PBMC polarization, we down-regulated alphaV beta6 and STAT1 in prostate cancer cells derived sEVs using siRNA as well as CRISPR-Cas9 strategies. The sEVs

isolated from these cells were used to analyse M2 polarization by measuring the levels of CD163 in PBMC.
Results: The results show that sEVs lacking alphaV beta6 inhibit CD163 levels in PBMC in a STAT1-independent manner. Analysis of cytokines released by PBMC upon incubation with sEVs lacking alphaV beta6, show that PBMC selectively up-regulate the levels of IL-10 and IL-6, which are predominantly anti-tumorigenic cytokines. In contrast, sEVs lacking alphaV beta6 do not upregulate pro-angiogenic cytokines, such as VEGF.
Summary/Conclusion: These findings suggest that cancer cell-derived sEVs containing alphaV beta6 integrin promote a pro-tumorigenic PBMC phenotype in the tumour microenvironment by regulating CD163, IL-6 and IL-10 levels.
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PF10.03

T cell activation by allogeneic EVs and allogeneic MHC cross-dressed cells in vitro and in vivo

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Introduction: The recognition of donor-MHC molecules by recipient T cells triggers the immune response leading to rejection of allografts. Our recent studies have documented the presence of high numbers of recipient APCs displaying donor-MHC molecules (cross-dressed) on their surface in the lymphoid organs of mice after skin, heart or pancreatic islet transplantation. In addition, we have reported that acquisition of allogeneic MHC molecules by host APCs (MHC cross-dressing) is mediated by donor-derived extracellular vesicles (EVs) trafficking through blood and lymphatic vessels (Marino et al. Science Immunology, 2016). In the present study, we investigated the ability of allogeneic EVs and allo-MHC-cross-dressed cells to initiate a T cell alloresponse in vitro and in vivo.
Methods: EVs were isolated (using differential centrifugation) from BALB/c Bone Marrow Derived Dendritic Cells (BMDCs). These EVs were used to cross-dress B6 splenocytes in vitro. The transfer of donor MHC class I and II on B6 cells was analysed by imaging flow cytometry. Next, T cells from B6 mice were cultured in vitro with either allogeneic BMDC-derived BALB/c EVs or B6 spleen cells cross-dressed with allogeneic BALB/c MHC. Alternatively, 2 × 109 BALB/c or B6 BM derived EVs or 20 × 106 BALB/c BM cells were injected IV to B6 mice. In both cases, the T cell response was assessed by activation markers detection, INFg production and cell proliferation.
Results: APCs cross-dressed with allogeneic MHC molecules can trigger a pro-inflammatory direct alloresponse by T cells in vitro and in vivo. On the other hand, allogeneic EVs alone were only able to induce early T cell activation but not proliferation in vitro. Furthermore, injection of mice with allogeneic EVs alone could induce some but suboptimal alloresponse in vivo and only when administered with complete Freund's adjuvant.
Summary/Conclusion: Blocking donor EVs release and subsequent recipient APC cross-dressing may represent a promising target to selectively inhibit anti-donor T cell inflammatory responses thus achieving long-term allograft survival.
Funding: R01DK115618.

PF10.04

Antifungal antibiotic activity of outer membrane vesicles from adherent Lysobacter enzymogenes C3 against therapeutic and biocontrol targets.

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Introduction: Lysobacter enzymogenes is a predatory gram negative bacterial species being studied for biocontrol activity against fungi. Planktonic L. enzymogenes C3 produces outer membrane vesicles (OMV) harbouring small molecule antifungal antibiotics (Meers et al. 2018). We show here that the more biologically relevant surface-associated C3 exerts remote antifungal activity via OMV as well. The results have important consequences regarding the natural mechanism of biocontrol of fungal pathogens by C3 as well as isolation and delivery of therapeutically relevant antifungal compounds.
Methods: OMV were isolated from scraped adherent C3 culture on agar by similar methods to Meers et al 2018. OMV were stained in some cases with fluorogenic SYTO 9 DNA stain for microscopic observation.

Fungal growth was monitored via turbidity readings in liquid culture or photomicrographs on agar. C3 was also grown on polycarbonate filter membranes with defined pore sizes to monitor growth of fungal cells on the opposite side. Vesicles were also labelled with an amine-reactive probe Alexa-555 and washed 4x by sedimentation. Binding of labelled OMV to fungal cells was observed by epifluorescence microscopy.

Results: SYTO 9-stained vesicles from surface-adherent C3 were similar to previously observed ~130 nm vesicles (Meers et al., 2018). The isolated vesicles inhibited growth of Saccharomyces cerevisiae or Candida albicans in liquid cultures at similar potency and were active against the filamentous species Fusarium subglutinans grown on agar or maize leaves. C3 cultures grown on filters with 400 nm pore size but not 30 nm were able to inhibit the hyphal growth of F. subglutinans on the opposite side. Similarly C3 on filters with a 200 nm pore size were able to inhibit growth of C. albicans. Observation of fluorescently-labelled C3 OMV after interaction with C. albicans showed binding specifically to hyphae or pseudohyphae and for F. subglutinans to the growing hyphal tips.

Summary/Conclusion: The OMV of C3 specifically bind and inhibit the growth of fungal hyphae of various species without direct C3 cell contact. These data elucidate mechanisms of biocontrol and suggest strategies for production of therapeutic antifungal antibiotics. Meers et al. (2018) Appl. Env. Microbiol. 84(20), e01353-18.

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PF10.05

Elucidating the cellular uptake and tissue distribution mechanism of cell derived vesicles, a novel therapeutic carrier

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Introduction: Cell derived vesicles (CDVs) are emerging as a novel therapeutic carrier. One of the crucial factors in the development and therapeutic applications of CDVs is to understand the precise mechanism by which vesicles find and enter the target cells. In this study, we aim to investigate the uptake mechanism of CDVs produced from natural killer (NK) cells using a manufacturing process established at MDimune Inc. Both in vitro uptake assay and in vivo distribution analysis were performed to provide precise insights into how CDV exert its effect at the cellular level.

Methods: NK cells were mainly used to produce CDVs. Breast cancer cells, BT549, and human and rodent endothelial cells, with a varying degree of ICAM-1 expression, were used to determine the effect of LFA-1 expressed on the surface of NK-CDVs in cellular uptake using FACS and confocal imaging analysis. Next, various inhibitors for uptake pathways, such as phagocytosis, dynamin dependent endocytosis, and receptor mediated endocytosis, were used to understand the underlying mechanism of cellular uptake of CDVs. Biodistribution profile of CDVs were characterized using both normal and tumour xenograft models by IVIS imaging.

Results: Using a recently established manufacturing process, we demonstrate that NK-CDVs can efficiently enter the target cells. This study also shows that the cellular uptake depends on the molecular interaction between ICAM-1 and LFA-1. In vivo distribution profile of NK-CDVs are also assessed using various tumour models. Furthermore, we present a cellular uptake mechanism involved in the entrance of CDVs into the target cells.

Summary/Conclusion: This study demonstrates that the CDVs produced at the manufacturing scale can be easily taken up by cells via specific cellular pathways. This finding will facilitate the development of more efficient therapeutics for cancer and other debilitating diseases.

PF10.06

Myofibroblasts-derived microvesicles increase dermal fibroblasts collagen production through PLGF-1

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Introduction: A proper wound healing of the skin involves angiogenesis, extracellular matrix (ECM) remodelling and re-epithelialization. These three mechanisms require well-organized interactions between different cell populations. A key role in this context is played by myofibroblasts (Wmyo), a cell population mainly differentiated from dermal fibroblasts. These cells contract wound edges and synthesize new ECM. We previously showed that myofibroblasts predominantly produces microvesicles (MVs) and can favour angiogenesis. However, proteomic analysis of MVs from our previous studies indicated some molecules that can potentially be implicated in ECM remodelling. In this study, we evaluated whether myofibroblasts-derived MVs could affect dermal fibroblasts who are highly responsible for ECM regulation.

Methods: MVs were isolated by differential centrifugation of medium collected from Wmyo cells. Number and size of MVs were characterized by transmission electron microscopy and NanoSizer. Multiplex assays of 45 cytokines were evaluated in MVs samples, Wmyo and MVs-depleted medium. To examine the interaction of MVs with fibroblasts, we evaluated the uptake of MVs isolated from Wmyo transduced with a fluorescent protein. We then treated fibroblasts cultures with MVs or a selected cytokine for 5 days and evaluated collagen production. Lastly, we neutralized the selected cytokine in MVs samples before evaluating collagen production.

Results: PLGF-1 was the cytokine detected in MVs samples in large amount (0.88 ± 0.63 pg/μg proteins in MVs). Fibroblasts treated with MVs or PLGF-1 significantly stimulated pro-collagen I level production with a fold change of 1.80 ± 0.18 and 2.07 ± 0.18. Moreover, the neutralization of PLGF-1 present in MVs significantly inhibited the production of pro-collagen I by dermal fibroblasts.

Summary/Conclusion: Our results indicated that MVs influence fibroblasts pro-collagen production through PLGF-1 signalling.

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PF10.07

Structural insights on fusion mechanisms of extracellular vesicles with model plasma membranes

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Introduction: Extracellular vesicles (EVs) represent a potent intercellular communication system. While their functional biological properties are more and more investigated, the biophysical aspects of their interaction with recipient cells are often overlooked. Small size (30 to a few hundred nanometres in diameter) of EVs and their heterogeneous origin still pose a great challenge for their isolation, quantification and biophysical/biochemical characterization. In particular the complex network of interactions between differently classified EVs and recipient cells remains to be further revealed. Here we deeply investigate the fusion mechanism between EVs and a model plasma membrane system by an interplay of different structural/morphological techniques to get a molecular description of the interaction helping to clarify the role of different membrane compartments on the EVs uptake mechanism.

Methods: Standardized protocols and Good Manufacturing Practice conditions were employed to derive highly stable vesicles of defined size and reproducible molecular profiles from Umbilical Cord multipotent Mesenchymal Stem (Stromal) Cells. After a thorough biophysical and biochemical characterization of EVs non-contact liquid imaging Atomic Force Microscopy (AFM) and, in parallel, Neutron Reflectometry (NR), as well as Small Angle Neutron Scattering (SANS) experiments were performed on EVs to determine their interaction with model plasma membranes in the form both of supported lipid bilayers and suspended unilamellar vesicles of variably complex composition.

Results: We observed that EVs tend to fuse with the model membranes with a preferential interaction with the external layer of the fluid membrane. Moreover we revealed a stronger interaction with the liquid ordered domains, strengthening the hypothesis of a critical role of lipid rafts in fusion mechanisms.

Summary/Conclusion: Our results on the analysis of the interaction of EVs with artificial lipid membranes could provide insights on the internalization mechanisms of EVs. The approach shown here can be further extended to convey incremental complexity, adding glycolipid and membrane proteins to the model lipid bilayers. This approach combined with data on the specific biological function of each EV subpopulation as retrieved by standard functional assays, will turn useful to select the crucial molecular aspects of EVs internalization by cells.

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PF11: EVs as Intrinsic Medicines

Chair: James G. Patton, PhD – Vanderbilt University

Chair: Chantal Boulanger – Université de Paris, Paris Cardiovascular Research Centre

PF11.01

Platelet enhanced plasma: a novel extracellular vesicle enriched resuscitation fluid?

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Introduction: Platelet-derived extracellular vesicles (PEV) are the most abundant circulating extracellular vesicle (EV) and exhibit platelet-like properties, hence the original term "platelet dust". Direct phenotyping of EV surface markers within biofluids is challenging often requiring time-intensive purification steps that can significantly alter resultant EV population characteristics. The ExoView™ (NanoView Biosciences) specifically captures EV sub-populations and was used to characterise the EV content of platelet free plasma (PFP) and a potential novel haemostatic agent designed for the treatment of severe trauma and haemorrhage, Platelet Enhanced Plasma (PEP).
Methods: Freeze-thaw cycling of platelet rich plasma/expired platelet concentrates was followed by centrifugation to remove platelet remnants and yeilded PEP. PFP controls were prepared by double centrifugation (2000 g for 20 minuntes followed by 13,000 g for 2 minutes). Rotational Thromboelastometry (ROTEM) and Calibrated Automated Thrombography (CAT) were used to assess EV driven haemostasis and thrombin generation. A dilutional and hypothermic model of coagulopathy was designed to assess PEP. EV capture arrays comprised of anti-CD41, anti-CD63, anti-CD81 and anti-CD9 were used (ExoView™, NanoView Biosciences). Captured vesicles underewent interferometric imaging and were quantified, sized and further probed with fluorescent tetraspanin markers, Annexin-V and intravesicular markers.
Results: PEP is highly procoagulant, exhibits enhanced thrombin generation and can restore haemostasis in a dilutional model of coagulopatic whole blood. PEP can be generated from expired platelet concentrates, potentially allowing for upscalable production. The predominant vesicle population were PEV with a large CD41/CD9 population that contained a smaller subpopulation of phosphatidyserine positive procoagulant vesicles. PFP as expected has a much lower number of PEV and a CD81 positive EV population.
Summary/Conclusion: PEP is a unique resuscitation fluid containing high PEV levels for the potential treatment of severe trauma and haemorrhage. Exoview measurements can be performed in unpurified plasma and may be useful for measuring circulating EV in health and disease.
Funding: Defence and Security Accelerator, DSTL

PF11.02

Therapeutic effect of exosomes in mice model of Autism

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Introduction: During the recent decade, exosomes that derived from mesenchymal stem cells (MSC-exo) have been spotlighted as a promising therapeutic target for various clinical indications, including neurological disorders. We have previously shown that intranasal administration of MSC-exo, cross the BBB and significantly ameliorate autistic-like behavioural phenotype in BTBR and SHANK3 animal models of autism, representing a potential therapeutic strategy to reduce symptoms of autism spectrum disorder (ASD). Our objective is to study the mechanism of action and the cellular pathways in which the MSC-exo activate their target, we performed RNA sequencing analysis of primary neurons isolated from SHANK3 mice treated with MSC-exo.
Methods: Primary neuronal cell cultures were prepared from newborn SHANK3 homozygotes mice model of autism. Cultures were treated with MSC-exo (10^7 particles/ul), isolated from human adipocytes, followed by RNA sequencing. The alterations in gene expression between the treated and intact neurons were analysed for gene ontology and pathways and were also

compared to proteomics analysis of the MSC-exo in order to find regulatory proteins that may lead to these differences.
Results: Bioinformatic analysis revealed several up-regulators proteins that might be responsible for the increase in anti-inflammatory and protective factors seen in the mice neurons treated with MSC-exo. One of them is BDNF which is known as an essential growth factor responsible for neuroprotection and neurogenesis. Importantly, no difference in the genetic expression of cancer-related genes was identified following MSC-exo treatment indicating for their safety.
Summary/Conclusion: Our data suggest that adipocyte-derived MSC-exo carry therapeutic potential in ASD via alternation in gene-expression related mainly to immuno-modulation, reduce neuroinflammation and increase neuroprotection and neurogenesis. The beneficial effects of the exosomes treatment in mice models is being translated into a novel, easy to administer, a therapeutic strategy to reduce the symptoms of ASD.
Funding: Stem Cell Medicine, LTD. Israel

PF11.03

miRNAs enriched in extracellular vesicles isolated from plasma- and serum-based autologous blood-derived products for osteoarthritis therapy

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Introduction: Autologous blood-derived products gain increasing focus in regenerative medicine, especially in orthopaedics and osteoarthritis therapy. This disease is characterised by cartilage degradation and inflammation among other symptoms, which are targeted by conventional therapies, but genuine cartilage regeneration is rarely achieved. Citrate-anticoagulated platelet rich plasma (CPRP) is often clinically applied to stimulate soft and hard tissue healing. Recently, cell-free alternatives to CPRP including hyperacute serum (hypACT™ serum) have been developed. CPRP and hypACT™ serum contain specific profiles of growth factors, however, they also contain extracellular vesicles (EVs) that harbour signal molecules including miRNA.
Methods: EVs were enriched by ultracentrifugation (UC) followed by size exclusion chromatography (SEC) to obtain purified EVs. Particle size and concentration of each fraction was measured by nanoparticle tracking analysis (NTA). Fractions with the highest amount of particles were pooled and concentrated via UC, before miRNA expression was assessed via screening with a panel of 372 miRNA-specific primer pairs by RT-qPCR. Presence of EVs was confirmed by cryo-electron microscopy.
Results: The EV concentration tended to be lower in hypACT™ serum than in CPRP as determined via NTA. Similarly, lower diversity of miRNA species was found in hypACT™ serum than CPRP EVs. Around 90% of detected miRNAs were found in both blood products, whereas only 30% of miRNAs were shared between EVs from CPRP and hypACT™ serum. While miRNAs such as miR-101 were consistently depleted in EVs compared to the corresponding blood product, others like miR-27a were in enriched in hypACT™ EVs, but not CPRP EVs, indicating release of specific miRNAs via EVs in response to clotting.
Summary/Conclusion: Although the purification resulted in high loss of EVs, we identified specific miRNAs enriched in EVs from CPRP and hypACT™ serum. Their functional spectrum with respect to osteoarthritis therapy focuses on inhibition of inflammation, inhibition of tissue remodelling via matrix degrading enzymes as well as preventing senescence. This renders blood product derived EVs as interesting candidates for in vitro and in vivo testing with respect to cartilage regeneration.
Funding: The work was jointly supported by the European Fund for Regional Development (EFRE) and the Fund for Economy and Tourism of Lower Austria, grant number WST3-F-5030664/003-2017.

PF11.04

Protective role of shiitake mushroom-derived exosome-like nanoparticles in D-galactosamine and lipopolysaccharide-induced acute liver injury in mice

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Introduction: Fulminant hepatic failure (FHF) is a rare, life-threatening liver disease with poor prognosis. New therapeutic interventions are urgently needed to treat this disease. Administration of D-galactosamine (GalN) and a low dose of lipopolysaccharide (LPS) triggers acute liver damage in mice, which simulates many clinical features of FHF in humans and therefore is widely used to investigate the molecular mechanisms and potential therapeutic interventions of FHF. Recently, suppression of the nucleotide binding domain and leucine rich repeat related (NLR) family, pyrin domain containing 3 (NLRP3) inflammasome was shown to alleviate the severity of LPS/GalN-

induced liver injury in animal models. Therefore, the goal of this study was to identify food-derived exosome-like nanoparticles (ELNs) with anti-NLRP3 inflammasome function to potentially control FHF.

Methods: Seven commonly consumed mushrooms were used to extract ELNs, which were examined for anti-NLRP3 inflammasome activities in primary macrophages.

Results: It was found that these mushrooms contained ELNs composed of biomolecules including RNAs, proteins, and lipids. Among these mushroom-derived ELNs, only shiitake mushroom-derived ELNs (S-ELNs) strongly inhibited NLRP3 inflammasome activation by blocking the inflammasome assembly. This inhibitory effect was specific for the NLRP3 inflammasome because S-ELNs had no impact on activation of the Absent in Melanoma 2 (AIM2) inflammasome. S-ELNs also inhibited the secretion of interleukin (IL)-6 and both protein and mRNA levels of the Il1b gene in macrophages. Remarkably, pre-treatment of S-ELNs protected mice from LPS/GalN-induced acute liver injury.

Summary/Conclusion: Therefore, S-ELNs, identified as potent inhibitors of the NLRP3 inflammasome, represent a new class of agents with the potential to combat FHF.

PF11.05

Approaches to assess clinically available exosomes' quality and safety

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Introduction: Recent adverse events resultant from an exosome product use in a Nebraska clinic, highlight the importance of assuring product quality and safety standards. An often-overlooked safety risk is ancillary reagents remaining within a finished product. When processes to obtain exosomes utilize cow proteins such as FBS or bovine sera albumin, failure to adequately remove these can result in significant adverse allergic reactions. We evaluated 3 different exosome products to test the hypothesis that purity of some products may not be consistent with actual product quality and safety profiles claimed.

Methods: Three different exosome products (manufacturer A, B, and C) were prepared per their instructions for use. Sample source identity was blinded from assaying scientists. An independent CRO service was used to conduct the experiments to ensure unbiased assay execution and data collection. Exosome suspensions were sampled undiluted for bovine protein content using commercially available bovine secretome protein arrays from Ray Biotech. A total of 30 different proteins found in bovine serum were quantified.

Results: Six of 30 proteins were not detected in any sample. 8 of 24 array antibodies were found to cross react with human antigens. Of the 16 bovine proteins that were acceptable for analysis, manufacturers A, B, and C exosomes contained 15 of 16 proteins,13 of 16 proteins, and 0 of 16 proteins, respectively. Concentrations of individual bovine proteins ranged from 0.2 to 1,220.1 ng/mL.

Summary/Conclusion: These results indicate manufactures A and B are selling potentially dangerous products. The successful implementation of exosome products into the clinic requires equivalent demonstrations of safety and quality. This requires adopting strict quality standards and safety testing during their production. Physicians must require safety data prior to clinical use.

PF11.06

Engineering pro-healing EV cargo using a closed-system bioreactor.

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Introduction: Chronic wounds, including diabetic ulcers and pressure ulcers, are difficult and expensive to treat. While tissue engineering approaches have largely failed as a viable treatment for chronic wounds, we hypothesize that stem cell-derived extracellular vesicles (EVs) may provide several unique advantages. ZenBio, Inc has developed a methodology to generate commercial-scale stem cell-derived exosomes using a closed-system hollow fibre bioreactor capable of continuous EV production. Additionally, we have shown that by manipulating the cellular environment, we can improve the pro-healing capacity of the EVs.This technology leverages the complex healing capabilities of stem cells without the obstacles of replicating cells.

Methods: We have demonstrated that a mild heat shock resulted in EVs enriched for stress-response proteins and increased pro-healing activities in vitro. We extended this innovative approach to include stimulating adipose stem cells with combinations of heat shock and growth factors to generate differential extracellular vesicle packaging that enhances pro-healing activity. To monitor reproducibility across lots and batches, we rigorously characterized tuned EVs for

particle size and number as well as surface marker and cargo composition.

Results: Our results using tuned EVs showed efficacy using cellular models of inflammation, motility, vascularization, collagen production and metalloprotease activity. We utilized an established murine model of pressure ulcers to assess the in vivo efficacy of the tuned EVs. These studies showed a single injection into the wound site activated a more rapid wound closure, increased collagen deposition and reduced dermal thickness compared to saline control.

Summary/Conclusion: These data strongly support our hypothesis that EVs may be selectively modified to improve their wound healing activity by modulating the culture or tissue microenvironment. Future studies will use chronic wound models to determine optimal dosing and routes of administration.

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PF11.07

Comprehensive analysis of methods and outcome reporting in preclinical animal studies of mesenchymal stem cell derived extracellular vesicles: A systematic review

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Introduction: Mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) can reduce inflammation, promote healing and improve organ function thereby providing a potential "cell-free" therapy. Prior to clinical translation, there is a critical need to synthesize existing preclinical evidence supporting their efficacy. This systematic review provides the most comprehensive evidence map of methods, safety and efficacy for MSC-EV research to date.

Methods: MEDLINE and Embase were systematically searched for in vivo interventional studies using MSC-EVs. Two reviewers extracted data for: 1) methodology, 2) study design, 3) intervention details and 4) efficacy/adverse events.

Results: After screening 754 articles, 206 studies met our eligibility criteria. MSC-EVs were used to treat a variety of diseases including renal (16%), neurological (12%) and cardiac (10%) conditions. Benefits were described in 95% of studies across all organ systems and adverse effects were seen in only three studies; two showing tumour growth. However, several key methodological concerns were evident. Based on size criteria for EV subtypes (exosomes/small EVs ~30-150 nm, microvesicles ~150-1000 nm) only 60% of studies used appropriate nomenclature. Ultracentrifugation (70%) and isolation kits (23%) were the most common isolation methods despite marked differences in yield and purity. EVs were inconsistently dosed by protein (68%), particle number (16%) or cell count (4%), hindering inter-study comparisons. Two-thirds of studies used xenogeneic EVs suggesting immunocompatibility. Techniques to determine size, protein markers and morphology was highly heterogeneous, and only 12 and 4 studies met the characterization standards recommended in the MISEV 2014 and 2018 guidelines, respectively. Finally, 50% of studies did not incorporate randomization which represents a high risk for bias and only a quarter performed biodistribution studies.

Summary/Conclusion: This systematic review reveals extensive heterogeneity in methods and intervention details for animal studies of MSC-EVs. Nonetheless, nearly all studies showed significant benefits in a wide range of distinct conditions. The knowledge gaps we identified highlight important opportunities for improving preclinical design and the need for more standardized approaches in this growing field of EV therapeutics.

PF11.08

MSC-exosomes as next generation therapeutics for atopic dermatitis

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Introduction: Atopic dermatitis (AD) is a systemic inflammatory disease with unknown cause. Recent approval of a targeted therapy, dupilumab, opens new era of AD management. However, current therapeutic options for AD are only targeting inflammation, a component of AD vicious cycle including itching and barrier disruption. Human mesenchymal stem cells (MSCs) have been highlighted as a novel therapy for suppressing allergic progress of AD in clinical studies. Unfortunately, phase III clinical study of human umbilical cord blood MSCs for AD was failed with unknown reason. Previously, our group reported that exosomes derived from human adipose tissue-derived MSCs (ASC-exosomes) alleviated the pathological symptoms in a murine AD model with concomitant reduction of inflammation.

Methods: Our group has further investigated the therapeutic effects of human ASC-exosomes in an alternative murine AD model with skin barrier defects. Large scale isolation of ASC-exosomes was performed by tangential flow filtration and isolated ASC-exosomes were characterized according to the recommendation by the ISEV. The protein and lipid cargo were also analysed.
Results: We found that ASC-exosomes induced restoration of skin barrier by inducing de novo lipid synthesis and reduced the levels of multiple inflammatory cytokines. In addition, ASC-exosomes suppressed the expression of itching-causing cytokines. Transcriptomic analysis of AD skin lesions revealed that ASC-exosomes reversed the abnormal expression of genes functioning in skin barrier function, lipid metabolism, and cell cycle.
Summary/Conclusion: Taken together, ASC-exosomes could be a promising cell-free therapeutic option for the treatment of AD, which affecting inflammation, skin barrier function, and itching.

PF11.09

Cell derived vesicles: unravelling the science of novel vesicles with therapeutic promises

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Introduction: Cell derived vesicles (CDVs) are nano-sized vesicles produced by serially extruding cells through small pores. A growing number of studies have implicated their therapeutic potentials, with superior yield compared to other extracellular vesicles (EVs). However, two key objectives remain to be accomplished to demonstrate the utility of CDVs in clinical applications. First, a manufacturing process has to be developed to allow a large-scale production of CDVs. Next, these novel vesicles need to be thoroughly characterized at multiple levels.
Methods: Manufacturing-scale extruders were developed to allow extrusion of large volume of cell suspension in a single process. CDVs with approximately 150–200 nm in diameter were obtained by a serial extrusion. Crude samples were then purified using the tangential flow filtration method to further remove cellular impurities. Finally, physical and biochemical characteristics of purified CDVs were analysed using DLS, NTA, Cryo-EM, and FACS analysis. Additionally, CDVs were subject to multi-omics profiling to comprehend our understanding in molecular contents of CDVs. Both mesenchymal stem cells (MSCs) and natural killer (NK) cells were used for this study.
Results: In this study, we first demonstrate that the large-scale extruder efficiently produce CDVs with consistent quality at the scale that are compatible for clinical applications. Surface marker and membrane composition analyses show that the CDVs are primarily formed using plasma membrane of source cells, with characteristic cellular markers enriched on the surface. Comprehensive profiling of molecular components reveals the unique properties of CDVs as well as the underlying mechanism of formation of CDVs.
Summary/Conclusion: Recently, we have established a manufacturing process to enable clinical applications of CDVs. This study also highlights key molecular features of CDVs that can be harnessed to offer a powerful tool for regenerative and anticancer medicine.

PF11.10

Antifibrotic properties of extracellular vesicles derived from human induced pluripotent stem cells

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Introduction: Fibrosis is a pathological condition resulting from abnormal healing of various tissues. It is triggered by activation of fibroblasts and their subsequent transition to myofibroblast. In consequence, excessive deposition of extracellular matrix proteins leads to impaired organ function. To revert this process, we employed extracellular vesicles (EVs) derived from human induced pluripotent stem cells (hiPSCs). As a model system, we used human cardiac fibroblasts (hCFs), since heart fibrosis constitutes a serious socio-economic problem worldwide.
Methods: We isolated EVs from conditioned media from three hiPSC lines using ultrafiltration combined with size exclusion chromatography methods. Next, we analysed the EVs by NanoSight, transmission electron microscopy, mass spectrometry and Western blot

methods. Finally, we treated TGF-b-stimulated hCFs with hiPSC-EVs and evaluated expression of fibrosis-related genes using real-time qPCR, Western blot and fluorescence microscopy.

Results: We detected anti-fibrotic properties of hiPSC-EVs exerted on hCFs pre-stimulated with TGF-b. The EVs significantly decreased the expression levels of ACTA2, FN, TNC, SNAI2, COL1A1 and reduced the number of myofibroblasts. The canonical profibrotic TGF-b-dependent SMAD2/3 pathway was significantly attenuated in response to EV-treatment.

Summary/Conclusion: In this study we demonstrated strong anti-fibrotic function of hiPSC-EVs. Our findings can further be exploited for future medical applications to treat fibrotic diseases, such as heart fibrosis.

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PF11.11

Induced pluripotent stem cells- derived extracellular vesicles ameliorates D-galactosamine and lipopolysaccharide induced acute liver failure

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Introduction: Liver failure is among the most causes of death in patients with liver disease. Promoting liver regeneration will help patients with liver failure recover on their own. Extracellular vesicles (EVs) can released by induced pluripotent stem cells (iPSCs) through paracrine effects and play a pivotal role in inter-cellular communication in the treatment of disease. In this study, we investigated whether the iPSCs-EVs have therapeutic effects on acute liver failure.

Methods: The iPSCs-EVs were isolated by ultracentrifugation and identified using nanoparticle tracking analysis, transmission electron microscopy and Western blotting. The isolated iPSCs-EVs were administrated D-galactosamine-injured HepRG cells in vitro and tail intravenously injected into D-galactosamine and lipopolysaccharide induced acute liver failure model mice in vivo, respectively. The anti-apoptosis role and potential mechanism were evaluated using flow cytometry and immunofluorescence staining. And Alanine transaminase (ALT) and aspartate transaminase (AST) in serum, H&E staining and TUNEL staining were explored the effect of iPSCs-EVs on liver injured and liver function. Finally, high throughput sequencing of small RNAs was performed to investigate miRNA expression profiles in iPSCs-EVs and iPSCs.

Results: The iPSCs-EVs that were all 50–200 nm, double-layered and oval or round cellular vesicles and expressed the marker proteins CD63, TSG101 and HSP70. In vitro, the iPSCs-EVs treatment inhibited HepRG apoptosis induced with D-galactosamine in a time- and dose-dependent manner and promote the proliferation of hepatic stem cells. In vivo results showed that iPSCs-EVs significantly alleviated liver failure, improved liver function and prolonged the survival period. TUNEL assay showed that iPSCs-EVs suppress apoptosis of hepatocytes. Moreover, miRNA expression profiles analysis found that miR17-92a cluster and miR302-367 cluster were enriched in iPSCs-EVs and iPSCs.

Summary/Conclusion: These findings indicated that iPSCs-EVs could ameliorate D-galactosamine and lipopolysaccharide induced acute liver failure to attenuate hepatocyte apoptosis, which will be benefit for therapy of liver disease in the future.

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PF11.12

MSC-derived extracellular vesicles promote human cartilage regeneration by control of autophagy

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Introduction: Osteoarthritis (OA) is a rheumatic disease leading to chronic pain and disability with no effective treatment available. Recently, allogeneic human mesenchymal stromal/stem cells (MSC) entered clinical trials as a novel therapy for OA. Increasing evidence suggests that therapeutic efficacy of MSC depends on paracrine signalling. Here we investigated the role of bone marrow MSC-derived extracellular

vesicles (BMMSC-EVs), an important component of MSC secretome, in cartilage repair.
Methods: To test the effect of BMMSC-EVs on OA cartilage inflammation the TNF-alpha-stimulated human OA chondrocytes were treated with BMMSC-EVs and inflammatory gene expression was measured by qRT-PCR after 48 h. To access the impact of BMMSC-EVs on cartilage regeneration the BMMSC-EVs were added to the regeneration cultures of OA chondrocytes, which were analysed after 4 weeks for glycosaminoglycan content by DMMB and qRT-PCR. Paraffin sections of the regenerated tissue were stained for proteoglycans (safranin-O) and type II collagen (immunostaining).
Results: We show that BMMSC-EVs promote cartilage regeneration in vitro. Treatment of OA chondrocytes with BMMSC-EVs induces production of proteoglycans and type II collagen and promotes proliferation of these cells. MSC-EVs also inhibit the adverse effects of inflammatory mediators on cartilage homoeostasis. Our data show that BMMSC-EVs downregulate TNF-alpha induced expression of pro-inflammatory COX-2, pro-inflammatory interleukins and collagenase activity in OA chondrocytes. The anti-inflammatory effect of BMMSC-EVs involves the inhibition of NFκB signalling, activation of which is an important component of OA pathology.

Autophagy, a cellular homoeostatic mechanism for the removal of dysfunctional cellular organelles and macromolecules, is essential to maintaining chondrocytes survival and differentiation. The expression of autophagy regulators is reduced in osteoarthritic joints, which is also accompanied by increased chondrocyte apoptosis. Our preliminary data indicate that BMMSC-EVs carry mRNA of natural autophagy inducers and promote autophagy in OA chondrocytes. Therefore, we hypothesize that MSC-EVs exert their beneficial effects on cartilage regeneration by restoring the expression of autophagy regulators.
Summary/Conclusion: In summary, our findings indicate that BMMSC-EVs have ability to promote OA cartilage repair by reducing the inflammatory response and stimulation of OA chondrocytes to produce extracellular matrix, the essential processes for restoring and maintaining cartilage homoeostasis. Thus, MSC-EVs hold great promise as a novel therapeutic for cartilage regeneration and osteoarthritis.
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PF11.13

Large-scale preparations of small extracellular vesicles from conditioned media of mesenchymal stromal cells modulate therapeutic impacts on a newly established Graft-versus-Host-Disease model in batch dependent manners

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Introduction: Extracellular vesicles (EVs) harvested from supernatants of humane adult bone marrow-derived mesenchymal stem/stromal cells (MSCs) can suppress acute inflammatory cues in a variety of different diseases, including Graft-versus-Host Disease (GvHD) and ischaemic stroke. Furthermore, they can promote regeneration of affected tissues. Following a successful clinical treatment attempt of a steroid refractory GvHD patient, we intend to optimize MSC-EV production strategies for further clinical applications. As we observed functional differences of independent MSC-EV preparations in vitro, we aimed to adopt an in vivo GvHD model for the more advanced functional testing of different MSC-EV preparations.
Methods: To this end we set up a bone marrow transplantation mouse model in which endogenous bone marrow was myeloablated by ionizing irradiation (IIR). GvHD was induced by the transplantation of major histocompatibility mismatched allogeneic spleen-derived murine T cells. If not treated otherwise, myeloablated mice developed severe GvHD symptoms.
Results: The GvHD symptoms were effectively suppressed, when MSC-EV preparations were applied at 3 consecutive days, which exerted immune modulatory effects in a mixed-lymphocyte reaction assay. MSC-EV preparations lacking in vitro immune modulating activities, however, hardly improved the symptoms of the GvHD mice. Thus, our results demonstrate that not all MSC-EV preparations harvested from adult bone marrow-derived MSCs contain the same therapeutic potential.
Summary/Conclusion: Thus, successful transplantation of MSC-EVs into the clinics requires a platform allowing identification of MSC-EV preparations with sufficient therapeutic, most probably immune modulating activities.
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PF11.14

Milk extracellular vesicles can repair malnutrition-induced gut barrier dysfunction

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Introduction: Malnutrition impacts approximately 50 million children worldwide and is linked to 45% of global mortality in children below the age of five. Severe acute malnutrition (SAM) is associated with intestinal barrier breakdown and epithelial atrophy. Extracellular vesicles including exosomes (EVs; 30–150 nm) can travel to distant target cells through biofluids including milk. Since milk-derived EVs are known to induce intestinal stem cell proliferation, this study aimed to examine their potential efficacy in improving malnutrition-induced atrophy of intestinal mucosa and barrier dysfunction.

Methods: Mice were fed either a control (18%) or a low protein (1%) diet for 14 days to induce malnutrition. From day 10 to 14, they received either bovine milk EVs enriched using differential ultracentrifugation and sucrose gradient purification or control gavage and were sacrificed on day 15, 4 hours after a Fluorescein Isothiocyanate (FITC) dose. Tissue and blood were collected for histological and epithelial barrier function analyses.

Results: Mice fed low protein diet developed intestinal villus atrophy and barrier dysfunction. Despite continued low protein diet feeding, milk EV administration improved intestinal permeability, intestinal architecture and cellular proliferation.

Summary/Conclusion: Our results suggest that EVs enriched from milk should be further explored as a valuable adjuvant therapy to standard clinical management of malnourished children with high risk of morbidity and mortality.

Funding: CB was generously awarded a catalyst grant from The Centre for Global Child Health at the Hospital for Sick Children to support this work.

PF11.15

The impact of spheroids culture on mesenchymal stem cells and EV production

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Introduction: Mesenchymal stem/stromal cells (MSCs) are now widely believed as bio-factories releasing bioactive products responsible for their therapeutic effect, i.e. cytokines, chemokines, and extracellular vesicles (EVs). MSCs are highly sensitive to physical stimuli from their surrounding microenvironment and can change their characteristics in response to their environment. The application of 3D spheroids cell culture allows MSCs to adapt to their cellular niche environment which, in turn, influences their paracrine signalling activity. We aim to determine how 2D and 3D culture microenvironments can modulate the EV production and investigate their anti-fibrotic activity.

Methods: For 2D culture, bone marrow-derived MSCs were cultured on standard tissue culture plastic. For 3D culture, MSCs were aggregated into spheroids using non-adherent 96-well plates and cultured with addition of 0.25% methylcellulose. To collect conditioned media, both 2D and 3D MSCs were cultured using serum free medium for 4 days. EVs were isolated by serial ultracentrifugation and were characterised on ExoView platform which allows simultaneous detection of particle size and expression of CD81/CD63/CD9. Cell lysates were collected for miRNA isolation and qRT-PCR was performed to analyse expression of candidate miRNAs. To model the progress of lung fibrosis, human lung fibroblasts (HLFs) were cultured with TGF-β1 to induce fibroblast activation, subsequently exposed to 2D and 3D EVs, and collagen production was measured. Further, 2D and 3D MSC-EVs were added into human lung MSCs isolated from healthy and IPF patients and cell proliferation was assessed using MTS assay.

Results: 2D and 3D MSC-EVs have similar EV characteristics in terms of particle size and EV tetraspanin markers expression. ExoView analysis showed expressions of CD81/CD63/CD9 and average particle diameters of <100 nm. On a cellular level, we identified a panel of anti/pro-fibrotic miRNAs which are differentially expressed in 2D and 3D MSCs. 2D and 3D MSC-EVs have similar anti-fibrotic activity shown by their ability to reduce collagen deposition in HLF cultures. Both 2D and 3D MSC-EVs could promote cell proliferation on IPF lung MSCs but no overall effect on healthy lung MSCs.

Summary/Conclusion: This concept of engineering the cellular microenvironment to promote EV production

is as yet untouched and we foresee that in 3D cultures, we can culture MSCs for longer timeframe and therefore maximising the overall EV production process. The outcome presents future potential for 3D culture of MSC to increase the efficiency and feasibility of scalable EV production.

PF11.16

Outer Membrane Vesicles from Photobacterium damselae subsp. piscicida: characterization and antigenic potential

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Introduction: Photobacterium damselae piscicida (Phdp) is a Gram-negative bacterium that causes a septicaemia in > 20 fish species worldwide. It represents a major drawback for aquaculture, whose importance has been sharply growing as a food supplier. Given the Phdp massive mortality and widespread antibiotic resistance, an effective vaccine is highly needed. Extracellular products (ECPs) have an essential role in Phdp virulence, containing important antigens. However, the ECPs' identity remain undisclosed. In our efforts to dissect their composition, we found that they contain high amounts of outer membrane vesicles (OMVs). These particles are potent weapons for bacteria and are being explored in the field of vaccinology, since OMVs present antigens in native conformations and are strongly immunogenic, without requiring adjuvants. This potential associated to the urgent need for an anti-Phdp vaccine prompted us to isolate and characterize the OMVs shed by Phdp.

Methods: In order to harvest high amounts of pure Phdp OMVs, a reproducible optimized protocol was developed: the bacteria-free supernatant from a Phdp overnight culture is concentrated, dialysed and ultracentrifuged to collect the OMVs.

Results: Analysis of the obtained OMVs preparations by Transmission Electronic Microscopy and Dynamic Light Scattering indicate that the main population of vesicles has sizes around 30–50 nm. Proteomic analysis of the vesicles revealed the presence of the apoptogenic AB toxin AIP56 that is known to play a major role in Phdp virulence, a putative pore-forming toxin, a putative adhesin/invasin and several Outer Membrane Proteins (OMPs), including a 64kDa OMP, predicted to be involved in iron acquisition, and 5 other OMPs (18–34kDa), with an OmpA-like structure that may act as adhesins. Moreover, preliminary in vivo studies suggest that some of those proteins may have important roles for virulence, since injection of knock-out strains in sea bass induced a decreased mortality comparing to the wt strains.

Summary/Conclusion: Our findings suggest that OMVs are a promising vaccine candidate and we are currently studying their biological activities and determining the antigenic potential of the identified proteins.

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PF12: Advances in Characterization of EV-associated Molecules: Omics

Chair: Marta Adamiak – Cardiovascular Research Centre, Icahn School of Medicine, Mount Sinai

Chair: Juan M Falcón-Pérez – CIC bioGUNE

PF12.01

Comparison of extracellular vesicle isolation methods from small volumes of urine for radiation metabolomics analysis.

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Introduction: Whole body exposure to high doses of ionizing radiation (IR) can potentially be lethal if radiation injury is not diagnosed and treated expeditiously. When considering a non-invasive approach for the identification of biomarkers of IR exposure, we and others have studied molecules in plasma, serum, saliva, and urine. However, these matrices can potentially have significant background noise, obscuring potential biomarkers of biological importance. Extracellular vesicles (EVs) are fast becoming a platform for biomarker discovery in radiation research as well as in other pathologies. However, no groups have investigated the use of metabolomics to analyse EVs derived from urine in the context of IR exposure. Furthermore, the dominant protocols for EV isolation from urine require a large (up to 30 mL) amount of starting volume, which may not be available for many studies. The aim of this study was to optimize EV isolation from rat urine and assess radiation-induced alterations in urine EV number and metabolic content.

Methods: As a proof of concept, we compared and optimized several EV isolation methods on small volumes of urine from male WAG/RijCmcr rats exposed to 0 Gy or 13 Gy X-rays to the whole body except the hind leg. Starting with either 500 µL or 1000 µL of urine, we isolated EVs using ultracentrifugation (UC) with filtration, size exclusion chromatography (SEC), and a proprietary bead-based isolation method developed by a 3rd party provider. EV samples were characterized using nanoparticle tracking analysis. Metabolomics profiles were measured using LC-QToF-MS.

Results: We found that SEC resulted in the highest yield of EVs from as little as 500 µL of urine, while UC was the poorest performing. LC-QToF-MS analysis revealed that SEC and UC had the most consistent identification of features, whereas the bead-based method contained artefacts likely as a result of the extraction method. We next used SEC to isolate EVs from a larger cohort of rats exposed to IR and analysed with MS. EV metabolic content will eb related to differences in survival and organ function between sham and irradiated groups.

Summary/Conclusion: We conclude that SEC is the preferred method for isolating EVs from small volumes of urine for broad-based mass spectrometric analysis, and that the EV metabolome may be a sensitive and specific early indicator of radiation injury.

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PF12.02

MALDI-TOF MS fingerprinting of potential oesophageal cancer biomarkers in exosomes isolated by LEAPS

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Introduction: There is growing evidence that contents (including RNA and proteins) of exosomes may serve as biomarkers for early diagnosis and prognostic prediction of cancers. Here we aim to identify potential protein markers for oesophageal cancer.

Methods: Using our newly developed label-free exosome automated preparation system (LEAPS), exosomes were isolated from 20 ml culture medium of various

oesophageal cancer cells with different differentiation profiles and different sources of metastasis. Exosomes from 20 µl plasma of cancer patients at different clinical stages or with/without relapse and healthy controls were also prepared by LEAPS. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed to directly analyse exosomes. Protein identities of exosomal fingerprint peaks were tentatively assigned by correlation with top-down and bottom-up proteomics.

Results: Start from 20 ml culture medium or 20 µl plasma, high-quality exosomes rapidly isolated by LEAPS are sufficient for MALDI-TOF mass spectrometry. It seemed that poorly differentiated cells showed more exosome release. MALDI-TOF MS fingerprints of exosomes in cells is cell line specific. MS profiles from poorly differentiated cells showed more peaks than that from highly differentiated cells. Fingerprints also allowed classification of cancer cell lines through software mathematical analysis. We identified different numbers of significantly differentially expressed peaks in exosomes of various cancer cells. Fingerprints of exosomes derived from the poorly differentiated cells showed more elevated peaks. Top four peaks (2,427 m/z, 3,596 m/z, 4,458 m/z, 4,537 m/z) were commonly down-regulated in exosomes of most cancer cells. Top four protein peaks (2,268 m/z, 5,713 m/z, 6,255 m/z, 6,337 m/z) that might be correlated to the differentiation profile of cancer cells were also identified. MALDI-TOF MS detection of exosomes in the plasma and clarifying identities of potential biomarker peaks will be done in the future.

Summary/Conclusion: The combination of LEAPS and MALDI-TOF mass spectrometry provides a fast and high-throughput tool for exosomal marker discovery. Potential biomarker identified in exosomes derived from oesophageal cancer cells or from plasma of cancer patients by this tool might be useful in cancer diagnosis and prognosis.

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PF12.03

Fraction-based Proteomic profiling of serum extracellular vesicles derived from cervical cancer patients

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Introduction: Current evidence indicates that extracellular vesicles (EVs) can release from most of cell types and affect adjacent or distant cells by circulating in all bodily fluids. Proteomic analysis of EVs from clinical samples is complicated by the low abundance of EV proteins relative to highly abundant circulating proteins. Size exclusion chromatography (SEC) has been overcome as a method to deplete protein contaminants and enrich EVs.

Methods: we collected serum of healthy women and cervical cancer patients with stage I–III and then counted concentration and size distribution of the EVs using nanoparticle tracking analysis (NTA). Differential ultracentrifugation combined with SEC was used to isolate and purify EVs from contaminant proteins. Isolated EVs were investigated their characteristic based on morphology using transmission electron microscope (TEM) and on expression of CD63, CD81, CD9 protein markers using western blot analysis. Fraction no.8–10 of isolated EVs in among sample groups were profiled by nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) analysis.

Results: NTA shows that the concentration of EVs is increased in patients compared with healthy women. Proteome profiles of EVs isolated by SEC were compared in each fraction. Moreover, we detected molecular evidence for fraction-specific molecular pathways in connection with cancer progression and complied a set of protein signatures that closely reflect the associated clinical pathophysiology.

Summary/Conclusion: These unique features in each fraction among sample groups would be the informative considering in order to select for further analysis as in vitro.

Funding: Faculty of Medicine, Prince of Songkla University, Thailand

PF12.04

Isolation methods of exosomes optimized for biomarker screening based on proteomic analysis using 2-D gel electrophoresis

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Introduction: Recently, diagnostic biomarkers from exosomes by proteomic analysis have been reported, but it is required to optimize the isolation protocol to screen out more effective biomarkers. For serum-originated exosomes, it has been also reported to isolate them selectively, however, it is observed that a different method resulted in different protein profiles in 2-D gel electrophoresis.
Methods: We isolated exosomes by two discrete methods, using ultracentrifugation and magnetic separation. Before ultracentrifugation and magnetic separation, precipitation using polymer materials was perforemd. The isolation of exosomes by these two methods followed by comparison of their size, total vesicle number, morphology, and protein markers. To identify protein biomarkers, proteomic analyis using 2-D gel electrophoresis was performed.
Results: Both methods induced enrichment of exosome-specific proteins, but protein profiles in each exosome fraction was totally different. The protein profiles showd that the magnetic seperation following a polymer-based precipitation step was more efficient to screen out candidate biomarkers, which showed nearly 200 protein profiles originated from exosomes.
Summary/Conclusion: In our study, magnetic separation of exosomes from serum fraction was optimized for 2-D gel electrophoresis to observe identifiable biomarkers.

PF12.05

An extracellular small RNA-seq data processing pipeline optimized for high-performance computing

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Introduction: A variety of RNA species is found in extracellular biofluids such as blood, bile, and urine, carried by extracellular particles including extracellular vesicles (EVs) and lipoproteins (e.g high density lipoproteins (HDLs)). The extracellular RNA (exRNA) carried by EVs and HDLs is of great interest for two reasons: 1) the exRNA within different carriers could be diagnostic of the state of the tissues from which the particles originate, and 2) exRNA has been shown to affect gene expression in target cells. Although the origin and functions of exRNAs remain largely unknown, there is growing interest in exRNA research for the development of diagnostics and new therapeutic targets. Small RNA sequencing is widely used to estimate the abundance of exRNAs in biofluid samples.
Methods: Here we present a data processing pipeline for extracellular small RNA sequencing. Sequencing data are pre-processed through quality control, and then aligned to the endogenous genome to obtain the gene counts for various RNA biotypes, including microRNA, tRNA, rRNA, PIWI-interacting RNA, long non-coding RNA (lncRNA) and protein coding RNA. It also aligns sequencing reads to exogenous databases, including the ribosomal RNA sequence database SILVA, and all sequenced bacteria genomes available on Ensembl, to estimate the abundance of exogenous genes.
Results: We analysed a publicly available small RNA-seq dataset of HDL from three Systemic lupus erythematosus (SLE) patients and three healthy controls using this pipeline. The miRNA hsd-mir-93, lncRNA AL137186.2 and AC108050.1 were elevated in SLE patients compared to controls. Exogenous RNA reads mapped to Bacteroidetes were also elevated in SLE patients.
Summary/Conclusion: Our pipeline is able to process exRNA sequencing data and estimate the abundance of major exRNA species, as well as exogenous RNA taxonomy. The pipeline is optimized for the job scheduler SLURM, and can therefore utilize the full computational power of high-performance computers. The pipeline is publicly available on github (www.github.com/zhuchcn/exceRNApipeline).
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PF12.06

Characterization of extracellular vesicles (EVs) derived from Peripheral Blood Mononuclear Cells (PBMCs) of Inflammatory Bowel Disease (IBD) patients

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Introduction: IBD is a chronic hyperinflammatory disorder that severely compromises the intestines. The aetiology of IBD is poorly understood. However, it has been associated with a dysregulation of the immune system and gut microbiota and with genetic and environmental factors. Cumulative evidence indicates that EVs play an essential role in modulating immune responses. Recent research suggests that EVs derived from dendritic cells, saliva and intestinal epithelial cells may be involved in the progression of IBD inflammation. However, little is known about the contribution of immune cells-derived EVs with this pathology. The goal of this study is to shed light on the contribution of PBMC-derived EVs on IBD pathogenesis.

Methods: Here we characterized and compared the composition of EVs derived from PBMCs of 3 IBD patients and 1 Healthy control. EVs were isolated by differential centrifugations from the supernatant of PBMC activated with CD3-CD28 beads for 6 days in serum-free media. Size and concentration were analysed using a Nano Sight 300 instrument, while the presence of known EVs markers (CD63, CD81, Hsp70) was analysed by immunoblotting. Whole EVs proteome was performed by MS/MS and functional-enrichment analysis was done using FunRich with Uniprot database.

Results: Proteomics analyses identified a total of 1299 proteins in the four groups. Of those, 673 (51.8%) were present in both the IBD patients and control. This group of protein was composed of several Ras-related proteins, eukaryotic initiation factors, granzyme, CD9, tubulin, and serpins among others. Patients' EVs shared 46 proteins in common such as proteasome subunit beta type-10, T cell receptor beta, and the amine oxidase containing copper 3. Interestingly, each patient sample had a unique group of proteins. Among these are myeloperoxidase, neutrophil elastase, proteasome subunit alpha type-3, and signalling lymphocytic activation molecule (SLAMF1).

Summary/Conclusion: These preliminary studies show that the EV composition from PBMCs of IBD patients is specific and differs from a healthy control. This exclusive composition has the potential to be used as a biomarker for diagnostics and progression of the disease, and it could also provide new insights into our understanding of the cellular pathways involved in the pathogenesis of IBD. The studies were performed with corresponding IRB approvals.

PF12.07

Proteomic analysis of exosomes isolated using precipitation and column-based approaches

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Introduction: Exosomes are a subtype of small extracellular vesicles (EVs) involved in various physiological and pathological processes with huge potential as biomarker resources or as therapeutic tools. Although several exosome isolation approaches are available, complementary studies focusing on optimizing the methods for human blood-derived exosomes isolation and method-specific comparative exosomal proteomic profiles will be of clinical value.

Methods: Blood-derived EVs were isolated through precipitation- and column-based methods and characterized by transmission electron microscopy, nanoparticle tracking analysis and western blot analysis. Serum-derived exosomal proteomes were analysed by mass spectrometry (MS). The resulting proteomes were then overlapped with the proteomes obtained from exosome-related databases, to determine the % of similar content. In addition, bioinformatic analysis, including Gene Ontology (GO) was carried out.

Results: Both methodologies tested isolated particles with the expected morphology and size range, although the column-based method isolated a higher number of particles. About 75% of the exosomal proteins identified through MS overlapped with the proteomes extracted from the databases. GO terms were similar for the proteomes isolated from the column- and precipitation-based methodologies. The top 3 GO terms identified for Molecular Function were ion binding, peptidase activity and enzyme regulator activity and for Biological Process were immune system process, transport and response to stress. Further, partial least square analysis revealed a clear segregation of proteomes obtained by the distint methodologies and complementary statistical analysis revealed the proteins differently expressed.

Summary/Conclusion: No major differences were found in the top 3 biological processes and molecular function based on GO analysis. Nonetheless, the two approaches result in different EVs yields and significant proteome differences were identified. Characterization of distinct methods for blood-derived exosomes isolation can be useful in the context of EVs potential in disease diagnostics/therapeutics.

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PF13: Separation and Concentration

Chair: Metka Lenassi – University of Ljubljana, Faculty of Medicine, Institute of Biochemistry

Chair: Fuquan Yang – Institute of Biophysics, Chinese Academy of Sciences

PF13.01

From total plasma EVs to immuno-captured neuronal EVs – yield, purity and enrichment

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Introduction: We and others are developing biomarkers for neurodegenerative diseases using neuronal-enriched EVs immunocaptured from a suspension of total plasma EVs. Here we assess how the isolation method for total EVs affects the yield, purity and enrichment of neuronal EVs.
Methods: For N = 5 subjects, total EVs were isolated by EV precipitation solution (ExQ), EV precipitation solution plus bipartite resin columns (ExU) and Size Exclusion Chromatography (qEV) from 0.5, 0.5 and 2.7 ml plasma, respectively. Then, neuronal-enriched EVs were immunoprecipitated using anti-L1CAM antibody. In total and L1CAM EVs, we measured particle concentration by Nanoparticle Tracking Analysis, protein concentration, and novel multiplex electrochemiluminescence immunoassays for tetraspanins CD81, CD63 and CD9 on intact EVs.
Results: For total EVs, yield followed the order of ExQ > qEV > ExU, assessed by particle ($p < 0.0001$) and protein concentrations ($p < 0.001$). L1CAM EVs immunocaptured after ExQ showed 16-fold higher particle ($p < 0.001$) and fivefold higher protein ($p < 0.01$) concentrations compared to L1CAM EVs after ExU, and 41-fold higher particle ($p < 0.001$) and 11-fold higher protein ($p < 0.001$) concentration compared to L1CAM EVs after qEV. L1CAM EVs after EV precipitation (ExQ) showed 48, 35 and 30-fold higher CD81, CD63, and CD9 concentrations ($p < 0.001$) compared to L1CAM EVs after ExU, and 59, 43 and 36-fold higher CD81, CD63, and CD9 concentrations ($p < 0.001$) compared to L1CAM EVs after qEV. L1CAM EVs following 3 different methods had equal purity assessed by ratios of particle/protein concentrations (p = ns), and tetraspanin/particle concentrations (p = ns).
Summary/Conclusion: L1CAM EV immunocapture preceded by ExQ exceeded the yield of immunocapture preceded by ExU or qEV. Recovered L1CAM EVs showed equal purity by particle/protein and tetraspanin/particle metrics. Neuronal enrichment results will be available by the time of ISEV. Immunoprecipitation following ExQ, often considered impure, purifies final isolates as effectively as more onerous methods typically considered purer. Balancing sensitivity, purity and scalability is essential for implementation of blood biomarkers in the clinical setting and may be achieved by combining techniques.
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PF13.02

Characterisation of breath exosomes: towards non-invasive diagnosis

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Introduction: Breath-derived exosomes present new potential for non-invasive diagnosis of lung cancer. However, breath-derived exosomes have not been well characterized and methodology for their purification has not been optimised. In order to exploit their potential for diagnosis, it is first necessary to develop methods that reproducibly provide high quality pure exosomes from breath. In this study, we optimise methods for their isolation and characterise them in comparison to exosomes derived from cell culture models.
Methods: In order to characterize exosomes from exhaled breath condensate (EBC) it was first necessary to optimize methods for isolation of pure, intact, and high quality exosomes. To this end, isolation methods

were optimised on cell-derived exosomes and then applied to EBC, yielding high quality exosomes from size exclusion chromatography (SEC). EBC exosomes were compared with those from A549 and WI-cells using DLS, TEM, and Cryo-SEM. An immunoblotting-grid technique was used to validate the presence of exosome-specific markers CD63 and CD81. Protein content of exosomes were quantified and compared.

Results: SEC-based isolation was more effective at isolation of pure and intact exosomes than ultracentrifugation, with the highest purity exosomes obtained in the middle fractions of the exosome-containing eluate. Exosomes from EBC had a size range (45–100 nm), protein content (20–40 ug/mL) and molecular markers typical of cell-derived exosomes.

Summary/Conclusion: Breath-derived exosomes isolated through size exclusion chromatography are sufficiently pure for diagnostic purposes and are phenotypically similar to exosomes derived from other sources. We foresee their use in non-invasive diagnostics for lung cancer as an important future application.

PF13.03

Ligand-based Exosome Affinity Purification (LEAP) is a rapid and reproducible method for the enrichment of functional EVs

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Introduction: Platelet-derived extracellular vesicles (pEVs) represent the next generation of therapeutic biologics as they enable a more refined and targeted approach when compared to crude blood derivatives currently used for treating diseases such as cancer, thrombocytopenia and chronic wounds. However, development of an EV-based therapeutic is hindered by the lack of a scalable, validated and reproducible purification process.

In this study, pEVs were isolated from activated platelet concentrates and purified using Exopharm's Ligand-based Exosome Affinity Purification (LEAP) technology to produce a functionally active EV therapeutic.

Methods: Platelet concentrates (n = 20) were obtained from the Australian Red Cross Blood Service and were activated by Exopharm's proprietary process. Activation was verified by measuring CD62p using flow cytometry. The resulting platelet releasate (500 ml) was subjected to LEAP purification to isolate pEVs. For characterization, protein concentration was determined by a bicinchoninic acid assay, microfluidic resistive pulse sensing (MRPS) was used to perform a particle count and transmission electron microscopy (TEM) enabled visualization of EV morphology. Key EV markers were detected using Mass Spectrometry (MS) and Western Blots.

To confirm biological activity, human dermal fibroblasts were subjected to serum starvation for 16 hours before treatment with pEVs (15 μg/ml). Cell growth was recorded by the real-time xCelligence system and differences in proliferation were statistically analysed using a one-way ANOVA.

Results: MRPS and TEM both revealed isolated pEVs to be 100–300 nm in size. The final product was positive for platelet markers (CD41, CD62p) and key EV markers (Tsg101, Alix, CD63). Treatment with purified pEVs significantly increased proliferation in serum-starved fibroblasts over 48 hours.

Summary/Conclusion: Exopharm's LEAP technology is a rapid and reproducible purification process which produces pEVs that adhere to MISEV 2018 guidelines and are functionally active.

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PF13.04

A Novel but simple method to obtain purified exosomes by one-step ultracentrifugation

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Introduction: Exosomes are Extracellular vesicles (EVs) that are derived from endosome membrane. They are usually 50–150 nm in diameter, actively secreted in most living cells. Originally, exosomes were thought to act as cellular garbage disposals. Recent studies showed that exosomes not only can serve as biomarkers for diagnosis, but also can be used as an ideal delivery vehicle for drugs in therapeutics. Exosomes are natural carrier for mRNA, miRNA, siRNA, protein, DNA and peptide for long distance intercellular communication. Isolation of exosomes is challenging due to their small size and heterogeneity. Traditional differential ultracentrifugation method is still the gold standard for exosome purification. To further explore the potentials of exosomes being as the therapeutic delivery vehicle or diagnostic reagent,

it is an essential step to purify them in high quality at high yield.

Methods: Here, we report a novel method to obtain intact shape, high- quality and high purity exosomes with one-step ultracentrifugation by using "Exojuice".

Results: Data of nanoparticle tracking analysis (NTA) and western blotting showed "Exojuice" can yield exosomes with a simpler method to obtain higher purity exosomes in comparison to previous method of cushion ultracentrifugation using Optiprep.

Summary/Conclusion: Our method can be used to purify exosomes from cell culture medium, serum, urine, saliva, and other biofluids.

PF13.05

A straightforward device to extract apoplastic fluid from succulent fruits for higher purity of extracellular vesicles

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Introduction: Edible plants are emerging as a sustainable source for extracellular vesicle (EV)-based drug delivery vehicles. However, current isolation methods (e.g. grinding or squeezing) may cause destruction of plants' biostructures, and in turn leads to unwanted effects in downstream applications and complicates the study of nanovesicles – cell. Therefore, we designed a simple device that allows the extraction of apoplastic fluid (AF) from succulent fruits, facilitating EV isolation as well as effective downstream applications.

Methods: An inner filter tube was designed to extract AF with a determined membrane pore size. AF was collected by low-speed centrifugation method and then filtered to eliminate the impurities from the cytoplasm and damaged cells. Minced juice (MJ) was homogenized by a blender and then centrifuged to remove large fragments. Subsequently, the differential centrifugation method was employed to extract EVs from AF and MJ. Fourier-transform infrared spectroscopy (FTIR), nanoparticle tracking analysis (NTA), and transmission electron microscopy (TEM) were performed to discriminate AF, MJ and their EVs.

Results: The "spectroscopic" protein-to-lipid (P/L) ratio of AF (1.23 ± 0.03) is significantly lower than that in MJ (1.27 ± 0.01), showing the higher lipid contents in AF, which may result from the loss of lipids in MJ obtained from grinding or juicing methods. Similarly, FTIR showed the difference in P/L ratio between AF and its EVs (1.23 ± 0.03 and 1.29 ± 0.02, respectively). NTA showed the sharper peak and smaller vesicle size in the following order: MJ (173.0 ± 21.3 nm), AF (154 ± 11.0 nm), AF-derived EVs collected at 40,000 × g and 10,000 × g (108.5 ± 2.4 nm and 95.7 ± 3.1 nm, respectively). Furthermore, TEM study indicated that the collected EVs exhibited a typical lipid bilayer of extracellular nanovesicles.

Summary/Conclusion: By using a reusable filter device, we successfully isolated AF from succulent fruits, paving the way to collect plant EVs without an interference of significant biodestruction or damaged cells, hence improving the purity of EVs and facilitating downstream applications. Moreover, this method is straightforward, reproductive, and can be potentially used in a large-scale production.

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PF13.06

Method to simultaneously capture multiple classes of intact extracellular RNA carriers including extracellular vesicles and lipoprotein particles

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Introduction: Extracellular particles including extracellular vesicles (EVs), lipoproteins, and free proteins are carriers of extracellular RNA (exRNA), which has been shown to regulate cellular function. Because these particles have different physiological origins, they have different RNA signatures, so the first step to understanding the biology of exRNA is to isolate individual particle fractions with high purity and efficiency. Current methods for isolating EVs are optimized for

increased yields and purity of EV fractions but typically require multiple millilitres of starting plasma and do not capture the other exRNA carrier particle types. Methods that can capture EVs from low starting plasma volumes and can also capture other exRNA carriers simultaneously are needed for analysing samples from previously conducted large cohort studies, biorepositories, and in populations where sample volume is limiting.

Methods: We have developed a method adapted from lipoprotein isolation that requires only 500 µl of starting plasma, and uses brief ultracentrifugation (UC) followed by fast protein liquid chromatography (FPLC) to capture 6 classes of purified exRNA carriers including EVs, LDL, HDL, lipidated albumin, proteins, and VLDL/chylomicrons. We have validated successful capture of EVs by Microfluidic Resistive Pulse Sensing (MRPS, Spectradyne), transmission electron microscopy (TEM), and Single Particle Interferometric Reflectance Imaging System (SP-IRIS; ExoView) with optional fluorescence.

Results: We have observed 1.02 × 1010 particles per ml from a 1 ml FPLC fraction of EVs measured from 25,000 events by MRPS, confirming that EVs are being captured by this method. There were also 1.26 × 1010 particles/ml and 2.70 × 1010 particles/ml in the two subsequent 1 ml fractions that are known to contain lipoprotein particles, though these were measured from 1,500 events each. By TEM we confirmed these observations that EVs are eluting before lipoprotein particles with some EVs eluting later in fractions containing lipoproteins.

Summary/Conclusion: These results confirm the efficacy of the method in isolating multiple exRNA carrier fractions simultaneously from a single 500uL plasma sample, making it amenable for the analysis of exRNA in samples from large cohort studies, biorepositories, and vulnerable populations such as the elderly and young children.

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PF13.07

Optimizing the isolation of placental mesenchymal stromal cell-derived extracellular vesicles in a 3D bioreactor system

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Introduction: Extracellular vesicles (EVs) derived from placental mesenchymal stromal cells (PMSCs) have the potential to provide neuroprotection at sites of injury. However, a rate limiting step in EV research is the low yield, high technical time, and high cost of current isolation procedures. To address this inefficiency, we cultured PMSCs in a unique bioreactor system to increase the absolute yield of EVs per mL of media and per cell. Future studies will determine if this system can improve PMSC EV yield without altering the demonstrated neuroprotective properties of PMSC-EVs.

Methods: PMSCs were cultured in the bioreactor for ten weeks. EV-conditioned media was collected weekly and EVs were isolated through differential centrifugation. Nanoparticle tracking analysis (NTA) measured EV size and concentration. Western blots tested for normal EV markers (CD9, CD63, and CD81, Calnexin(-)) and enzyme-linked immunosorbent assays (ELISA) measured levels of characteristic growth factors in conditioned media including vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and hepatocyte growth factor (HGF).

Results: EVs remained consistent until week eight, after which a decrease in both EV size and concentration was seen. Western blots revealed normal positive expressions of CD9, CD63, and CD81 and negative expressions of calnexin. Levels of VEGF, BNDF, and HGF were comparable after 10 weeks. Cost analysis revealed an overall increase in EV yield for shorter labour time and lower material cost.

Summary/Conclusion: This initial study uses a bioreactor system for a unique source of cells and has brought us closer to optimizing PMSC EV isolation protocols for increased yield, lower cost and time commitment, and maintained sample purity. Preliminary data suggests the EV phenotype and cell secretome are consistent with those present in current culture settings. Future experiments will assess the preserved neuroprotective properties of the PMSC EVs.

PF13.08

A novel method for isolating extracellular vesicles from cell culture media and plasma using polyethylenimine

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Introduction: Due to their ability to transport DNA, RNA, and protein cargoes between cells, extracellular vesicles (EVs) are becoming popular for biomarker discovery as well as for therapeutic delivery. Here we

describe the development of a novel precipitation method for the isolation of EVs from cell culture media and plasma that is based on polyethylenimine (PEI), an inexpensive, water-soluble, and biocompatible cationic polymer. PEI is a group of hydrophilic cationic polymers that are synthesized as either linear or branched forms of varying molecular masses (1,000 to 750,000 Da) and are widely used in the biomedical field as a coating and transfection agent.

Methods: Linear and branched PEI of varying molecular weights (MW) were tested for their ability to precipitate EVs from either conditioned culture media (CCM) or human plasma. Isolated EVs were characterized by Western blotting and nanoparticle tracking analysis (NTA). The small RNA profile of EVs isolated using PEI from human plasma was analysed by NGS and EV-specific miRNAs were confirmed by digital droplet PCR (ddPCR). Mass spectrometry (MS) was used to analyse the proteome of PEI-captured EVs from plasma. HEK293 cells producing GFP+ EVs were used to optimize conditions for release of EVs from both linear and branched PEI by fluorescent spectrophotometry and flow cytometry measure-ments.

Results: Linear and branched PEI were both able to precipitate EVs as determined by Western blotting for EV protein markers; however, branched PEI with MW > 10,000 Da and linear PEI with MW > 25,000 Da were more efficient for EV precipitation than lower MW forms. Despite its known ability to bind nucleic acids PEI was unable to capture cell-free DNA from plasma, although RNA and in particular EV-associated miRNAs such as mir-142-3p were recovered. MS revealed that PEI enriches extracellular exosome proteins from plasma. EVs captured from CCM by PEI could be released from the complex using heparin or high salt conditions.

Summary/Conclusion: PEI has an unexpected preference for associating with EVs compared to nucleic acids in complex biological samples and has a hitherto unrecognized application for EV precipitation.

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PF13.09

Assessment of various extracellular vesicle isolation methods through chemical analysis with surface-enhanced Raman spectroscopy

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Introduction: There is ongoing debate about which is the most appropriate method for isolation of EVs, with most labs using some combination of differential ultracentrifugation (UC), size-exclusion chromatography (SEC), and/or density gradient ultracentrifugation (DG). Here we applied a surface-enhanced Raman spectroscopy (SERS) analysis platform to compare chemical composition of the isolate from each method against lipoprotein standards to assess the relative purity of the EV preps.

Methods: 2–3 mL of plasma was separated from whole blood collected from head and neck cancer patients. Each sample was split into 3 batches and EVs were isolated by either UC, SEC, or DG. Following isolation, samples were incubated on commercial SERS substrates and Raman spectra were collected. Lipoprotein standards were purchased and also measured for comparison. Using principle component analysis (PCA), spectra were analysed for chemical variability.

Results: SERS analysis of SEC, UC, and DG isolated EVs were chemically distinguishable using simple PCA. The chemical changes could in large part be attributed to fitting the differences in spectra to lipoprotein standards. We found that UC isolated populations clustered with the high-density lipoproteins (HDL), SEC populations with the low-and very low-density lipoproteins (LDL, VLDL), and DG populations were more variable, but mainly clustered together with the high-density-lipoproteins (HDL).

Summary/Conclusion: This set of experiments matches our expectation that various lipoprotein would contaminate EV preps according to their relative size and density distributions. No single isolation method could separate pure EV samples. This study also illustrates the utility of label-free SERS analysis for rapid chemical characterization of EVs.

PF13.10

Bioreactors: lessons to develop an extracellular vesicle factory

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Introduction: High density mammalian cell culture systems (bioreactors) provide valuable advantage for large scale production of secreted products such as Extracellular Vesicles (EV). However, optimisation of design selection, handling and operational costs can be

quite challenging. Here we provide our experience with a CELLine Bioreactor system.

Methods: Cultures of 6 adherent cell lines were established in CELLine AD 1000 bioreactors and propagated for up to 19 weeks. Media was changed twice weekly and cells shed into serum-free conditioned medium were counted and assessed for viability. NanoEVs were isolated by sequential centrifugation (2000 g – 10,000 g – 100,000 g) and Size Exclusion Chromatography (SEC). NanoEVs were characterised in their protein (BCA) and particle (Nanoparticle Tracking Analysis) amount, EV markers (Western blotting) and morphology (Transmission electron microscopy, TEM).

Results: The viability of shed cells varied between cell lines and through time, suggesting a changing dynamic during reactor establishment and continuous growth phases, that was specific to each cell line. HDFa, BT20 and BT474 consistently shed mainly dead cells (60–100%), as opposed to MCF7 and MDA-MB-231 which predominantly shed live cells. SEC fractionation of nanoEVs identified a dominate EV-rich peak and significant quantities of smaller proteins, highlighting the need for further purification. NanoEV yields from each 3–4 day culture averaged 2–7 × 1010 particles, representative of yields obtained from cells grown in 8 to 28 conventional T175 tissue culture flasks. EV markers and TEM confirmed the protein profiles and morphology of EVs obtained from bioreactors.

Summary/Conclusion: High density bioreactor cultures offer a physiologically relevant, cost and space efficient approach to produce significant amounts of EVs, providing sufficient material for numerous experimental uses. In our hands, with careful twice weekly management, they can be propagated for up to 19 weeks without significant changes to the EVs.

Funding: Breast Cancer Foundation NZ

PF13.11

Rapid and efficient isolation of extracellular vesicles by Phosphate–DNA –Cholesterol functionalized defected Metal Organic Framework

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Introduction: Extracellular vesicles (EVs) have potential applications for clinical theranostics. Ultracentri-fugation is most commonly adopted to the EVs isolation, which is recommended as a gold standard method. However, ultracentrifugation is time-consuming and expensive equipment requirement, resulting in the co-isolation of contaminants such as protein aggregates. Therefore, our aim is to develop a rapid and efficient platform to isolate heterogonous EVs based on the insertion of lipid molecules into the EVs membrane to avoid co-isolation of non-membranous protein particles.

Methods: Herein, a defected nanoscale functional Metal Organic Framework (MOF) was constructed as an efficient platform for EVs isolation. Typically, one single-stranded DNA was designed and modified with a phosphate group at the 5′-end and cholesterol at the 3′-end to form a capture DNA named Phosphate–DNA–Cholesterol (PDC). The phosphate group forms a strong covalent bond with the designed defeated site of Zr (IV) in MOF UiO-66-NH2 and the cholesterol inserts into the phospholipid bilayer to capture EVs without non-membranous particles contamination. The formed MOF–Phosphate–DNA –Cholesterol–EVs (MOF@PDC@EVs) system was further treated with DNase I for DNA hydrolysis to give high pure EVs.

Results: A rapid and efficient isolation platform of EVs based on a defected MOF functionalized with Phosphate-DNA-Cholesterol (MOF@PDC) has been constructed successfully. Compared with Ultracentrifugation, MOF@PDC platform promises to isolate size heterogeneous EVs i) without non-membranous particles contamination, maintaining EVs intact membrane structure, protein components, and biological functions; ii) with the ability to capture EVs with 78% isolation efficiency; iii) makes EVs isolation process simple and fast, which could be finished in 40 minutes without requirement of the expensive equipment.

Summary/Conclusion: In conclusion, this rapid and efficient platform is suitable for isolation EVs from biological fluid for downstream protein analysis. This work opens a new perspective in MOF-based separation researches and may shed light on further studies towards EVs isolation.

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PF14: EVs as Delivery Vehicles

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Chair: Riccardo Alessandro – University of Palermo School of Medicine

PF14.01

Site-specific integration enables rapid cell engineering for the development of precision extracellular vesicle therapeutics

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Introduction: Incorporation of pharmacologically active molecules on the surface or the lumen of extracellular vesicles (EVs) is an important strategy for maximizing the therapeutic potential of EVs. Genetic engineering of producer cells by introducing DNA through random or site-specific integration are promising strategies for creating engineered EVs. Long-term stability with consistent transgene expression in the EV producer cells and therapeutic potency of resulting engineered EVs are crucial for biomanufacturing. We present a comprehensive study to investigate stability of transgene expression and potency of two potential therapeutic engineered EVs derived from stably selected pools transfected by either random integration (RI) or site-specific integration (SSI).
Methods: Producer cells were engineered to make EVs displaying interleukin 12 (IL12) or interferon gamma (IFNg) by RI or nuclease-mediated SSI into AAVS1 locus. Following puromycin (puro) selection, long-term cellular stability and transgene expression without selective pressure was investigated. EVs were generated from stable cell pools at 0, 1, and 2 months post-thaw and purified by density gradient ultracentrifugation. Purified EVs were biochemically characterized by NTA, BCA, Western blot, and cholesterol quantitation. Transgene expression and biological activity of EVs displaying IL12 and IFNg were assessed by AlphaLISA and in vitro reporter assays.
Results: Transfection by SSI resulted in faster recovery in puro selection compared to RI. All stable cell pools, regardless of integration method, resulted in comparable cell culture performance, EV yield, and lipid and protein content at all time points tested. The engineered EVs also demonstrated long-term stability of IL12 and IFNg transgene expression and in vitro activity from both integration strategies.
Summary/Conclusion: Both methods for generating stable cell lines were comparable in terms of cell stability, transgene expression, EV titre and potency, with SSI having the advantage of speed, allowing for more rapid iteration cycle times. Thus, both methods are suitable for the precision engineering of therapeutic EVs. This work demonstrates feasibility to manufacture therapeutic engineered EVs from stable cells from either integration strategy for clinical development.

PF14.02

Transport of outer membrane vesicles as a model therapeutic delivery system in pathogenic and commensal bacteria

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Introduction: Outer membrane vesicles (OMVs) in Gram-negative bacteria have been shown to be important carriers of biomolecules, including toxins and other virulence factors, peptidoglycan, and nucleic acids. It has been shown that OMVs play an important role in the delivery of these biomolecules to host cells and bacterial cells. While many thorough studies have explored OMV delivery to host cells, few studies have explored the mechanisms of delivery of OMVs to bacterial cells. Our goal was to study the delivery of OMVs to other bacterial cells. Specifically, we were studying the oral pathogen Aggregatibacter actinomycetemcomitans (A.a.), a Gram-negative organism associated with localized aggressive periodontitis, to study the process by which vesicles from this organism communicate with other bacterial cells. Overall, we want to

understand the roles specific surface components of OMVs play in the transport of these OMVs to other bacterial cells.
Methods: We studied OMVs from two strains of A.a.: JP2, a highly pathogenic strain, and 33384, a natural commensal strain. AF488-labelled OMVs were incubated with fresh bacterial cultures. Association of the OMVs with the bacterial cells was quantified using flow cytometry. To examine the role of surface-associated DNA in this process, DNA was digested with DNase, and the amount of surface-bound DNA was quantified with the membrane impermeable DNA stain, TOTO-1.
Results: Using flow cytometry, we observed JP2 OMVs were delivered to 33,384 cells, and at a lesser amount to JP2 cells. Alternatively, 33,384 OMVs associated readily with JP2 cells, more than to 33,384 cells. This suggests that the delivery of OMVs to bacterial cells may be a targeted delivery mechanism. Furthermore, we hypothesized surface-associated DNA may play a role in this interaction. We next digested the surface-associated DNA on the OMVs with DNase, and observed a decrease in association between the OMVs and bacterial cells. This supports our hypothesis that DNA on the surface of the OMVs plays a role in association. Current experiments are investigating this interaction in more detail.
Summary/Conclusion: We have demonstrated that OMVs are selectively delivered to bacterial cells, and surface-associated DNA plays a role in this process. We propose to investigate this process to further understand OMVs delivery to bacterial cells.
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PF14.03

Utilizing a Gaucher's disease cell line for the evaluation of a novel exosome-based replacement therapy

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Introduction: Engineered nano-scale exosomes have great potential as new and targeted delivery vehicles for the treatment of Gaucher's disease, the most common lysosomal storage disease. Recently, we have reported the design, production, and isolation of exosomes loaded with lysosomal β-glucocerebrosidase (GBA). People suffering from Gaucher's disease do not have functional GBA, which results in toxic build-up of undegraded substrates within the cell.
Methods: To evaluate the efficacy of this exosome-based therapy, a human Gaucher's disease model is required. Here, we have utilized near-haploid human cells (Hap1) modified via CRISPR-Cas9 to model Gaucher's disease in vitro. These cells contain a 479bp insertion in the 6th exon of the GBA gene, resulting in non-functional GBA. PCR, enzyme activity assays, and flow cytometry have been employed to confirm the diseased genotype and phenotype.
Results: Characterization of GBA-knock out cells shows a total loss of GBA enzyme activity. Further characterization demonstrates a normal growth rate but an increased number of lysosomes, indicating a diseased phenotype.
Summary/Conclusion: The utilization of a human GBA-knock out cell line will enable the evaluation of the efficacy of our engineered exosomes. Disease models will be an important resource for the evaluation of new biologic therapeutics, including exosomes.
Funding: We would like to acknowledge the Santa Clara University School of Engineering for their support.

PF14.04

ThRxosomes: a novel exosomes based theranostic for lung cancer

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Introduction: Chemotherapy is the first-line of treatment for lung cancer. However, inefficient bio-distribution and reduced accumulation of drugs in the tumour results in treatment failure. Therefore, improved drug delivery and diagnostic systems are warranted. Herein, we propose a novel theranostic system "ThRxosomes" where exosomes are loaded with Super Paramagnetic Iron Nanoparticles (SPIONs) conjugated to an anticancer drug via a pH-responsive linker for controlled release. We hypothesize that ThRxosomes will exert profound anticancer tumour activity that can be concurrently be monitored by Magnetic Resonance Imaging (MRI).
Methods: ThRxosomes were produced by combining normal human lung fibroblast (MRC9) cell-derived exosomes with SPIONS conjugated to and anti-cancer drug (Chemodrug or miRNA) via a pH cleavable linker. The physical and biological properties of ThRxosomes were determined using Transmission

Electronic Microscopy (TEM), Nanotracker-Analysis (NTA), Inductively Coupled Plasma Mass Spectrometry (ICPMS), Western blotting, cell viability, and MRI.

Results: Exosomes used in preparing ThRxosomes were 130 nm in size with a typical lipid bilayer structure, and were positive for CD63, CD81, Flotillin and negative for Annexin A1 confirming presence and purity of exosomes. Charge analysis, TEM, and ICMPS data showed successful loading of SPION-drug conjugate. Biological studies showed selective and enhanced drug release under acidic condition (pH 5.5) compared to drug release at pH 7.2. Cell uptake and viability studies demonstrated increased uptake and killing of ThRxosome-treated human A549 lung cancer cells compared to MRC-9 cells. In vivo studies demonstrated accumulation and detection of SPIONs by MRI in in-situ tumours of A549 tumour-bearing mice.

Summary/Conclusion: Our study demonstrates ThRxosomes will produce profound anticancer activity in lung cancer that is measurable by MRI.

Funding: The study was supported by grants from the Department of Defence (W81XWH-18-1-0637 & W81XWH-19-1-0647), Department of Veterans Affairs Merit Grant (101BX003420A1), by the National Cancer Institute Cancer Center Support Grant (P30CA225520), Oklahoma Center for Advanced Science and Technology (OCAST; HR18-088) and Presbyterian Health Foundation Bridge Grant.

PF14.05

Exosome-modified nanoparticles as an alternative delivery system for small RNAs in cancer therapy

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Introduction: Gene knockdown by RNA interference (RNAi) is an alternative, non-invasive method for inhibiting proliferation or promoting apoptosis in tumour cells. This technique allows the specific targeting of key signalling proteins or mutated genes. Most of the available transfection compounds suffer from rather profound cytotoxicity in vitro. The aim of our study was to establish a novel targeted small nucleic acid delivery system to the cells, with good cellular biocompatibility and applicability for in vivo studies. For this aim, we used native, cell own vesicles-exosomes. Since exosomes are known to transport peptides and different RNAs between cells and tissues, these unique, small extracellular vesicles (EV) may also be useful as transport vehicles for therapeutic siRNA.

Methods: As detected by multiple cell surface protein expression analysis, exosomes carry specific surface expression markers, allowing the cellular uptake by the most of tissues. We established an EV purification protocol from tumour cell culture supernatants and a strategy for the efficient EV loading with our test siRNAs or antimiRs. Here we used the combination of polyethylenimine (PEI)-complexation of the RNAs with ultrasound treatment for their loading into the EVs. Our EV-modified, ultrasound-treated nanoparticles were tested in vitro by measuring knockdown efficacies in luciferase reporter cell lines or by RT-qPCR gene expression analysis.

Results: More efficient cellular siRNA uptake was observed upon EV-modification of our PEI/RNA nanoparticles, accompanied by efficient inhibition of gene expression. Biological efficacies were retained also after storage for several days at room temperature. The monitoring of the EV-based particles by FACS revealed a different time resolution of cellular uptake and nucleic acid release compared to the classically formulated PEI-nanoparticles. In an in vivo therapy study in tumour xenograft-bearing mice, high biocompatibility, significant biological knock-down and tumour inhibition were observed after injection of anti-Survivin siRNAs formulated in our ECV-modified PEI nanoparticles.

Summary/Conclusion: Our data demonstrate the usability of ECV-modified nanoparticles as efficient delivery system for small RNAs in cancer therapy.

PF14.06

Microglial extracellular vesicles as therapeutic vector for neuroinflammation

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Introduction: Microglia is considered an eligible target against the progressive multiple sclerosis (MS), but currently available therapies do not allow its efficient targeting. As many cell types, microglia communicate with the neighbouring cells through a complex system of extracellular vesicles (EVs) exchange. Recently my group described that microglia derived-EVs, engineered to encapsulate IL4, are taken up by microglia itself,

mediating a phenotype switch to a protective phenotype. In vivo studies suggest that these EVs can ameliorate established neuroinflammation, thus making them a promising drug-delivery tool to target CNS in MS. My project focuses on understanding the mechanism of action and the signalling pathway of EVs delivery and to exploit this knowledge to specifically deliver different potential therapeutic molecules. For this purpose, we decided to characterize the EVs through TRPS technology.

Methods: A murine microglia cell line (BV2) was engineered to stably overproduce endogenous IL4. This cell line was cultured in exosome-depleted RPMI and stimulated with PMA(20 mg/mL) for 30 min. EVs isolation was carried out by collecting supernatant and subjecting it to consequential centrifugation of 300 g,10 min, RT and 2000 g,20 min, 4°C. The resulting supernatant was filtered (5 μm) and ultracentrifuged at 100,000 g for 2h at 4°C. The EVs pellet was re-suspended in ice-cold PBS.

Results: The EVs analysis with TRPS shows two populations of EVs, one with a mean diameter of 60–80 nm and a broad zeta potential ranging from −10 mV to −60 mV, while the second population has a mean diameter of 120–140 nm and a zeta potential of −10/-20 mV. This difference can be consistent with the different pathway formation of exosomes and microvesicles. We demonstrated in vivo the strong phenotypic change induced by our EVs to resting microglia in a dose- and time-dependent effect. Then, impairing the physiological procedure of the endosome acidification, the effect of our EVs on recipient cells is higher. Thus, suggesting an endocytic pathway for the internalization of the vesicles. We further demonstrate with gradient ultracentrifugation the capability of our formulation to vehicle endogenous IL4 inside the vesicles. Even if some protein is co-purified in the procedure, we know that the half-life of this cytokine is too short to elicit a strong in vivo response. Consequently, we assume that the anti-inflammatory effect of our EVs in vivo is a result of the IL4 internalized in our formulation.

Summary/Conclusion: These data help us understand more in detail the process of internalization and phenotype change mediated by these EVs. Our next goals are to discriminate between different internalization pathways and further validate the efficacy of our therapy on the EAE mouse model.

PF14.07

Targeting IL-3Rα on tumour-derived endothelial cells blunts metastatic spread of triple negative breast cancer via extracellular vesicle reprogramming

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Introduction: The lack of an approved targeted therapy and the early onset of metastasis highlight the need for new treatments for triple-negative breast cancer (TNBC) patients. Interleukin-3 acts as an autocrine factor for tumour-endothelial-cells (TEC), and exerts pro-angiogenic paracrine action via extracellular vesicles (nEVs). IL-3Rα blockade on TEC changes TEC-EV (anti-IL-3R-EVs) microRNA cargo and promotes the regression of established tumour vessels. As TEC are the doorway for "drug" entry into tumours, we have aimed to assess whether IL-3R blockade on TEC impacts tumour progression via their unique EV cargo.

Methods: 27 human TNBC samples, MDA-MB-231, MDA-MB-453 and MCF10 cell lines were evaluated for the expression of IL-3Rα. nEVs and anti-IL-3R-EVs were characterized by electron-microscopy, MACSPlex-Exosome-Kit and western blot. Proliferation, migration, apoptosis and sphere formation were evaluated. SCID mice were used for in vivo experiments.

Results: We noticed that, besides TEC and inflammatory cells, tumour cells from 55.5% of the human TNBC samples expressed IL-3Rα. MDA-MB-231 and MDA-MB-453, but not MDA10 cells, expressed IL-3Rα. In vitro, nEVs provide survival and migratory signals, while anti-IL-3 R-EVs promoted apoptosis as well as reduced cell viability and migration of human TNBC cell lines. In vivo anti-IL-3 R-EV treatment induced vessel regression in established tumours formed of MDA-MB-231 cells and almost abolished the spread of liver and lung metastasis. Moreover, decreased β-catenin and TWIST1 were found in tumours from animals treated with anti-IL-3 R-EVs. In addition, anti-IL-3 R-EVs reduced lung metastasis that was generated via the intravenous injection of MDA-MB-231 cells. nEVs that were depleted of miR-24-3p (antago-miR-24-3p-EVs) were effective as anti-IL-3 R-EVs in down-regulating TWIST1 and reducing lung-vessel density and metastatic lesions in vivo.

Summary/Conclusion: Overall, these data provide the first evidence that IL-3 Rα is highly expressed in TNBC cells, TEC and inflammatory cells, and that IL-3 Rα blockade on TEC impacts tumour progression.

Funding: AIRC, associazione Italiana per la Ricerca sul Cancro.

PF15: Evs in Cancer Metasis and Tumour Angiogenesis

Chair: Elena S Martens-Uzunova – Department of Urology, Erasmus Medical Centre

Chair: Richard Zieren – The Brady Urological Institute, Johns Hopkins University School of Medicine

PF15.01

Statins decrease the implantation and invasion of high-grade serous ovarian cancer cells induced by exosomes by altering its composition and its cell uptake and the endocytic traffic at the metastatic niche.

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Introduction: High-grade serous ovarian cancer (HGSOC) is the deadliest gynaecologic cancer. Its lethality is explained for late diagnosis at advanced stages and frequent recurrences despite achieving complete response with standard therapy. Most of recurrences occurs at abdominal cavity with multiple metastasis. Therefore, identifying key determinants of metastatic process remains as priority to find better therapies. Current evidence assigns a central role of the exosomes in conditioning the metastatic niche in epithelial cancers. Recently, we demonstrated that statins reduce metastasis in HGSOC in preclinical models. Here, we decided to study the effects of statins on HGSOC-derived exosomes and its capability to condition the metastatic niche.
Methods: Exosomes were isolated from HeyA8 ovarian cancer cell line and primary tissue cultures established from advanced-stage HGSOCs (with signed informed consent and IRB approval) by differential ultracentrifugation and quantified by nanoparticle tracking analysis (NTA). Enriched-cancer initiating cells (CIC) spheroids were established from HeyA8 cells by using stem-selecting conditions. The paracrine effect of exosomes on CIC migration/invasion was studied using either 3D migration or Boyden chamber invasion assays. Previous to exosome isolation, HeyA8 cells were treated with simvastatin (5uM, 24 h) or solvent and proteins involved in exosome biogenesis/uptake (Alix, Tsg101), its trafficking (Rab7a, Rab27a) and in conditioning the metastatic niche (EMMPRIN) were measured by immunoblotting.
Results: Exosomes isolated from HeyA8 cells or HGSOCs enhance the metastatic potential of HeyA8-established spheroids in 3D migration or Boyden chamber invasion assays. Upon simvastatin treatment, we observed a significant reduction in migration/invasion induced by equivalent number of exosomes in HeyA8-derived CICs. Under same treatment, we observed a significant decrease in protein levels of Alix and Tsg101 and an increase in the inactive forms of Rab7a and Rab27a in HeyA8 cells. We also observed a decrease in EMMPRIN levels in HeyA8-derived exosomes.
Summary/Conclusion: Here, we demonstrated a paracrine effect of HGSOC-derived exosomes that favour the metastasis process. In addition, we demonstrated that simvastatin reduces metastasis induced by cancer-derived exosomes. Such an effect is partially explained by changes in the expression of proteins involved in exosome biogenesis/uptake, its endocytic trafficking and in the content of proteins conditioning the metastatic niche. Thus, simvastatin arises as potential therapeutic target to improve outcomes in this disease.
Funding: This research was supported by Fondecyt granted to Mauricio A. Cuello

PF15.02

Label-free optical imaging and characterization of cancer-associated extracellular vesicles in tissues

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Introduction: Cancer-associated extracellular vesicles (EVs) visualized in the tumour microenvironment have been identified as a potential biomarker for cancer-related tissue changes. Analyses of EVs have

traditionally been performed in cells or isolated EVs, with no temporal or spatial information that could be critically important for elucidating their roles in carcinogenesis. Since the unperturbed distribution and organization of EVs in the tumour microenvironment is associated with their cellular function and can potentially serve as a diagnostic and prognostic biomarker, there is a strong need for visualizing EVs in freshly isolated tissue specimens. Currently, only fluorescent labelling methods enable visualization and tracking of EVs. We used a custom label-free multimodal multiphoton optical imaging system to detect and characterize EVs and classify them using their optical signatures both in isolated tissues and in situ tumours.

Methods: Label-free multimodal multiphoton imaging was used to provide simultaneous, co-registered structural and functional images of EVs in untreated samples. Heterogeneous populations of EVs could be identified from their unique optical signatures.

Results: The intrinsic metabolic and structural properties of EVs enabled reliable visualization and optical characterization of EVs from cell cultures and in situ imaging of tumour-bearing rats. Unique optical signatures were then used for identification of cancer-related EVs in tissues from human breast cancer patients, and their density was found to be highly correlated with clinical diagnosis. In the current study, EVs were isolated from urine of tumour-bearing dogs, and urine and serum from breast cancer patients. Analysis of EV content showed higher concentration of NAD(P)H in EVs isolated from cancer subjects, than from healthy subjects, which reflects the reprogramming of cellular metabolism in carcinogenesis.

Summary/Conclusion: These results suggest a potential label-free optical methodology to detect and characterize EVs by their optical signatures, which can be utilized as possible diagnostic and prognostic biomarkers for cancer.

Funding: This research was conducted under protocols approved by the IACUC and IRB at the University of Illinois and Carle Foundation Hospital, and supported by funding from NIH.

PF15.03

Novel potential anticancer therapies based on interference with nuclear entry of cancer cell-derived extracellular vesicle components in recipient cells

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Introduction: The intercellular communication mediated by extracellular vesicles (EVs) in the tumour microenvironment plays an important role in tumour progression. Experimental evidence indicates that EVs derived from highly metastatic cells influence the behaviour of less aggressive cancer cells. We have previously described a novel intracellular pathway where a fraction of endocytosed EV-associated proteins and nucleic acids is transported into the nucleoplasm of the host cell via a subpopulation of late endosomes penetrating into nucleoplasmic reticulum (NR). Here, we better characterize this pathway and report that it is required for the induction of an aggressive behaviour induced by EVs released from highly metastatic SW620 colon cancer cells in isogenic primary cancer cells.

Methods: Super resolution-structured illumination microscopy and magnetic-based co-immunoisolation studies were employed to identify the protein components of the nuclear pathway and to monitor the entry of EV-containing late endosomes into the nucleoplasmic reticulum. Human SW480 carcinoma cells expressing ER-GFP and Rab7-RFP were exposed to EVs from SW620 cells and then live imaged.

Results: We have previously reported that the tripartite protein complex, containing VAP-A, ORP3 and Rab7 orchestrates the localization of EV-carrying late endosomes into NR. We now report that silencing of ORP3 or VAP-A, but not its homologue VAP-B, reverses the pro-metastatic changes induced by EVs isolated from metastatic cells on their non-metastatic counterpart, including transition to an ameboid phenotype, cell rounding and blebbing. Moreover, we found that certain nuclear pore complex proteins and importin-beta1 are co-immunoisolated with ORP3, VAP-A and Rab7 suggesting the formation of a large protein complex at the entry of nuclear pores.

Summary/Conclusion: Interfering with the mechanisms regulating this novel intracellular pathway may find therapeutic applications particularly in EV field and oncology.

PF15.04

Educated osteoblasts regulate breast cancer proliferation via small extracellular vesicles

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Introduction: Breast cancer commonly traffics to bone, where breast cancer cells (BCCs) can survive undetected for years before metastatic outgrowth. In bone, BCCs interact with surrounding stromal cells, including osteoblasts (OBs), to shape the metastatic niche. Our lab discovered there are at least two subpopulations of OBs in the bone-tumour niche, based on protein marker expression. One group, "educated osteoblasts" (EOs) have engaged in crosstalk with BCCs whereas another group, naïve OBs, have not. We have novel evidence that EOs regulate BCC proliferation. The purpose of this study was to determine if extracellular vesicles (EVs) produced by EOs play a role in regulating BCC proliferation. We hypothesized EVs produced by EOs would decrease BCC proliferation.
Methods: EO-derived small EVs from culture media were isolated via ultracentrifugation and characterized EVs for size, protein marker expression, and density floatation to validate the purity of EV samples. The functionality of EO-derived EVs on BCC proliferation was examined using EdU and checkpoint proteins p21 and p27. BCC protection from chemotherapy induced cell death was also examined.
Results: We found that EVs produced by EOs, but not naïve OBs, decreased both triple negative and ER-positive BCC proliferation in a concentration dependent manner. Furthermore, using an EdU assay, we found that exposure to EO-derived EVs induces BCCs to undergo cell cycle arrest. Interestingly, the cell cycle arrest was reversible and BCC proliferation was restored upon removal of EO-derived EVs. In addition, exposure to EO-derived EVs leads to increases in BCC expression of the G1 checkpoint proteins, p21 and p27.

We next wanted to investigate proliferative signalling pathways that may be deregulated in BCCs following exposure to EO-derived EVs. We found that EO-derived EVs reduce BCC levels of ERK1/2.

Because our data indicate EO-derived EVs induce sustained cell cycle arrest in BCCs, we desired to know if EO-derived EVs protected BCCs from chemotherapy-induced cell death. We found that BCCs exposed to EO-derived EVs and the chemotherapy drug, doxorubicin, have decreased cell death compared to BCCs exposed only to doxorubicin.
Summary/Conclusion: Altogether, our data suggest EOs play a crucial role in bone-tumour microenvironment by regulating BCC proliferation.
Funding: Supported by NIH R00 CA178177 and Commonwealth of Pennsylvania – Department of Health SAP 4100072566 for KMB.

PF15.05

Phosphorylation of Tyrosine 23 in annexin A2 is essential for its association with exosomes and for imparting invasive and proliferative capacity to other cells

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Introduction: Triple negative breast cancer (TNBC) accounts for 15%-20% of all breast cancer cases. The lack of targeted-based therapies highlights the importance of studying TNBC. Elevated levels of Annexin A2 (AnxA2), a Ca+2-dependent phospholipid binding protein, has been correlated with worse overall survival in TNBC patients. Our previous data implicate that exosomal AnxA2 is involved in creating a pre-metastatic niche and facilitating metastasis in TNBC. Moreover, N-terminal phosphorylation of Tyrosine (Tyr) 23 in AnxA2 has been implicated in regulating several AnxA2 activities in cancer progression. Here, we demonstrated that N-terminal phosphorylation of AnxA2 at Tyr23 is important for its association with exosomes which imparts invasive and proliferative phenotype to other cells. Hence, dissecting the regulatory pathway will be critical for verifying the value of AnxA2 as a therapeutic, diagnostic or prognostic marker in TNBC.
Methods: pN1-EGFP plasmids expressing the constitutive phosphomimetic (AnxA2-Y23E) and non-phosphomimetic mutant (AnxA2-Y23 F) gene expressing mutation at Tyr23 site were overexpressed in MDA-MB-231 TNBC cells. Mutant cells were experimentally validated for AnxA2 specific functions like migration, invasion and proliferation. Exosomes were isolated from the mutant phosphomimetic (exo-AnxA2-Y23E-GFP) and non-phosphomimetic (exo-AnxA2-Y23 F-GFP) cells and were analysed for exosomal surface expression of AnxA2 by immunoprecipitation and flowcytometry. CAL-148 breast adenocarcinoma epithelial cells were treated with exo-AnxA2-Y23E-GFP and exo-AnxA2-Y23 F-GFP to analyse the rate of invasion and proliferation by transwell invasion and proliferation assay, respectively. Transfer of exosomal AnxA2 in CAL-148 was studied using immunofluorescence and its implications on signalling pathways were studied by Western blot.
Results: MDA-MB-231 phosphomimetic TNBC mutant cells showed increased migratory, invasive and proliferative capacity compared to non-phosphomimetic TNBC mutant cells. Exo-AnxA2-Y23E-GFP had elevated surface AnxA2 expression compared to

exo-AnxA2-Y23 F-GFP. CAL-148 cells treated with Exo-AnxA2-Y23E-GFP showed high migratory, invasive and proliferative characteristics, with a higher expression of p-AnxA2(Tyr23), p-Src(Tyr416) and p-Paxillin(Tyr31) compared to Exo-AnxA2-Y23 F-GFP treated cells.

Summary/Conclusion: N-terminal phosphorylation of Tyr23 in AnxA2 in MDA-MB-231 TNBC cells (Phosphomimetic mutant cells) is essential for its association with exosomes and for conferring increased invasive and proliferative capacity to other breast cancer cells.

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PF15.06

A novel method for epithelial-derived extracellular vesicle isolation and enrichment in patients with advanced prostate cancer

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Introduction: Evaluation of changes in prostate cancer biology is difficult due to presence of lymph nodal or bony metastatic disease in a majority of patients. A number of liquid biopsy assays have shown clinical utility in prostate cancer, but are limited by low sensitivity (e.g. circulating tumour cells-based assays) or inability to perform transcriptome sequencing (cell-free DNA-based assays). Epithelial-derived extracellular vesicles (epi-EV)-based assays are uniquely positioned overcome both these limitations as EVs are abundantly secreted into the blood and have RNA-cargo that mirrors the cell of origin. However, a reliable method to enrich for epi-EVs is currently lacking.

Methods: Plasma was isolated from the peripheral blood collected from 15 patients with metastatic prostate cancer enrolled in an institutional biobanking study before initiation of systemic antineoplastic therapy. EVs were isolated from 500 µl of plasma using a commercially available qEV 35 size exclusion column (Izon Inc.). Without subjecting the EVs to any physical stressors such as centrifugation, CD61 magnetic beads were used to fractionate the EVs into CD61+ (platelet derived) and CD61- (non-platelet derived) fractions. Multiparameter flow cytometry was used to evaluate EVs that expressed CD9 and EpCAM and were negative for Calnexin. Nanotracking Analysis (NTA) was used to quantify both total EV and CD61+ and CD61- fractions in all patient samples.

Results: The average ± standard deviation of total EVs obtained from the 15 patients was 6.39x10^11 ± 4.60x10^11 EVs/ml of plasma (coefficient of variation [CV]: 72%) while the average and standard deviation of CD61- EVs was 1.24x10^10 ± 3.37x10^10 (CV: 271%). The CD61- EV fraction represented a variable amount of the total EVs in prostate cancer patients ranging from 0.0001% to 32.21%. Multiparameter flow cytometry showed that over 80% of total EVs were CD9+ and calnexin-, suggesting an endosomal origin for a vast majority of the EVs in these plasma samples. However, the proportion of EVs expressing EpCAM (marker of epi-EVs) was higher among the CD61- fraction (5% – 30%) as compared to the CD61+ fraction (0.04% – 1%).

Summary/Conclusion: Our novel method was able to isolate and enrich the epi-EV from the plasma of advanced prostate cancer patients. Correlation between clinical characteristics and EV quantity is being evaluated to identify the reason(s) for large variations in CD61- EV fraction. Future studies are planned to use our method in improving the sensitivity of EV-based assays and increase the RNA yield to facilitate transcriptome sequencing.

Funding: This work was funded by grants from Randy Shaver Community and Research Fund, Minnetonka, MN.

PF15.07

Exosomes drive medulloblastoma metastasis in a MMP2 and EMMPRIN dependent manner

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Introduction: Recurrent/metastatic medulloblastoma (MB) is a devastating disease with an abysmal prognosis of less than 10% 5-year survival. The secretion of extracellular vesicles (EVs) has emerged as a pivotal mediator for communication in the tumour microenvironment during metastasis. The most investigated EV's are exosomes, nanovesicles secreted by all cell types and able to cross the blood-brain-barrier. Matrix metalloproteinases (MMPs) are enzymes secreted by tumour cells that can potentiate their dissemination by modification of the extracellular matrix. We hypothesise that exosomal MMP2 and its inducer EMMPRIN could enhance metastasis of MB.

Methods: Proliferation, invasion and migration assays were used to evaluate the phenotypic behaviour of

primary cell lines pre-treated with metastatic tumour cell-derived exosomes. Gelatin zymography and western blotting were performed to confirm MMP2 functional activity in cell lines and exosomes. Nanoscale flow cytometry was used to measure surface exosomal EMMPRIN levels. Exosomal MMP2 and EMMPRIN were modulated at the RNA level.

Results: Number of exosomes is directly related to the migratory behaviour of parental MB cell lines ($p < 0.01$). Notably, functional exosomal MMP2 and EMMPRIN levels also correlate with this. Furthermore, exosomes from metastatic cell lines conferred enhanced migration and invasion on their matched isogenic primary (non-metastatic) cell line pair by ~3.8-fold ($p < 0.01$). Exosomes from metastatic cell lines also conferred increased migration on poorly migratory foetal neuronal stem cells.

Summary/Conclusion: Together this data suggests that exosomal MMP2 and EMMPRIN may promote medulloblastoma metastasis and supports analysis of exosomal MMP2 and EMMPRIN levels in patient cerebral spinal fluid samples.

Funding: The James Tudor Foundation, The Children's Brain Tumour Research Centre, The University of Nottingham Life Sciences

PF15.08

Anti-migratory effect of Nm23-H1 via exosomes

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Introduction: Exosomes secreted from cancer cells harbour the potential to regulate intracellular signalling and promote metastasis. Wherein, metastasis suppressor genes (MSGs) play a pivotal role in regulating such signalling cascades. However, the regulation gets hampered due to low expression of MSGs under metastatic conditions. Nm23-H1, product of first identified metastasis suppressor gene NME1, is significantly downregulated under metastatic conditions. Nm23-H1 serves as a regulator of small GTPases. Several evidences have highlighted an involvement of small GTPases (such as Rab5, Rab7 and Rab27) in the biogenesis of exosomes. In addition, bacterial homolog of Nm23 has been shown to interact with Rab5 and Rab7. However, experimental evidence supporting a relationship between exosomes and Nm23-H1 is lacking. Our current focus is to deduce the relationship between exosomes and MSGs.

Methods: Breast cancer cell lines were used to assess the effect of exosomes isolated from highly metastatic cells (MDA-MB-231 cells) on lower/non metastatic cells (MCF-7 cells). NME1 was overexpressed in MDA-MB-231 cells and subsequently used to isolate exosome fractions. Equivalent amount of isolated exosome fractions from MDA-MB-231 cells and MDA-MB-231/NME1 cells were utilized to access their effect on migration and difference in exosome markers.

Results: We observed an enrichment of Nm23-H1 in the exosomes isolated from MDA-MB-231 cells upon overexpression of NME1. Proteinase K protection assay confirmed the packaging of Nm23-H1 inside the exosomes isolated from MDA-MB-231/NME1 cells and excluded the possibility of membrane association of Nm23-H1. Additionally, overexpression of Nm23-H1 led to a significant reduction in the ability of MDA-MB-231 exosomes to stimulate movement of MCF-7 cells as confirmed by wound healing assays. Our data also highlights a clear reduction in the protein levels of exosome markers such as CD63, CD9 and Alix in the exosome fraction isolated from MDA-MB-231/NME1 cells as compared to MDA-MB-231 cells. Interestingly, Rab7A, a protein involved in the endosome-lysosome fusion was also present in lower amount in the exosomes isolated from Nm23-H1 overexpressing cells.

Summary/Conclusion: Our data highlights an anti-migratory effect of Nm23-H1 via exosomes. These findings support a regulatory role of Nm23-H1 in the packaging or release of exosomes in highly metastatic breast cancer cells, and further suggest that metastasis suppressor proteins may be involved in the regulation or packaging of exosomes. Additional studies will be required to decipher the downstream signalling of Nm23-H1 which affects the biogenesis of exosomes as well as to assess the effect of Nm23-H1 overexpression on the content of exosomes. These insights could help us delineate the complex exosome biogenesis pathway and provide new potential drug targets for exosome regulation.

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PF16: EVs in Tissue Injury and Repair

Chair: Cristina Grange – Deparment of Medical Sciences,University of Torino

Chair: Felix Kim – Thomas Jefferson University

PF16.01

Moderate exercise has beneficial effects on mouse ischaemic stroke by enhancing the functions of circulating endothelial progenitor cell-derived exosomes via activation of the miR-126/BDNF/PI3 k pathway

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Introduction: Exosomes (EXs) are emerging as novel players in the beneficial effects induced by exercise on vascular diseases. Our recent study has revealed that moderate exercise enhances the function of circulating endothelial progenitor cell-derived exosomes (cEPC-EXs) on protecting endothelial cells against hypoxia injury. In this study, we aimed to investigate whether exercise-regulated cEPC-EXs contribute to the beneficial effects of exercise on ischaemic stroke (IS).
Methods: C57BL/6 mice performed moderate treadmill exercise (10 m/min, 4-wks) before IS induced by middle cerebral artery occlusion surgery. Acute injury was evaluated at day 2 by determining neurologic deficit, infarct volume, cell apoptosis in the penumbra and neurologic recovery was assessed by determining angiogenesis/neurogenesis, sensorimotor functions at day 28. The correlations of cEPC-EXs and their carried miR-126 with neurological parameters were analysed. The underlying mechanism of the effects of cEPC-EXs isolated from exercised mice was explored in a hypoxia neuron model. Cellular miR-126 level, apoptosis, axon growth ability and gene expressions (cas-3, BDNF and Akt) were measured.
Results: 1) Exercised mice had a smaller infarct volume on day 2, which was associated with decreased cell apoptosis and cleaved cas-3 level, and a higher microvessel density than those in control; 2) The elevated cEPC-EX level positively correlated with tEPC-EXs in ischaemic brain of exercised mice on day 2. The upregulated miR-126 level positively correlated with the numbers of tEPC-EXs in ischaemic brain; 3) The numbers of cEPC-EXs and their carried miR-126 level negatively correlated with the infarct volume, cell apoptosis and positively correlated with the microvessel density in the peri-infarct area on day 2; 4) Exercised mice had decreased infarct volume, increased microvessel density, promoted angiogenesis/neurogenesis and improved sensorimotor functions on day 28, accompanying with upregulated levels of BDNF, p-TrkB/TrkB and p-Akt/Akt; 5) cEPC-EXs of exercised mice protected neurons against hypoxia-induced apoptosis and compromised axon growth ability which were blocked by miR-126 and PI3 k inhibitors.
Summary/Conclusion: Our data suggest that the protective effects of moderate exercise intervention on the brain against MCAO-induced ischaemic injury are ascribed to cEPC-EXs and their carried miR-126.
Funding: This work was supported by American Heart Association (18POST33990433) and NIH (1R01NS 102720).

PF16.02

Syndecan-1 regulates alveolar type 2 epithelial cell senescence mediating through extracellular vesicles during lung fibroproliferation

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Introduction: Alveolar type 2 epithelial cell (AT2) senescence is implicated in the pathogenesis of lung fibrosis, a progressive fatal condition. Syndecan-1, a heparan sulphate proteoglycan, is overexpressed by AT2 cells of human idiopathic pulmonary fibrosis (IPF) and bleomycin-injured WT mice and the overexpression of syndecan-1 is profibrotic. Moreover, syndecan-1 deficient (Sdc1-/-) mice had less lung fibrosis after bleomycin injury. We reported that extracellular vesicles (EVs) in bronchoalveolar lavage (BAL) of bleomycin-injured WT mice augmented lung fibrosis whereas the Sdc1-/- -BAL-EVs attenuated the process. Moreover, WT-BAL-EVs expressed lower level of anti-fibrotic miRNAs (miR-34b-5p, −142-3p, −144-3p, and −503-5p) compared to the Sdc1-/–BAL-EVs. These miRNAs targeted genes in the cellular senescence pathway indicating that syndecan-1 altered microRNA profiles in the BAL-EVs to promote cellular senescence during lung fibrogenesis.

We investigate how syndecan-1 regulates AT2 senescence through EVs.
Methods: Bleomycin was intratracheally given into WT and Sdc1-/- mice. At day 21, lungs were processed for single-cell RNA sequencing (scRNAseq) and western blot (WB). EVs were isolated using ultrafiltration centrifugation method. Human (A549) and mouse (MLE-15) lung epithelial cell lines were used for in vitro experiments.
Results: scRNAseq analysis indicated while bleomycin stimulated an overexpression of cellular senescence-specific genes on AT2 cells of WT mice, these genes were significantly downregulated on Sdc1-/- AT2 cells. Senescence proteins, p16 and p21, were also less expressed in the lungs of Sdc1-/- than of the WT mice by WB. To determine the functional effects of EVs in BAL, A549 cells were treated with human IPF or control lung wash-EVs and evaluated for beta-galactosidase activity. We found that IPF-EVs markedly increased beta-galactosidase enzymatic activity. Corroborating with these data, bleomycin-injured BAL-WT-EVs also significantly upregulated senescence marker, p21, by WB on MLE15 cells whereas Sdc1-/--BAL-EVs inhibited p21 expression.
Summary/Conclusion: Our data indicate that syndecan-1 regulates lung fibrosis through the senescence signalling pathway on AT2 cells. Furthermore, syndecan-1 controls AT2 senescence mediating through extracellular vesicles in the BAL. Lastly, the most likely cargo molecules mediating this process are microRNAs.
Funding: This research was funded by the National Institute of Health (NIH)/National Centre for Advancing Translational Sciences UCLA-CTSI-KL2-UL1TR001881 (TP), R01 HL13707 (PC), and the Parker B Francis Foundation Fellowship (CY).

PF16.03

Immortalized cardiosphere-derived cell EV-associated piRNA, imEV-Pi, protects against ischaemic injury in the heart

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Introduction: Cardiosphere-derived cells (CDCs) are a population of heart-derived progenitors with demonstrated therapeutic efficacy in preclinical and clinical settings. CDCs function by secreting extracellular vesicles (EVs), lipid-bilayer nanoparticles laden with bioactive molecules. Recently our group developed a strategy for immortalizing CDCs (imCDC) that retains their therapeutic potential and enhances CDC function indirectly through their secreted EVs. imCDC show a different RNA content(miRNA, mRNA, rRNA, tRNA and piRNA) compared to primary CDC. In particular, we focus on Piwi RNAs (PiRNAs), small RNAs bound by Piwi proteins, important regulators of both the epigenome and transcriptome. We seek to explore the role of a PiRNA highly enriched in imCDC-EVs (imEV-Pi).
Methods: EVs are prepared by conditioning cells for 24 hrs in serum-free basal media, in hypoxic culture. After 24 hrs conditioned medium is cleared of cellular debris and EVs isolated using ultrafiltration by centrifugation (ufc). Fractions were analysed in terms of particle size, number, and concentration and piRNA content. In vitro, bone marrow derived-macrophages (BMDM) were exposed to imCDC-EV, imEV-Pi and control and transcriptomic profile and potentially activated pathways were assessed. In vivo, 8–10 week-old Wistar-Kyoto female rats received 10^10 imCDC-EV, imEV-Pi, scramble or vehicle intracoronary 20 minutes after ischaemia-reperfusion(I/R). Cardiac Troponin I levels, scar size and monocytes were assessed at 24 and 48 hrs.
Results: By small-RNA sequencing analysis we found that piRNAs are enriched in both CDC-EV and imCDC-EV. imCDC show a different PiRNA composition compared to primary CDC. imEV-Pi was identified as one of the most highly-expressed non-coding RNAs (the number of reads were 35X higher in imCDC-EV compared to CDC-EV). In vitro, imExo-Pi-conditioned BMDM exhibit a different transcriptomic profile compared with control, with upregulation of pathways involved in the inflammatory response, cell death, and cell-to cell signalling. In vivo, imEV-Pi is cardioprotective, as shown by reduced scar size and lower cardiac troponin levels compared to vehicle- and scramble-injected animals at 48 hrs post I/R. imEV-Pi only minimally alters neutrophil counts profile in blood but it alters monocytes profile with a decreased number at 24 hrs and an increase at 48 hrs.
Summary/Conclusion: We posit that imEV-Pi is a key determinant of imCDC-EV therapeutic efficacy. Our results indicate that target cells may be macrophages/monocytes, given that imEV-Pi exposure modifies their composition and mRNA profile both in vitro and in vivo.
Funding: This work was supported by funding from the National Institutes of Health (NIH R01 HL124074 (to EM)

PF16.04

Enhancement of extracellular vesicles from umbilical stem cell in hair follicle regeneration

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Introduction: Extracellular vesicles (exosomes, EVs) are cell membrane particles (30–200 nm) secreted by virtually cells. During intercellular communication in the body, secreted EVs play crucial roles by carrying functional biomolecules (e.g., microRNAs and enzymes) into other cells to affect cellular function, including disease progression and tissue regenerations. Literature previously reported that the macropinocytosis pathway contributes greatly to the efficient cellular uptake of EVs. The activation of growth factor receptors, such as epidermal growth factor receptor (EGFR), induces macropinocytosis. In this study, we demonstrated the effects of EVs on demal papilla and hair follicle regeneration.
Methods: Identification of distinct nanoparticles and subsets of extracellular vesicles from umbilical cord blood stem cell by asymmetric flow field-flow fractionation.
Results: The effects of EVs from umbilical cord blood stem cell on the propagation of demal papilla and hair follicle regeneration were observed.
Summary/Conclusion: The enhancement of extracellular vesicles from umbilicalcord blood stem cell the propagation of demal papilla and hair follicle regeneration were observed and confirmed.

PF16.05

Mechanisms of host resistance to plasma membrane damage induced by pneumolysin attack

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Introduction: Bacterial pore-forming toxins (PFTs) are major virulence factors produced by pathogens. PFTs target host plasma membrane (PM) and create transmembrane pores, which allow uncontrolled flux of ions and small molecules across the PM disrupting cellular homoeostasis. To survive, cells display poorly understood repair mechanisms to recover the cell homoeostasis. Several mechanisms were proposed to participate in cell recovery: exocytosis of cortical lysosomes; endocytosis of PFTs pores; PM blebbing and shedding.
Methods: We used increasing concentrations of purified PLY to intoxicate cells. PM permeability was assessed by flow cytometry using propidium iodide dye. Cytoskeleton rearrangements were investigated by confocal immunofluorescence microscopy.

Extracellular vesicles released during PM repair were isolated by high-speed centrifugation and characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and mass spectrometry/liquid chromatography analysis.
Results: PLY triggers a complete reorganization of the actomyosin cytoskeleton inducing the formation of cortical actomyosin bundles at sites of PM remodelling. These structures assemble upon loss of PM integrity and disassemble as PM recovers. We detected the release of microvesicles during the recovery of PM integrity. Vesicle population is heterogeneous with sizes ranging from 100 to 500 nm, with the majority of them measuring 100–200 nm. Vesicle proteomic analysis revealed that they contain PLY, suggesting they participate in pore removal, proteins involved in vesicle trafficking, PM repair and exosome biogenesis.
Summary/Conclusion: Our data demonstrate that cells are able to recover from the damage induced by sublytic concentrations of PLY. Actomyosin cytoskeleton undergo massive changes with the assembly of cortical bundles possibly at sites of PM damage. We showed that cells produced extracellular vesicles during the process of repair. We are now focusing on understanding the biogenesis of those vesicles and its importance during the process of repair.
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PF17 = OP2

Oral with Poster Session 2: Cancer and Technology

Chair: Lizandra Jimenez – Postdoctoral Research Fellow, Vanderbilt University

PF17.01 = OP2.01

Development of scalable processes to produce therapeutic mesenchymal stromal cell-derived extracellular vesicles and their characterization

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Introduction: Despite of high expectations, mesenchymal stromal cell (MSC)-based therapies still lack efficacy, partially due to loss of cell viability and function upon administration. MSC-derived extracellular vesicles (MSC-EV) emulate the regenerative potential of MSC, shifting the field towards cell-free therapies. Clinical applications require the establishment of a scalable and GMP-compliant processes for the production and isolation of MSC-EV, combined with robust characterization platforms.
Methods: To develop a well-established process for the production of therapeutic MSC-EV, we compared different MSC sources (bone marrow, adipose tissue, umbilical cord matrix), culture media compositions (DMEM supplemented with foetal bovine serum (Thermo Fisher Scientific), DMEM supplemented with human platelet lysate (AventaCell Biomedical) and StemPro MSC SFM Xeno Free medium (Thermo Fisher Scientific)) and culture parameters (oxygen tension and shear stress) in two different culture platforms (2D static tissue culture flask vs 3D dynamic spinner vessels). Subsequently, MSC-EV were isolated by ultracentrifugation or a commercially available isolation kit and characterized according to ISEV guidelines.
Results: MSC derived from different sources/donors were able to grow under normoxia and hypoxia in 2D T-flasks and 3D spinner vessel culture systems, while maintaining their immunophenotype and differentiation potential, according to the minimal criteria defined by the ISCT. The time point for pre-conditioning and collection of conditioned medium for MSC-EV isolation was also optimized for both 2D and 3D culture systems. MSC-EV were characterized according to MISEV 2018 guidelines, using techniques as NTA, protein and lipid quantification, western blot, imaging and Fourier-Transform Infrared Spectroscopy (FTIR). The results indicate that MSC-EV derived from different sources/donors have similar size distribution, however, EV yields tend to be higher for the 3D culture system. Of notice, several spectral regions were identified by FTIR, enabling the detection of differences in the biomolecules present in MSC-EV, MSC-conditioned media and cells produced under different conditions.
Summary/Conclusion: In summary, this study contributes to the establishment of a scalable process for MSC-EV production.

PF17.02 = OP2.02

Evaluation of three different isolation methods for small extracellular vesicles from human plasma in prostate cancer diagnosis

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Introduction: Extracellular vesicles (EVs) have great potential in prostate cancer (PCa) diagnosis and progression monitoring to complement the inaccurate prostate specific antigen (PSA) screening and invasiveness of tissue biopsy. However, current methods cannot isolate pure EVs and therefor EVs characteristics remain largely unknown. In order to develop an accurate approach for EV isolation, we aimed to compare three emerging methods with different characteristics of small EVs (sEVs) from human PCa plasma samples and to choose the best one for diagnostic and functional studies.
Methods: PCa patients and age-matched healthy controls (HC) plasma (n = 6 in each group) were used to isolate sEVs with 3 different isolation methods including commercial ExoQuick Ultra Kit, qEV 35 and qEV 70 size exclusion chromatography (SEC). Isolated sEV were characterized by nanoparticle tracking analysis,

immunoblotting, cyrogenic electron microscopy, flow cytometry (FC) and proteomics analysis. For FC characterizing surface marker expression, the sEVs were further purified by CD9 and CD81 commercial immunoaffinity magnetic beads . Lipoprotein was captured by streptavidin biotinylated ApoB magnetic beads to measuring the lipoprotein contamination.

Results: The sEV size, morphology, surface protein and protein cargo with proteomics were analysed between the three isolation methods. sEVs isolated from SEC methods had a lower particle size, protein amount, protein/sEV marker ratio and ApoB+/sEV marker ratio than those from ExoQuick Ultra method. In addition, sEVs isolated from qEV35 demonstrated a significantly higher sEV content, more up-regulated and down-regulated PCa proteins from proteomics but lower sEV marker/protein ratio and a higher protein contamination than those from qEV70. Furthermore, sEV marker signal also showed a good correlation with particle numbers instead of protein content in all the methods.

Summary/Conclusion: qEV 70 method demonstrated better performance in isolating relatively pure sEVs from human plasma; qEV35 has the better performance in isolating samples with higher sEV content; ExoQuick Ultra isolated samples with closely sEV content to the qEV35 but with the highest non-sEV protein contaminations. People can choose higher sEV content or higher sEV purity according to the downstream analysis.

Funding: St. George Hospital Cancer Research Trust Fund, UNSW Sydney-University International Postgraduate Award, Cancer Institute NSW Early Career Fellowship.

PF17.03 = OP2.03

Multiplexed surface protein profiling of tumour-derived extracellular vesicles by an electrokinetic sensor

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Introduction: Small extracellular vesicles (sEVs) (30–200 nm in diameter) are secreted by most cells, including tumour cells. They have attracted interest as biomarker for cancer diagnostics based on liquid biopsies, because they are abundant in body fluids and their content (proteins, RNAs and other cargos) reflects their parent cells. Moreover, sEVs may also be used for treatment monitoring, as recent studies suggested that the expression levels of certain markers may change during therapy, reflecting tumour response. For cancer diagnostics and therapeutic purposes in clinical settings, it is important to have a device which allows multiplexed measurements, in order to scan a large number of markers simultaneously and compare the expression levels of different patients, or same patients at different treatment stages, in a time efficient manner.

Methods: Herein, we propose a multiplexed platform for label-free detection and surface protein profiling of sEVs. The technique is based on the electrokinetic phenomena of streaming current and zeta potential (\zeta*) and measures the\zeta* change upon sEV binding on functionalized microcapillary surfaces. For the purpose, we used sEVs derived from lung cancer cells. In its current form, the platform can measure up to 5 channels simultaneously, however, it can be further expanded.

Results: Having demonstrated that our electrokinetic sensor successfully detects sEVs in a specific way, we tested its ability to measure the expression level of membrane proteins. The analysis showed that it could detect differences in the expressions of EGFR on sEVs, with a sensitivity of 10%. We then extended the platform for multiplexed analysis, by connecting and measuring four capillaries, functionalized with different capture probes, simultaneously. For the purpose, we targeted specific tumour markers, i.e. EGFR, and exosomal tetraspanin family proteins, such as CD9 and CD63. The results showed successful multiplexed EV detection.

Summary/Conclusion: Being the sensor suitable for multiplexed sEV detection, we shall present our investigation on a set of pleural effusion samples collected from a cohort of lung-cancer patients with different genetic makeup.

Funding: Erling Persson Foundation.

PF17.04 = OP2.04

Optimized immunocapture methods for the direct detection of EV tumour associated proteins in biological fluids: playing around with biophysics

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Introduction: Extracellular vesicles (EVs) are released to biological fluids from different tissues and organs and they contain molecules proposed as biomarkers for multiple pathological conditions. However, most EV biomarkers have not been validated due to the lack of sensitive techniques compatible with high-throughput analysis required for routine screenings. Using immunocapture techniques, combining antibodies against tetraspanins and candidate tumour-specific markers we have recently optimized several assays that greatly facilitate EV characterization.
Methods: We have improved flow cytometry and ELISA assays, increasing substantially the sensitivity for EV detection. Using DLS, EM and analytical ultracentrifugation, we have characterised the biophysical basis of this enhancement. The final methodology can be performed in any laboratory with access to conventional flow cytometry or ELISA reader.
Results: Using combinations of antibodies specific for the tetraspanins CD9, CD63 and CD81, it is possible to detect EVs in minimal volumes of urine and plasma samples without previous enrichment. Additionally antibodies against other less abundant markers, like the epithelial marker EpCAM, have been used to capture and identify EVs directly in minimal volumes of urine or plasma with sensitivity higher than Western Blot analysis of isolated EVs. Furthermore, we demonstrate that additives altering the biophysical properties of an EV suspension, increased detection of tumour antigens in these immune-assays.
Summary/Conclusion: The development of sensitive, high-throughput methods, easily translatable to clinical settings, as ELISA and flow cytometry described here, opens a new avenue for the systematic identification of any surface marker on EVs, even scarce proteins, using very small volumes of minimally processed biological samples. These methods will allow the validation of EV biomarkers in routine liquid biopsy tests.
Funding: MINECO, IMMUNOTHERCAN, TENTAC LES, Immunostep

PF17.05 = OP2.05

Normalized extravesicular protein expression profiles on antibody microarrays reveal protein associations in EVs of organotropic and metastatic breast cancer cell lines

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Introduction: When EV subpopulations are enriched on antibody microarrays and probed for their surface proteins, the detection signal is biased towards abundant subpopulations as it is dependent on both the protein expression level and the number of EVs captured. To address this challenge, we developed a novel normalization approach allowing: 1) the estimation of a target signal independent of EV subpopulation size through dye-based EV quantification, and 2) the assessment of subpopulation target enrichment relative to the population average by leveraging TIM4 as an unbiased, lipid-based EV capture. Here, we investigated the expression of cancer-associated proteins, particularly metastasis-associated integrins (ITGs), in breast cancer EVs with varying metastatic potential and organotropism.
Methods: The relative protein enrichment profiles for various EV subpopulations were established from EVs of SkBr3 (HER2+), T47D and MCF-7 (ER+PR+), BT549 and MDA-MB-231 (triple negative) breast cancer cell lines, as well as five MDA-MB-231-derived cell lines of four different organotropisms (brain, bone, lung, liver) using our custom antibody microarrays with our normalization approach.
Results: As expected, HER2 was broadly detected in HER2+ SkBr3 EVs. Interestingly, HER2- T47D and MCF-7 EVs also expressed HER2 where it was highly enriched in its EpCAM+ subpopulations. ITG α6, β3 and β4 were only found in triple negative and organotropic EVs with ITG β3 and β4 differentially enriched based on the organotropism. The population average of MDA-MB-231 and lung-tropic EVs had high expression of ITG β4, where subpopulations of CD44+ EVs showed positive enrichment while CD9+ and CD63 + EVs showed negative enrichment. ITG α5, β3 and β4 were absent in the bone-tropic CD81+ EV subpopulation, a profile atypical in other organotropisms. Lastly, EGFR was negatively enriched in Tetraspanin+ subpopulations in MDA-MB-231 EVs, but positively enriched in these subpopulations in organotropic EVs, especially for brain-tropism.
Summary/Conclusion: Following normalization, we were able to quantify specific protein associations, uncovering a multitude of co-enrichment profiles that characterize specific metastatic and organotropic cell lines. Notably, we found enrichment signatures that distinguish between different organotropisms derived from the same parental cancer line.
Funding: This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Genome Canada Distruptive Innovation program.

PF17.06 = OP2.06

Heparan sulphate proteoglycans are required for EV-mediated delivery of multiple growth factors

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Introduction: The tissue microenvironment surrounding tumours is complex and the cross-talk between cancer and non-cancer cells is essential for tumour growth and progression. We have previously shown that heparan sulphate proteoglycans (HSPGs), on the surface of prostate cancer EVs, are required for delivery of TGFβ and initiation of a disease-supporting fibroblast phenotype. However, HSPGs are known to bind numerous growth factors, so here we have explored the repertoire of such proteins tethered to EVs by HSPGs.
Methods: EVs were isolated from DU145 prostate cancer cell conditioned media by ultra-centrifugation onto a sucrose cushion. Vesicular HSPGs were modified either by removal of heparan sulphate (HS) glycosaminoglycan (GAG) chains using the enzyme Heparinase III (HEPIII), or attenuation of HSPG core protein expression using shRNAs to knockdown specific HSPGs within the parent cell. Differences in proteins present in control vs modified EVs were identified by a sensitive protein array, based on proximity-ligation technology, and selected targets validated by ELISA. Functional delivery of growth factors by EV-associated HSPGs to recipient fibroblasts is being explored using a variety of in vitro techniques.
Results: Proteome analysis identified 49 targets that bind to HS-GAG chains, and also 108 different proteins that showed altered expression following the loss of one or more HSPGs from EVs. Using ELISA, we have been able to quantify selected candidates on wild type vesicles, some of these are lost following HS-digestion. We were also able to validate proteins on HSPG-deficient vesicles. Gene ontology analysis suggests that EV HSPG-mediated delivery of growth factors is important for control of processes such as angiogenesis, tumour invasion and immune regulation. Functional validation of proteins identified is ongoing.
Summary/Conclusion: Here we demonstrate that HSPGs play a key role in loading of EVs with a complex assortment of growth factors, and therefore subsequent EV-mediated growth factor delivery. We anticipate that loss or damage of EV-associated HSPGs will result in attenuation of EV induction of a tumour-supporting fibroblast phenotype.
Funding: Cancer Research Wales

PF17.07 = OP2.07

Robust exosomal biomarker panel discovery in ovarian cancer using machine learning approaches and studying miRNA & miRNA-target interactions

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Introduction: Ovarian cancer (OC) is the fifth leading cause of cancer-related death in women, partly due to difficulty in early diagnosis. Extracellular vesicles (EVs) show promise for use in early diagnostics of OC. Here, EVs from cervical mucus (CM) of ovarian cancer patients were used for discovery of OC biomarkers for diagnostics. Machine learning was used to mine EV miRNA data to develop an OC biomarker panel (validation via The Cancer Genome Atlas). Examination of the miRNA targets reveal that the panel is a sufficiently accurate predictor of OC.
Methods: EVs from the CM of 48 patients (15 high-grade serous, 24 low-grade, 7 benign) were isolated for small RNA-sequencing. The top differentially expressed miRNAs were used in a random forest and "voom" (variance modelling at the observational level) model. Unsupervised approaches were used and then vetted against patient symptomology data. A TCGA ovarian cancer dataset (n = 100) was used for validation.
Results: An OC biomarker panel of 10 microRNAs (voom: 96.55% accuracy; random forest: 88% accuracy) was generated. The panel consists of members from the mir-200 family and the mir-16 family, among others. The miRNA targets are associated with molecular functions and pathways specific in OC progression.
Summary/Conclusion: Our method has identified EV miRNA biomarkers that may be crucial for early, non-invasive detection of OC. Data science has been used to develop a feedback system integrating biochemical experiments, smaller datasets, and previously available data to identify and verify a biomarker panel for OC diagnostics.
Funding: Support from the National Science Foundation, Eppley Foundation for Scientific Research, Gibson Foundation, Prisma Health System and ITOR Biorepository are gratefully acknowledged.

PS01: EVs in Infectious Diseases and Vaccines

Chair: Susmita Sil – University of Nebraska Medical Centre

PS01.01

Ethanol and HIV-induced exosome from hepatocytes activate hepatic stellate cells

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Introduction: Liver disease has become a significant cause of morbidity and mortality among HIV patients. Alcohol exposure can further exacerbate liver damage by activating hepatic stellate cells (HSCs), leading to hepatic fibrosis or cirrhosis, often seen at all levels of alcohol exposure among people with HIV. Due to the potentiating effects of alcohol on HIV-induced hepatocytes (Hep) damage, as well as the effect of ethanol in HSC-mediated extracellular remodelling, it is imperative to understand the interplay of Hep and HSCs. Here, we focus on the exosomes released by HIV-and ethanol exposed Hep and how these exosomes modulate the functional behaviour of HSCs.
Methods: Human hepatocyte Huh7.5CYP2E1 [hepatoma cells stably transfected with CYP2E1 designated as RLW cells] were infected with HIV in the presence or absence of alcohol metabolite, acetaldehyde using the acetaldehyde-generating system (AGS). The conditioned medium was collected from 4 groups of cells: untreated, HIV-, AGS- and HIV+AGS. Quantification of exosomes number and size were evaluated with ZetaView or Nanosight and further characterized for exosome markers following the guideline from Minimal information for studies of EVs 2018 (MISEV2018). The human hepatic stellate LX-2 cell line was exposed to hepatocyte-derived exosomes and assessed for the activation using pro-inflammatory markers IL-1β, IL-6, TNFα, and fibrotic markers ACTA2, and TIMP1 using quantitative PCR. We also analysed exosome miRNA content in primary human hepatocytes (PHH), which potentially regulates the function of recipient cells by "programming" their inflammation/fibrosis status. The network analysis for mRNA and miRNA were carried out using Gene ontology consortium, and mirror 2.0 and DAVID bioinformatics resources 6.8.
Results: AGS treatment further enhanced the release of HIV-induced exosome from hepatocytes. Size distribution assessed by zeta view or Nanosight revealed that approximately 85–90% of particles distributed in the range of 50 to 200 nm, with a peak at ~90 nm. Enriched expression of HIV protein P24 was observed in fractions F9-F12. Western blotting of hepatocyte-derived exosome demonstrated positivity for exosome-enriched proteins Alix, TSG 101 and CD9 specifically in F2-F8 fractions and negative for endoplasmic reticulum protein calnexin. The uptake of Hepatocyte-derived exosomes by HSCs was apparent as demonstrated by immunofluorescence. The internalization of hepatocyte-exosome induced activation of HSCs as evidenced by increased expression of pro-inflammatory IL-1β, IL-6, TNFα markers in the latter cells.
Summary/Conclusion: We conclude that AGS treatment in HIV-infected hepatocytes potentiates the release of exosomes, which, following uptake by the HSCs, leads to their activation.
Funding: This work is supported by NIH-1 R01 AA027189-01A1.

PS01.02

Antimicrobial peptide LL-37 induces neutrophil-derived extracellular vesicles with antibacterial potential and protects murine sepsis

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Introduction: Extracellular vesicles (EVs) released from immune cells or other host cells upon microbial infection modulate the immune response and thereby regulate the infection. Sepsis is a life-threatening multiple organ dysfunction caused by systemic dysregulated inflammatory response to infection. Nevertheless, numerous therapeutic trails concerning immune dysfunction have still been disappointing outcomes. We have previously shown that LL-37, a human cathelicidin antimicrobial peptide, improves the survival of caecal ligation and puncture (CLP) septic mice. Here,

we investigated the induction of EV release by LL-37 and functions of LL-37-induced EVs in murine sepsis.
Methods: EVs were isolated from peritoneal exudates of CLP mice and the supernatant of LL-37-stimulated mouse bone marrow neutrophils by differential centrifugation or size exclusion chromatography. Isolated EVs were analysed by flow cytometry, western blotting, and nano particle analysis. Neutrophil-derived EVs were injected into CLP mice to assess the protective function of EVs against septic mice. The antibacterial activity of EVs was evaluated by incubating with Escherichia coli.
Results: In CLP mice, LL-37 augmented the level of EVs. EVs from LL-37-injected CLP mice contained higher amounts of neutrophil-derived antibacterial proteins (lactoferrin and CRAMP, cathelicidin-related antimicrobial peptide) and exhibited higher antibacterial activity compared to EVs from PBS-injected CLP mice. Furthermore, LL-37 stimulated mouse bone marrow neutrophils to release EVs with antibacterial potential, and administration of the LL-37-induced EVs reduced the bacterial load and improved the survival of CLP mice.
Summary/Conclusion: LL-37 induces the release of antimicrobial EVs from neutrophils in CLP mice, thereby reducing the bacterial load and protecting mice from lethal septic condition.

PS01.03

Identification of miRNA profiles of serum exosomes in active tuberculosis

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Introduction: Tuberculosis (TB) has exceeded HIV as the most lethal infectious disease globally for two consecutive years, mainly due to difficulties in achieving early and definitive diagnosis, and timely treatment. Exosomes carrying RNA, particularly miRNA, have demonstrated their functional and diagnostic potential in diseases including TB. However, few published studies have explored whether exosomal miRNAs could be used for diagnosis of TB. Thus, more systematic and comprehensive study of exosomal miRNAs with regard to their potential as non-invasive TB biomarkers is still urgently needed.
Methods: We searched the Gene Expression Omnibus database for datasets published before December 2019, and performed meta-analysis on available exosomal miRNA profile data for healthy control (HC) and active TB clinical specimens . Reprocessing next generation sequencing data under uniform parameters and utilizing state-of-the-art bioinformatics analysis.
Results: We identified many distinct up-regulated and down-regulated differentially expressed exosomal miRNA across multiple studies, and further screened the top 10, which might provide a potential panel for differentiation of HC and TB. We classified all differentially expressed miRNAs into six expression patterns and identified two persistently up-regulated miRNA (hsa-miR-140-3p, and hsa-miR-423-3p) as potential markers during TB progression. Moreover, the differential expressed exosomal genes that we screened from the datasets were consistent with the genes overlapped with predicted mRNA targets of differentially expressed miRNA. Pathway and function analysis further demonstrated down-regulated signalling pathways/immune response and up-regulated metabolism and apoptosis/necrosis.
Summary/Conclusion: Our findings demonstrated the selective packaging of RNA cargoes into exosomes under different stages of Mycobacterium tuberculosis (Mtb) infection, as well as facilitating development of potential targets for the diagnosis, prevention and treatment of TB.
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PS01.04

The new therapy to treatment experimental Acute Chagas Disease

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Introduction: Trypanosoma cruzi is a protozoan parasite that causes Chagas disease, a relevant source of morbidity in Latin America, which has spread to many countries as result of immigration of the people from endemic areas. Many studies have been showed that trypomastigote forms of T. cruzi release extracellular vesicles (EV) that increase parasite infection.

Objectives. Here, we aim to test if previous immunization with EVs in adjuvant can generate a protective immune response by decreasing the effects of EVs in experimental Chagas disease.

Methods: Female BALB/c mice were immunized by intra peritoneal (ip) administration with 5 × 105 or 107 EVs isolated from trypomastigotes forms, with aluminium hydroxide adjuvant (AlOH). Injections were administered intravenous in 3 doses during 45 days (15 days interval). After immunization, mice were infected intra-peritoneally with 500 trypomastigotes forms. Parasitaemia was quantified by counting motile parasites in fresh blood sample drawn from lateral tail veins. Mortality and weight were analysed during the infection. In control group, the mice were immunized with AIOH.

Results: The immunization with EVs with AlOH decreased the blood parasitaemia and the animals survived, while all animals died in the group AlOH alone. The animals immunized with EVs had an increase of F4/80+ CD11b+ and CD80/CD86 expression in cells isolated from the peritoneum.

Summary/Conclusion: These results indicate that T. cruzi EV antigens can induce an immune response that controls the development and establishment of the experimental Chagas disease.

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PS01.05

Characterization of outer membrane vesicle-carried proteins as pathogenicity factors from Acinetobacter baumannii

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Introduction: Acinetobacter baumannii (Ab) is a nosocomial pathogen, of major concern due to its multidrug resistance (MDR) and the recent appearance of hyper-virulent strains in the clinical setting. The World Health Organization included Ab as a critical priority pathogen for the development of novel antibiotics. Ab pathogenesis is associated with a multitude of potential virulence factors (VF) that remain poorly characterized. There is growing evidence that outer membrane vesicles (OMV) are used as vehicles to transport bacterial proteins that contribute to set up the conditions for the infections. In the present work we studied the physiopathology of MDR Ab. We focused on the contribution of non-characterized outer membrane proteins (OMPs) associated to OMVs, with special focus on lipoproteins (LP).

Methods: We conducted a bioinformatic prediction using available datasets to construct a list of OMV-associated OMPs putatively acting as VF in AB5075. Seven genes were selected and the corresponding mutants were obtained from Manoil Lab collection. Physiological analyses of the mutants were performed, and the involvement of the selected proteins in Ab pathogenesis was evaluated by adherence, invasion, and cytotoxicity assays on human lung cells A549.

Results: Biochemical analysis indicated similar growth rates in rich media, as well as similar levels of OMV production for all the mutants as compared to WT. Also, no differences in susceptibility to chaotropic agents were observed, indicating no alteration of the OM function as a general permeability barrier. All mutants similarly reduced A549 cell viability, but to a lesser extent than the WT. Moreover, three of them exhibited less adhesion and invasion compared to the WT, and OMV isolated from these mutants displayed variable levels of cytotoxicity.

Summary/Conclusion: These results suggest roles for the mutant gene products in Ab pathogenesis and contribute to the better understanding of Ab virulence mechanisms, revealing novel possible targets for therapeutic development.

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PS01.06

Talaromyces marneffei yeasts-derived Extracellular Vesicles Mediate Inflammatory Response and Modulate Macrophage Functions

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Introduction: Talaromyces marneffei (T. marneffei) grows as a mycelial form in the environment but multiplies rapidly as a yeast form in the host and within macrophages. The yeast can cause disseminated and progressive infections or lethal Talaromycosis. But the mechanisms of pathogenicity of T. marneffei are poorly understood. Fungal extracellular vesicles (EVs) have previously been shown to transmit a proinflammatory message to macrophages. However, the characteristics and effects of T. marneffei EVs on the progress of infection have not yet been investigated.
Methods: In this study, EVs of T. marneffei yeasts were isolated by ultracentrifugation method. EVs were detected and confirmed by Electron microscopy and nanoparticle tracking analysis (NTA). The RAW 264.7 murine macrophages were incubated with the T. marneffei vesicles to observe the changes of macrophage morphology and function, especially in inflammatory response. The proteins, DNAs, RNAs of T. marneffei vesicles were respectively removed with protease, DNase and RNase. All treated EVs were used to incubate with murine macrophages observe the effect on macrophages in inflammatory response.
Results: We observed that EVs secreted by T. marneffei have a typical spherical shape with a diameter of 30 to 200 nm. T. marneffei EVs were internalized by RAW 264.7 murine macrophages and promoted the production of NO and proinflammatory cytokine by macrophages in a dose-dependent manner. T. marneffei EVs stimulate macrophages to generate Reactive oxygen species (ROS). Addition of T. marneffei EVs to macrophages also promoted transcription of the M1-polarization marker CD86 and diminish that of the M2 markers CD206. Incubation of T. marneffei vesicles with murine macrophages resulted in increased levels of extracellular interleukin-1β(IL-1β), interleukin-6 (IL-6) and interleukin-12 (IL-12). The proinflammatory effect of vesicles was weakened when the proteins of the vesicles were destroyed. In contrast, no similar changes were observed in degraded DNA and RNA.
Summary/Conclusion: Our results indicate that the extracellular vesicles of T. marneffei can stimulate macrophage towards to M1 polarization phenotype and promote proinflammatory function.

PS01.07

Plasma-derived extracellular vesicles as potential biomarkers in chronic Chagas disease patients

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Introduction: Chagas disease (CD), caused by the parasite Trypanosoma cruzi (T. cruzi), is a neglected tropical disease affecting about 8 million people worldwide. Currently, one of the main clinical problems is the lack of effective biomarkers for therapeutic response and disease prognosis during chronic infections. In that context, extracellular vesicles (EVs) are raising attention as novel, minimally invasive, and inexpensive method for diagnostic and screening of diseases, as well as a new source to identify new biomarkers. The main objective of this study is to use EVs derived from biological fluids of chronic CD patients for identifying novel biomarkers, specifically in the context of therapeutic response and disease prognosis.
Methods: Plasma, saliva and urine from a cohort of chronic CD patients are being collected before and at the end of benznidazole treatment. As negative controls, healthy donors have been also included. The purification and characterization of the EVs was performed by size exclusion chromatography, followed by nanoparticle tracking analysis, bead-based flow cytometry assay and transmission electron microscopy. A proteomic analysis of the EVs was also performed.
Results: Proteins associated with EVs secreted by infective T. cruzi have been previously identified in cell culture, but never in human samples. Our results, based on the analysis of a single heart-transplanted patient with chronic CD, showed the presence of T. cruzi and human proteins specifically associated with plasma-derived EVs. Noticeably, several human and parasite proteins identified in EVs obtained from plasma samples, were present or upregulated before chemotherapy and were absent or downregulated following treatment. Currently, proteomics analyses are being performed with higher numbers of CD plasma samples.
Summary/Conclusion: To the best of our knowledge, this is the first proteomic profiling of plasma-derived EVs from a heart-transplanted patient with chronic CD. These results thus open the possibility of using EVs from biological fluids as a tool for the identification of new biomarker candidates in chronic CD. These biomarkers are essential for assessing disease

prognosis, evaluating the recovery of chronic patients after treatment, and for testing new drugs in clinical trials.

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PS01.08

Eukaryotic extracellular vesicles and prokaryotes – a different perspective

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Introduction: Eukaryotic cells communicate with one another through multiple pathways. An established route of communication between eukaryotic cells is via the production of a range of different membrane bound signalling "packages", called extracellular vesicles (EVs). EVs are produced by all domains of life and carry proteins, nucleic acid (RNA and DNA), and other biological material, travelling between cells and around the body to deliver a range of chemical messages. Bacteria can also produce EVs that communicate with each other to coordinate population behaviour, as well as with eukaryotic cells to stimulate host defence or induce tolerance. Here I investigate the poorly explored axis where EVs are the vehicle for communication between eukaryotic cells and bacteria.

Methods: As a first step, I have isolated EVs from tissue cultured eukaryotic cells grown in Advanced RPMI media with minimal EV-depleted FBS. NanoEVs were isolated from spent culture media using sequential centrifugation (2,000 × g, 10,000 × g) and concentration (100 kDa filter) before purifying using size exclusion chromatography columns. NanoEV-rich fractions were pooled based on particle (Nanoparticle Tracking Analysis) and protein quantity data. NanoEVs were characterised by electron microscopy and expression of exosomal markers. Eukaryotic nanoEVs were then characterised in their effect upon the growth of Escherichia coli as a model bacterium, also grown in tissue culture media to mimic relevant in vivo conditions.

Results: Further experiments with increased dosages are required to determine the effect of human EVs on bacteria.

Summary/Conclusion: Our work will investigate whether human EVs communicate with the resident and pathogenic microbiota, while examining the mechanisms behind this communication.

Escherichia coli
pathogenic bacteria
commensal bacteria

PS01.09

Hydrogen sulphide (H2 S) derived extracellular vesicles: a potential protective role in response to respiratory syncytial virus (RSV) infection

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Introduction: Hydrogen sulphide (H2 S) is as a critical endogenous gasotransmitter with antiviral and anti-inflammatory activity against respiratory syncytial virus (RSV) infection. Extracellular vesicles (EVs), novel mediators of cell–cell communication, have the potential to modulate cellular responses of recipient cells following a variety of stimuli. In this study, we investigated the composition/function of EVs derived from airway epithelial cells treated with GYY4137, a slow-releasing H2 S donor, and whether GYY-derived EVs can alter airway epithelial cell response in recipient cells following RSV infection.

Methods: EVs were isolated from untreated (control EVs) and GYY4137 treated (GYY-EVs) A549 cells, a human alveolar type II–like epithelial cell line. EVs were purified using a two-step enrichment procedure. EVs were characterized using particle sizing (size and concentration) and Western blot for the EV markers. Electron microscopy and immunofluorescence staining were used to investigate presence of multivesicular bodies (MVBs), EVs precursors, in both groups. Recipient A549 cells were cultured for 24 hours in the presence or absence of control- or GYY- EVs, then infected with RSV for 24 hours. Viral titres by plaque assay were measured in recipient infected A549 cells.

Results: We confirmed the presence and purity of our EVs. We found that GYY4137 reduced the particles number of EVs, but did not change EV size. A549 cells treated with GYY4137 showed an accumulation of MVBs/lysosomes-like structures, as well as an increase in CD63 expression, a MVBs marker, compared to untreated cells. Recipient A549 cells treated with GYY-EVs showed lower viral replication than control EV-treated cells in response to RSV infection. We are currently investigating the potential mechanism for this observation and characterizing the RNA cargo composition of GYY-EVs.

Summary/Conclusion: No vaccine or effective treatment is currently available for RSV. Cellular pretreatment with GYY-EVs reduced the RSV replication in airway epithelial recipient cells, suggesting that H2 S could exert its antiviral activity in the context of RSV infection potentially through modulation of EV composition. Therefore, GYY-EVs could represent a future novel pharmacological approach for ameliorating virus-induced lung disease.

PS01.10

Effects of extracellular vesicle-mediated transmission on Reoviridae infection

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Introduction: The Reoviridae family contains important viruses that infect humans and other animals. Reovirus is in advanced clinical trials as an oncolytic therapeutic and has been linked to onset of coeliac disease. Rotavirus is a leading cause of diarrhoeal gastroenteric mortality in children aged 5 years or younger in the developing world. Traditionally, viruses have been thought to transit between cells as independent particles. This viewpoint is being challenged with the recent discoveries that many virus families utilize extracellular vesicles (EVs) for non-lytic transport. EV-mediated transmission potentially enables viral evasion of immune responses and collective transmission to drive enhanced productive infection. Recently, rotavirus was shown to egress host cells in large EVs, and EV-mediated transmission enhances rotavirus virulence in vivo. Mechanisms of reovirus egress are incompletely defined, and effects of multiparticle transmission on rotavirus and reovirus genetic complementation are unknown.
Methods: Using cultured cells, microflow cytometry, and genetically barcoded viruses, we provide evidence that i) reovirus particles egress in large EVs in a virus strain- and cell type-dependent manner, ii) rotavirus upregulates EV release from infected cells, and iii) EV-mediated transmission increases the frequency of reovirus genotype mixing.
Results: Taken together, these data suggest that multiple particles of reovirus and rotavirus egress in large, virus-modulated EVs, and that transmission in EVs increases segment complementation compared to transmission as free particles.
Summary/Conclusion: These discoveries may be broadly applicable to viruses that travel in EVs and will contribute to general principles of virus transmission and diversification. Continued studies will illuminate the specific cellular pathways reovirus and rotavirus utilize for successful egress. These pathways may prove to be critical targets for the improvement of vaccines and oncolytic therapy.

PS01.11

Multiparameter flow cytometry analysis of the human spleen and its interaction with plasma-derived EVs from Plasmodium vivax patients

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Introduction: The spleen is a secondary lymph organ that filters blood and elicits immune responses against blood-borne pathogens, such as malaria parasites. Extracellular vesicles (EVs) are membrane-bound particles involved in intercellular communication. EVs play several roles in malaria ranging from modulation of immune responses to induction of vascular alterations. Here, we report the first integrated characterization of human spleen cells using multiparameter flow cytometry (MFC) describing subpopulations of splenic leukocytes and red blood cells (RBCs), and studied their interaction with plasma-derived EVs from P. vivax patients (PvEVs).
Methods: Human spleens were obtained from organ transplantation donors. Myeloid, lymphoid, erythroid and haematopoietic stem cells (HSCs) were immunophenotyped by MFC. T cells, dendritic cells (DCs) and RBCs were enriched by density centrifugation and immunomagnetic isolation. PvEVs and healthy donors EVs (hEVs) were purified by size-exclusion chromatography (SEC) and characterized by bead-based flow cytometry. Enriched EVs were labelled with fluorescent lipophilic dyes and incubated with total splenocytes or enriched populations. EVs-cells interaction was assessed by flow cytometry.
Results: Human spleen immunophenotyping showed that CD45+ cells included B (30%), CD4 + T (16%), CD8 + T (10%), NK (6%) and NKT (2%) lymphocytes. Myeloid cells comprised neutrophils (16%), monocytes (2%) and DCs (0.3%). Erythrocytes

represented 70% whereas, unexpectedly, reticulocytes were 0.93% of total cells. In addition, we also detected HSCs, which accounted for 0.02%. SEC separated EVs from the bulk of soluble plasma proteins as shown by the enrichment of CD63, CD5 L and CD71 markers. Interaction studies showed an increased proportion of T cells (CD4 + 3-fold and CD8 + 4-fold), monocytes (1.5-fold), B cells (2.3-fold) and erythrocytes (three-fold) interacting with PvEVs as compared to hEVs.

Summary/Conclusion: The integrated cellular analysis of the human spleen and the methodology employed here allowed in vitro interaction studies of human spleen cells and EVs. A larger proportion of monocytes, T and B lymphocytes as well as erythrocytes was found to interact with PvEVs compared to hEVs. Future functional studies of these interactions can unveil pathophysiological processes involving the spleen in vivax malaria.

PS02: EVs in Cancer Metastasis and Tumour Angiogenesis

Chair: Vincent Hyenne – Tumour Biomechanics INSERM UMR_S 1109 Institut d'hématologie et d'immunologie, CNRS SNC5055, Strasbourg, France

Chair: Shiv Ram Krishn – Thomas Jefferson University, Philadelphia, USA

PS02.01

Activating protein-1 and extracellular vesicles in cancer metastatic behaviour phenocopying

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Introduction: Activating protein 1 (AP-1) is a dimeric transcription factor that is formed by one member of the Fos family and one member of the Jun family of proteins. The expression of Fra-1 and c-Jun, two members of the AP-1 family, is highly increased in triple-negative breast cancer. AP-1 regulates several soluble factors which play autocrine and paracrine roles to enhance cancer progression and metastasis. My previously published work showed that factors secreted by the metastatic MDA-231 cells and regulated by AP-1 induced proliferation and migration of non-tumorigenic MCF10A cells and the non-metastatic MDA-468 cells through the induction of Fra-1. Previous studies showed that exosomes from MDA-231 cells increased the migration and invasion of the less invasive MCF7 cells. Proteomic comparison of the cargo of the exosomes from these two cell lines found that exosomes from MDA-231 tended to carry proteins that enhance the metastatic process like vimentin. So, I hypothesized that AP-1 controls the cargo of the MDA-231 released exosomes so it directs its oncogenic and metastatic phenocopying behaviour.
Methods: To study the role of AP-1, I utilized the dominant-negative version of Fos protein named A-Fos. MDA-MB-231 cells were infected with the gene for a flag tagged A-Fos cloned to a retroviral vector under doxycycline (Dox) inducible promoter to produce a stable cell line (MDA231/Afos). Exosomes were extracted from conditioned medium acquired from Dox(-) and Dox(+) MDA231/Afos cells using ultracentrifugation. The presence of exosomes was confirmed using TEM and nanoparticle tracking analysis. The exosomes were added to serum-starved MDA468 cells.
Results: Exosomes from Dox(-) MDA231/Afos cells increased the Fra-1 level in serum-starved MDA-468 cells. This effect was reduced in the case of exosomes acquired from Dox(+) MDA231/Afos.
Summary/Conclusion: These results suggest a role of AP-1 both in controlling and in mediating the action of exosomes from MDA-231 cells on non-metastatic cells. My plan is to explore the cargo of the exosomes regulated by AP-1 to transfer the malignant and the metastatic properties of MDA-231 cells. I will use RNAseq and proteomic analysis to analyse the content of MDA-231 exosomes in the presence and absence of A-Fos.

PS02.02

Neuroblastoma-secreted exosomes carrying miR-375 promote osteogenic differentiation of bone marrow mesenchymal stromal cells

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Introduction: Bone marrow (BM) is the major target organ for neuroblastoma (NB) metastasis and its involvement is associated with poor outcome. Yet, the mechanism by which NB cells invade BM is largely unknown. Tumour microenvironment represents a key element in tumour progression and mesenchymal stromal cells (MSCs) have been recognized as a fundamental part of the associated tumour stroma. Here, we explore the potential role of NB-derived exosomes in induction of a pro-osteogenic phenotype on BM-MSCs.
Methods: In this prospective study, we assessed the osteogenic differentiation of BM-MSCs isolated from patients with and without BM metastasis. Expression profiling of exosomal microRNAs was performed on 8 NB cell lines by Q-PCR. MiR-375, represented the

main candidate related to the osteogenic phenotype. Its over- and down-regulation was performed in vitro. MiR-375 was then evaluated in plasma (peripheral and BM derived) and BM biopsies of NB patients.
Results: BM-MSCs isolated from NB patients with BM involvement exhibit a greater osteogenic potential than MSCs from non-infiltrated BM. We show that BM metastasis-derived NB cell lines secrete higher levels of exosomal miR-375, which promotes osteogenic differentiation in MSCs. Clinical data demonstrate that high level of miR-375 correlate with BM metastasis in NB patients.
Summary/Conclusion: Our findings suggest a potential role for exosomal miR-375 in determining a favourable microenvironment in BM to promote metastatic progression. MiR-375 may, thus, represent a novel biomarker and a potential target for NB patients with BM involvement.
Funding: This work was supported by grants from the Ministero della Salute (GR-2016-02364088 to ADG), the Associazione Italiana per la Ricerca sul Cancro (AIRC)-Special Program Metastatic disease: the key unmet need in oncology 5 per mille 2018 Id. 21147 to F.L. and Ministero dell'Istruzione, Università e Ricerca (MIUR, grant PRIN 2017 to F.L).

PS02.03

Effects of Small-Extracellular Vesicles on cell biomechanical properties in metastatic breast cancer

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Introduction: Extracellular Vesicles (EVs) are nano-sized particles delimited by a lipid bilayer which transfer functional molecular cargos from the cells of origin to target cells. This intercellular crosstalk controls both physiological and pathological conditions. Given their presence in body fluids and their characteristics, these nanocarriers might be potentially used in diagnostics and/or therapy. Breast Cancer is the most frequently diagnosed malignancy and ranks as the leading cause of cancer mortality in women worldwide; the triple negative breast cancer, in particular, is the most aggressive subtype with a poor prognosis. Since it is recognized that cell stiffness of cancer cells play a crucial role during the metastatic spreading, we set ourselves the goal of clarify the effects and the activity of small-EVs (i.e. with a diameter below 200 nm) in metastatic breast cancer, with a special attention on their correlation with the biomechanical properties of cells.
Methods: Functional assays were performed on the non-invasive MCF7 breast cancer cell line, before and after the cellular uptake of small-EVs originating from the invasive MDA-MB-231 triple negative breast cancer cell line. The mechanical properties (cell stiffness, cytoskeleton organization and focal adhesions) of MCF7 cells were investigated before and after the vesicle uptake.
Results: The uptake of small-EVs derived from MDA-MB-231 significantly reduces the Young's modulus values of MCF7 cell line making them more invasive. Moreover actin and focal adhesion variations were observed in MCF7 cells before and after small-EV's uptake, suggesting a molecular rearrangement inside MCF7 cells upon uptake.
Summary/Conclusion: Our results evidence that small-EVs play a key role in altering biomechanical properties of target cells and underline their relevance in cell-cell crosstalk. Our approach is very promising to identify new molecular mechanisms through which EVs perform their oncogenic function.

PS02.04

Stratification of angiogenic or non-angiogenic lesions in colorectal cancer liver metastases patients using extracellular vesicle miRNA

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Introduction: Colorectal carcinoma (CRC) is the second leading cause of cancer death in the western world. Over 50% of the CRC patients develop liver metastasis (LM) and 90% will die from metastatic disease. In the current clinical setting, liver resection provides the only possible cure, but only 20% of CRCLM patients are resectable. The combination of angiogenic inhibitors with chemotherapy is used to downsize CRCLM with the goal of converting unresectable patients to resectable ones. However, only 10–15% of these patients can be successfully converted to a resectable state. We have no way of identifying those CRCLM patients that would respond/benefit to the addition of anti-angiogenic therapies (e.g. Bevacizumab: Bev)). Proper stratification of patients into Angiogenic Inhibitor responders and non-responders will permit a proper assessment of the efficacy of Angiogenic Inhibitors. CRCLM forms 2 distinct histopathological growth patterns (HGP): angiogenic (desmoplastic) and non-angiogenic (replacement) HGP. We demonstrated

that CRCLM patients with predominant angiogenic lesions receiving Bev plus chemotherapy have a more than double 5-year overall survival compared to patients with non-angiogenic lesions. Therefore, non-angiogenic lesions do not respond to angiogenic inhibitors. Our study focuses on stratifying angiogenic vs non-angiogenic lesions of CRCLM through extracellular vesicle miRNAs. We are using two approaches in the selection of miRNAs to target: 1. Text mining of published EV miRNA from CRCLM patients; and 2. Differentially expressed miRNAs present in tumour tissue from both lesion types, we have obtained by sequencing 50–60 patients. These two strategies will generate a list of miRNAs that we will target using qPCR on plasma-derived EV miRNA from the patients used in approach 2, where we have classified the lesions in the patients. Preliminary data on 10 patients will be presented.

Methods: EV isolation was performed using the gold standard centrifugation method. RNAseq and qPCR are used to generate the expression profile for angiogenic vs non-angiogenic type of CRCLM.

Results: The research is under progress.

Summary/Conclusion: The research is under progress.

PS02.05

The role of extracellular vesicles from cancer cells in bone metastasis

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Introduction: It is known that bone metastasis causes a reduction in the quality of life of cancer patients due to fractures and nerve compression. Therefore, it is important to elucidate the mechanism of bone metastasis and develop new treatments. Metastatic bone tumours occur at particularly high rates in cancers of the prostate, breast, and lung. In this study, we focused on extracellular vesicles (EVs) in bone metastasis, and investigated that the role of EVs derived from cancer cells in osteolysis.

Methods: The prostate, breast, and lung cancer cell-derived EVs were added to osteoclast precursors with RANKLs. The osteoclast differentiation was evaluated by Tartrate-resistant acid phosphatase (TRAP) stain and by measuring the expression level of osteoclast markers using by qRT-PCR. A proteome analysis (LC-MS/MS) and siRNA approaches were used to identify molecules which are responsible for promotion of osteoclast differentiation in the prostate cancer cell-derived EVs. To investigate whether the molecules are suitable for the detection of bone metastasis in serum EVs, we isolated EVs from serum of prostate cancer patients, and analysed the protein level of the molecules by western blot analysis.

Results: We found that the prostate cancer and lung cancer-derived EVs significantly promoted the RANKL-stimulated osteoclast differentiation. Our analysis revealed that CUB domain-containing protein 1 (CDCP1), which is a membrane protein on the prostate cancer cell-derived EVs, was responsible for promotion of osteoclast differentiation. Moreover, CDCP1 was markedly detected in the EVs-derived from serum of prostate cancer patients who had bone metastasis than that of normal subjects. We also found that CDCP1 exits on the breast and lung cancer cell-derived EVs.

Summary/Conclusion: We showed that the EVs-derived from bone metastatic tumours have a role in activation of osteoclastogenesis. Moreover, we revealed that CDCP1 in the EVs is responsible for promoting of osteoclast differentiation. These EVs could be the novel diagnostic and therapeutic target for bone metastasis.

PS02.06

Increased expression of chemokine receptor CXCR4 in non-invasive colorectal cancer cells after incorporation of platelet-derived extracellular vesicles.

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Introduction: Blood platelets and platelet-derived extracellular vesicles (P-EVs) play a crucial role in tumour growth and metastasis. P-EVs, also referred to as platelet microparticles, are recognized as a carrier for proteins and nucleic acids that control cell-to-cell communication, mediate the formation of metastatic niches and affect tumour invasion and metastasis. Among the other factors, P-EVs contain the chemokine receptor CXCR4, known as a co-receptor for HIV entry but also regarded as important in cancer development due to the importance of CXCR4/CXCL12 signalling. Overexpression of CXCR4 was reported in various, especially in invasive cancers, including colorectal cancer (CRC). CRC, the third most commonly diagnosed cancer, is usually diagnosed at the late stage and patient's death is mainly related to metastasis. Increased levels of CXCR4 has been reported as a poor prognostic factor for survival of CRC patients and its blocking has been suggested as therapeutic

approach. The aim of this study was to analyse the effect of P-EVs on the levels of CXCR4 in CRC cells on various epithelial-to-mesenchymal transition stage.
Methods: We used CRC cell lines HT29 and SW620, which represent distant invasive potential and different phenotypes, epithelial and strongly mesenchymal, respectively. P-EVs were isolated from outdated concentrates of human blood platelets after activation by thrombin in the presence of calcium ions, by subsequent centrifugation and ultracentrifugation. The P-EVs were labelled using PKH67 fluorescent dye to visualize their uptake into cell lines by confocal microscopy. We also quantified the levels of CXCR4 in HT29 and SW620 by Western blot analysis. The effect of P-EVs uptake on the migration of CRC cells was studied by "wound healing" method.
Results: We found that the levels of CXCR4 in CRC lines used in the study were correlated with their EMT stage. We show here that P-EVs released by activated platelets were incorporated into both HT29 and SW620 cell lines. The expression of CXCR4 in HT29 was increased after the uptake of P-EVs. Additionally we observed that migration rate of HT29 cells with incorporated P-EVs was elevated as compared to control cells.
Summary/Conclusion: We posit that circulating P-EVs can be incorporated into yet not invasive CRC cells to significantly increase the level of CXCR4 receptors and that may lead to the their more invasive characteristics.
Funding: Supported by 2018/29/B/NZ5/01756 from the National Science Centre, Poland.

PS02.07

Changes of miRNA profile in extracellular vesicles released by colorectal cancer cells during early EMT

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Introduction: For cancer therapy it is important to identify markers and key processes induced during cancer progression. One of them is epithelial-mesenchymal transition (EMT) which is associated with cell acquisition of invasiveness, stem cell characteristics and resistance to apoptosis and therapy. Also the extracellular vesicles (EVs) released from tumour cells, which can be taken up by cells constituting pre-metastatic niches, can alter cancer progression by promoting cells' reprogramming. Our group has recently reported that Snail transcription factor, a key factor of EMT, when overexpressed in CRC HT29 cells, drives their early EMT and alters the expression of microRNA (miRs). In the present study we analysed the miRs profile of EVs released from those cells.
Methods: EVs from three HT29 clones stably overexpressing Snail and from control HT29-pcDNA were isolated by differential centrifugation and ultracentrifugation of conditioned media after 24 h of culturing in serum-free medium. Total RNA was isolated and next-generation sequencing (NGS) analysis of the miRNAs was performed followed by Gene Ontology (GO) Enrichment Analysis.
Results: MiRs profiling shows that overexpression of Snail in HT29, while does not affect the global miRNAs content of EVs, significantly triggers changes in individual miRs levels. Three miRs: miR-205, let-7i and miR-130b were upregulated and five miRs: miR-1246, miR-3131, miR-375, miR-552-3p, miR-552-5p were decreased in HT29-Snail-EVs in comparison to control EVs. We also report the top Biological process and Molecular functions and cellular compartment that are GO terms associated with differentially expressed microRNAs.
Summary/Conclusion: We show here that EVs from CRC cells undergoing partial EMT driven by Snail, have different miR profile as compared to control cells. Particular miRNAs are considered to be either tumour suppressor or oncogenic thus changes in their profile need to be taken into account during the cancer treatment. The EVs miRNA profile may also serve as a marker of CRC progression.
Funding: Supported by the project DEC-2011/02/A/NZ3/00068 from the National Science Centre, Poland.

PS02.08

Extracellular vesicles from prostate cancer cells deliver microRNAs to promote osteogenesis

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Introduction: Prostate cancer (PCa) is the most common malignant tumour in male urinary system and osteoblastic bone metastasis is the most observed metastasis in prostate cancer patients. It has been demonstrated that circulating microRNAs contained in extracellular vesicles are potential early biomarkers and therapy targets for many diseases. However, the potential role of microRNAs in prostate cancer bone metastasis, is not yet to be fully explored.

Methods: After isolation and purification EVs using ultracentrifugation from conditioned media of bone metastatic co-opting prostate cancer cells and normal cells, total RNA was extracted. Subsequent to library preparation and small RNA-Seq, differential gene expression analysis was performed. Data were filtered by mean miRNA expression of ≥ 50 reads, two fold up or down regulation between 2.5 − 7.5 and adjusted p-value ≤ 0.05. The uptake of PCa-sEVs was performed. Three candidate miRNAs (has-miR-200 c-3p; has-miR-1275; has-miR-383-5p) were internalized and osteoblast differentiation were detected by qPCR, histochemical staining and protein activity detection.
Results: Total reads of miRNAs in bone metastatic co-opting PCa-EVs exceeded significantly than that in normal EVs ($p < 0.001$), indicating that miRNAs delivered by PCa cells play critical role in PCa bone metastasis. PCa-CM enhanced osteoblast differentiation and can be reversed by GW4869. The uptake of PCa-EVs by MC3T3-E1 was efficient. The high expression of the three candidate miRNAs in PCa-EVs was verified by qPCR. All the three candidate miRNAs promoted osteogenesis, verified by mRNA expression of osteoblastic markers (ALP, OCN, RUNX2, OSX), ALP activity, ALP staining and Aliza Red S staining.
Summary/Conclusion: These findings suggest that miRNA cargos in PCa-EVs play a pivotal role in the development of osteoblastic bone metastasis of PCa, which can be potential early biomarkers and therapy targets for prostate cancer bone metastasis.
Funding: This work was supported by grants from the National Natural Science Foundation of China (81872347); Xijing Hospital Science and Technology Foundation Project (XJZT19PTK14).

PS02.09

Proteomic profiling of retinoblastoma-derived exosomes reveals potential biomarkers of vitreous seeding

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Introduction: Retinoblastoma (RB) is the most common intraocular cancer of childhood. Despite recent advances in conservative treatment have greatly improved the visual outcome, local tumour control remain difficult in presence of massive vitreous seeding. Thus, the identification of new biomarkers is crucial to design more effective therapeutic approaches. Traditional biopsy has long been considered unsafe in RB, due to the risk of extraocular spread. Exosomes, nano-sized vesicles containing nucleic acids and proteins, represent an interesting alternative to detect tumour-associated biomarkers. The aim of this study was to determine the protein signature of exosomes derived from RB tumours (RBT) and vitreous seeding (RBVS) primary cell lines.
Methods: Exosomes from 4 RBT (HSJD-RBT1, HSJD-RBT2, HSJD-RBT5, HSJD-RBT14) and 3 RBVS (HSJD-RBVS1, HSJD-RBVS3, HSJD-RBVS10) cell lines were isolated by high speed ultracentrifugation. Vesicles number and size were confirmed by NanoSight and Scanning Electron Microscopy. Protein content was analysed by bicinchonic-acid assay and high resolution mass spectrometry.
Results: A total of 5666 proteins were identified. Among these, 5223 and 3637 were expressed in exosomes RBT and one RBVS group respectively. Gene enrichment analysis of exclusively and differentially expressed proteins and network analysis identified identified in RBVS exosomes upregulated proteins specifically related to invasion and metastasis such as proteins involved in Extracellular Matrix (ECM) remodelling and interaction, resistance to anoikis and metabolism/catabolism of glucose and aminoacids.
Summary/Conclusion: In conclusion, in this study, we isolated exosomes from RB primary tumour and vitreous seeding cell lines and characterized their content with a proteomic approach. This is the first evidence describing a proteomic exosome signature specifically associated with vitreous seeding in RB. This characterization may represent a starting point for future analyses that allow defining exosomal markers as promising diagnostic and potential prognostic markers in RB as well as therapeutic targets.

PS02.10

Activation of hepatic stellate cells by extracellular vesicles released by uveal melanoma cells

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Introduction: Uveal melanoma (UM) is the main intraocular tumour in adults, and is particularly resistant to treatments when disseminated to the liver. Our hypothesis is that extracellular vesicles (EVs) released by the primary tumour are priming the liver stroma for metastatic cell colonization by activating hepatic stellate cells (HSteCs). This study aimed to characterize EVs from UM cells, and to determine their interactions with liver cells.
Methods: EVs were isolated from cell lines derived from ocular tumours and liver metastases by differential centrifugation. Their concentration/diameter range were determined by high-sensitivity flow cytometry. Cryo-TEM combined with receptor-specific gold labelling was used to reveal the morphology/size of melanomic EVs. The presence of melanoma and EV markers was assessed by Western blotting. The internalization of fluorescent melanomic EVs in HSteCs and their subsequent activation were assessed by confocal imaging using alpha-smooth muscle actin (alpha-SMA) and phalloidin stainings. EV impact on invasion was measured with a tumour spheroid model embedded in extracellular matrix. Melanomic EVs were inoculated into the retro-orbital sinus of immunodeficient mice to study their selective organ distribution.
Results: Melanomic EVs were positive for Annexin-5, tetraspanins, as well as some melanoma markers. Stellate cells with internalized melanomic EVs expressed more alpha-SMA, reflecting their activation. Adding EVs on tumour spheroids increased the invasion process. Melanomic EVs were localized into different murine organs, but mainly into the liver, as observed by in vivo fluorescent imaging.
Summary/Conclusion: Metastatic UM cells produce EVs that can reach the liver and activate stellate cells. Analysis of plasmatic EVs collected from UM patients should help designing new strategies for monitoring the progression of this cancer and its therapeutic response.
Funding: Vision Health Research Network; Fonds de recherche du Québec – Santé (FRQS); Fondation du CHU de Québec; Canada Foundation for Innovation

PS02.11

Exosome of cancer-associated fibroblast induce anti-cancer drug-resistance of NSCLC

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Introduction: The understanding of interaction mechanisms between cancer cells and the tumour microenvironment (TME) is crucial for developing therapies that can arrest tumour progression and metastasis. CAFs are the major constituent of the TME in many cancers. Recent studies indicate that exosomes harbour the potential to regulate proliferation, survival and immune status in recipient cells. Most of the current studies are focused on cancer cell secreted exosomes; and little is known about CAF-derived exosomes and their influence on cancer cells.
Methods: NSCLC cell lines (PC9GR) and MRC5 (normal fibroblast cells) were grown in culture with exosome-free FBS. Cutured media was filtrated by Tangential Flow Filtration Systems. Exosomes in supernatant were isolated with the ExoQuick-TC™ system. Considering the important role of cell extrinsic factors on cell growth and survival, we assessed whether factors contained in the MPA exosome could affect proliferation and survival of recipient cancer cells. Cells were then treated with 1 μM Osimertinib or PBS for 3 days prior to cell quantification of live cells.To investigate mechanisms of resistance to osimertinib mediated by MA or MPA-Exosome in NSCLC cell lines, we test cell viability by crystal violet assay in Trametinib or Osimertinib treated after pretreated MA or MPA-exosome, PC9GR during 6 days. We will investigate how MPA-exsomes activate ERK signalling pathway in PC9GR cells to induce antitumor effects by western blot.
Results: MPA exosome increased proliferation of PC9GR cells by more than 50% compared to control PBS. PC9GR cells grown in MPA-exosome and subsequently treated with osimertinib showed a significant increase in cell survival compared to PC9GR cells grown in MA-exosome. Osimertinib is used to treat EGFR-mutant non-small cell lung cancer (NSCLC) with tyrosine kinase inhibitor resistance mediated by the EGFR T790 M mutation.These data show that "MRC5-PC9GR-crosstalk factors" affect proliferation and adaptive drug resistance of cancer cells. MPA-Exosome mediates ERK signalling activation and attenuated after treatment of 1uM osimertinib.
Summary/Conclusion: CAFs support cancer growth and invasion. Co-cultured NSCLC with MRC5 lung fibroblast increased cell viability and exosomal miR-21 through the TGF-ß pathway in treatment Osimertinib. Exosomal miR-21 up-regulation in co-cultured NSCLC with MRC-5 induced drug resistance to drug-induced apoptosis. Thus, exosomal miR-21 expression in co-cultured NSCLC with MRC5 may support drug tolerance persister cells.
Funding: This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korea government(MEST) (No. 2019R1A2C1005593)

PS03: EVs in Intrinsic Medicines

Chair: Ewa K. Zuba-Surma – Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University

Chair: Cristina Grange – Deparment of Medical Sciences, University of Torino

PS03.01

Proteomic analysis of amniotic fluid-derived exosome cargo reveals a therapeutic potential for regenerative therapies.

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Introduction: Exosomes are being tested for their use as therapeutic agents in degenerative and chronic diseases. However, the optimal source of exosomes is currently under investigation. Amniotic fluid (AF) is a naturally-rich source of exosomes that is easily obtained for use in regenerative medicine. Organicell Flow™ is a minimally-manipulated, acellular product derived from human AF and consist of over 300 cytokines/chemokines as well as exosomes derived from the amniotic membrane and surrounding tissues. We characterized the exosome fraction of our product to elucidate the protein cargo of AF exosomes and demonstrate the therapeutic potential as a novel regenerative therapy.

Methods: The exosome fraction of our product was analysed using Nanosight nanoparticle imaging and MACsplex exosome surface marker array analysis. Exosomes were precipitated using size-exclusion filtration followed by ultracentrifugation from 3 independent products (in triplicate) and subjected to protein lysis and preparation for mass spectrometry analysis using the Easy nLC 1000 and Q Exactive instruments. Tune (version 2.9) and Xcalibur (version 4.1) was used to collect data while Proteome Discoverer (version 2.2) was used to analyse data. Protein expression lists were created by merging the 3 sample replicates together and commonly expressed proteins were determined using Vinny 2.0 vin diagram analysis. WebGestalt tool kit classification system was used to identify top protein function and pathway hits.

Results: Organicell Flow™ contain a mean concentration of 5.24x10^11 particles/mL (n = 12) with a mean mode size of 125.2nm (n = 12). Surface marker analysis confirms the presence of exosome associated proteins CD63, CD81, and CD9 in addition to a high expression of CD133 (n = 3). The completed analysis revealed 1225 commonly detected proteins across 3 products. The top molecular functions of identified proteins included protein-binding, ion-binding, and nucleic acid-binding with enzymes, transcription regulators, and transporter proteins representing the most abundant protein groups. Pathway enrichment analysis revealed top hits for Integrin, PDGF, and P53 pathways. A deeper dive into the enzyme category of the protein cargo further demonstrates the presence of proteins that promote DNA repair such as DNA polymerase (beta and lambda), telomerase reverse transcriptase, and BRCA1.

Summary/Conclusion: Organicell Flow™ characterization demonstrates the therapeutic potential of AF-derived exosomes. Proteomic analysis revealed protein cargo that may regulate various growth factor and cell-cycle associated pathways. Furthermore, the presence of DNA damage response proteins suggests a possible mechanism for induction of cellular repair.

PS03.02

Generation of CAR-T and γδT cell-derived exosomes for future cell free immunotherapies

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Introduction: T cell therapies have predominantly focused on treating cancers, with the more recent example of CAR-T cells targeting CD19+ cells in lymphoma patients. Despite recent success, challenges including demand for reduced off-target toxicities, reduced donor-donor variabilities and the targeting of multiple malignant cell types, still remain.

γδT cells are a subset of T cells with dual innate and adaptive qualities. This duality provides various advantages over their more studied and used counterpart, αβT cells. In the present study, we sought to compare

the immunotherapeutic potential of CAR-T cell and γδT cell-derived exosomes as novel cell-free based alternatives.

Methods: CD19-targeting CAR-T cells were obtained following the isolation, expansion and transduction of αβT cells using a lentiviral vector bearing the CAR construct. γδT cells were isolated and expanded from Peripheral Blood Mononuclear Cells (PBMCs) following innate or adaptive stimulation.

Exosomes from both cell sources were isolated after a 3-day culture in serum-free media using ultracentrifugation-based methods. Exosomes were characterized by Nanoparticle Tracking Analysis (determination of size) and western blot assays (detection of the appropriate surface markers).

Nalm-6 (B cell precursor leukaemia) cells were used as target cells for assessment of exosome cytotoxic/killing function. CAR-T cell and γδT cell-derived exosomes were incubated at 1000 particles/target cell for 24-hours. Total viable cell counts were assessed via imaging-based cytometry (NC-3000) utilizing acridine orange and DAPI staining.

Results: Exosomes derived from γδT cells activated via innate mechanisms showed significant killing of Nalm-6 as compared to exosomes from non-activated or adaptively activated γδT cells. In comparison, CAR-T cell-derived exosomes showed minor killing capabilities of the target cells.

Summary/Conclusion: Here, we report for the first time that exosomes derived from CD19 CAR-T cells and innately activated-γδT cells show/exert inhibitory action on Nalm-6 cells. Further studies are currently underway to identify the underlying mechanism(s) responsible.

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PS03.03

Characterization, composition, and biological properties of extracellular vesicles generated from human induced pluripotent stem cell-derived neural stem cells

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Introduction: Neural stem cell (NSC) therapy has shown promise for brain repair after injury or disease mostly through bystander effects. Nevertheless, the translation of NSCs derived from human induced pluripotent stem cells (hiPSCs) to the clinic remain constrained due to safety issues, which include immunogenic risks, tumorigenesis potential, and incomplete differentiation. A way to avoid these issues is by using extracellular vesicles (EVs) generated from NSCs, as NSC-EVs likely have similar neuroprotective properties as NSCs and are amenable for non-invasive administration as an autologous or allogeneic off-the-shelf product. However, this would require reliable purification and characterization processes, and testing of EVs for composition and biological properties.

Methods: We generated EVs from hiPSC-derived NSCs using a combination of ion-exchange chromatography (IEX) and size-exclusion chromatography (SEC) and investigated their composition through small RNA sequencing and proteomics. We also performed in vitro and in vivo experiments to determine their biological and functional properties.

Results: IEX and SEC facilitated purification of hiPSC-NSC EVs nearly to homogeneity, which expressed EV markers such as CD9, CD63, CD81, and ALIX with a mean size of 145 nm. Small RNA sequencing revealed enrichment of miRNAs related to different neuroprotective signalling pathways and diverse metabolic functions consistent with their role in cell-cell communication. The proteomic analysis identified >1,000 proteins, including EV markers and many other proteins involved in central nervous system function and cellular processes. The EVs also displayed antiinflammatory activity in an in vitro mouse macrophage assay. Intranasal (IN) administration of NSC-EVs resulted in their rapid incorporation by neurons, microglia, and astrocytes in virtually all regions of the brain. Functionally, IN administration of NSC-EVs reduced inflammatory activity in the brain in a model of status epilepticus, and increased hippocampal neurogenesis in the adult brain.

Summary/Conclusion: Biologically active EVs with antiinflammatory and neurogenic properties could be purified and harvested from hiPSC-NSCs. Such EVs also contain many miRNAs and proteins that are of interest for brain repair after injury or disease.

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PS03.04

Promise of neural stem cell-derived extracellular vesicles for combating age-associated cognitive and mood impairments

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Introduction: Age-related cognitive dysfunction is associated with increased oxidative stress, low-level chronic neuroinflammation, and waned hippocampal neurogenesis in the brain. From this perspective, biologics capable of modulating oxidative stress and neuroinflammation, and stimulating neural stem cell activity in the brain might be useful as anti-ageing interventions.

Methods: We investigated the efficacy of intranasal administration of extracellular vesicles (EVs) generated from cultures of rat subventricular zone neural stem cells (SVZ-NSCs) in the middle-aged mice to alleviate cognitive and mood dysfunction, increased oxidative stress, neuroinflammation, and neurogenesis decline in old age. Mice were treated intranasally with NSC-EVs once weekly for three weeks (50 billion per administration) starting from 18.5 months of age. A month later, the animals were examined for cognitive, memory, and mood function using multiple behavioural tests, and brain tissues were examined for oxidative stress, neuroinflammation, and neurogenesis.

Results: Object-based tests revealed that aged animals receiving vehicle displayed cognitive impairments for discerning minor changes in the environment as well as for distinguishing similar but not identical experiences. These animals also exhibited spatial memory dysfunction and anhedonia. In contrast, aged animals receiving NSC-EVs showed improved cognitive and mood function. Biochemical analyses of brain tissues revealed that NSC-EV treatment normalized elevated concentrations of oxidative stress markers malondialdehyde and protein carbonyls and the proinflammatory cytokine interleukin-1 beta. Moreover, NSC-EV treatment stimulated increased production of antiinflammatory protein interleukin-10 and the antioxidant superoxide dismutase. Immunohistochemical analysis revealed modulation of neuroinflammation typified by reduced activity of reactive astrocytes and activated microglia and improved hippocampal neurogenesis.

Summary/Conclusion: The results suggest that the intranasal administration of NSC-EVs is a promising approach for maintaining better cognitive and mood function in ageing through modulation of oxidative stress, neuroinflammation, and neurogenesis.

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PS03.05

Chemically modified myocytes-derived EVs for the treatment of cardiac fibrosis.

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Introduction: Myocardial fibrosis is a disorder that may occur after cardiac injure due to a malfunction of the cardiac remodelling. Fibroblasts resident in myocardium are erroneously activated causing an excessive accumulation of extracellular matrix, which decreases cardiac function and eventually, leads to death. It is known that cardiomyocytes communicate with the surrounding cells such as fibroblast and endothelial cells by extracellular vesicles (EVs). The loss of this communication is thought to play a central role in cardiac fibrosis. Therefore, cardiomyocytes-derived EVs may be a promising a cell-free system for the treatment of fibrosis inhibition.

Methods: A novel culture medium was stablished to improve the expansion of primary cardiac myocytes. This was tested using two commercially available primary myocytes cell lines. EVs were collected by serial ultracentrifuges, and their effect on fibrosis was tested. For that, prior to any treatment, and to mimic fibrosis, primary cardiac fibroblast were activated overnight with TGFβ.

Results: By the use of a defined conjunct of chemicals, mature cardiomyocytes culture was highly improved to ensure a high collection of EVs. Terminal differentiation markers, as well as senesce apparition was delayed in comparison to predetermined culture medium. Interestingly, those primary cells secreted a rather large amount of EVs, which expressed common EVs membrane marker. TGFβ-treated cardiac fibroblasts were co-cultured with myocytes showing a decrease of fibroblast activation markers both at mRNA and protein levels. Similar results were found when activated fibroblast were treated with EVs.

Summary/Conclusion: Our findings indicate that the use of EVs derived from chemically modified myocytes

is a promising treatment for ischaemic myocardial fibrosis. However, further molecular experiments have to be done to identify the molecules within EVs responsible for the inactivation of fibroblast.

PS03.06

Evaluation of osteoinductive and anti-inflammatory properties of spine-derived exosomes

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Introduction: Over the last 2 decades, Mesenchymal Stem Cell-derived exosomes have been shown to play a crucial role in a myriad of cell function such as extracellular matrix synthesis, proliferation, differentiation or cell migration. Biological sources of exosome (heterogeneous or homogeneous cell population, serum, urine etc.) have a direct influence on the content of their cargo and their therapeutic application and potential. In this study, we evaluated exosomes excreted from cadaveric spine-derived cells. We hypothesized that exosomes derived from a bone source such as the spine, will drive the osteogenic differentiation of progenitor cells. We also investigated their effects on inflammation in Nucleus Pulposus cells using an in-vitro assay.

Methods: After their isolation and characterization, exosomes derived from cadaveric human spines were assayed for osteoinductive properties. A C2C12 myoblast cell line was treated with different concentrations of exosomes and expression of alkaline phosphatase was measured after 5 days incubation. Treatment with BMP-4 was used as positive control.

Anti-inflammatory properties were assessed by incubating TNF-treated Nucleus Pulposus cells with exosomes for 3 days. qPCR analysis of mRNA expression of inflammatory cytokines (IL-6, IL1-beta, IL-8) metalloproteinases (MMP3 and ADAMTS4), and apoptotic genes (BAX, BCL2) was used to determine the effects of exosomes on inflammation.

Results: Spine-derived exosomes positively expressed the exosome flow cytometry markers tested (CD81, CD63 and CD9). The mean number of exosomes per microgram of protein was 3.31 ± 2.33 x 108 indicating a relatively high purity. Osteoinductive (OI) testing was performed using different concentrations of exosomes. The OI index of treatment of C2C12 cells with BMP-4, 2 x 108, 1 x 109, 2 x 109, 5 × 109 or 1 × 1010 exosomes alone was 28.5, 1.0, 3.7, 7.4, 11.8 and 27.6 respectively. Anti-inflammatory properties of exosome are currently being assessed and will be presented at the time of the poster presentation.

Summary/Conclusion: Administering exosomes alone or in combination with an exogenous scaffold has the potential to repair injured tissue and to restore bone function. The clinical significance of this application is aimed to promote the patients' bone healing process and provide a cell-free therapeutic platform that is safe and effective.

PS03.07

Administration of human Mesenchymal Stem Cell derived extracellular vesicles modulates the abnormal plasticity of newly born neurons and neuroinflammation in a rat model of status epilepticus

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Introduction: Extracellular vesicles (EVs) generated from human bone marrow-derived mesenchymal stem cells (hMSCs) display anti-inflammatory and neuroprotective properties. Our recent study has shown that intranasally (IN) administered hMSC-EVs incorporate into significant percentages of neurons and microglia in virtually all regions of the intact as well as the injured forebrain within 6 hours (Kodali et al., Int J Mol Sci, 2019). In this study, using a rat model, we investigated the efficacy of a low dose of hMSC-EVs administered intranasally for alleviating the abnormal plasticity of newly born neurons and the activation of microglia after SE.

Methods: Approximately 10 billion EVs were dispensed bilaterally into both nostrils of young F344 rats that experienced two hours of Kainate-induced SE. Animals were euthanized seven days after SE, and brain tissue sections were processed for immunohistochemical staining of NeuN (a neuronal marker), DCX (a marker of newly born neurons), IBA-1 (a microglial marker), and parvalbumin (PV) and reelin (markers of subclasses of interneurons). In addition, activated microglia were quantified using IBA-1 and CD68 dual immunofluorescence.

Results: IN administration of EVs reduced the SE-induced loss of pyramidal neurons in the hippocampal CA1 subfield. Also, EV administration after SE maintained higher levels of PV+ interneurons in the dentate gyrus. Furthermore, EV treatment after SE modulated abnormal neurogenesis, which was evidenced by a

decline in the percentage of newly born neurons displaying basal dendrites. Besides, EV treated animals displayed higher percentages of resting microglia (ramified microglia), reduced percentages of activated microglia (microglia expressing IBA-1 and CD68), in comparison to animals receiving vehicle after SE. Interestingly, diminished abnormal plasticity of newly born neurons was accompanied by the preservation of interneurons positive for reelin; a protein believed to guide newly born neurons to their correct locations.

Summary/Conclusion: The results suggest that even a low dose IN administration of MSC-derived EVs after SE can limit neurons loss, dampen the abnormal plasticity of newly born neurons, and modulate the activation of microglia.

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PS03.08

Intranasal administration of exosomes improves the symptoms in three mice model of Autism

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Introduction: Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by three core symptoms that include social interaction deficits, cognitive inflexibility, and communication disorders. They have been steadily increasing in children over the past several years, with no effective treatment. Two percent of all ASD patients are suffering from a disorder caused by a mutation in the shank3 gene. Shank3 is an important synaptic protein, disruption of this gene directly leads to cognitive and motor impairments. During the recent decade, exosomes that derived from mesenchymal stem cells (MSC-exo) have been spotlighted as a promising therapeutic target for various clinical indications, including neurological disorders. Here we test three different autistic mice models. BTBR as a multifactorial mice model of autism and two different Shank3 mutated mice. The first is a complete deletion of exon 22 (22q13.3) and the second is a specific insertion mutation of Guanine to 3680 position in the gene (insG3680) that leads to stop codon.

Methods: Exosomes were isolated using differential centrifugation protocol and characterized using the 2018MISEV guideline recommendations. Each animal received an intranasal administration of 20ul containing 107 exosomes/μl. For intravenous administration, the same number of exosomes, were used, injected in 100μl.

Results: All three animal models showed significant improvement in their autistic behavioural phenotypes following intranasal administration. The improvement seems to be dose-dependent and was better achieved via intranasal vs intravenous administration. Biodistribution of MSC-exo showed accumulation in the brain within 72 hours, yet the reduction of the signal was observed in the kidneys, heart and lungs.

Summary/Conclusion: Our data suggest that Exosomes derived from adipose MSC, carry a therapeutic potential in ASD, via non-invasive intranasal administration in three different mice models. These data further emphasize our potential therapeutic strategy to reduce symptoms of autism in clinical trials.

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PS03.09

Equine tendon injury treatment by EVs: an in vitro study

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Introduction: Current treatment options for tendinopathies (chronic, painful tendon disorders), are not able to restore the functional properties of native tendons. Hence, new treatment options are sought.

The efficacy of mesenchymal stem cells (MSCs) therapies, which combined with a rehabilitation programme including controlled exercise is the current gold standard in equine tendon treatment, has been shown to be largely due to the cells´ paracrine activity.

The aim of this study was therefore to evaluate the effect of bone marrow MSC derived autologous and allogeneic conditioned medium (CM, full secretome) and their extracellular vesicles (EVs) on "tendon healing" in vitro.

Methods: To compare the "therapeutic" effect of MSC derived EVs and CM, a standardized scratch assay (wound healing assay) was performed. CM from equine tenocytes, EV depleted medium and medium with or without FCS served as controls.

Tendons and bone marrow aspirates were obtained from three horses (6, 19 and 23 years) which were euthanized for reasons unrelated to this study. MSCs were isolated by Ficoll density gradient centrifugation and tenocytes were obtained by migration from tendon explants. For CM and EV production, cells were cultured in EV depleted medium.

EVs were harvested by a stepwise ultracentrifugation approach and characterized by Nanoparticle Tracking analysis (NTA), Western Blot (CD9, CD63) and Transmission-Electron Microscopy (TEM).

Results: Western blot, NTA and TEM confirmed successful isolation of EVs from equine MSCs. The strongest positive effect on wound healing (fastest gap closure) was achieved by MSC-CM ($p < 0.0071$). The gap closure achieved with MSC-EVs was slower than with MSC-CM ($p < 0.7985$) but faster than with CM of tenocytes ($p < 0.8289$). Donor specific differences in wound healing capability were shown for both autologous and allogeneic application.

Summary/Conclusion: Treatment with MSC-CM resulted in significantly faster wound healing of adult tenocytes in vitro than MSC- EVs or tenocyte-CM.

MSCs donor age shows a significant effect on gap closure following autologous but not allogeneic administration.

PS03.10

EV-enriched secretome fraction from GMP-compatible, scalable, human iPSC-derived cardiac progenitors improve heart function in chronic heart failure mice

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Introduction: We have shown that research-use-only grade (res) human iPSC-derived cardiac progenitors (CPCres) can produce a secretome whose small-EV-enriched fraction (sVF) can treat chronic heart failure (CHF) in mice. GMP-compatible, scalable processes for a CPC-derived sVF suitable for human therapeutic use is needed.

Methods: iPSC-derived CPC were produced and cultured using GMP-compatible, scalable processes (CPCtx). Media without cells were "cultured" in parallel for "virgin media" controls (MV). CPCres were cultured as previously described. As a proof of concept, sVFs were isolated from conditioned media by ultracentrifugation: CPCtx-EV, CPCres-EV and MV. Particle size distributions/concentrations (nanoparticle tracking analysis), protein levels (BSA), and the presence of CD-63 (ELISA) were determined. In vitro activity was assessed by HUVEC scratch wound healing assay, and by rat and human cardiomyocyte (CM) survival assays. C57BL/6 mice in CHF received echo-guided myocardial injection of PBS vehicle control (60uL, n = 11), CPCtx-EV (60uL, n = 11), or CPCres-EV (45uL, n = 11). Change in cardiac function was assessed by echocardiography.

Results: CPCtx-EV particle sizes were polydisperse (mode ~72 nm) at a concentration of ~1.6e11 particles/mL (~2,600 particles/cell) and ~0.975mU CD63/ug protein. CPCtx-EV increased wound healing, human CM survival, and rat CM survival in vitro by 4.75x, 1.4x, and 2x, respectively over MV controls. In CHF mice, significantly less CPCtx-EV mice, and less CPCres-EV mice had severely progressive heart failure (Left Ventricular End Systolic Volume, LVESV, increased >14%) than PBS control mice (PBS vs CPCtx-EV, $p < 0.05$; PBS vs CPCres-EV, $p < 0.056$), and the Average Ejection Fraction of the PBS group deteriorated 2.5x more than the CPCtx-EV group (−4% vs −1.6%, respectively; ns).

Summary/Conclusion: We have a process for CPC differentiation and production of conditioned media suitable for use in human clinical trials from which can be made an sVF with the potential to treat CHF, possibly through re-vascularization or preservation of CM viability.

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PS03.11

Electrospun collagen coupled with spine bone marrow-derived exosomes

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Introduction: Exosomes are nanoscale vesicles that mediate cell-to-cell communication via exchanging molecular cargo. Mesenchymal stem cell (MSCs) modification towards an osteogenic path can occur by uptake of exosomes from other cells. It is less clear whether vesicle placement in the absence of cells will facilitate site-specific delivery through acellular transfer of osteogenic activity. An electrospun fleece was combined with bone marrow-derived exosomes in the absence of cells to evaluate osteoinductive potential

that might be thermo-stable and be used in a biologically neutral collagen carrier. Comparisons were made of standard laboratory assay of osteoinductivity (OI), and in vivo expression in a mouse calvarial defect model.

Methods: Electrospun Type-I collagen was prepared with and without hydroxyapatite (HA) (SpinPlant GmbH, Leipzig) as a foundation base for application of the bone marrow-derived exosomes. Individual discs of the collagen enhanced scaffolds (3-mm) were prepared and placed in a mouse calvarial skull defect. Animals were followed for 4 and 8 weeks. Exosomes were isolated from qualified cadaveric human spines by differential ultracentrifugation. Microscopic observation, quantitative assessment of OI with an alkaline phosphatase assay, and flow cytometry were used to evaluate the composition, the hybrid nature of the addition to the nano-collagen fibres. A fluorescent protein reporter transgenic mouse model expressing osteocalcin, Type-I collagen, Phex, and SP7 (Osterix) was evaluated at 4 and 8 weeks to determine bone formation across the defect.

Results: ALP activity on the scaffold with HA demonstrated an approximate tenfold increase to that of the collagen scaffold alone. While a dose-dependent effect, with higher doses of exosomes resulting in a greater amount of alkaline phosphatase expression, expression that exceeded that of the 50ng BMP-4 control. Dose escalation from 1.5, 2, and 4E9 resulted in similar increases in expression that was statistically greater with the combination of the fleece with the exosome component. Bone formation in the mouse calvaium did not demonstrate gap closure at 4 or at 8 weeks, but did demonstrate enhanced osteoclastivity and robust bone remodelling at the margins of the defect.

Summary/Conclusion: Bone marrow-derived exosomes dried into an electrospun fibrillar collagen demonstrated in vitro osteoinductive potential that might provide site-specific placement that could enhance biologic potential. With the capacity for ambient temperature storage, the provision of site-specific placement becomes a technical consideration. Placement of the human tissue derived exosomes in a transgenic mouse calvarial defect model did not demonstrate bridging bone across the defect.

PS03.12

Exosomes loaded with PTEN-Interfering RNA enables functional recovery in rats after complete spinal cord transection

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Introduction: Complete spinal cord transection is a debilitating disease that usually leads to permanent functional impairments, with various complications and limited spontaneous recovery. The current investigation of molecular mechanisms controlling axon regeneration, (e.g., signalling networks and environmental cues), led to new strategies to enhance axonal regeneration. We have previously shown that intranasal administration of mesenchymal stem cells derived exosomes (MSC-exo), cross the blood-brain barrier and significantly ameliorate motor and behavioural phenotype in several animal models of neurotrauma and neuropsychiatric disorders.

Methods: MSC-exo were isolated from human bone marrow and were loaded with phosphatase and tensin homolog small interfering RNA (PTEN-siRNA). The exosomes were given intranasally to rats two hours after complete spinal cord transaction. Eight weeks later we followed the motor function and histology and electrophysiology study was performed in order to reveal the connectivity and the biochemical changes in the treated rats.

Results: We demonstrate that Intranasal (IN) administrations of MSC-derived exosomes could penetrate the blood-brain barrier, home selectively to spinal cord lesion via chemotaxis, and integrated in neurons within the lesion. Furthermore, in rats with complete spinal cord transection, MSC-exo loaded with PTEN-siRNA silenced PTEN protein expression in the lesion and promoted robust axonal regeneration and angiogenesis, companied with decreased astrogliosis and microgliosis. Moreover, the intranasal treatment partially restored electrophysiological and structural integrity, and most importantly, enabled the remarkable functional recovery and significant improvement in their movements.

Summary/Conclusion: This rapid, non-invasive, approach, using cell-free nano-swimmers carrying molecules to target pathophysiological mechanisms suggest novel strategy for clinical translation to spinal cord injury and beyond.

PS03.13

A novel umbilical cord derived wharton's jelly formulation for regenerative medicine applications

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Introduction: Musculoskeletal injuries have traditionally been treated with activity-modification, physical therapy, pharmacological agents and surgical procedures. These modalities have limitations, as well as potential side-effects. Over the last decade, there has been an increased interest in the use of biologics for regenerative medicine applications (RMA), including umbilical cord (UC) derived Wharton's Jelly (WJ). Despite this increase, there is insufficient literature assessing the amount of growth factors, cytokines, hyaluronic acid (HA) and extracellular vesicles (EV) including exosomes in these products. The purpose of this study was to develop a novel WJ formulation and evaluate the presence of growth factors, cytokines, HA and EV including exosomes.

Methods: WJ was isolated from human-UC obtained from consenting C-section donors and formulated into an injectable form. Randomly selected samples from different batches were analysed for sterility testing and quantified for presence of growth factors, cytokines, HA and particles in EV size range.

Results: The results showed all samples passed the sterility test. Growth factors including IGFBP 1, 2, 3, 4 and 6, TGF-α, PDGF-AA were detected. Expression of several immunomodulatory cytokines, RANTES, IL-6 R, IL-16, were also detected. Expression of pro-inflammatory cytokines MCSFR, MIP-1a; anti-inflammatory cytokines TNF-RI, TNF-RII, IL-1 RA; and homoeostatic cytokines TIMP-1 and TIMP-2 were observed. Cytokines associated with wound-healing, ICAM-1, G-CSF, GDF-15, and regenerative properties, GH were also expressed. High concentrations of HA were observed. Particles in the EV size range (30–150 nm) were detected and were enclosed by the membrane, indicative of true EV.

Summary/Conclusion: Our results confirmed the presence of numerous growth factors, cytokines, HA and EV in the WJ formulation. More studies are underway to confirm the presence of exosomes in detected EV using exosome-specific markers. We believe the presence of multiple factors within one WJ formulation may play a role in reducing inflammation, pain and augment healing of musculoskeletal injuries. This offers a potential expanded use for RMA.

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PS03.14

Collagen sponge loaded with mesenchymal stem cell-derived small extracellular vesicles promote robust bone regeneration

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Introduction: Mesenchymal stem cell (MSC) therapy has demonstrated effective bone regeneration in clinical studies. However, the therapeutic efficacy of MSCs have been attributed to the secretion of extracellular vesicles (EVs), particularly 50–200 nm small EVs (sEVs). Here, we investigate the efficacy of MSC-sEVs loaded in collagen sponge in the regeneration of critical-sized calvarial defects in immunocompetent rats.

Methods: sEVs were isolated from conditioned medium of human MSCs and stored at −20 C. Calvarial defects of 8-mm diameter were surgically created on thirty-two 10-week-old male Sprague-Dawley rats. These rats were then randomly assigned to 2 groups (n = 16 rats/group): defects treated with collagen sponge containing 100 μg of sEVs in 100 μl saline (CS/sEVs) and defects treated with control collagen sponge containing an equivalent volume of saline (CS/Control). At 1 and 8-week post-surgery, the calvarial bone samples was harvested for analyses by micro-computed tomography (micro-CT), histology, immunohistochemistry and histomorphometry.

Results: At 1-week post-surgery, micro-CT analysis showed little bone formation at the defect site in both CS/sEVs and CS/Control groups. No statistical differences were observed in micro-CT and histology scores in both groups. Interestingly, CS/sEVs group showed significantly higher osteocalcin (OCN)+ area of 5.7 ± 2.4% than that of CS/Control group (2.1 ± 1.3%; P = 0.003). CD31+ microvessels at sizes ≤50 μm and >50 μm in CS/sEVs group (34.4 ± 2.9 and 8.6 ± 2.1 microvessels/HPF) were also significantly higher than that of CS/Control (18.8 ± 1.1 and 5.2 ± 1.4 microvessels/HPF; P = 0.002 and P = 0.038 respectively). By 8 weeks, CS/sEVs group displayed enhanced new bone formation that completely bridged the calvaria defect. In contrast, rats in CS/Control showed limited bone formation. Consequently, CS/sEVs group displayed a micro-CT score of 3.9 ± 0.2 which was significantly better than that of CS/Control group (2.5 ± 0.8; P = 0.001). CS/sEVs group also exhibited >twofold increase in bone volume, and improved bone quality with higher trabecular thickness and number, and smaller separation (P < 0.01),

compared to CS/Control group. Consistently, CS/sEVs group displayed a significantly better histology score of 5.4 ± 1.0 than that of CS/Control (2.6 ± 1.8; P = 0.001). Moreover, CS/sEVs group showed significantly higher OCN+ area of 48.9 ± 7.9% than that of CS/Control group (20.4 ± 4.4%; P = 0.004).

Summary/Conclusion: This study demonstrates that single-stage implantation of collagen sponge loaded with ready-to-use MSC sEVs can promote robust bone regeneration in a rat calvarial defect model.

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PS03.15

Immunomodulatory potential of extracellular vesicles derived from Mesenchymal stromal cells

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Introduction: Extracellular vesicles (EVs) derived from mesenchymal stem/stromal cells (MSCs) are promising new agents in regenerative medicine and immunotherapy. Considering that independent MSC-EV preparations might differ in their therapeutic function, we have set up a functional assay allowing testing for the potential immunomodulatory properties of independent MSC-EV preparations.

Methods: Human peripheral blood-derived mononuclear cells (PBMCs) were pooled from up to 12 different healthy donors warranting high allogeneic cross-reactivity, even following an optimized freezing and thawing procedure. After thawing, mixed PBMCs were cultured for 5 days in the absence or presence of MSC-EVs. Thereafter, cell morphologies were documented, supernatants were harvested for cytokines quantification and cells were phenotypically characterized by flow cytometry. By analysing the expression of a collection of different lineage and activation markers, we selected a panel of antigens apparently being regulated by MSC-EV preparations considered to be therapeutically active.

Results: We observed that in the presence of active MSC-EV preparations more CD14+ (monocytes) are recovered from the MLR assay than in corresponding control samples. Focusing on T cells, we learned that active MSC-EV preparations reduced the content of CD4 and CD8 T cells expressing activation markers like CD54 and CD25.

Summary/Conclusion: The MLR assay allows elaborated functional testing of immunomodulatory activities of given MSC-EV preparations. Currently, we are comparing the immune modulatory capabilities of EVs derived from distinct sources and optimize the marker panel to distinguish discrete immune cell subtypes such as different CD4 cell types, i.e. TH1, TH2, TH17 and TRegs.

PS03.16

Extracellular vesicles in platelet-rich plasma: dependency on sample processing

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Introduction: Platelet-rich plasma (PRP) proved effective in regenerative medicine. Numerous protocols for its preparation and application are available in the published literature. PRP possesses important immune, haemostasis and regenerative factors, however, the mechanisms of their action are yet poorly understood. Extracellular vesicles (EVs) could be one of the important factors that would contribute to the beneficial effects of preparations.

This study was performed as a part of a registered randomised controlled clinical trial (Nr: NCT04281901). PRP was used to treat chronic middle ear inflammations. Here we present the results of PRP analyses from 29 blood samples of volunteers with no record of disease.

Methods: Plasma obtained from 20 ml of blood was depleted of erythrocytes and enriched with other particles by repetitive centrifugation of samples. Flow cytometry (FCM) was employed to monitor particle contents (cells and smaller particles) throughout the sample processing. The platelet gate was divided into two parts: intact platelets and smaller particles. Identity and morphology of particles in the preparations were examined by scanning electron microscopy (SEM). Standard laboratory tests of blood were performed.

Results: SEM images revealed the presence of heterogeneous population of particles in the preparation of PRP, most of which were activated and partially fragmented platelets. The population of smaller particles measured with FCM, was identified as EVs. The erythrocyte sedimentation rate was statistically significantly correlated to the volume of plasma obtained in the initial centrifugation step (R = 0,70, p < 0,001) and to the concentration of EVs (R = 0,82; p < 0,0001). Time from sample collection to the preparation of PRP was negatively correlated with the concentration of platelets in PRP and positively with the concentration of EVs (R = 0,75, p < 0,001). Platelet concentration in preparation samples was found to depend on the concentration of platelets in the blood and parameters of sample processing connected with larger centrifugal and shear forces on the samples during centrifugation. These include: sample volume, the size and shape of the centrifuge tube and the distance of the sample from the rotor axis.

Summary/Conclusion: EVs are gradually forming upon activation and degradation of cells in the sample throughout the sample processing. Optimal processing may importantly contribute to the healing properties of preparation.

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PS03.17

Satellite cell-derived extracellular vesicles as a therapeutic for mitochondrial dysfunction in duchenne muscular dystrophy

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Introduction: Satellite cells (SCs) are muscle stem cells that play a central role in muscle remodelling. Despite their therapeutic potential, SC-based therapies have met numerous logistical challenges in the treatment of systemic muscle diseases, such as Duchenne muscular dystrophy (DMD). SC-derived extracellular vesicles (SC-EVs) may unlock the therapeutic potential of SCs by overcoming these limitations. To investigate their therapeutic potential, we assessed the ability of SC-EVs to reverse mitochondrial dysfunction, a key pathological feature of DMD, in oxidatively-damaged C2C12 and primary DMD myotubes.

Methods: SCs from C57 mice were isolated and cultured. EVs were isolated from the supernatant of SCs via polyethylene glycol precipitation and characterized using nanoparticle tracking analysis. The ability of SC-EVs to deliver protein cargo to C2C12 myotubes, and the localization of the cargo once delivered, were analysed using fluorescence microscopy. To examine SC-EV potential to restore the function of damaged mitochondria, C2C12 myotubes were treated with 50 μM H2O2 for 24 h followed by treatment with 3.12x108 SC-EVs for 24 h. Separately, cultured dystrophic myotubes were treated with 3.12 × 108 EVs every 24 h for 72 h. In both sets of experiments, maximal oxygen consumption rate (max OCR) was measured via Seahorse XF Cell Mito Stress Test. Where appropriate, a t-test was performed to test for statistical significance ($p < 0.05$).

Results: Based on estimated cell number and EV quantification, each SC released approximately 2.35 × 105 ± 3.10x104 EVs/day. EVs delivered protein cargo into myotubes within 2 h. Fluorescent labelling of intracellular mitochondria showed co-localization of delivered protein and mitochondria. Incubation of myotubes with H2O2 resulted in a 42% decline in max OCR relative to untreated myotubes. Subsequent treatment with SC-EVs resulted in a 76% increase in max OCR. Treatment of undamaged myotubes with SC-EVs had no effect on max OCR. Primary DMD myotubes treated with SC-EVs showed a 78% increase in max OCR relative to untreated DMD myotubes.

Summary/Conclusion: SC-EVs rapidly deliver proteins into myotubes, much of which co-localizes with mitochondria, and reverses mitochondria dysfunction in oxidatively-damaged and dystrophic myotubes.

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PS04: Advances in EV Quantification

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PS04.01

Quantitation of extra-cellular vesicle surface markers with MESF liposomes

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Introduction: Flow cytometry has been used extensively for analysis of EV particles stained with fluorescent antibodies directed to the known cell surface markers. Quantitation of the surface markers in terms of the number of molecules or the number of antibodies bound per specific marker has remained one of the largest challenges in the EV research field. Changes in instrument setup as well as changes in fluorescent antibodies from different vendors, all impact the relative MFI values for the same EV sample. In this work we report a standardization method of quantitating extra-cellular vesicle surface markers with MESF liposomes.

Methods: Liposomes labelled with FITC fluorescent dye were prepared with a BD proprietary technology. Dynamic light scattering analysis was used for size determination of the liposomes. BD FACSAria™ Fusion system, modified with a small particle side scatter module (SP SSC), was used for analysis of the labelled liposomes by flow cytometry.

Results: We created a set of 180 nm FITC-modified liposomes of various fluorescent intensities with a known number of FITC molecules incorporated in each liposome intensity. The MFI values of each liposome population (intensity) had a linear relationship to the amount of FITC used for labelling the liposome nanoparticles, suggesting that no self-quenching of FITC fluorescence had occurred. The number for the FITC fluorophores for each liposome intensity was expressed in the units of Molecules of Equivalents Soluble Fluorochrome (MESF). A plot of MESF vs. the fluorescent intensity of the liposomes (MFI values) obtained from flow cytometry analysis provided a calibration curve, from which the fluorescent intensity (MFI value) of a stained EV sample can be converted to the number of fluorophores bound (MESF value) to the surface of the EV particles.

Summary/Conclusion: By this approach, the MFI values of stained EV particles are converted to standardized MESF values that are independent of instrument variation, resulting in further improvement of inter-laboratory standardization. Furthermore, utilization of liposomes with similar size and refractive index to EV particles simplifies the data evaluation and improves the accuracy of EV surface marker quantitation by flow cytometry. Currently, other fluorescent dyes are being explored to expand the utility of MESF liposomes with other fluorescent colours.

PS04.02

Measuring cholesterol as a high-throughput method for quantifying extracellular vesicles

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Introduction: The extracellular vesicle (EV) field currently lacks a high-throughput method for accurately quantifying EVs in solution. EV quantification has traditionally relied on nanoparticle tracking analysis (NTA), which is time intensive and indiscriminately counts non-EV particles, such as membrane fragments and protein aggregates. We have rigorously assessed two commercially available methods for measuring cholesterol, a major lipid component of the EV lipid bilayer, and evaluated the utility of these assays to quantify EVs in minimally processed samples.

Methods: The Amplex® Red Cholesterol Assay and Cedex Bio HT were used to quantify cholesterol in EV samples via enzymatic oxidation, with dynamic ranges of 80–8,000 ng/mL and 4–800 µL/mL, respectively. Samples throughout various stages of purification were analysed, from clarified cell culture medium to highly purified EVs separated on an iodixanol gradient. We evaluated several pre-processing methods, to remove non-EV cholesterol content prior to analysis.

Results: The Amplex® and Cedex Bio HT assays were found to perform comparably for quantifying cholesterol in purified EVs (R2 = 0.92). Importantly, cholesterol quantification on purified EV samples, ranging from 1e11 to 4e13 particles/mL, correlated well with NTA measurements (R2 = 0.96). Both 45 μM filtration or an additional 16,000 RCF centrifugation step following clarification removed cholesterol associated with cellular debris or other non-EV sources, allowing for accurate quantification of conditioned medium samples or ultracentrifugation pellets (UCP) instead of needing to rigorously purify samples with an iodixanol density gradient.
Summary/Conclusion: Cholesterol quantitation can be used to accurately estimate EV concentration, allowing for rapid characterization of samples from clarified cell culture supernatant to highly purified EVs. This high-throughput analytical capability may enable more comprehensive assessment of methods to boost EV yield through mass screening of cell culture conditions.

PS04.03

Optimization of nanoparticle tracking analysis of extracellular vesicles isolated from plasma and bronchopulmonary lavage fluid of patients with non-small cell lung cancer

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Introduction: Recent studies show that tumour-derived extracellular vesicles (EVs) greatly influence the tumour microenvironment and impact the therapy. In non-small cell lung cancer (NSCLC), bronchopulmonary lavage fluid (BALF) appears to be a good source of tumour-derived EVs, providing more accurate information about the tumour microenvironment than EVs from plasma. So far there is a lack of accurate and standardized methods for EV quantification. Fluorescence nanoparticle tracking analysis (FL-NTA) is an emerging method of EV-analysis, allowing discrimination of EVs and exosomes from impurities. Here we perform an optimization of the FL-NTA method to compare EVs from plasma and BALF of NSCLC patients and healthy controls (NC).
Methods: EVs were isolated using homemade size-exclusion chromatography (SEC) columns (plasma) and ultrafiltration or differential ultracentrifugation (BALF). NTA was performed using ZetaView PMX220 (Particle Metrix) after EV-staining with membrane dyes or fluorescence-labelled antibodies against typical EV-marker (CD9, CD81, CD63).
Results: NTA scatter measurements showed a higher total particle concentration in plasma than in BALF. However, membrane-specific staining showed a much greater purity of EV-preparations from BALF, where nearly 100% of the particles detected in scatter mode showed positive membrane-staining. In contrast, only around 40–50% of particles in the plasma EV-preparations were positive for the membrane dyes. Fluorescence-staining for EV surface marker requires further optimization to obtain reproducible results.
Summary/Conclusion: Classical NTA using only the scatter mode fails to discriminate between EVs, lipoproteins and protein aggregates. For EV-analysis from complex biofluids like plasma, FLA-NTA and staining for specific EV marker is necessary to receive reliable data. BALF seems to be a better source of tumour-derived EVs than plasma, since the obtained EV-preparations show a higher purity. Improving conditions for fluorescence-staining and NTA measurement of EVs from plasma and BALF of NSCLC patients will provide an additional method for quantifying and phenotyping of EVs.
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PS04.04

Introducing a universal fluorescence extracellular vesicle stain for NanoView Bioscience's Exoviewer platform

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Introduction: The ExoViewer platform currently enables the user to capture extracellular vesicles (EV) by means of surface antigen-specific antibodies (e.g. targeting tetraspanins), making possible the enumeration of individual particles using single-particle interferometric reflectance imaging sensor (SP-IRIS, interferometric) imaging as well as fluorescence. Currently, through interferometric imaging particles smaller than 50 nm cannot be detected, while fluorescently stained EV smaller than 50 nm can be well resolved. Further, it is conceivable that small EV contain antigen numbers in the single digits, making antigen-specific immunostaining a challenge. To further

characterize EV populations of different sizes and surface marker composition, it would be highly advantageous to target the vesicular nature of the detected particles linked to a fluorescence readout.

Methods: The goal of this project is to detect EV with a probe that is ubiquitously distributed across the surface (or lumen) of the vesicle. Small (30–150 nm) EV present fairly distinctive lipid membrane features in the extracellular environment, turning the EV membrane into a "universal" marker, and as such may serve as an alternative marker that is complementary to canonical EV surface markers.

Results: Here we present data on successfully staining EV with the membrane dye Di-8-ANEPPS (Di-8) and the luminal dye Calcein-AM. We demonstrate that EV from different sources can be efficiently stained with either dye, allowing the quantitative characterization of EV in an unbiased manner using Exoviewer's fluorescence mode. While both dyes certainly have their own unique strengths, they exhibit the wanted linear correlation of EV staining versus concentration. Further, both dyes are compatible with subsequent immunostaining applications, allowing the user to target specific surface or luminal markers (Di-8).

Summary/Conclusion: While a large-panel screening featuring other powerful dyes is continuously ongoing, the current data support the notion of providing the experimenter with a reference for total particle count and at the same time fully exploring the larger dynamic range of the fluorescence mode. Moreover, the universal probe will enable the user to correlate intensity and particle size measurements, thereby significantly improving the ExoViewer platform and its applications.

PS04.05

Membrane labelling is essential for the identification and quantification of extracellular vesicles via FACS

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Introduction: Extracellular vesicle (EV) research is challenged by the lack of standard protocols to identify and distinguish between exosomes and ectosomes being released via exocytosis or plasma membrane shedding, respectively. Analysis of small EV populations requires high-resolution technology and can be further improved using fluorescent labels such as carboxyfluorescein diacetate succinimidyl ester (CFSE). At the inner leaflet of the plasma membrane, CFSE is cleaved enzymatically resulting in covalent binding of the dye. In this study we optimized the conditions for membrane labelling of EVs and their subsequent detection by flow cytometry to obtain a maximum yield of intact EVs.

Methods: Using sequential centrifugation, we separated EV subpopulations from supernatants of COLO 357 pancreas carcinoma cells based on size and mass. After 10,000x g centrifugation, we reconstituted EVs from the pellet. We used CFSE for EV detection and analysed the expression of tetraspanins by FACS to confirm the lipid bilayer structure. Furthermore, we determined size distribution of EVs by nanoparticle tracking analysis (NTA) and electron microscopy. Detecting EVs as CFSE+ events, we quantified our samples and investigated the impact of threshold adjustment on EV quantification.

Results: After high speed centrifugation of cell free supernatants, we identified CFSE+ events as EVs, which appeared as round structures under the microscope, and ranged from 80 to 40 nm in size. Interestingly, tetraspanin markers CD9 and CD81 were detectable only on a subpopulation of purified EVs, suggesting heterogeneity of our preparations. For sufficient labelling of EVs, minimal temperature variations and short incubation times correlated with EV stability. Of note, threshold adjustment significantly improved the sensitivity of the flow cytometer for the detection of labelled EVs and hence, is central for data comparability.

Summary/Conclusion: Protocol standardization is of major importance for the use of EVs as diagnostic markers in liquid biopsies.

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PS04.06

Surface plasmon field-enhanced fluorescence spectroscopy (SPFS) system for quantitative and qualitative extracellular vesicles total evaluation without any sample pretreatment

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Introduction: The function of extracellular vesicle (EV) is interested in the immunology and oncology fields as a key transmitter for cellular communication. However, the conventional EV evaluation methods

are required complicated EVs preconcentration from the sample, its leads EV analysis uncertainty. In this study, we applied the SPFS highly sensitive automated system for quantitative and qualitative EV evaluation without any sample pre-concentration and preparation step.
Methods: SPFS automated system and plastic disposable sensor had been developed by Konica Minolta corporation in house. Anti-membrane protein (CD9, CD63, CD83) antibody was chemically bonded on hydrophilic polymer which was immobilized through the gold thin film on the SPFS sensor. The concentration of standard EV materials was evaluated by the Q-nano system before using. EV detection without preconcentrating was achieved by sandwich immunoassay step in microchannel round-trip flow reaction (TAT 120 min) with the SPFS system, and ELISA was adapted as a conventional standard method. After SPFS highly sensitive fluorescent measurements step, extracted and detected EV were effectively recovered by using the recovery buffer reaction.
Results: The EV sensitivity performance between SPFS and ELISA clearly showed a significant difference, and the LOD of SPFS (8.3 particles/μl) method was estimated 3000 times superior to the LOD of conventional ELISA (26,000 particles/μl). The SPFS calibration curve showed a wide dynamic range at least over 5 logs as an additional specificity. SPFS method also showed fine results in the dilution linearity test with high reproducibility under the serum/plasma sample condition. The data for recovery test of EV expected us that highly accurate measurement can be guaranteed under the condition of dilution about 10 times or less even in the whole blood sample. After the SPFS measurement, extracted EV on the SPFS sensor chip could be effectively recovered and could be analysed nucleic acid which contains micro RNA.
Summary/Conclusion: SPFS system might have great potential for quantitative and qualitative EV evaluation. Our strategy with SPFS system for EV proteomic and genomic profiling will be possible for applying to EV quality control as well as a novel biomarker development.

PS04.07

Identification of a novel compound that inhibits small EV secretion and tumour progression by a sensitive ELISA screening.

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Introduction: Small EVs from tumour cells are known to promote tumour progression, therefore, it is expected to develop drugs that regulate small EV secretion, which can be used in clinical applications.
Methods: To identify such regulators, we first developed a sensitive ELISA system for the quantification of small EV secretion using a high-affinity EV binding protein Tim4. By using this ELISA system, we screened for small compounds that promote or inhibit small EV secretion using a drug-repositioning compound library (about 1,600 compounds).
Results: As a result, we identified eight promoters and two inhibitors, including compound A, which significantly reduced small EV secretion from various cell types without affecting cell growth. We further investigated the effects of compound A on a mouse model of osteosarcoma and found that compound A suppressed tumour progression efficiently.
Summary/Conclusion: These data suggest that compound A would be useful not only for the characterization of small EV function but also for the clinical therapy against tumour progression, by inhibiting small EV secretion.
Funding: JST CREST

PS04.08

Quantitative proteomics identifies proteins enriched in microvesicles and exosomes respectively

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Introduction: For many years it was believed that several proteins such as CD63, CD9 and Flotillin-1 were unique for exosomes, however recent studies have shown that several of these markers also can be present in other subpopulations of EVs (Kowal et al PNAS 2016). Furthermore, few markers have been identified as uniquely present in microvesicles. The aim of this study was to in depth compare the proteome of microvesicles and exosomes.
Methods: MDA-MB-231-luc-D3H1, -D3 H2LN and -BMD2a were cultured in EV-depleted media. Microvesicles (16,500 x g, 20 min) and exosomes (118,000 x g 2.5 h) were isolated using a combination of differential ultracentrifugation and a density cushion (~1.1 g/ml). Purity and yield of EVs were determined by nanoparticle tracking analysis (NTA), Western blot, and electron microscopy (EM). Quantitative mass

spectrometry (TMT-LC-MS/MS) was used to identify differently enriched proteins in microvesicles and exosomes (n = 3 x 3 cell lines).

Results: In total 6493 proteins were quantified, with 4851 being quantified in all samples. In total 818 and 1567 proteins were significantly upregulated in exosomes and microvesicles, respectively. GO Terms associated with the proteins significantly upregulated in exosomes were "Extracellular Exosome" and "Plasma membrane", while the microvesicle proteome was associated with "Membrane" and Mitochondrion". In exosomes tetraspanins, annexins, ESCRT and rab proteins were significantly upregulated. In contrast, proteins that were upregulated in microvesicles were involved in protein translocation into the mitochondrial membrane (TIMM and TOMM proteins), in cytokinesis, and in MICOS complex. However, Flotillin-1 was not differently expressed in the EV subtypes.

Summary/Conclusion: This study identifies several proteins to be differently enriched in exosomes and microvesicles. Several of the proteins suggest recently by Kowal and colleagues, such as ADAM10 and Mitofilin could be validated. Additionally several novel proteins could be identified. Identifying markers separating microvesicles and exosomes is of high importance for the EV field and future studies will have to validate them also in other cells to determine if they are generic.

PS05: EVs in the Central and Peripheral Nervous Systems

Chair: Norman Haughey – Department of Neurology, Johns Hopkins University School of Medicine

Chair: Sowmya V. Yelamanchili, PhD – Department of Anaesthesiology

PS05.01

Vesicle secretion by ependymal cilia in brain ventricle

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Introduction: The cellular elements composing the lining of brain ventricles have drawn much attention from neuroscientists, especially the role of subependymal cells in neurogenesis, but the role of ependymal cells in brain function and disease is still neglected.

Our objective is to study the morphological aspects of rat brain ventricles and the ependymal cells as analysed by transmission and field emission scanning microscopy in normal or ischaemic rats.

Methods: For this purpose, male Wistar rats were submitted to 10 minutes of global brain ischaemia and divided into two groups: a) sham-operated animals and b) saline-treated ischaemic animals. All animals were allowed to survive for seven days. All procedures were approved by the ethics committee of the Federal University of São Paulo (2018/7633081117). Transmission and scanning electron microscopic analysis of lateral brain ventricles were done in buffered 2,5% glutaraldehyde/2%formaldehyde perfused brains. Cerebrospinal fluid was collected for NTA analysis.

Results: The morphological characterization of brain ventricle revealed a slight rarefaction of ciliary tufts of animals submitted to ischaemia when compared to normal animals. Field emission electron microscopy revealed the secretion of vesicles by the ependymal cilia in the lateral ventricle. Size and concentration of particles in the cerebrospinal fluid was confirmed by NTA and transmission electron microscopy.

Summary/Conclusion: Our results are unprecedented and bring innovative potential regarding the role of extracellular vesicles in both the physiology and pathogenesis of the nervous system. These data may also contribute to the development of new technologies for diagnosis and therapy of chronic degenerative diseases.

Funding: FAPESP and CAPES

PS05.02

Mitochondrial vesicles in neurons

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Introduction: The function of mitochondria relies on precise and effective quality controls. Neurons have high metabolic demands and employ multiple mechanisms to ensure functional mitochondria. We investigated mitochondrial vesicles – a less understood quality control mechanism for mitochondria – and assessed the effect of cellular stress.

Methods: We surveyed mitochondrial vesicles in rat and planaria brains with electron microscopy. We quantified these vesicles with serial-section electron microscopy (FIB-SEM). We also conducted confocal microscopy with Airyscan analysis of cultured neurons expressing fluorescently tagged mitochondrial markers.

Results: Electron microscopy showed the ultrastructure of various types of mitochondrial vesicles. Serial-section electron microscopy revealed the 3D ultrastructure of mitochondrial vesicles and their prevalence in neurons. Confocal microscopic analysis showed increased numbers of mitochondrial vesicles in neurons under mild stress.

Summary/Conclusion: Our findings provide direct structural evidence for mitochondrial vesicles in neurons and their abundance in response to neuronal stress. Their detection in the extracellular compartment (evidence for which is expected to be presented by the time of ISEV) may allow for development of biomarkers for mitochondrial health, with relevance to numerous pathologic conditions.

PS05.03

The role of small extracellular vesicles in chronic neuropathic pain

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Introduction: Chronic pain is the most prevalent, disabling, and expensive public health condition in the USA. Exosomes are 30–150 nm extracellular vesicles that can transport RNAs, proteins, and lipid mediators to recipient cells via circulation. Exosomes can be beneficial or harmful depending on their source and contents. We hypothesized that the composition of small extracellular vesicles (sEVs) can be altered following nerve injury and these alterations can provide insight into how the body responds to neuropathic pain.

Methods: To characterize changes following nerve injury, small extracellular vesicles (sEVs) were purified by ultracentrifugation from mouse serum four weeks after spared nerve injury (SNI) or sham surgery. miRNA profiling and proteomics analysis using tandem mass spectrometry were performed to determine differential expression of miRNAs and protein cargo respectively. For in vivo studies, sEVs were administrated intrathecally into the mouse lumbar region. Animals were evaluated for mechanical and thermal hypersensitivity over 21 days after injection.

Results: Our miRNA profiling showed a distinct miRNA signature in SNI model compared to sham control. Proteomics analysis detected 274 gene products. Of these, 24 were unique to SNI model. Neuropathic pain can induce the activation of the complement cascade and we observed significant upregulation of complement component 5a (C5a) in sEVs from SNI model. Intercellular Adhesion Molecule 1 (ICAM-1), required for the leukocyte recruitment, adhesion and homing of exosomes was also upregulated in sEVs from SNI model compared to sham control. Administration of sEVs from SNI model increased paw withdraw threshold in naïve recipient mice and inflammatory pain model, indicating a protective role for sEVs in attenuating chronic pain.

Summary/Conclusion: Our preliminary studies suggest a critical role for sEVs cargo in regulating pain. Additional studies are ongoing to determine the functional significance of alterations in sEVs composition using mouse models of pain.

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PS05.04

Large and small extracellular vesicles in ALS: friends or foes?

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Introduction: Amyotrophic Lateral Sclerosis (ALS) is a progressive adult-onset neurodegenerative disease caused by selective motor neurons (MNs) death. The rapid disease progression strongly suggests that cell-to-cell spreading of noxious factors could take place in ALS pathogenesis. Extracellular vesicles could potentially spread the disease. In this study, we characterized large (lEVs) and small extracellular vesicles (sEVs) isolated from plasma of sporadic ALS patients and healthy controls and determined their different composition in order to understand their neuroprotective or neurotoxic role in ALS pathogenesis.

Methods: lEVs and sEVs were isolated from plasma of 40 ALS patients and 30 healthy volunteers by differential centrifugation and characterized by Nanosight NS300. CD45, CD31, CD61, CD235a and Annexin V were used for flow cytometry. SOD1, TDP43, FUS protein level was investigated by Western Blot. For Raman Spectroscopy, EVs were dried on top of a CaF2 slide and Raman spectra were acquired using a 633 nm laser line. miRNA libraries were prepared by TruSeq Small RNA Library kit (Illumina).

Results: The mean size both for lEVs and for sEVs resulted increased in ALS patients compared to controls. lEVs derived from ALS patients were enriched in SOD-1, TDP-43 and FUS proteins compared to CTRLs. sEVs showed a distinct spectral pattern from lEVs. In addition, lEVs of ALS patients were richer in lipids and had less intense bands relative to aromatic aminoacids compared to healthy controls. We also found a great presence of leukocyte derived lEVs (LMVs) in ALS patients compared to AD patients and healthy donors and significant correlation with the Progression Rate of the disease. On the other hand, miRNA and RNA whole transcriptome sequencing identified a specific signature of miRNAs in plasma derived sEVs of ALS patients compared to a group of healthy controls and three neurological groups of control.

Summary/Conclusion: These data may suggest that lEVs derived from ALS patients, enriched in lipids and toxic proteins, might play a role in prion-like propagation and immunity of ALS disease, while sEVs, deriving

from endosomes, might be involved in the impairment of RNA, specific feature of ALS disease.

Funding: This work was supported by Italian Ministry of Health (Grant No. RC2017-2019); Fondazione Regionale per la Ricerca Biomedica for TRANS–ALS (Translating Molecular Mechanisms into ALS risk and patient's well-being: FRRB 2015–0023) and by Italian Ministry of Health (GR-2016-02361552).

PS05.05

Combining high-resolution flow cytometry and surface marker analysis using an automated platform to study extracellular vesicle in cerebrospinal fluid

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Introduction: There is growing enthusiasm that extracellular vesicles (EVs) carry the potential for a variety of applications in medicine. As biomarkers, EVs may aid clinicians in the evaluation of diagnoses, disease progression, or even response to therapy. However, proper characterization of the amount, size, and phenotype of EVs in a given sample remains challenging due to their sub-micrometre size and heterogeneity. Over the last years, technologies, including high-sensitivity flow cytometry and automated platforms that simultaneously assess EV amount, size, and phenotype, have matured, providing new opportunities to study EVs for future clinical applications. Using such technologies to analyse cerebrospinal fluid (CSF), which is in direct contact with the brain and spinal cord, may yield valuable insights into neurological disease processes. While there is often uncertainty about the exact source of EVs in a biological sample, CD171 has emerged as a surface marker that suggests a neuronal origin.

Methods: CSF samples that had been stored at – 80 degrees Celsius for advanced biomarker studies were analysed using two distinct approaches. A Becton, Dickinson and Company (BD) Aria III flow cytometer was converted into using violet side scatter (SSC) for improved detection of EVs with 405 instead of 488 nm SSC. For the combined analysis of amount, size, and phenotype, samples were analysed with the NanoView Bio R100 platform. Phenotype analysis included probing for the classic tetraspanins associated with exosomes (CD9, CD63, CD81) and the neural cell adhesion molecule L1 (CD171).

Results: Flow of CSF samples showed similar vesicle counts in control vs. disease and an increase of counts in later disease stages when neurodegeneration is thought to be more prominent. All CSF samples showed some binding to classic exosomal markers (CD9, CD63, CD81). The sample taken at the latest time point showed relatively high vesicle counts, overall larger vesicle size, and abundant CD171 binding. Interestingly, the CD171 positive EVs were not positive for any of the classic exosomal markers (CD9, CD63, and CD81).

Summary/Conclusion: This data supports the notion that analysing the amount, size, and surface markers of EVs in CSF can reveal intriguing dynamics in such basic EV characteristics over time and suggests important differences between EV populations in different disease stages. While previous studies indicated that CD171 could identify an EV to be of neuronal origin, it remains to be determined whether such specific surface markers will emerge as clinically relevant tools to support the evaluation of people affected by neurological diseases.

PS05.06

A distinct microRNA signature in plasma derived small extracellular vesicles of different neurodegenerative diseases

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Introduction: Exploring Identifying robust biomarkers is essential for early diagnosis of neurodegenerative diseases. Blood stream transports large (lEVs) and small extracellular vesicles (sEVs), which are extracellular vesicles of different sizes and biological functions that are transported in blood. Aim of our study was to investigate mRNA/miRNA signatures in plasma derived lEVs and sEVs of Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD), Parkinson's disease (PDPD), Fronto-temporal Dementia (FTD) and Alzheimer's Disease (AD) patients.

Methods: lEVs and sEVs were isolated from plasma of patients and healthy volunteers (CTR) by ultracentrifugation and RNA was extracted. Whole transcriptome and miRNA libraries were prepared with TruSeq Stranded Total RNA kit and TruSeq Small RNA Library kit (Illumina).

Results: Our data suggested that the RNA cargo in lEVs and sEVs varies among different diseases. miRNA analysis in sEVs provided the most informative disease specific signatures, while whole transcriptome analysis did not show any specific signature. ALS was

characterized by a small but specific group of circulating miRNAs. miRNAs profiling revealed that PD and FTD can be subgrouped in two classes while AD appears to be a homogeneous disease population. Furthermore, miRNAs profiling show the presence of overlaps in the signatures between the analysed diseases. miRNA profiling in lEVs is similar to that observed in sEVs, although in lEVs the overall differences between diseases are less marked.

Summary/Conclusion: In this study we have demonstrated that miRNAs are the most interesting subpopulation of transcripts transported by plasma derived sEVs since they discriminate a disease from the other and they can provide a signature for each neurodegenerative diseases.

Funding: This work was supported by Italian Ministry of Health (Grant No. RC2017-2019); Fondazione Regionale per la Ricerca Biomedica for TRANS–ALS (Translating Molecular Mechanisms into ALS risk and patient's well-being: FRRB 2015–0023) and by Fondazione Cariplo 2017 (Extracellular vesicles in the pathogenesis of Frontotemporal Dementia 2017–0747).

PS05.07

Role of exosomes in the development of dendritic filopodia, spines and synapses

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Introduction: Dendritic spines are actin-rich structures at the postsynaptic sites of most excitatory synapses in the central nervous system. They are highly important structures for higher brain functions such as learning and memory. Several live imaging studies have shown that long, thin, actin-rich protrusions called dendritic filopodia are precursors of dendritic spines in hippocampal and cortical neurons. So far, many intracellular factors that regulate filopodia formation have been identified. However, extracellular mechanisms of filopodia formation are largely unknown. Also, detailed molecular mechanisms by which astrocyte secreted factors regulate synaptogenesis are not well understood. Small extracellular vesicles (SEVs)/exosomes have potential to regulate filopodia, spine and synapse formation in autocrine or paracrine manner due to their unique cargo composition. Here, we examine role of exosomes in filopodia, spine and synapse formation.

Methods: Primary rat hippocampal and cortical neurons were transiently transfected with the multivesicular body (MVB) docking regulator GFP-Rab27b or with shRNAs against the exosome secretion and biogenesis regulators Rab27b and Hrs. Transfected neurons were immunostained for synaptic proteins and analysed for filopodia at day in vitro (DIV) 6 or spines at DIV12. For rescue experiments, exosomes were isolated using differential ultracentrifugation method from conditioned media of DIV9 cortical neurons or primary astrocytes and characterized for their size, common protein markers and morphology.

Results: Here, we find that MVB docking factor GFP-Rab27b localizes to both the tips and bases of actin-rich filopodia and spines in primary neurons. Furthermore, genetic regulation of exosome secretion by overexpression or knockdown of Rab27b or Hrs leads to respective increases or decreases in the number of filopodia, spines and synapses. The defects of exosome-inhibited neurons in filopodia density are rescued by add-back of neuronal exosomes. Additionally, treatment of primary neurons with exosomes isolated from primary astrocyte cultures leads to enhanced spine and synapse formation.

Summary/Conclusion: These results indicate that autocrine and paracrine communication via exosomes are a key part of the process of neuronal filopodia, spine and synapse formation.

PS05.08

Effects of apolipoprotein E genotype on protein and small RNA profiles of brain tissue-derived extracellular vesicles of Alzheimer's disease patients

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Introduction: In Alzheimer's disease (AD), three Apolipoprotein E (APOE) polymorphic alleles contribute to risk: ε4 represents higher risk, ε2 is protective, and ε3 is neutral. To determine if extracellular vesicles in brain

may be linked with APOE genotype, we investigated the possible effect of APOE genotype on brain-derived EVs (bdEVs) and their protein and RNA molecular cargo.

Methods: Cortical brain tissues of AD patients with different APOE genotypes [ε2/ε3 (n = 5), ε3/ε3 (5), ε3/ε4 (6), ε4/ε4 (6)] and non-AD controls (n = 7) were obtained. bdEVs were separated by size exclusion chromatography plus ultracentrifugation (UC) and characterized per MISEV2018. Proteins were analysed by mass spectrometry. After protein identification, data were normalized using the Cyclicloess method and analysed by principal component analysis (PCA). Nested Factorial design highlighted differentially expressed proteins. RNA from bdEVs was extracted by miRNeasy Mini kit. Small RNA libraries were constructed using the Ion Total RNA-Seq kit and sequenced on the Ion Torrent S5™ using Ion™ 540 chips. Reads were aligned to human reference transcriptomes using bowtie. Differential gene expression was quantified by edgeR and limma.

Results: Among 28 proteins dysregulated in AD bdsEVs, several have reported roles in AD, e.g., microtubule-associated protein tau and peroxiredoxin-6. Regarding APOE genotypes, 16 proteins were differentially expressed between ε4 carriers (ε3/ε4 and ε4/ε4) with non ε4 carriers (ε2/ε3 and ε3/ε3). However, EV markers did not differ by APOE genotype. In contrast to protein cargo of bdEVs, the overall small RNA expression pattern was similar among AD patients with different APOE alleles and non-AD patients. Only a few miRNAs showed different abundance level between ε2/ε3 and ε4/ε4 groups, or between AD and non-AD groups.

Summary/Conclusion: bdEVs carry proteins and miRNAs related to AD development and APOE genotypes. Further verification of protein and RNA expression in brain and plasma derived EVs may reveal mechanisms of EV function in neuroinflammation and develop biomarkers for AD disease.

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PS05.09

Efficient pathology spread by extracellular vesicles from human brain tissues in mouse brain and tissue cultured neurons: Transmission and propagation to GABAergic neurons

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Introduction: Tau-containing extracellular vesicles (EVs) are transmissible and induce tau phosphorylation and conformational change in the recipient neurons. However, whether human brain-derived EVs induce tau pathology has not yet been characterized in the mouse brain. Here, we assess the mechanisms of disease spread after intrahippocampal injection of human brain-derived EVs into the aged mouse model.

Methods: EV-enriched fractions were isolated from unfixed frozen human brain samples from AD, prodromal AD (pAD), control (CTRL) cases, and tau knockout (TKO) mouse brains. Isolated EVs containing 300 pg of human total tau were sterotaxically injected into the right outer molecular layer of the dentate gyrus of 18 months-old C57BL/6 female mice. 4.5 months after the injection, hippocampal slices were prepared for whole-cell patch clamp recordings of CA1 pyramidal neurons were undertakent. Hippocampi were analysed with immunohistochemistry using phosphorylated-tau (p-tau) epitopes including AT8. EVs were examined for protein composition by protein mass-spectroscopy, the neuronal uptake in vitro, and structural analysis by the atomic force microscopy (AFM).

Results: Semiquantitative brain-wide immunohistochemistry of p-tau revealed that inoculation of AD or pAD-EVs induced tau propagation throughout the hippocampus, including the dentate gyrus, CA3 and CA1 subregions. AT8 was localized primarily in GAD67 + GABAergic neurons in pAD and AD EVs groups, accompanied with reduced amplitude of inhibitory postsynaptic currents and Excitatory-Inhibitory ratio in amplitube of postsynaptic currents in CA1 pyramidal neurons in pAD EVs. AFM analysis showed higher density of tau oligomers in both AD and pAD EVs while only AD EVs showed significantly higher neuronal uptake compared to CTRL EVs. Finally, proteomic analysis showed that AD EVs are enriched in disease and glia-related molecules compared to CTRL EVs, which may contribute to their enhanced neuronal uptake.

Summary/Conclusion: Intracranial injection of AD or pAD EVs induced p-tau accumulation primarily in GABAergic neurons throughout the hippocampus, resulted in higher uptake by neurons, and tau oligomer conformation, indicating of their pathogenic potency as seeding factors. GABAergic neuronal dysfunction in the hippocampal neuronal circuitry reported in early AD brains could be attributed to specific EV mediated tau propagation in this cell type, a phenomenon meriting further investigation and validation.

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PS05.10

Isolation of extracellular vesicles from cerebrospinal fluid and characterization of their bioactive compounds to investigate multiple sclerosis pathogenesis

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Introduction: Multiple sclerosis (MS) is the most frequent chronic inflammatory disease of the young adult central nervous system. Nevertheless, the pathogenesis remains largely unknown. It is therefore relevant to better characterise in cerebrospinal fluid (CSF), which irrigates the brain, novel bioactive compounds whose dysregulation could be involved in MS pathology. The concentration of extracellular vesicles (EVs) has been already found affected in MS patient fluids but the content in bioactive molecules, particularly the microRNAs (miRNAs), remains barely investigated. The miRNA are short oligonucleotides that are major posttranscriptional regulators and we previously showed the dysregulation of specific miRNAs in CSF of MS patients. EVs can potentiate miRNA effects by allowing remote action through the shuttling within biological fluids such as CSF while providing a protection from circulating RNAse. Nevertheless, CSF remains a challenging fluid to analyse due to limited access, low volume and presence of lipoproteins (other putative miRNA carrier) that can be co-isolated with EVs.

Methods: We performed a comparative analysis of EV isolation from CSF by size-exclusion chromatography (SEC), density-gradient ultracentrifugation, ultrafiltration or chemical precipitation (ChemP) to determine the optimal technique(s) to enrich EV.

Results: SEC applied on CSF of control patients showed optimal EV purification with sufficient EVs from 0.5 ml of CSF for downstream EV characterization. Furthermore, we were able to isolate miRNAs from CSF and determined their enrichment in EVs by RNAse-sensitivity treatments. Finally, we have combined ChemP and SEC to enable a fast and large-scale isolation of EVs from > 5 ml of CSF, which successfully provided an increase in particles detected by nanoparticle tracking analysis. We are currently characterising the particles to confirm that they are purified EVs, cleared from contaminants.

Summary/Conclusion: This work opens perspective to analyse EVs from MS patients and to determine whether miRNAs participates in MS pathogenesis through their transit in EVs.

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PS05.11

Differences in circulating number of extracellular vesicles between contact sport athletes with and without acute mTBI: a pilot study

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Introduction: Extracellular vesicles (EVs) are released by cells of the central nervous system as a result of injury, including mild traumatic brain injury (mTBI). Since mTBI may alter circulating levels of EVs, this study aimed to investigate differences in circulating EV numbers between contact sport athletes with and without acute mTBI.

Methods: Circulating EVs containing CD63 (CD63 + EV), CD81 (CD81+ EV), and neural cell adhesion molecule (L1CAM+EV) were analysed in young, male athletes with or without mTBI (18–29 yo, n = 6 per group). Sodium citrate-treated blood samples were obtained from athletes with mTBI within 48-hours of injury and from control athletes free of mTBI for one year. Athletes were best matched for age and history of prior mTBI. Samples were double-centrifuged to obtain platelet-poor plasma and stored at −80°C until analysed. Quantification of EVs was performed using a spectral flow cytometer. The study was approved by Temple University's IRB, and all athletes provided written informed consent.

Results: Mann-Whitney U tests showed that population percentages of small size (179–304 nm) CD63 + EV, CD81+ EV and L1CAM+EVs were significantly higher in mTBI athletes (mean rank: 8.0, 9.5, 9.3) than controls (mean rank: 3.6, 3.5, 3.7) ($U = 3.0$, $p = 0.03$; $U = 0.0$, $p > 0.01$; $U = 1.0$, $p > 0.01$, respectively). Population percentages of large size (500–800 nm) CD63+ EV, CD81+ EV and L1CAM+EVs were also significantly higher in mTBI athletes (mean rank: 8.2, 9.2, 9.5) than controls (mean rank: 3.4, 3.8, 3.5) ($U = 2.0$, $p = 0.02$; $U = 2.0$, $p > 0.01$; $U = 0.0$, $p > 0.01$, respectively). There were no significant differences between percentages of EVs associated with blood brain barrier function (CD144+ EV) or platelets (CD42a+EV) among mTBI athletes or controls.

Summary/Conclusion: Athletes with acute mTBI demonstrate different EV profiles than contact sport athlete controls. Further investigation of EV biomarkers is necessary to determine their potential for future, diagnostic usage.

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PS05.12

Plasma-derived Extracellular Vesicles profiling as a biomarker for Parkinson's Disease

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Introduction: Parkinson's disease (PD) is characterized by clinical heterogeneity, different rates of progression and absence of definitive biomarkers. Extracellular vesicles (EVs) are easily isolated from plasma and play a central role in intercellular communication which is highly relevant for inflammatory processes implicated in protein misfolding-related neurodegenerative disorders. Thus, we characterized distinctive plasmatic EV subpopulations of PD and atypical parkinsonisms (AP) patients, with the aim to identify candidate biomarkers among EVs surface membrane-proteins.

Methods: Plasmatic EVs were collected from 27 PD, 19 matched healthy controls (HC), 9 AP with multiple system atrophy (MSA) and 9 AP with tauopathies (AP-Tau). EVs were quantified by Nanoparticle Tracking Analysis. The expression of 37 EV-surface markers, related to inflammatory and immune cells, were measured by MACSPlex and correlated to clinical scales. A diagnostic model based on EV markers expression was built via supervised machine learning algorithms and validated in an external cohort (10 PD, 20 HC, 5 MSA, 5 AP-Tau). The Cantonal Ethics committee approved the study protocol. All enrolled subjects gave written informed consent.

Results: PD showed the highest EV concentration compared to others groups. PD and MSA displayed a greater pool of overexpressed immune markers compared to AP-Tau. EV antigens correlate to cognitive impairment and disease gravity in PD and MSA. The ROC curve analysis of a compound EV marker showed optimal diagnostic performance for PD (AUC 0.908; sensitivity 96.3%, specificity 78.9%) and MSA (AUC 0.974; sensitivity 100%, specificity 94.7%) and good accuracy for AP-Tau (AUC 0.718; sensitivity 77.8%, specificity 89.5%). A diagnostic model based on EV markers expression, correctly classified 88.9% of patients with reliable diagnostic performance after validation in an external cohort (77% of accuracy).

Summary/Conclusion: This analysis of multiple immune surface markers of circulating EVs in PD and AP well captured the clinical heterogeneity of PD and showed optimal diagnostic performance. Furtherly it suggests a different immune dysregulation in PD and MSA vs. AP-Tau, to be confirmed by functional analysis in experimental models of disease.

Funding: Supported by ABREOC.

PS05.13

Separation and characterization of extracellular vesicles from human cerebrospinal fluid

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Introduction: Extracellular vesicles (EV) are released from cells to the surroundings and are found in human biofluids, where they constitute promising targets for novel biomarker identification. EV have been found in cerebrospinal fluid (CSF) where they may provide with markers for neurological diseases.

Here, we aimed at purifying and characterizing EV from human CSF.

Methods: CSF was collected by lumbar puncture from patients with amyotrophic lateral sclerosis. Patients gave written consent and studies were agreed by the local ethics committee. CSF was fractionated by ultrafiltration (Vivaspin, cut-off 3,000), and size-exclusion chromatography (SEC; qEVsingle Izon Science). Eluted fractions were analysed by dynamic light scattering (DLS) and electron microscopy. Proteins were analysed by immunoblotting and nano-liquid chromatography-tandem mass spectrometry.

Results: EV eluted in early fractions (3 + 4) after the SEC void volume as evaluated by detection of CD63

and CD9 markers (immunoblotting) and annexin A2 (peptide mapping by nanoLC-MS/MS). There, nanoparticles around 150 nm were identified by DLS. In agreement, electron microscopy showed EV with characteristic shape and sizes typically between 55 and 165 nm, with average diameter 94 ± 31 nm. CD63 was visualized by immunocytochemistry at the surface of EV around 80 nm. On the other hand soluble proteins IgG and albumin eluted in later fractions. Curiously, galectin-3 binding protein (LGALS3BP or 90 K) was also partially detected in early-eluting fractions as nanoparticles of irregular shapes and heterogeneous sizes typically between 15 and 60 nm; some of those nanoparticles had ring-like appearance. Occasionally 90 K also appeared on EV of variable dimensions.

Summary/Conclusion: In conclusion, EV from the CSF may be separated from soluble proteins and small molecules by a combination of ultrafiltration with SEC fractionation. However, using this strategy a population of 90 K-containing nanoparticles co-eluted with EV from the CSF. Further separation techniques need to be applied to separate EV from 90 K nanoparticles to investigate their individual physiological relevance and biomarker potential.

Funding: Euronanomed 2 ERA-NET project GlioEx (ENMed/0001/2013), FCT, Portugal; iNOVA4Health Research Unit (LISBOA-01-0145-FEDER-007344.

PS05.14

Separation and characterization of extracellular vesicles from human cerebrospinal fluid

Julia Costa[a], Ana Pronto-Laborinho[b], Susana Pinto[b], Marta Gromicho[b], Sara Bonucci[c], Erin Tranfield[c], Catarina Correia[d], Bruno Alexandre[d] and Mamede de Carvalho[e]

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Results: EV eluted in early fractions (3 + 4) after the SEC void volume as evaluated by detection of CD63 and CD9 markers (immunoblotting) and annexin A2 (peptide mapping by nanoLC-MS/MS). There, nanoparticles around 150 nm were identified by DLS. In agreement, electron microscopy showed EV with characteristic shape and sizes typically between 55 and 165 nm, with average diameter 94 ± 31 nm. CD63 was visualized by immunocytochemistry at the surface of EV around 80 nm. On the other hand soluble proteins IgG and albumin eluted in later fractions. Curiously, galectin-3 binding protein (LGALS3BP or 90 K) was also partially detected in early-eluting fractions as nanoparticles of irregular shapes and heterogeneous sizes typically between 15 and 60 nm; some of those nanoparticles had ring-like appearance. Occasionally 90 K also appeared on EV of variable dimensions.

Summary/Conclusion: In conclusion, EV from the CSF may be separated from soluble proteins and small molecules by a combination of ultrafiltration with SEC fractionation. However, using this strategy a population of 90 K-containing nanoparticles co-eluted with EV from the CSF. Further separation techniques need to be applied to separate EV from 90 K nanoparticles to investigate their individual physiological relevance and biomarker potential.

Funding: Euronanomed 2 ERA-NET project GlioEx (ENMed/0001/2013), FCT, Portugal; iNOVA4Health Research Unit (LISBOA-01-0145-FEDER-007344.

PS06: EVs in Cardiovascular Diseases and Vascular Disorders

Chair: Ahmed Ibrahim, Ph.D., MPH – Smidt Heart Institute, Cedars-Sinai Medical Centre

PS06.01

Release of extracellular vesicles from platelets requires platelet-platelet interaction

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Introduction: Arterial thrombosis is a major and global cause of human death and disability, but a biomarker for early-diagnosis of thrombosis is absent. Platelet activation and aggregation are the first steps of platelet-rich thrombus formation, but their relative contribution to platelet extracellular vesicles (PEVs) release is unknown.

Methods: To study the relation between PEV release and platelet interaction (aggregation), citrate-anticoagulated whole blood (WB) from healthy donors was diluted 2, 4, 8, 16 and 32-fold and activated by 30 μM thrombin-receptor activating peptide (TRAP). In addition, undiluted WB and 10-fold diluted WB, which totally blocked PEV release, were activated with various TRAP concentrations. Concentrations of PEVs (CD61 + and CD61+, CD62p + >1000 nm) and activated platelets (CD61+, CD62p+ >1000 nm) were measured by flow cytometry (Apogee A60-Micro). Platelet aggregation was assessed using impedance aggregometry.

Results: A 10-fold dilution of WB blocked both aggregation and the release of PEVs. Compared to baseline, activation of undiluted WB with TRAP increased the concentrations of CD61 + 2.2-fold and CD61+-CD62p + PEVs 7.2-fold. The concentration of CD61+ (R2 = 0.71) and CD61+-CD62p+ (R2 = 0.78) PEVs as well as platelet aggregation (R2 = 0.8) scaled inversely (reciprocal) with the dilution of WB. Further, we found a linear correlation between the % of activated platelets and the concentration of CD61+ (R2 = 0.80) and CD61 +, CD62p+ (R2 = 0.64) PEVs in undiluted WB, which was absent in 10-fold diluted blood (R2 < 0.05).

Summary/Conclusion: The absence of aggregation and PEV release upon platelet activation in 10-fold diluted blood shows that aggregation directly depends on the distance between platelets, which is confirmed by the reciprocal relationship between PEV release and blood dilution. Because PEVs are only released when platelet activation is followed by aggregation, PEVs are a potential early biomarker of thrombosis.

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PS06.02

Age-dependent alteration in concentration and size distribution of extracellular vesicles in plasma of normotensive and hypertensive rats

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Introduction: Spontaneously hypertensive rats (SHR) are the most widely used animal model of human essential hypertension. We previously reported that plasma small extracellular vesicles (sEVs) in SHR regulate systolic blood pressure, however, the mechanism has not been clarified. In the present study, we compared the concentration and size distribution of plasma EVs (sEVs and large EVs) from young and aged normotensive Wistar Kyoto rats (WKY) and SHR.

Methods: Heparin-anticoagulated plasma was collected from male WKY and SHR at 5 ~ 7- (young) and 15- (aged) week-old. Large EVs were isolated from the plasma by centrifugation (10000 x g). sEVs

were isolated by ultracentrifugation (164,071 x g) following precipitation with polyethylene-glycol. The concentration and size distribution of sEVs and large EVs were measured by a tunable resistive pulse sensing analysis.

Results: There was no significant difference in the total concentration of plasma sEVs between WKY and SHR or between young and aged rats. The mean diameter of plasma sEVs from aged rats was larger than that from young rats in both WKY and SHR. Also, the number of particles with a diameter of smaller than 150 nm in plasma sEVs from aged rats was lower than that from young rats. The concentration of plasma large EVs from aged rats was higher than that from young rats in both WKY and SHR. There was no significant difference in the size distribution of plasma large EVs between WKY and SHR or between young and aged rats.

Summary/Conclusion: The present results for the first time demonstrate that the concentration of plasma large-sized EVs is increased by ageing, while there is no difference in the concertation and size distribution of EVs between WKY and SHR. Further research is required to clarify the cause of age-dependent alternation in plasma EV size distribution and its physiological meaning.

PS06.03

microRNA profiling of circulating extracellular vesicles is involved with susceptibility to age-related diseases: relevance to cardiovascular signalling in ageing process

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Introduction: Ageing represents a central risk factor for several diseases, such as cardiovascular diseases. Our hypothesis is that extracellular vesicles (EVs) can be potential mechanism of spreading molecules, such as microRNAs, involved with susceptibility to chronic age-related diseases and geriatric syndromes. In this context, the role of microRNAs in age-induced detrimental changes in the cardiovascular system has been suggested. Although EVs can protect microRNAs from endogenous RNAses and internalization of these vesicles into cells is involved with cell communication, delivering microRNAs even to distant tissues, the relationships between EVs microRNAs profile and chronic age-related diseases has not been evaluated. Our aim was to investigate the microRNA profile of circulating EVs during ageing process and their downstream signalling pathways.

Methods: The Ethics Committee (CEUA – Comissão de Ética no Uso de Animais – UFRGS; nr. 29,818) approved all animal procedures and experimental conditions. Male Wistar rats of 3- and 21-month-old were used, and plasma was obtained from the trunk blood. EVs were isolated with ExoQuick following the manufacturer's instructions. microRNA was isolated from EVs and then amplified. microRNA was labelled using the FlashTag Biotin HSR RNA Labelling Kit and profiled on Affymetrix GeneChip microRNA 4.0 Arrays. Ingenuity Pathway Analysis (IPA) was used to identify pathways regulated by significantly altered microRNAs.

Results: Microarray analysis revealed 728 microRNAs. Of these microRNAs, 48 were differentially expressed between aged and young-adult animals, 18 microRNAs were significantly upregulated and 30 were downregulated in aged animals compared to young adult ($p < 0.05$; fold change of $|1.1|$). A conservative filter was applied on IPA and only experimentally validated and highly conserved predicted mRNA targets for each microRNA was used. IPA analysis showed that cardiac hypertrophic signalling is ranked as highly predicted targets for these differentially expressed microRNAs ($p < 0.0001$). Moreover, IPA demonstrated that this canonical pathway is upregulated in aged animals when compared to young adult. In addition to cardiac hypertrophic signalling, other relevant cardiovascular canonical pathways, such as endothelin-1 signalling and intrinsic prothrombin activation pathway have predicted targets.

Summary/Conclusion: Our results showed for the first time that microRNAs profile in circulating EVs has a potential role to drive heart senescence and consequent cardiac diseases which represents the leading cause of death.

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PS06.04

Endothelial cell-derived extracellular vesicles induce a smooth muscle cell pro-inflammatory phenotype via HMGB1

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Introduction: Introduction: The vascular endothelium and smooth muscle form adjacent cellular layers that comprise part of the vascular wall. Here, we examined the extent to which extracellular vesicles (EVs) vesicles participate in endothelial-vascular smooth muscle cell communication.
Methods: Methods: EVs were collected from rat aortic endothelial and smooth muscle cell serum-free media by ultracentrifugation. Vesicle morphology, size and concentration were evaluated by transmission electron microscopy and nanoparticle tracking analysis. Endothelial cell and vascular smooth muscle cell cultures were subjected to various concentrations of EVs for various times. Functional assays were performed.
Results: Results: Western blot as well as shot gun proteomic analyses revealed sets of proteins common to both endothelial- and smooth muscle-derived EV as well as proteins unique to each vascular cell type. Functionally, endothelial-derived EVs stimulated vascular cell adhesion molecule-1 (VCAM-1) expression and enhanced leukocyte adhesion in vascular smooth muscle cells while smooth muscle EVs did not elicit similar effects in endothelial cells. EVs from endothelial cells also induced protein synthesis and senescence-associated β galactosidase activity in vascular smooth muscle cells. Proteomic analysis of vascular smooth muscle cells following exposure to endothelial cell-derived EVs revealed upregulation of several proteins including pro-inflammatory molecules, high-mobility group box (HMGB) 1 and HMGB2. Pharmacological blockade of HMGB1 and HMGB2 and siRNA depletion of HMGB1 in smooth muscle cells attenuated NF-kb (p65) phosphorylation and nuclear translocation, VCAM-1 expression and leukocyte adhesion induced by endothelial cell EVs.
Summary/Conclusion: Conclusions: These data suggest that endothelial cell-derived EVs can enhance signalling pathways that induce a pro inflammatory in vascular smooth muscle cells.
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PS06.05

Double value of Microvesicles in CABG: patency predictive capacity and tool for personalized antiplatelet therapy

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Introduction: Graft patency is one of the major determinants of long-term outcome following coronary artery bypass graft surgery (CABG). Biomarkers, if indicative of the underlying pathophysiological mechanisms, would suggest strategies to limit graft failure. Many studies have generated compelling data on the sensitivity of MVs as biomarkers of cardiovascular disease progression and events. The MV usefulness in CABG has been tested only in a study that highlighted their importance in surgical haemostasis. No information is so far available on the association between the amount or pattern of circulating MVs and CABG outcome. We aimed to evaluate whether MV pre-operative signature could predict mid-term graft failure.
Methods: This was a nested case-control substudy of the CoronAry bypass grafting: factors related to late events and Graft patency (CAGE) study that enrolled 330 patients undergoing elective CABG. Of these, 179 underwent coronary computed tomography angiography 18 months post-surgery showing 24% graft occlusion. Flow cytometry MV analysis was performed in 60 patients (30/group with occluded [cases] and patent [controls] grafts) on plasma samples collected the day before surgery and at follow-up.
Results: Before surgery, cases had two-fold ($p = 0.020$) and four-fold ($p = 0.042$) more activated platelet-derived and TF+ MVs, respectively than controls. The MV thrombin generation capacity was also significantly greater ($p < 0.05$). This MV signature predicted graft occlusion (AUC of 0.897 [95%CI: 0,77–0,96], $p = 0.02$). By using a MV-score (0–6), the OR for re-occlusion for a score above 3 was 16.3 (95% CI 4.1–65.3, $p < 0.001$).
Summary/Conclusion: The pre-operative signature of MVs is an independent predictor of mid-term graft occlusion in CABG patients and a cumulative MV-score stratifies patient's risk. Since the MV signature mirrors platelet activation, patients with a high MV-score would benefit from a personalized antiplatelet therapy.

PS06.06

Exosomes from engineered immortalized human heart cells improve ventricular function and attenuate fibrosis in mice with arrhythmogenic cardiomyopathy

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Introduction: Arrhythmogenic cardiomyopathy (AC) is characterized by progressive loss of cardiomyocytes and fibrofatty tissue replacement. Currently, there is no effective treatment for this disease. Exosomes (imEXOs) secreted by heart stromal cells, engineered to be immortal and overexpressing β-catenin, exert anti-inflammatory and anti-fibrotic effects and improve ventricular function in models of ischaemic injury (Ibrahim et al., Nature BME 2019).
Methods: To investigate the effectiveness of imEXOs in a murine model of AC, four-week old homozygous DSG2 knockout (DSGKO) mice and wild type (WT, age- and strain-matched) mice were compared. DSGKO mice were randomized to receive weekly imEXOs or vehicle via intravenous injection for 4 weeks. Neonatal rat ventricular myocyte (NRVM) proliferation and apoptotic assays were performed to explore potential effects of exosomes.
Results: Biodistribution studies of DiR-labelled imEXOs revealed some cardiac uptake, along with strong signals in spleen. At 4 weeks, DSGKO mice which had received intravenous imEXOs showed improved cardiac function (echocardiographic ejection fraction 73 ± 2 VS 59 ± 5% in vehicle mice, P = 0.03), with an underlying attenuation in myocardial fibrosis by histology. Electrophysiology test showed shorter QRS duration (4.0 ± 2.0 ms imEXO VS 6.5 ± 2.9 ms vehicle, P = 0.02) and effective refractory period. Programmed ventricular stimulation showed DSGKO mice which had received imEXOs were remarkably less prone to ventricular tachycardia induction (10.0 ± 32% VS 55.0 ± 52% in vehicle, P = 0.031). In vitro study showed NRVM exposed to imEXOs for 2 days exhibited higher BrDU expression relative to vehicle group, and less Annexin-V expression after oxidative stress induced by 10-minute illumination with 254 nm UV.
Summary/Conclusion: Intravenous administration of imEXOs improved cardiac function, reduced cardiac fibrosis, and suppressed arrhythmogenesis in AC. Our findings motivate clinical testing of imEXOs in AC, an orphan disease with great unmet medical need.
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PS06.07

Cardiac-derived extracellular vesicles contribute to communication between heart and brain in chronic heart failure (CHF) and target Nrf2/ARE signalling

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Introduction: miRNAs regulate the translation of proteins that are involved in redox homoeostasis in the heart and brain. Intra- and/or inter-organ communication takes place by multiple mechanisms including extracellular vesicular (EV) transport. Our previous studies suggested that cardiac derived miRNA-enriched EVs contribute to the dysregulation of Nrf2/antioxidant enzyme (ARE) signalling in the myocardium via intercellular cross-talk, and result in the decreased Nrf2/ARE signalling in the sympatho-regulatory areas of the brain in CHF. However, it is unclear if cardiac derived EVs circulate to the central nervous system evoking sympatho-excitation by disrupting central redox homoeostasis.
Methods: Cardiac-specific membrane GFP+ mice were generated to track the brain distribution of cardiac EVs in rats with CHF (coronary ligation). The isolation and characterization of EVs were carried out by differential ultracentrifugation, TEM, NanoSight, western blotting, and qRT-PCR. Transfection, labelling, and microinjection of EVs into the rostral ventrolateral medulla (RVLM) were performed.
Results: Nrf2 protein was reduced in the RVLM of CHF rats consistent with an upregulation of Nrf2-targeting miRNAs. Nrf2-targeting miRNAs were enriched in cardiac and circulating EVs of CHF rats. Nrf2-targeting and cardiac-specific miRNAs were abundant in brain-derived EVs. Circulating EVs were taken up by neurons in sympatho-regulatory areas of the brain. miRNA-enriched EVs from CHF animals increased sympathetic tone which was prevented by a cocktail of Nrf2-targeting miRNA inhibitors.
Summary/Conclusion: Myocardial infarction-induced miRNA-enriched EVs mediate the inter-organ cross-talk between heart and brain in the oxidative regulation of sympathetic outflow through targeting the Nrf2/ARE signalling pathway. These findings suggest that cardiac-derived EV miRNAs targeting Nrf2/ARE signalling may act as an endocrine signalling mediator of CHF that has potential as a novel therapeutic target.
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PS06.08

Ischaemia impairs the ubiquitin-mediated secretion of Cx43 into extracellular vesicles

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Introduction: A fine-tuned communication between cardiac cells is vital to maintain myocardial integrity and contractility. Not only an impairment of gap junction (GJ)-mediated intercellular communication, but also defects in EV-mediated communication have been associated with ischaemic heart disease, a major causative factor of heart failure. We have previously shown that Cx43, the main ventricular GJ protein, assembles into channels at the EVs surface, mediating the release of vesicle content into target cells.The main objective of this work was to characterize the signals underlying protein sorting into extracellular vesicles (EVs) in a human pathophysiological context, using connexin43 (Cx43) as a model substrate.
Methods: Animal models of ischaemia/reperfusion (I/R) injury by ligation of the left anterior descending coronary artery, ex vivo and in vitro ischaemia models and human patients were used to investigate the secretion of EV-Cx43.
Results: Release of Cx43 was downregulated in circulating vesicles from I/R-injured mice and patients with ST-segment elevation myocardial infarction, as well as in intracardiac and cardiomyocyte-derived EVs. Additionally, we show that ubiquitin signalled the release of Cx43 in basal conditions but appeared to be dispensable during ischaemia. Depletion of the autophagy adaptor p62 partially restored the secretion of Cx43, suggesting an interplay between ischaemia-induced Cx43 degradation and secretion.
Summary/Conclusion: Overall, we demonstrated that ischaemia impairs the sorting of Cx43 into EVs, which may ultimately affect long-distance communication. Through the identification of the underlying molecular mechanisms and players, these results pave the way towards the development of innovative diagnostic and therapeutic strategies for cardiovascular disorders.
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PS06.09

Cardioprotection mediated by calcium-ionophore induced extracellular vesicles

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Introduction: Remote ischaemic conditioning is a cardioprotective intervention which protects the heart against ischaemia/reperfusion injury. Transient activation of Toll-like receptor 4 (TLR4) and its downstream regulators (TNFα and IL-6) have been implicated in cardioprotective interventions. Extracellular vesicles (EVs) play a role in cardioprotection through the activation of the TLRs. However, since isolation of EVs in high amounts with suitable purity from blood is a challenge, our aim was to develop a cellular model system from which TLR-inducing, cardioprotective EVs can be isolated in a reproducible manner.
Methods: EV release from HEK293 cells was induced by calcium-ionophore A23187. EVs were characterized, cytoprotection by EVs against simulated ischaemia/reperfusion injury and its mechanism were investigated in H9c2 and AC16 cell lines.
Results: A23187 induction of HEK293 cell induced EV release and the isolates contained mostly large EVs. EVs decreased cytotoxicity and apoptosis due to 16 h ischaemia followed by 2 h reperfusion in H9c2 and AC16 cells in a dose-dependent manner. EVs activated TLR4 and its downstream signalling pathway in H9c2 and AC16 cells as well as the expression of cytoprotective haem oxigenase 1 (HO-1) in H9c2 cells.
Summary/Conclusion: A23187-induced EVs exert cytoprotection in H9c2 and AC16 cells by inducing TLR4 signalling and HO1 expression. Therefore, EVs released via calcium-ionophore treatment may serve as a basis of an efficient carpdioprotective therapy.

PS07: EV Nucleic Acid Biomarkers

Chair: Kendall Van Keuren-Jensen – Translational Genomics Research Institute

PS07.01

Extracellular-Vesicle microRNAs for detecting Pancreaticobiliary Cancers

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Introduction: Biliary strictures may be benign or malignant. The major malignant causes of biliary stricture are a primary cholangiocarcinoma (CCA) or pancreatic ductal adenocarcinoma (PDAC). There is ongoing debate about adequate diagnostics in biliary strictures of unknown aetiology. MicroRNAs (miRNAs) are small non-coding RNAs important in tumourigenesis. MiRNA have been found to be enriched in exosomes, small membrane-bound extracellular vesicles (EV) of endocytic origin, which is a novel pathway for intercellular signalling within the tumour microenvironment and have been implicated in loco-regional pre-metastatic niche formation. This project aims to investigate circulating-free and EV miRNAs as biomarkers that can aid diagnosis in patients with a biliary stricture. We will (1) isolate and characterise EVs in plasma and bile from patients with benign and malignant biliary strictures (i.e. pancreaticobiliary cancers); and (2) identify differentially expressed circulating-free and EV miRNAs in plasma and bile suitable for detecting malignancy.
Methods: Sample size (n = 126) was calculated for a study power of 90% and α error of 5% for the ability of extracellular miRNAs to discriminate benign from malignant biliary strictures. Prospective matched plasma and bile samples will be collected from patients with benign (n = 63) and malignant (n = 63) biliary strictures undergoing endoscopic retrograde cholangiopancreatography (ERCP). EVs will be isolated from the biofluids by ultracentrifugation and/or size exclusion chromatography and then characterised (TEM, NTA and immunoblotting). Circulating-free and EV-associated miRNAs will be profiled using small RNA sequencing. Extracellular miRNA "signatures" will then be validated by RT-qPCR, and diagnostic accuracy confirmed (sensitivity, specificity, AUC).
Results: EVs derived from patient samples have been characterised using NTA, Western blotting and TEM. SEC derived EVs appear to be more well-defined than UC EVs with marker positivity for CD63, CD81 and CD9. Ongoing work will be focused on RNA profiles of EVs from both malignant and benign cohorts.
Summary/Conclusion: There is currently no effective method to differentiate benign from malignant biliary strictures. Novel plasma and bile circulating-free and EV-associated miRNA biomarkers may improve the speed and accuracy of diagnosis, resulting in considerable patient benefits. Furthermore, as little is known about the EV-associated function of these tumours, candidate EV-miRNAs could be taken from "bedside to bench" and their function further investigated using in vivo, vitro and silico models.

PS07.02

Optimization of urinary extracellular mRNA profiling during pregnancy

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Introduction: Urine is a source of extracellular RNA (exRNA) biomarkers that can be obtained non-invasively throughout pregnancy. Several studies have profiled extracellular miRNAs in biofluids during pregnancy, but few have profiled extracellular mRNAs (ex-mRNAs) in urine. Objective: To optimize methods for ex-mRNA isolation and RNA-Seq library preparation from urine of healthy pregnant and non-pregnant females.
Methods: RNA was isolated from pooled non-pregnant urine using kits based on EV precipitation (miRCURY Exosome kit for CSF/urine, SeraMir), EV affinity

purification (ExoRNeasy), and protein precipitation (miRNeasy Serum/Plasma Advanced). Next, long (>200nt) and short RNAs were isolated from EV enriched urine of pregnant (n = 5) and non-pregnant (n = 5) individuals using the mIRCURY kit followed by the miRNeasy Micro kit. RNA-Seq libraries were prepared using the SMART-Seq v4 Ultra Low Input RNA (Oligo(dT) priming) and the SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input (random priming) methods (Takara). Preliminary data were obtained using the Illumina MiSeq, and aligned using STAR v.2.7.3.a.

Results: Overall, RNA isolation using miRCURY followed by the SMART-Seq v4 library preparation kit yielded the highest % of mapped reads: 42% in pooled non-pregnant, 27% in individual non-pregnant, and 31% in individual pregnant urine. For RNA extracted using the miRCURY kit, the SMART-Seq v4 libraries had higher % of mapped mRNA reads compared to Pico libraries ($P < 0.05$, t-test). In contrast for miRNeasy Advanced it was reversed (38% vs 21%).

Summary/Conclusion: Early results from low-depth sequencing show the highest mRNA mapping rates for miRCURY followed by the SMART-Seq v4 kit. High-depth sequencing data are now being generated, which will enable us to perform detailed comparisons of different RNA species from the RNA profiles obtained using different library preparations and RNA isolation methods from urine of pregnant and non-pregnant subjects.

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PS07.03

IL-2 mutein-induced changes of exosomal miRNA cargo in a humanized mouse model

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Introduction: Regulatory T cells (Tregs) are key contributors to immune homoeostasis. Decreased number and/or function of these cells are frequent features of many autoimmune diseases linked to the development of tissue inflammation. While interleukin-2 (IL-2) is essential for pan T cell proliferation and performance, low dose IL-2 treatment has been shown to preferentially affect Tregs and is being evaluated as an intervention in autoimmune diseases. PT101 is a novel IL-2 mutein Fc fusion molecule (IL-2 M) designed to selectively engage with Tregs. Using a humanized NOD-scid IL2R■-null (NSG) mouse model we have shown that PT101 expanded Tregs without significant effects on other immune cells. We have also shown that Tregs from PT101-dosed humanized mice exhibit increased expression of FOXP3 and CD25, and demethylation of FOXP3 and CTLA-4 genes, suggesting enhanced function and stability. In the current study we investigated the miRNA content of plasma exosomes isolated from PT101- or vehicle-treated mice in order to identify Treg specific miRNAs from the IL-2 M treated animals.

Methods: CD34+ haematopoietic stem cell humanized NSG mice were dosed once subcutaneously with PT101 or vehicle. Plasma samples from 8 mice were collected at Day 7 and exosome isolation was conducted using the ExoQuick method. Small RNA was extracted and quantified using the Bioanalyzer Small RNA assay. An Illumina NextSeq instrument was used for library preparation and sequencing with 75bp single end reads at an approximate depth of 10–15 million reads per sample. Raw sequences were mapped to human genome GRCh37 and analysed via a pipeline provided by the University of California Santa Cruz.

Results: RNA within the exosomes from vehicle and IL-2 M-treated groups was mostly comprised of miRNA and tRNA. Plasma was pooled from 8 animals per treatment group and differential expression was determined using a twofold change cut-off. We found that PT101 treatment actively altered the miRNA content of plasma exosomes, compared to exosomes from vehicle-treated mice. Many of the differentially expressed miRNAs are involved in immunoregulation.

Summary/Conclusion: Plasma exosomes from PT101-treated humanized mice encapsulated treatment-specific miRNAs which can potentially be used as systemic biomarkers of Treg expansion and function.

PS07.04

Identification of potential biomarkers in microglial specific exosomes isolated from prion-infected serum

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Introduction: Transmissible spongiform encephalopathies (TSE) are neurodegenerative disorders caused by the misfolding of the cellular prion protein (PrPc) to the beta-sheet rich abnormal prion protein (PrPsc). PrPsc aggregates in the brain and causes amyloid plaques, neuronal loss, spongiform degeneration and microglial activation. Currently, definitive diagnosis of TSE diseases is only confirmed post-mortem thus a diagnostic test in accessible

body fluid is of interest. Exosomes are a good resource for biomarker discovery since they cross the blood-brain barrier easily and contain protein, lipids and nucleic acids from the cells of origin. The goal of this study was to look at biomarkers from brain-originating exosomes (specifically microglia) isolated in the serum of prion-infected animals.

Methods: Westerns and nanoparticle tracking analysis (NTA) were used to look at the composition of microglial-specific exosomes. As proof of principle, exosomes were isolated from a microglial cell line (BV2 cells). A CD63 antibody was labelled with a fluorophore and binding to exosomes was visualized via NTA. Exosomes were isolated from serum of both prion-infected and mock-infected mice throughout disease course. A macrophage specific antibody (F4/80) was bound to beads which were used to isolate exosomes which includes those of microglial origin. microRNA was extracted from these exosomes and Next-Generation Sequencing (NGS) was performed using the Illumina platform. CLC Genomics Workbench was used for bioinformatics analysis.

Results: Microglial and macrophage proteins (TMEM119 and Iba1) were identified in exosomes isolated from BV2 cells and prion-infected mouse serum. Macrophage exosomes were isolated via a novel antibody-bead based system. Results of the NGS analysis of the microRNA isolated from these exosomes indicated a series of miRNA that could differentiate between control and infected samples as well as age-specific markers.

Summary/Conclusion: To our knowledge, this is the first time microglial-specific exosomes have been isolated from prion-infected serum from early and end stage disease. The results of this analysis could facilitate the diagnosis of prion disease in easily-accessible biofluids pre-mortem.

PS07.05

Comparison of urinary extracellular vesicle isolation methods for transcriptomic biomarker research in diabetic kidney disease

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Introduction: Urinary Extracellular Vesicles (uEVs) are emerging as a source for early biomarkers of kidney damage, holding the potential to replace the conventional invasive techniques including kidney biopsy.

Several methods are available for uEV isolation. Our aim was to compare different workflows and isolation by Hydrostatic Filtration Dialysis (HFD), ultracentrifugation (UC) and a kit based isolation method for their subsequent use in miRNA-seq and RNA-seq for biomarker discovery in diabetic kidney disease.

Methods: Type 1 diabetic patients (T1D) with macroalbuminuria and normoalbuminuric healthy controls were included in the study. Sample collection and all experiments were performed in accordance with the declaration of Helsinki. EVs were isolated from 10–50 ml of 24 h urine collections by UC, HFD, or a commercially available kit (Purification based on spin column chromatography, Urine exosome purification and RNA isolation Midi kit, Norgen Biotech, Canada) each with different established urine clarification steps.

Quality control of the EVs was performed with negative staining EM, NTA and Western blotting. Isolated RNAs were profiled with Bioanalyzer Pico kit and subjected to miRNA and mRNA sequencing.

For RNA-seq, cDNA library was prepared using SMART-seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio, Japan). RNA-seq was performed using HiSeq 2000 (Illumina).

miRNA-seq library was prepared using QIAseq miRNA Library Kit (Qiagen, Germany). miRNA-seq was performed on the Illumina HiSeq 4000 platform (Illumina).

Results: Our data showed that uEV yield, morphology and size distribution were closely similar in HFD and UC preparations, while lower yields were obtained using the kit.

By Western blot, EV markers were detectable in samples isolated by HFD and UC but not readily in samples isolated with the kit. Tamm-Horsfall Protein was detected in all the samples and albumin levels appeared higher in HFD and Kit isolated samples relative to UC samples.

The number of paired-end reads for RNA-seq in HFD and UC samples (in both > 5 M) were closely similar. Instead, RNA reads were lower than 2 M for the kit samples. For miRNA-seq, the number of reads as well as the molecular biotype distribution were similar for the three methods. By principal component analysis of the RNA-seq data, we observed that HFD and UC grouped together showing similarities. However, for miRNA-seq data such similarities were not obvious. This suggests that the three different workflows and isolation principles may enrich different miRNA-rich uEV preparation components.

Summary/Conclusion: Our transcriptomics data shows that HFD and UC are suitable methods to isolate

uEVs for miRNA-seq and RNA-seq. The kit based method appears better suited for miRNA-seq.
Funding: This work was supported by the H2020-IMI2 consortium BEAt-DKD

PS07.06

cfDNA distribution in bioliquids: exosome-associated vs. free circulating form

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Introduction: Exosomes contain a variety of biomolecules including DNA. Knowledge of cfDNA distribution and localization in bioliquid is important for understanding both biological function of cfDNA and exosomes. Some publications state that a large proportion of plasma cfDNA is localized in exosomes. To quantify cfDNA content in free vs. exosomal form in human plasma, urine, and saliva, we employed SubX technology, which allows affinity capture DNA via phosphates groups of the polynucleotide chain and exosomes via membrane surface phosphate moiety clusters. SubX is a proprietary compound that can simultaneously bind to both cfDNA and exosomes in bioliquids, thus allowing precipitation of the [SubX-DNA/SubX-Exosomes] complexes without ultracentrifugation.
Methods: Detection of SubX-DNA and exosomes binding was done by measurement of particle sizes using Zetasizer Nano ZS and Nanosight NS300. The samples were processed with the SubX Exo-DNA isolation kit following the standard protocols. DNA, protein and lipid concentrations were measured by fluorescent assays using Qubit Fluorometer.
Results: SubX efficiently and selectively captures and co-precipitates cfDNA and exosomes directly from bioliquids. Exosomes are easily extracted from the pellet in exosome reconstitution buffer (ERB), followed by subsequent isolation of tightly bound cfDNA from the SubX pellet. ERB does not extract DNA form the [SubX -DNA] pellet and thus does not contaminate reconstituted exosomes with cfDNA. Thus, we separate two distinct types of extracellular material – intact exosomes and purified cfDNA in a single protocol from the same sample. Over 90% of DNA in plasma and urine exist as a free circulating pool, while in saliva up to 30% is associated with exosomes. Thus, cfDNA distribution is probably bioliquid-specific and must be evaluated by methods that eliminate cfDNA-outer exosomal membrane aggregation.
Summary/Conclusion: SubX technology is suitable for simultaneous isolation of both cfDNA and exosomes from the same bioliquid sample. SubX separates cfDNA fragments non-specifically attached to the outer lipid layers of the exosome membrane from the true intra-exosomal cfDNA. In contrast, salting-out PEG technique is associated with aggregation of macromolecules and vesicles and thus leads to overestimation of exosome-associated polymers content, including cfDNA.

PS07.07

Tracing extrachromosomal DNA inheritance patterns in glioblastoma using CRISPR

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Introduction: Glioblastoma multiforme (GBM) is the most lethal brain tumour; it is characterized by poor response to standard post-resection radiation and cytotoxic therapy, resulting in a dismal prognosis with a five-year survival rate of 10%. Recurrence after therapy for GBM is unavoidable. There are substantial differences among the cells of GBM tumours in the abundance and types of genetic material. This heterogeneity likely is the major cause of therapy failure, the development of treatment resistance, and ultimately recurrence. A recent study has suggested that the amount of a particular type of DNA – extrachromosomal DNA (ecDNA) – differs substantially among different GBM tumours, and differs within a given GBM tumour over time. Despite the speculation that ecDNA is a key factor of tumour heterogeneity, how ecDNA is propagated and distributed among – and how it behaves within – cancer cells is completely unknown.
Methods: To address this gap in knowledge, this study focused on developing a novel cytogenetic CRISPR-based tool that enables visualization and tracking ecDNA behaviour in live GBM cells.
Results: We found breakpoint sequences resulting from genome rearrangements during ecDNA formation by performing computational analysis from Whole Genome Sequencing data. And each breakpoint was regarded as a unique target sequence for ecDNA-specific labelling. The uniqueness of each breakpoint was validated by breakpoint-PCR (BP-PCR). Furthermore, the location and the amount of each breakpoint were observed by breakpoint-FISH (BP-FISH) analysis in GBM cells.
Summary/Conclusion: This results will be strong evidence to make ecDNA-specific CRISPR system in

further research. Tracing ecDNA dynamics will provide new insight into the impact of ecDNA on cancer evolution.

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PS07.08

RNA signatures of mouse primary neurons, astrocytes and their small extracellular vesicles

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Introduction: Small extracellular vesicles (sEVs) are 30–150 nm vesicles that mediate intercellular communication by transferring RNA and proteins to the recipient cells. These cargo molecules are selectively sorted into sEVs and mirror the physiological state of the donor cells. Given that sEVs can cross the blood-brain barrier and their composition can change in neurological disorders, there is an increasing interest in elucidating the molecular signatures of sEVs in circulation as disease biomarkers. However, circulating sEVs are derived from multiple cellular sources and determining their source is challenging. Information on sEV composition can be beneficial in predicting whether these sEVs are released predominantly from central nervous system cells. We hypothesized that differentially expressed miRNAs between neuronal sEVs and astrocytic sEVs could be used as cell-type-specific signatures.

Methods: Small extracellular vesicles were isolated from cell culture media of postnatal mouse primary neurons and astrocytes using differential centrifugation and characterized using nanoparticle tracking analysis, transmission electron microscopy and western blotting. RNA from neurons, astrocytes, and their respective sEVs were used for transcriptome and small RNA sequencing.

Results: We observed that only a subset of cellular miRNAs was packaged into sEVs; differential expression of specific miRNAs between sEVs and their corresponding cells suggest that cells employ special mechanisms to sort miRNAs into sEVs. These mechanisms could be cell-type specific since neuronal sEVs showed a different miRNA profile compared to astrocytic sEVs. EXOmotifs, the short sequence motifs that control the loading of RNA into sEVs, were present in differentially expressed miRNAs. We also observed that five RNA-binding proteins, which are associated with passive or active RNA sorting into sEVs, were differentially expressed between neuronal and astrocytic cells.

Summary/Conclusion: miRNA signatures of sEVs from neurons and astrocytes could be beneficial in determining if these cell types contribute to the alterations of sEV composition in circulation in neurological disorders. Cell-type-specific selectivity in RNA loading might be attributed to the differential expression of RNA-binding proteins.

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PS07.09

Isolation and characterization of Extracellular vehicles (EVs) and EV RNA in human urine samples

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Introduction: Analytes present in the extracellular fraction of bodily fluids (ex. blood, urine) have utility as a tool for uncovering the molecular landscape of tumours and hold great potential for discovery of individualized cancer medicine. Urine, being non-invasive as a sample type, has an obvious advantage over blood when used for liquid biopsy purposes. However, potential for microbial proliferation and the labile nature of host cells and extracellular vesicles (EVs) at the point of sample collection/transport to the lab drives the need for stabilization of urine samples. Development of such sample stabilization opens up capability for the detection of various biomarkers present in the extracellular fraction to be used in liquid biopsy. This is of particular concern as studies around urinary analytes for cancer diagnosis, progression and therapeutic effect are rapidly expanding in cohort sizes. Multi-site collections and at-clinic collections are increasingly prohibitive for large scale recruitment and also lead to variability in the time between collection and processing.

Methods: In this study, we have analysed two commercially available EV extraction kits and compared them with ultracentrifugation technique for size, concentration and specificity of the isolated EVs from human urine samples with and without our proprietary preservation solution using nanoparticle tracking analysis and western blot analysis for exosomal membrane markers. EV RNA contents in various urine fractions (first morning first void, random first void and midstream) were compared using RT-qPCR assay to provide better understanding of the collection techniques and fractionations that are ideal for EV research work.

Results: In our current work, we have bench-marked human urine collection and EV extraction in order to provide recommendations in standardization of sample acquisition and processing for urinary EV studies. We have utilized these standardization in order to develop a novel and efficient sample stabilization principle for preservation of EVs and EV RNA in urine samples during an ambient temperature hold.
Summary/Conclusion: Taken together, we have established a framework for evaluating technologies and techniques in the EV sample processing space, which can be utilized by other research groups.

PS07.10

Vn96-isolated plasma extracellular vesicles improve tumour mutation detection by next-generation sequencing compared to cell-free DNA and correlate with tissue biopsy of NSCLC patients

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Introduction: Liquid biopsy is a minimally-invasive diagnostic method that detects circulating biomarkers and has the potential to improve access to molecular profiling for NSCLC patients when tissue biopsy material is unavailable or insufficient. Although isolation of cell-free DNA (cfDNA) from plasma is the standard liquid biopsy method for detecting DNA mutations in cancer patients, the sensitivity can be highly variable. Vn96 is a 27 amino acid peptide with an affinity for heat shock proteins that are exposed on the surface of extracellular vesicles (EVs); peptide-EV aggregates readily sediment using a benchtop centrifuge and therefore the Vn96 peptide provides a rapid, clinically-amenable procedure for EV isolation. In this study, we determine whether isolation of EVs from NSCLC patient plasma improves the sensitivity of single nucleotide variants (SNVs) detection compared to cfDNA and correlate genetic changes observed by liquid biopsy with tumour FFPE tissue biopsy.
Methods: Blood was collected from stage III/IV NSCLC patients with informed consent in either EDTA or Cell-Free DNA BCT® collection tubes and plasma was harvested within 30 minutes. Total nucleic acid (TNA) was extracted from either Vn96-isolated EVs from EDTA plasma or directly from plasma collected in EDTA or Cell-Free DNA BCT® tubes (cfDNA). SNVs were detected by next-generation sequencing (NGS)
Results: Vn96 isolation of EVs from plasma resulted in higher recovery of DNA than cfDNA isolation. The SNVs detected in both EV-DNA and cfDNA correlated well with those reported in matched FFPE tumour tissue using NGS, including 100% specificity for EGFR mutations. No improvement in SNV detection was observed using Cell-Free DNA BCT® collection tubes compared to EDTA tubes. Isolation of EVs with the Vn96 peptide prior to sequencing improved a number of NGS parameters including library yield, total reads, median read coverage and molecular coverage, resulting in improved sensitivity of SNV detection.
Summary/Conclusion: In summary, our research demonstrates that Vn96-based EV isolation is useful for molecular profiling of NSCLC patients for whom tissue biopsy is not an option, thereby improving access to molecular profiling and targeted therapies.
Funding: Atlantic Canada Opportunities Agency

PS07.11

Novel markers for neuroendocrine prostate cancer

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Introduction: Prostate cancer (PCa) is fuelled by androgens and androgen receptor (AR) signalling. Therefore, ablation of AR signalling by Androgen deprivation therapy (ADT) is the goal of first-line therapy that results in cancer regression initially. However, two to three years post-ADT, the disease develops into castration-resistant prostate cancer (CRPC). As a second-line of therapy, next generation of AR pathway inhibitors (API) such as Enzalutamide (ENZ) are used that are effective initially followed by emergence of drug resistance. A subset of API-resistant tumours emerges to an AR independent state via undergoing a trans-differentiation to neuroendocrine lineage, a process referred to as neuroendocrine differentiation (NED). Due to lack of AR signalling, these PCa variants, referred to as neuroendocrine prostate cancer (NEPC), are impervious to anti-androgen therapy and constitute an aggressive variant of advanced CRPC with poor prognosis. Currently, there is a lack of effective molecular biomarkers for predicting API therapy resistance and emergence of therapy-induced NED.
Methods: Exosomes/EVs were isolated from sera of a patient cohort with/without NED. The study was conducted in accordance with ethical guidelines of US Common Rule and was approved by the institutional committee on human research. Written informed consent was obtained from all patients. Following extensive characterization of EVs by electron microscopy, nanosight tracking analyses and Western blotting of

exosomal markers, small RNA sequencing was carried out on Illumina HiSeq platform to identify differentially expressed transcripts. Machine learning algorithms were applied to clinical sequencing data to train a "miRNA classifier". Further, we probed the proteomic profile of exosomes isolated from NEPC cellular model NCI-H660 and enzalutamide resistant CRPC cell lines by mass spectrometry.

Results: We identified that transition from CRPC-Adenocarcinomas to neuroendocrine states is associated with significant EV-miRNA dysregulation, with a specific dysregulation in certain miRNA families. With the application of machine learning algorithm, we identified an EV-based "molecular classifier" that can robustly stratify CRPC-NE tumours from CRPC-Adenocarcinomas. Proteomic analyses identified novel NEPC-specific, glycosylated proteins that can be exploited for NEPC diagnosis.

Summary/Conclusion: Our data suggest that EV miRNA and protein profile can predict neuroendocrine differentiation in advanced castration-resistant prostate cancer patients.

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PS07.12

Exosomal mRNA in diagnosis strategy for hepatocellular carcinoma

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Introduction: Exosomal cargo is informative source illustrating the genetic events happening in cells, what can be especially advantageous in case of cancer development for disease progression or treatment effectiveness monitoring.

Methods: 10 plasma samples of hepatocellular carcinoma (HCC) patients, 10 plasma samples of patients with liver cirrhosis 3–4 on the hepatitis C virus (HCV) background, 5 healthy donors' plasma samples. Exosomes were isolated with ultracentrifugation, western blot (CD63, CD9) was performed. Total mRNA was isolated with exosomal RNA isolation kit, Norgen Biotec Corp. Sequencing was carried out on a MinIon sequencer. Housekeeping genes (GAPDH, B2 M, ACTB, TUBA1A). Detected mutations were confirmed by real-time PCR with specific highly sensitive LNA probes.

Results: Significant changes in expression levels were identified for 50 genes in HCC and liver cirrhosis groups (increasing up to X200 compared to control samples and decreasing up to no detected expression). In 6 out of 10 patients with HCC mutant burden was significant increased compared to mutant burden in groups with cirrhotic samples. In 8 out of 10 patients with HCC increased expression for mRNA LINE-1 was identified compared to cirrhotic patients.

Summary/Conclusion: Exosomal mRNA expression levels may serve as a prognostic and diagnostic marker for patients with liver cirrhosis caused by HCV for HCC risk development.

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PS07.13

Circulating extracellular vesicle signatures in small cell lung cancer

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Introduction: Lung cancer is the leading cause of cancer deaths worldwide and classified primarily as either non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). Compared to NSCLC, SCLC has a faster growth rate, earlier widespread metastasis, and shorter overall survival. The early diagnosis of SCLC and the development of novel therapeutics have proven challenging. Thus, progression and recurrence rates remain high. Non-invasive methods for cancer detection are increasingly being used to inform clinical decision making. Extracellular vesicles (EVs) have recently emerged as potential carriers of genetic contents such as microRNAs (miRs) to induce reprogramming of components of the microenvironment in cancer initiation and progression. Moreover, extracellular miRs expression profiles have been shown to have signatures related to tumour classification, diagnosis, and progression.

Methods: We selected a cohort of patients divided into 4 groups: high-risk smokers, adenocarcinomas, squamous carcinomas, and SCLC. We extracted total circulating EV and plasma RNA from plasma (38 patients in total) and RNA from plasma in a separate group (24 patients in total). Utilizing both next-generation sequencing (NGS) and nanostring platforms, we analysed for global microRNA (miRs) expression patterns. Candidate miRs were then validated by qRT-PCR.

Results: We identified several deregulated miRs in both EVs and plasma of SCLC patients compared to the other groups. For EVs, we validated miR-1285-5p as a significant biomarker for the late stage of SCLC compared to controls. In the case of plasma, we validated the upregulation of miR-375 in SCLC compared to controls.

Summary/Conclusion: Our results indicate that a potential combination of plasma (miR-375) and EV-based (miR-1285-5p) miRs be valuable biomarkers for SCLC detection and serve as a basis for a non-invasive SCLC classifier.

Funding: Virginia Commonwealth University, DOIM – NIH/NCI

PS08: Separation and Concentration

Chair: Annalisa Radeghieri – Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy and Consorzio Sistemi a Grande Interfase, Department of Chemistry, University of Florence

Chair: Aleksandra Gąsecka – 1 st Chair and Department of Cardiology, Medical University of Warsaw

PS08.01

A fast and reliable in vitro cell culture system reveals that acidification of bovine milk extracellular vesicles impairs their functionality

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Introduction: The isolation of EVs from milk is technically challenging due to the complexity of milk. Currently used separation procedures allow for the removal of milk fat globules and cells (by low speed centrifugation of fresh milk), removal (by acidification), or disruption (by addition of EDTA) of casein micelles. Using these protocols the integrity, composition and targeting of bovine milk EVs has been evaluated and has led to believe that milk EVs might withstand these conditions. However, the effects on functionality of milk EVs (i.e. immunomodulatory properties) after processing and isolation have not been studied. Therefore, we have set up an in vitro culture system using a human T cell line that allows for the rapid screening of milk EV functionality.
Methods: Fresh bovine milk was defatted and cells were removed after 2x3,000 g centrifugation, followed by differential centrifugation at 5,000 g and 10,000 g. This milk was either subjected to acidification with HCL, or EDTA was added, or the milk supernatant remained untouched. Top down Optiprep density gradient separation followed by SEC was used to further purify EVs. These highly purified milk EVs were added to human Jurkat T cells, which were simultaneously stimulated using anti-CD3 and anti-CD28 antibodies. After 24 h T cell activation was measured by IL-2 cytokine production.
Results: Precipitation or disruption of casein micelles allowed for the substantial removal of proteins during isolation compared to directly isolated EVs, which aids in the purification of milk EVs. In vitro analysis revealed that in the presence of directly isolated, or EDTA isolated milk EVs, Jurkat cells were suppressed in their activation as measured by IL-2 production. Remarkably, EVs isolated from HCL-acidified milk were impaired in their suppressive capacity to inhibit IL-2 production.
Summary/Conclusion: Although casein removal from bovine milk greatly improves purity of isolated milk EVs, the detrimental effects on EV functionality should be considered. Interestingly, EVs exposed to acidic conditions lost their ability to modulate T cell activation, which is in contrast with the general believe that milk EVs could withstand the gastro-intestinal tract.
Funding: This work is funded by the European Union's Horizon 2020 Framework Programme under the grant FETOPEN-801367 evFOUNDRY.

PS08.02

Optimising methods for separation and characterisation of extracellular vesicles from skim milk and infant milk formula

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Introduction: Infant milk formula (IMF) is intended to impart nutrition to infants, similar to breast milk. However, although industrial IMF production involves harsh treatment, potential consequences on extracellular vesicles (EV) in IMF are not yet established. This study aimed to optimise methods for separating EVs from IMF and skim milk (SM) and to characterise the EVs in accordance with MISEV2018.
Methods: SM and IMF were either not treated (NT) or treated with acetic (AA) or HCl acid (isoelectric precipitation, IP), to remove caseins. Samples were then subjected to differential ultracentrifugation (DUC) or gradient ultracentrifugation using iodixanol solution (GUC). For DUC, 38 mL samples were centrifuged at 12 K g, 35 K g, 75 K g, 100 K g and 200K g sequentially for 75 min each and pellets re-suspended in 1 mL PBS.

For bottom-up GUC, increasing iodixanol gradients with 2.3 mL of samples were centrifuged at 186 K g for 18 h. Fractions were then pooled based on densities (1.1–1.2 g/mL). BCA and SDS-PAGE were used to analyse total protein; nanoparticle tracking (NTA) and transmission electron microscopy (TEM) for EV presence; and immunoblotting and imaging flow cytometry (IFCM) to evaluate EV specific markers. (EV-TRACK ID: EV190096).

Results: Immunoblotting showed absence of Actinin4 from all samples, while CD63 and TSG101 were detected for all samples; apart from IMF_IP. NT_samples were not analysed reliably by NTA and IFCM, due to the high concentration of casein micelles present (~10^14/mL in milk) that otherwise would be co-counted with EVs. As expected, following IP, which most efficiently removed casein micelles, BCA showed that samples had lowest total protein. This was confirmed by SDS-PAGE. Thus, most effects were then focused on the IP casein-depleted samples. IFCM indicated that, post-GUC, SM_IP EVs had significantly ($P < 0.05$) more CD81-positive particles/mL of milk vs all other GUC and 200 KDUC samples. While there were no significant differences in sizes of EV separated from SM or IMF, directly comparing the IP pre-treated samples, SM had significantly ($P < 0.01$) higher quantities of EVs when compared to IMF. Additionally, TEM indicated that EVs separated from SM by GUC were intact with limited background debris, whereas those separated from SM by DUC and all IMF EVs were not.

Summary/Conclusion: In conclusion, regardless of the method used, IMF has fewer intact EVs compared to SM. Also, to obtain purest SM EVs, IP followed by GUC separation is optimal.

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PS08.03

A novel platform to isolate extracellular vesicles based on surface molecule expression uncovers functional differences between EV subpopulations

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Introduction: Extracellular vesicles (EVs) exist as subpopulations with heterogeneous content. The surface heterogeneity of EVs may reflect differences in functionality between EV subpopulations, as interactions with recipient cells may differ between EV subpopulations with different surface profiles. However, it is currently challenging to study functional differences between EV subpopulations due to the lack of suitable techniques to purify intact EVs based on their surface signature. Here, we showcase a novel capture-and-release platform to enrich intact EV subpopulations by their surface profile and compare their characteristics.

Methods: MDA-MB-231 and SKOV-3 cell-derived EVs were isolated using size exclusion chromatography. EV subpopulations were enriched based on surface markers CD9, CD63, CD81 or phosphatidylserine (PS) using a novel magnetic bead-based capture-and-release platform. Obtained EVs were characterized by transmission electron microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and western blotting. EVs were fluorescently labelled using PKH67 and CellTracker Deep Red (CTDR) and their uptake by recipient cells was examined using flow cytometry.

Results: Western blot analysis showed that EV subpopulations enriched for the selected tetraspanins and PS were successfully isolated using a novel capture-and-release platform. Interestingly, EVs isolated based on PS exposure (PS+) lacked most canonical EV markers. All EV subpopulations showed intact, cup-shaped morphology when analysed by TEM, but contained less protein contaminants compared to the initial EV isolate. PS+ EVs were slightly larger than other EV subpopulations when analysed by TEM and NTA. To test the capacity of EV subpopulations to interact with recipient cells, EVs were labelled with PKH67 and CTDR prior to subpopulation fractionation. After fractionation, PS+ EVs showed a significantly higher CTDR/PKH67 ratio than other EV subpopulations as determined by fluorescence spectroscopy, suggesting higher esterase activity of PS+ EVs compared to other tested subpopulations. Furthermore, MDA-MB-231-derived EVs isolated based on CD9 and CD81 expression were taken up more efficiently by HMEC-1 and MDA-MB-231 cells than EVs isolated based on presence of CD63 or PS.

Summary/Conclusion: Using a novel technology to isolate EV subpopulations based on their surface profile, we here show that composition and cellular uptake efficiency differs between EV subpopulations. Theoretically, this technology is applicable to any surface marker of interest, allowing its use to further establish EV surface-functionality relationships and enrich EVs with desirable characteristics for therapeutic purposes.

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PS08.04

Preparation of agarose microspheres for high-efficient separation of extracellular vesicles

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Introduction: Size exclusion chromatography (SEC) is becoming a widely used technique for separating of extracellular vesicles. Various commercially available products were launched on the market, however, their separation efficiencies were not fully disclosed. Herein, novel porous agarose microspheres with the tunable diameter and pore size were synthesized by emulsion reaction. The performance was evaluated and compared with commercial products. The modified SEC column packing materials were shown to exhibit advantages for rapid, high-recovery and high-purity separation of extracellular vesicles from cell culture-conditioned medium and human plasma.
Methods: The homemade SEC column was packed by gravity flow. 100 μL of the sample was loaded and the PBS buffer was used as eluent. 24 factions were collected and analysed by CD9/CD9 sandwich ELISA assay and by micro BCA assay for determining respectively extracellular vesicles and total protein content.
Results: Agarose microspheres were prepared by emulsification. The particle size can be controlled by the types and concentrations of surfactants. The product was collected by desired screen meshes and used as packing materials of the SEC column. Our results showed that the extracellular vesicles were clearly separated from proteins. More than 99.8% of proteins were removed while the recovery of extracellular vesicles was close to 70%, which is much higher than 32% of the commercial product. The total separation time was less than 15 min.
Summary/Conclusion: We have established an approach for generating spherical agarose microspheres as packing materials of homemade SEC columns, which are capable of separating extracellular vesicles from complex samples with high efficiency. Further validations with additional samples are currently ongoing.

PS08.05

Immunomagnetic Sequential Ultrafiltration (iSUF) platform for enrichment and purification of extracellular vesicles from large and small volumes of biofluid

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Introduction: EVs derived from tumour cells have the potential to provide a much-needed source of non-invasive molecular biomarkers for liquid biopsies. However, compromises have to be made when using a particular technology/methodology for the isolation of EVs. Currently, there is a trade-off between sample volume and specificity in EV isolation technologies that limits quantitative molecular analysis of EV contents, ultimately impacting the utility of EVs in cancer diagnostics. Here, we present an approach called immunomagnetic sequential ultrafiltration (iSUF). Our platform combines ultrafiltration and immunoaffinity separation. Using iSUF, we demonstrate that small or large volumes of biofluid can be processed (~ 100 μL or > 100 mL) while concomitantly removing 99.9 % contaminating proteins. We also processed serum from breast cancer patients enabling the characterization of different tumour and immune biomarkers on the isolated EVs.
Methods: Human samples were collected under an approved IRB. Size distribution and concentration of EVs were measured using a tunable resistive pulse sensing (TRPS) method. EV proteins and RNAs were extracted and quantified using a BCA protein assay and UV spectroscopy. iSUF and other EV isolation methods were compared for EV concentration, protein, and RNA quantity.
Results: 50 mL of cell culture media (CCM), 0.5 mL serum, and 100 mL urine samples were processed with the iSUF platform and recovered in 100 μL. For all cases, EVs were enriched with recovery efficiency greater than 95%. The processing time for a 100 mL sample was 120 min with over 99% of purity. We compared EV concentration and purity isolate from 0.5 mL serum using iSUF and other commercially available methods, iSUF demonstrated superior performance on isolating EVs at high concentrations and purities. Analysis of total RNA amounts in the isolated EVs using different methods was corresponding to higher EV recovery efficiency of iSUF. We also compared protein and RNA levels of EVs enriched with iSUF present in urine and serum samples from the same donors (n = 10), and we found that for the same number of EVs, the EV RNA concentration from both biofluids showed no significant difference. Finally, we have processed serum samples from 10 metastatic breast cancer patients and demonstrated that their isolated EVs have expression levels of HER2, CD24 and miR21 biomarkers at significantly higher levels than healthy controls.

Summary/Conclusion: The iSUF platform can be scale-down or -up to work with small or large volumes of biofluids for the isolation of EVs. Using the iSUF platform with clinical samples shows the potential of our platform to be used for cancer diagnosis or monitoring treatment response.

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PS08.06

Challenges in exosomes isolation from primary biological samples derived from multiple myeloma patients

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Introduction: Multiple myeloma (MM) remains incurable despite advances in its treatment and research progress on the crosstalk between MM and surrounding host cells. Exosomes are important regulators of the cellular niche. Their importance for diagnostic and therapeutic applications has been proven in many cancers. In this context we hypothesized that a better understanding of the molecular role and features of MM-derived exosomes would provide a basis for their use for both risk stratification and as predictive biomarkers of response to anti-MM drugs already in use in clinical settings, given the optimization and validity of their isolation/purification method.

Methods: Exosomes were isolated from human MM cell lines (HMCLs) supernatants and peripheral blood plasma (PBPL) isolated from healthy donors, MM and MGUS (monoclonal gammopathy of undetermined significance) patients. Both fresh and frozen samples were tested. We evaluated 3 commercially-available kits, density-based separation and ultracentrifugation.

Results: Higher purity and recovery, evaluated by western blotting, nanoparticle tracking analysis and electron microscopy, were observed for supernatant density-based purification and for PBPL resin-based isolation.

Exploring the function of MM-derived exosomes, we observed an increase in proliferation of the immortalized stromal cell (SC) line HS5 treated with exosomes when compared to untreated cells, and a higher increase in proliferation of SCs treated with MM-exosomes when compared to exosomes derived from normal and MGUS PBPL samples.

Summary/Conclusion: The method of isolation represents a critical step in the study of exosomes as many factors can affect the purity, yield and downstream application. Our data demonstrated that density and resin-based isolation methods provided functional MM-derived exosomes with proliferative effects on SCs. Altogether our findings may serve as a guide to choose exosome isolation methods for MM studies. Further optimization steps, including albumin-depletion from plasma samples and use/type of serum in cell cultures, should be taken into consideration when planning proteomics and genomics as downstream applications.

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PS08.07

A rigorous method for exosome isolation from post-mortem eyes

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Introduction: In order to determine and validate the tissue-specific content of extracellular vesicles (EVs) in biofluids, robust EV isolation methods from tissues must be developed. However, to date very few rigorous methods to isolate or enrich for intact EVs from tissues have been reported. We present a comprehensive exosome isolation method with a sufficient level of characterization to unequivocally demonstrate true EV identity from ex vivo eyes.

Methods: Iodixanol (OptiPrep) buoyant density gradient ultracentrifugation (DGUC), cushioned DGUC (C-DGUC), and our newly developed C-DGUC immunocapture (C-DGUC-IP) method were used to compare yield and enrichment of exosomes isolated from porcine eyes between 3 to 5 hours post-mortem. Yield was assessed by Nanoparticle Tracking Analysis (NTA) and immunoblotting for exosomal markers along with total protein quantitation. Enrichment was assessed by comparison of exosomal markers, ocular-specific markers and known contaminant markers, plus in-depth proteomic mass spectrometry analyses.

Results: High enrichment of posterior eyecup small EVs (sEV) were achieved by DGUC and C-DGUC, with C-DGUC resulting in an eightfold increase in yield by NTA and two to fivefold increases of exosomal protein markers such as Syntenin-1 and CD81 by immuno-blotting compared to DGUC. Interestingly,

in-depth proteomic analyses revealed that a majority of these sEVs with densities of 1.07–1.11 g/ml isolated by DGUC and C-DGUC were likely of endoplasmic reticulum (ER) and Golgi origin, suggesting ER-to-Golgi transport vesicles resulting from post-mortem tissue cell rupture. In order to enrich further for sEVs (including exosomes) we subjected sEVs isolated by C-DGUC to anti-CD81 immunocapture. The resulting sEV proteome was enriched 1.5- to 4-fold for bona fide sEV and exosome markers compared to C-DGUC.

Summary/Conclusion: The C-DGUC method provides an enhanced yield and purity of sEVs and exosomes from ex vivo eye tissue. However, to avoid significant contamination with ER and Golgi-derived vesicles from post-mortem eyes, a final EV-specific immunocapture step is required to achieve sufficient purity for subsequent analyses. Our highly rigorous method paves the way for identification and validation of ocular-derived exosomes in blood and their potential use as eye disease biomarkers.

Funding: This work was supported by NIH grants EY026161 (CBR), EY023287 (WDS), EY022359 (WDS), EY019696 (WDS), a grant from Foundation Fighting Blindness (CBR), a Research to Prevent Blindness (RPB)/International Retinal Research Foundation Catalyst Award (CBR). A Core Grant for Vision Research (P30; EY005722) from NEI (to Duke University) supported mass spectrometric analyses carried out by NPS. In addition, Duke University Department of Ophthalmology is supported by an unrestricted grant to the Duke Eye Centre from RPB.

PS08.08

Characterization of the extraction of extracellular vesicles using a lab-on-a-disc filtration system

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Introduction: Personalized treatment for cancer is a promising way to face the multiplicity of the disease, to increase the efficacy of drugs and to decrease their toxicity. As part of this strategy, liquid biopsy explores a new non-invasive approach to diagnose cancer, guide treatment and monitor its efficacy. Extracellular vesicles (EVs) are nanometric lipid bilayers micelles with high potential as biomarkers. They are involved in the transfer of information (proteins, RNA and DNA) between cells. EVs include a broad spectrum of particle sizes, from the tens to thousands of nanometres.

The isolation of EVs from complex matrices is the first step of any protocol and is particularly important for the reproducibility and fidelity of the results presented, as it could bring bias in further analysis. In order to explore the heterogeneity of EVs, a full characterization (physical and biological) of the extracted EVs is needed. We evaluate and compare 3 EVs purification methods, including ultracentrifugation, size-exclusion chromatography (SEC) column and an emerging microfluidic technology: LabSpinner filtration lab-on-a-disc device isolating EVs between two filters of 600 and 20nm.

Methods: A431 cell supernatant was used as a model matrix. We compared three methods of extraction of EVs: ultracentrifugation with two cycles of 1 h10 at 110,000 g at 4 degrees Celsius (rotor type 70Ti, Beckman Floor Ultracentrifuge Optima L90 K), qEV size exclusion chromatography columns from Izon (qEVoriginal/70 nm) and lab-on-a-disc filtration system (LabSpinner, Exodisc C). EVs characterization was conducted with NTA (NanoSightNS500), TRPS (Izon), nanodrop (spectrometerND1000), TEM (FEI Tecnai 12 120 kV) and custom micro- immuno-assay.

Results: In this study, we characterize a filtration system made of two serial filters of 600 nm and 20 nm pores for isolation of EVs. Compared to ultracentrifugation and chromatography columns, yield of extraction is up to 10 times higher and the size of the extracted particles is smaller. TEM imaging was used for assessment of the quality of the extracted EVs. However, albumin concentration measurement tends to show that the purity of the solution is decreased. The immuno-labelling analysis shows that the proteomic signature of the extracted EVs differs according to the extraction methods. The new filtration technology seems to give us access to a broader range of EVs compared to standard methods.

Summary/Conclusion: In this study, we characterized 3 purification methods including lab-on-a-disc filtration, and were able to demonstrate an increase of the concentration of EVs by a factor of 10, a decrease of the size of the accessible extracted particles and access to new proteomic signatures.

Funding: We acknowledge the support of Génome Québec and Action Marie Skłodowska-Curie.

PS08.09

Effects of sample processing on isolation of extracellular vesicles from blood plasma by centrifugation

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Introduction: The isolation of extracellular vesicles (EV) from body fluids is still controversial and the poor understanding of vesicle stability and effects of sample processing is probably one of the core issues preventing the breakthrough of this field into applicative practices.

Methods: We performed an in-depth study of sample changes in blood, blood plasma and samples throughout the increasing speed of centrifugation, considering the number, size, contents and shape of particles in the isolates. Flow cytometry, light scattering, mass spectrometry and scanning electron microscopy were employed to reveal the properties of material in the samples.

Results: The particles of size about 100–500 nm with characteristic topology of membrane vesicles without internal structure were observed by the scanning electron microscope only in EV isolates prepared from fresh blood sample. Inspection of the tube surface in which the isolation took place suggests that those particles are likely formed from activated platelets tearing at the tube wall due to the centrifugal pull.

The isolates prepared from frozen blood plasma prepared by centrifugation with different forces contained different amounts of particles with similar protein contents, predominated by highly abundant human plasma proteins, including albumins and immunoglobulins. Some lipoprotein clearance and fibronectin precipitation were however observed through increased speed and time of centrifugation.

Summary/Conclusion: The results of this study [1] contribute to the understanding of stability and dynamics of membrane particles. The reported evidence provides the support for viewing EV isolates as a product, shaped by uniqueness of the starting samples and the thermal and mechanical stress applied upon processing. We believe this kind of insights strengthen our ability of reading the story of EVs.

Reference:

[1] Božič D., Hočevar M., Kononenko V., et al.: Pursuing mechanisms of extracellular vesicle formation. Effects of sample processing, Ch 17 in: Biological Membrane Vesicles: Scientific, Biotechnological and Clinical Considerations 32, Elsevier 2020, in press.

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PS09: EVs in Cancer Pathogenesis

Chair: Liang Dong – The Brady Urological Institute, Johns Hopkins University School of Medicine

Chair: Jeffrey Franklin – Vanderbilt University Medical Centre

PS09.01

Imaging extracellular vesicles arising from apoptotic tumour cells for cancer diagnosis and monitoring

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Introduction: Apoptosis is a form of programmed cell death with diverse roles in the tumour microenvironment and emerging data show that, besides its role in tumour suppression, it can also promote oncogenic proliferation. Highly aggressive tumours such as Burkitt Lymphoma (BL) show high levels of apoptosis, which has a diagnostic and prognostic value for classifying and staging the disease. We hypothesize that amongst other elements, extracellular vesicles (EV) are key mediators of apoptotic cell-derived tumour microenvironment signals. Here, we report on EV released in vitro by apoptotic BL cells (Apo-EV) in relation to their potential use as cancer biomarkers.
Methods: Basic physical properties of Apo-EV such as structure, size distribution, surface charge and membrane fluidity are discussed using Cryo Electron Microscopy (EM) and Tomography, Nanoparticle Tracking Analysis, Dynamic Light Scattering and fluorescence anisotropy respectively. For phenotypic analysis we apply immunocapture and flow cytometry, immunogold labelling on transmission EM, fluorescence microscopy and quantitative PCR. In addition, we study the interaction of Apo-EV with blood components such as platelets, leucocytes and red cells, in order to understand their effects in the circulation and therefore their potential for analysis in blood samples.
Results: Looking at the differences between Apo- and non-Apo-EV, Apo-EV have larger diameter, while structurally are not different. However, we have identified distinct Apo-EV markers such as active caspase 3 and histones, or DNA and small non-coding RNA-Y. There is also strong interaction of EV with platelets and leucocytes but not with red cells, indicating potential routes of transfer of EV cargo in the circulation.
Summary/Conclusion: It is concluded that for the characterization of the heterogenous EV populations, combination of multiple techniques is often required, and also, understanding the strengths and limitations of each method is essential for choosing the appropriate set of analytical tools. Finally, we consider that monitoring free circulating Apo-EV or blood cells with which they have interacted is a promising approach to improve cancer diagnosis, prognosis and evaluation of therapeutic response.
Funding: Engineering and Physical Sciences Research Council & Medical Research Council [grant number EP/L016559/1].

PS09.02

Casting a small netrin: functional roles of a novel surface factor on stroma-derived extracellular vesicles in pancreatic cancer

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Introduction: Pancreatic Ductal Adenocarcinoma (PDAC) is a devastating disease driven and supported by changes in its microenvironment, or stroma. Here we dissect the intercellular communication that exists between the primary stromal component, cancer-associated fibroblasts (CAFs) and PDAC. PDAC communicates with its microenvironment, in part, through the exchange of specific types of extracellular vesicles (EVs). Specifically, we focus on the mechanism by which CAF-secreted EVs support PDAC survival, with an additional goal to identify biomarkers suitable to generate a future "liquid biopsy" test for early PDAC detection and prognosis.
Methods: EVs are isolated from Patient-Derived PDAC-associated fibroblasts via differential ultracentrifugation and validated by ISEV standards. Human PDAC cell lines used as recipient cells are treated with CAF-EVs to assess their role in supporting PDAC survival. Recombinant proteins, neutralizing peptides,

and non-functional mutant proteins are used to block EV interaction with target cells.

Results: We observe sub-types of CAF-EVs containing unique surface receptors. One EV sub-population of interest contains a novel surface protein (NSP) expressed on the plasma membrane of pancreatic CAFs, but not their healthy counterparts. Further, PDAC cells up-regulate NSP's lone binding partner, suggesting a role for these factors in PDAC-selective EV uptake. Functional assays designed to test PDAC viability suggest these NSP(+)-EVs protect PDAC cells from programmed cell death as a result of physiological stress. This EV-mediated survival benefit can also be inhibited by blocking the interaction of NSP and its binding partner, suggesting the engagement of these two factors is necessary for CAFs to support PDAC via EVs. Pursuing our biomarker goal we confirm stromal NSP expression increases during early PanIn stages prior to tumour development, and we are currently seeking to validate NSP(+)-EVs in blood of PDAC patients.

Summary/Conclusion: This research shines light on a novel mechanism of tumour-stroma communication that may be crucial for cancer progression during early disease stages and a potential target for disrupting the supportive role of the tumour microenvironment. Additionally, we describe a sub-population of NSP (+)-EVs that have the potential to serve as biomarkers for identifying PDAC development.

PS09.03

Exosomes carry distinct miRNAs that drive medulloblastoma progression

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Introduction: Extracellular vesicles (EVs) represent an ideal source of functional biomarkers due to their role in intercellular communication and their ability to protect cargo, including RNA, from degradation. The most investigated EV's are exosomes, nanovesicles secreted by all cell types and able to cross the blood-brain-barrier. Here we characterised the RNA of exosomes isolated from medulloblastoma cell lines, with the aim of investigating exosomal RNA cargo as potential functional biomarkers for medulloblastoma.

Methods: Exosomes derived from a panel of matched (original tumour and metastasis) medulloblastoma cell lines were isolated and characterised by NanoSight, electron microscopy, western blotting and Nanoscale flow cytometry. Exosomal miRNA and mRNA from our matched cell lines and foetal neuronal stem cells, which were used as a normal control, were analysed by RNA-sequencing technology.

Results: Based on hierarchical clustering, malignant derived exosomes were distinctly separated from normal control exosomes. miRNA profiling revealed several established oncomiRs identified in our malignant derived exosomes compared to control samples. Using interaction pathway analysis, we identified that our malignant exosomes carry numerous miRNAs implicated in migration, proliferation, cellular adhesion and tumour growth. Several previously identified oncomiRs were also identified to be present at higher levels in metastatic exosomes compared to primary and normal, including hsa-miR-455-3p and hsa-miR-92a-3p.

Summary/Conclusion: This study shows that exosomes from MB cells carry a distinct miRNA cargo which could enhance medulloblastoma progression. The use of circulating exosomes as markers of metastatic disease could be an innovative and powerful non-invasive tool.

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PS09.04

Leukaemic extracellular vesicles induce inflammatory regulators and suppress haematopoietic stem and progenitor cell function

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Introduction: Inflammatory changes in the bone marrow (BM) and suppression of haematopoietic stem and progenitor cell (HSPC) function during acute myeloid leukaemia (AML) significantly contribute to patient morbidity and mortality. Our laboratory has previously shown that AML-derived extracellular vesicle (EV-AML) trafficking confers a state of enforced quiescence and leads to lasting DNA damage in HSPCs. Here we explore the underlying cause. Specifically, we hypothesize that EV-AML incite inflammatory regulators as potential mediators of DNA damage.

Methods: As a validated model of AML, we utilized the murine TiB49 cell line as a source of EV-AML. EV-

AML were harvested from TiB49 cells cultured in EV-free medium using serial ultracentrifugation. HSPC (KSL; Lin-Sca1+ cKit+) clonogenicity and inflammatory responses were assessed using colony-forming unit (CFU) assay and real-time polymerase-chain reaction, respectively. IFN-alpha receptor 1 (IFNAR1) expression and intracellular reactive oxygen species (ROS) levels were assessed by flow cytometry. DNA damage were assessed by quantifying nuclear γ-h2ax using immunofluorescent microscopy.

Results: Similar to EVs derived from AML patients, TiB49 EV-AML elicited double-stranded breaks in HSPCs, and actively suppressed HSPC clonogenicity. Transcriptional profiling revealed that exposure to EV-AML induced the upregulation of several inflammatory mediators in HSPCs, including ISG15, IL-6, IFNα, CH25 H. Inflammatory signalling triggered by EV-AML did not depend on IFNα signalling as evident from suppression of clonogenicity in IFNAR1-null HSPCs as well as the lack of EVs-induced STAT1 phosphorylation or IFNAR1 downregulation. Instead, we found increased levels of ROS following EV-AML exposure.

Summary/Conclusion: Our findings support a model whereby EV-AML inflammatory signalling and oxidative stress lead to DNA damage in HSPCs.

PS09.05

SARI prevents glioblastoma multiforme progression by inhibiting the recruitment of myeloid-derived suppressor cells via SARI-bearing extracellular vesicles

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Introduction: Basic Leucine Zipper ATF-Like Transcription Factor 2 (BATF2) is implicated in inflammatory response and anti-tumour effects. Although the tumour suppressive function of BATF2 has been reported, its extracellular role in maintaining a non-supportive cancer microenvironment has not been explored.

Methods: In this study, we established GBM orthotopic and subcutaneous tumour models in nude and balb/c mice and Flow cytometry analysis determined the BATF2 inhibitory effects of MDSCs recruiting. We used transwell assay to determine BATF2-positive EVs (EVs-BATF2) inhibitory of the chemotaxis of myeloid-derived suppressor cells (MDSCs) in vitro. In addition, exo-counter detection during the development of the GBM-BATF2 model to demonstrate EVs-BATF2 crosstalk with distant tissues. AMD3100 blocking in tumour model confirms that EVs-BATF2 dominated by the SDF-1a/CXCR4 signalling pathway. In addition, Exo-counter detection of EVs in 32 pairs of gliomas in different stages proposes plasma-EVsBATF2 (plEVs-BATF2) as a prognostic marker.

Results: We found that tumour-derived EVs-BATF2 regulate crosstalk between glioma cells and tumour microenvironment by inhibiting MDSCs recruitment. EVs-BATF2 can be detected in plasma and bone marrow of glioma-bearing mice, this provides direct evidence that glioma-derived EVs can communicate with distant site by crossing blood-brain barrier. Besides, EVs-BATF2 injection significantly reduced SDF-1α expression in the tumour tissues. After blocking SDF-1α signalling by AMD3100, the inhibitory effects of BATF2 overexpression on MDSCs recruitment were rescued. EVs-BATF2 inhibit MDSCs recruiting and secreting MMP2, MMP9, and VEGFA which promote GBM progression. Strikingly, Exo-counter detection of EVs in 32 pairs of gliomas in different stages reveals that the number of plEVs-BATF2 can distinguish stage III–IV glioma from stage I–II glioma and healthy donors.

Summary/Conclusion: Our results suggest that EVs-BATF2 may be an effective circulating biomarker associated with glioma progression. Of note, we are the first to determine the regulatory role of EVs-BATF2 in regulating tumour microenvironment and propose plEVs-BATF2 as a prognostic marker predicting glioma progression and candidate target for GBM therapy.

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PS09.06

Desmoglein 2 modulates extracellular vesicle microRNA content in squamous cell carcinoma

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Introduction: miRNAs are short, non-coding RNAs that regulate gene expression post-transcriptionally.

Previous work has indicated that miRNAs, notably miR-146a and miR-155, play a critical role in SCC tumour development. EVs are membrane-bound vesicles involved in cell-cell communication carrying actively sorted cargo, protected from degradation. The potential pathways these vesicular miRNAs modulate and the implication they have on cancer biology is under active investigation. We have previously shown that the cadherin Dsg2, a stem cell marker, modulates EV release. Dsg2 is upregulated in a number of cancers, including SCC, and correlates with poor prognosis. Here we aim to elucidate the impact of EV-associated miRNAs in SCCs by bioinformatic analysis.

Methods: SCC cells stably expressing Dsg2 were generated and EVs isolated by sequential ultracentrifugation. Total cellular and EV RNA was isolated by miRNeasy, analysed using RNAseq and identified by GRCh37 alignment. Results were confirmed by qPCR. Altered pathways based on targets were identified using miRNet and KEGG pathway analysis. Potential cancer-associated cytokine targets were confirmed by antibody array.

Results: RNAseq revealed 87 cellular and 15 EV miRNAs that were differentially expressed in response to Dsg2 with 7 overlapping. The highest altered miRNAs were validated by qPCR. KEGG pathway analysis determined that these miRNAs have the highest number of shared targets in Cancer, Cell Cycle, and p53 Signalling pathways. Interestingly, miR-155 was upregulated while miR-146a was dramatically downregulated in EVs. Targets of miR-146a, ICAM-1, IL-6, and IL-8, cytokines critical for cancer progression were upregulated.

Summary/Conclusion: These results suggest that the miRNA content of EVs is tightly regulated. By altering the miRNA profile, Dsg2 contributes to the pathogenicity of these EVs by increasing levels of cytokines important for cancer stem cell renewal and metastasis. In addition, these miRNAs may serve as non-invasive diagnostic markers for SCCs.

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PS09.07

Cancer cells grown in 3D release distinct extracellular vesicles during tumour growth and invasion

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Introduction: Cancer cells secrete extracellular vesicles (EVs) that affect tumour progression. The characteristics of EVs produced during tumour growth and invasion are however poorly understood. In this study, we identify the composition and characteristics of EVs produced by non-invasive and invasive tumours and correlate these characteristics with the invasive status of the tumour. For that purpose, we established a protocol for isolating EVs from extracellular matrix (ECM)-based three-dimensional (3D) cancer cell cultures.

Methods: Human prostate cancer PC3 cells were grown in 3D cultures using ECM-based hydrogel, in standard 2D culture conditions and in bioreactor. EVs were isolated from these cultures with differential and density gradient centrifugation. The isolated EVs were characterized with nanoparticle tracking analyses, electron microscopy, immunoblotting and mass spectrometry (MS).

Results: Our results demonstrate that 3D ECM-based hydrogel cell cultures secrete EVs that can be isolated from both the conditioned media and the hydrogel. The invasive 3D cultivated PC3 organoids were found to secrete large amounts of EVs compared to the non-invasive organoids. Interestingly, our MS results revealed that non-invasive and invasive organoids secrete EVs with partially distinct protein cargo.

Summary/Conclusion: We have established a novel protocol for EV production in a 3D cell culture system utilizing ECM-based hydrogel, in which invasive tumour growth can be mimicked. Our method allows the specific isolation and characterization of EVs derived from different stages of 3D culture, such as non-invasive and invasive organoids. Importantly, we found that tumour-derived EVs change in composition during the tumour progression. Taken together, our method can be used to define the distinct EV characteristics involved in cancer invasion.

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PS09.08

Exploring Extracellular Vesicles release inhibition in Prostate Cancer

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Introduction: Although pharmacological treatment options are available for prostate cancer, drug resistance can occur leading to limited survival for patients.

We previously showed extracellular vesicles (EVs) to be causally involved in transmitting drug resistance. This study aimed to evaluate compounds proposed to reduce/block EV release. Specifically, we selected calpeptin and Y2763 (proposed to inhibit EVs budding from the cell membrane) and manumycin A and GW4869 (proposed to inhibit EVs deriving from MVBs). Associated effects on -and consequences of- EV release were then investigated.

Methods: Suitable compounds concentrations that were non-toxic to cells were first selected by performing cytotoxicity assay and flow cytometry (FC). Conditioned medium (CM) was collected from docetaxel-resistant PC3 (PC3 RD) cells after 48 h incubation in dFBS-medium with or without the 4 compounds. EVs were separated from tangential flow filtration concentrated CM using Optiprep density gradient. 1.03–1.16 g/mL fractions were then pooled and washed. EVs were characterised using NTA, immunoblot, TEM and lipid assay and FC. Influences on growth and migration, of EVs continuing to be released (at 1x105EVs/3x103cells, 1x107EVs/3x103cells), were evaluated on recipient DU145 and 22Rv1 cells.

EVTRACK ID EV190113, score 88%

Results: Calpeptin and Y27632, alone and in combination, did not significantly affect quantities of EVs released. However, GW4869 significantly ($p < 0.004$) increased quantities of released EVs, of a larger size; very high protein to lipid ratio; and carrying GRP94 compared to control EVs ($p < 0.006$). This effect was reverted when GW4869 was combined with manumycin A ($p < 0.004$).

Following all compounds treatments, 1x105EVs/3x103cells inhibited 22 RV1 proliferation ($p < 0.0014$), while at 1x107EVs/3x103cells only EVs from manumycin A ($p < 0.05$) and Y27632 ($p < 0.00036$) treated cells reduce 22 RV1 proliferation. EVs following GW4869 treatment significantly ($p < 0.001$) inhibited DU145 migration compared to bulk non-treated control and compared to the effect obtained using the entire pool of EVs ($p < 0.001$).

Summary/Conclusion: While none of the 4 proposed inhibitors significantly reduced EV release, the resulting EVs were less potent in transmitting aggressive behaviour, such as proliferation and migration, to receiving cell lines.

Funding: H2020-MSCA-ITN-TRAIN-EV grant [722148]

PS09.09

Patient-derived organoids represent a novel tool to study the effect of intra-tumoral heterogeneity on EV release in non-small cell lung cancer

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Introduction: Lung adenocarcinoma (LUAD) is the leading cause of cancer-related death with a low 5-year survival. Although the importance of intra-tumoral cellular heterogeneity of solid tumors in the clinical outcome and treatment is emerging, proper models to study its effects on EV release and cargo in human tissues still lack. The 3D organoid technology maintains the cellular and genetic heterogeneity of in vivo tissues and has proved to be so far the best ex vivo model of human cancers. By using patient-derived and mouse organoids we set out i) to compare the EV release from normal and tumor tissues and ii) to follow changes in EV secretion when the relative ratio of tumor cell subpopulations is shifted.

Methods: We used mouse and LUAD patient-derived normal and tumor organoids. The Medical Research Council of Hungary approved our experiments with human samples and informed consent was obtained from patients. EVs were detected by antibody-coated beads, NTA and TEM. Intra-organoid heterogeneity was proved by immunostaining and RT-qPCR.

Results: We provide evidence that both mouse and human normal organoids contain all the bronchiolar cell types. Interestingly, LUAD organoids selected for TP53 mutation contained not only Ki67+ proliferating cells, but differentiated cell types as well. Furthermore, all the lung organoid cultures produced EVs and this was shifted to the smaller size range. Interestingly however, when modifying the proportion of organoid cell types, we observed an increased EV release when more Ki67+ proliferating cells were present both in normal and in LUAD samples.

Summary/Conclusion: Our data show that patient-derived lung organoids represent a novel model to study the role of intra-tissue heterogeneity in EV functions in the humans, leading to improved diagnosis.

Funding: This work was funded by the National Competitiveness and Excellence program NVKP_16-0007 (National Research, Development and Innovation Office, Hungary) and by the National Excellence Program in Higher Education (Ministry of Human Resources, Hungary).

PS09.10

Exosome mediate heart-adipocyte communication after myocardial Ischaemia/Reperfusion and impairs adipocyte endocrine function

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Introduction: By incompletely understood mechanisms, MI patients sustain systemic metabolic disorder. Adipocytes are an important cellular type regulating energy homoeostasis. The impact of MI upon adipocyte function remains unknown. Exosomes (Exo) are critical vehicles mediating organ-organ communication. However, whether and how Exo may mediate post-MI cardiomyocyte/adipocyte communication have not been previously investigated.
Methods: Adult male mice were subjected to MI/R. Serum Exo were isolated 3 hours after R and incubated with 3T3 L cells for 24 hours. The effects of Exo upon adipocyte function were determined.
Results: Compared to control, MI/R Exo significantly altered the expression of 17 genes known to be important in adipocyte function. GO analysis revealed that genes associated with endoplasmic reticulum (ER) function and adipocyte endocrine function are the primary two pathways altered by MI/R Exo. Venn analysis identified 11 mi-RNAs as cardiac-enriched, adipocyte-poor, and ER function-related miRNAs. RT-qPCR confirmed the miR-23a/27a/24-2 family members are the most markedly increased mi-RNAs in MI/R Exo. Incubation of 3T3 L cells with mi-R27a mimic significantly downregulated EDEM3, DsBA-L, and PPAR■, and upregulated PERK and CHOP. Conversely, mi-R27a inhibitor significantly decreased the impact of MI/R Exo upon ER function genes. Additional studies demonstrated EDEM3 and PPARγ (two critical molecules maintaining ER function and adipocyte endocrine function) to be direct targets of mi-R27a. One of the most significant endocrine molecules of adipocyte origin, adiponectin is regulated by PPAR■ at the transcriptional level and by DsBA-L at the post-translational level. We next determined whether MI/R Exo may affect adiponectin expression/assembly. Incubation of 3T3 L cells with MI/R Exo significantly inhibited total and high molecular weight adiponectin expression, an effect blocked by miR27a mimic. Finally, in vivo administration of GW4869 (Exo biogenesis inhibitor) or miR27a inhibitor attenuated adipocyte ER dysfunction and restored plasma adiponectin level in MI/R animals.
Summary/Conclusion: We demonstrate for the first time that MI/R causes significant adipocyte ER and endocrine dysfunction by Exo mediated cardiomyocyte/adipocyte communication via miR-23a/27a/24-2.

Funding: NIH and American Diabetes Association

PS09.11

Pancreatic cancer cell extracellular vesicles drastically alter the behaviour of recipient normal pancreas cells

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Introduction: Pancreatic cancer (PaCa) is predicted to become the 3rd leading cause of cancer-related deaths by 2030. Patients diagnosed with pancreatic ductal adenocarcinoma (PDAC) have a 5-year survival rate ~9%. Detection of pre-neoplastic lesions can potentially improve survival. However, there is currently no screening test for early stage detection. Importantly, PaCa tumours are 90% non-tumorigenic cells. A better understanding of early PaCa oncogenesis is needed. Cancer cells shed extracellular vesicles (EVs) that are internalized by neighbouring and distant cells to induce a myriad of cancer progression events. We hypothesize that in early PaCa oncogenesis, EVs mediate a behavioural change in surrounding normal cells, leading to the formation of this unique stroma. The purpose of this study was to develop a model to examine the phenotypic changes undergone by normal human pancreas cells when they are exposed to PaCa cell EVs.
Methods: EVs were isolated using differential ultracentrifugation with filtration from established (PANC-1, SW-1990, Capan-1 and MiaPaCa-2) and patient-derived xenograft (PPCL-46 and PPCL-68) PaCa cell lines. Cells were grown using EV-depleted FBS. EV isolations were validated and quantified using transmission electron microscopy, quantitative ELISA, immunoblot and nanoparticle tracking analysis. Normal pancreas cells (hTERT-HPNE and HPDE-H6c7) were co-cultured with cancer cell EVs for 24–48 hours. Metabolic activity was measured using a Mito Stress Test on a Seahorse XFe96 Extracellular Flux Analyser.
Results: We discovered that normal cells undergo vast behavioural transformations, including significant morphological changes, increased proliferation and an uncharacteristic invasive capability, when co-cultured with PaCa cell EVs. These responses were EV dose dependent. Further, PaCa cell EVs metabolically reprogrammed normal cells, causing a bioenergetic switch, from a quiescent, aerobic profile to a highly energetic and glycolytic profile.

Summary/Conclusion: Our results indicate that PaCa cell EVs confer enormous transformational properties to normal human pancreas cells in vitro. We hypothesize that EVs impart distinct transformational properties to normal cells in vivo and this influence could unveil novel mechanisms regulating cancer onset and progression. These signals may be detectable before progression of early-stage PaCa to PDAC, leading to the development of assays for earlier diagnosis in patients. Further studies are underway to identify the biochemical mediators of these changes.

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PS09.12

Plasma extracellular vesicles-miRNAs released by hypoxic cells are associated to pro-tumorigenic and immunosuppressive microenvironment in lung cancer

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Introduction: Extracellular vesicles (EV) containing specific subset of functional biomolecules, such as microRNAs (miRNAs) are released by all cell types. It has been widely demonstrated that EV-miRNAs from cancer cells can manipulate the tumour microenvironment modulating the gene expression of recipient cells. We previously identified a three levels risk classifier (MSC) based on 24 plasma-miRNAs associated with lung cancer development and prognosis. The aim of this study was to investigate the potential role of EV-24 miRNAs as mediators of pro-tumorigenic features.

Methods: EVs were isolated from plasma of heavy-smoker individuals with high (MSCpos) or low (MSCneg) risk of lung cancer by differential centrifugation method. Purity of EVs isolated was confirmed by sizing using NTA and TEM analysis and expression of EV-enriched proteins. miRNA levels were analysed by dPCR. In vitro and in vivo analyses were used to assess the biological effect of plasma EVs on different recipient cells.

Results: Levels of miRNAs in EVs correlated with determination of whole plasma (80% of correlation with MSC classifier). MSCpos-EVs stimulated 2D and 3D proliferation of non-tumorigenic epithelial cells through c-myc transfer into recipient cells. Furthermore, MSCpos-EVs increased the ability of HUVEC to form tubular structures compared to MSCneg-EVs. In vivo co-injection of MSCpos-EVs-treated HUVEC with A549 lung cancer cells resulted in an increase of tumour growth compared to MSCneg-EVs-treated HUVEC. miR-126 modulation in EVs with miRNA mimics or in recipient cells using miRNA inhibitors demonstrated that this miRNA is implicated in the autocrine proangiogenic modulation of HUVEC phenotype. MSCpos-EVs induced M2 polarization of macrophages both in vitro and in vivo. We demonstrated using synthetic oligonucleotides that miR-320 is responsible for the immunosuppressive modulation of these cells. Regarding the potential origin of EVs in MSCpos individuals, we observed that hypoxia stimulated the secretion of EVs containing c-myc from fibroblasts, miR-126-EVs from endothelial cells and miR-320-EVs from granulocytes.

Summary/Conclusion: These data show that plasma EVs of high-risk individuals display pro-tumorigenic features, as documented by their ability to induce a pro-angiogenic and immunosuppressive microenvironment suggesting an involvement of EVs in lung cancer development.

PS09.13

Exploration of EV-associated metastatic targets on melanoma cells

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Introduction: Cancer cells secrete EVs that may harbour metastatic proteins. Previous studies have demonstrated the decrease of CD63 tetraspanin expression in melanoma cells are correlated with enhancing its metastatic potential. However, other proteins, such as CD44, CD47, MET and NRP2 which are overexpressed in melanoma cells, are also associated with the spread of cancer. In this study, we sought to investigate the expression of metastatic proteins in EVs derived from murine melanoma B16F10 lineage.

Methods: B16F10 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The cells

supernatant were harvested each 24 hours, filtered through 0.22 μm and ultracentrifuged at 110000 x g for 90 min at 4ºC to pellet EVs. Next, size and concentration was determined using nanoparticle tracking analyses technique, and the morphology of EVs were analysed by negative-staining transmission electron microscopy (TEM). The EV's surface protein were characterized by flow cytometry and protein content was profiled by mass spectrometry.

Results: Our flow cytometry results have shown the presence of tetraspanins markers CD63, CD9 and CD81 on vesicle´s surface. In addition, our assay demonstrated a diminished expression of CD63 molecule in comparison to CD9 and CD81. We have profiled melanoma-EVs by mass spectrometry, identifying the presence of proteins that may be associated to metastasis, such as CD44, CD47, MET and NRP2.

Summary/Conclusion: These preliminary results are consistent with the literature and suggest that melanoma-derived EVs harbour proteins, which may contribute to promote tumour metastasis. In our next step, we plan to generate B16F10 lineages harbouring shRNA vectors, in order to knockdown gene expression of CD63, CD44, CD47, MET and NRP2 to investigate the metastatic potential in vitro, in comparison to parental cells. We also may use syngeneic mice models to explore metastatic potential of genetically modified B16 F10-derived cells.

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PS09.14

Analysing extracellular vesicles from drug-resistant and drug-sensitive cancer cells as potential predictive biomarkers in the liquid biopsy

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Introduction: Overexpression of HER2 occurs in ~ 20% of breast cancers and confers aggressive behaviour and poorer prognosis. Thankfully, a number of drugs such as neratinib have been developed to target HER2, potentially providing substantial benefit for many patients. Nevertheless, it is estimated that up to 70% of patients with HER2-overexpressing tumours do not gain benefit, as a result of innate or acquired drug-resistance. This study aimed to investigate if nano-sized membrane-surrounded extracellular vesicles (EVs) released from drug-sensitive and drug-resistant cells reflect the HER2 status of their cells of origin and thus have potential as minimally-invasive biomarkers.

Methods: EVs were isolated from conditioned media (CM) of HER2-positive cell lines (HCC1954 and SKBR3) and their neratinib-resistant counterparts (HCC1954-NR and SKBR3-NR) that we developed in our laboratory. EVs from CM of a triple-negative breast cancer (TNBC) cell line variant, Hs578 Ts(i)8, were evaluated as negative control for HER2. In brief, CM was centrifuged at 300 g, for 10 min x3 to get rid of any cells. The supernatant was then centrifuged at 200,000 g for 6 h at 4ºC to collect EVs. The resulting EVs were washed in PBS, centrifuged as before, and re-suspended in 100 μL PBS. EV quantities were estimated by nanoparticle tracking analysis (NTS). EV lysates were characterised by immunoblots, for established positive and negative EV markers. Particle concentration as well as size distribution of EVs were measured using the ZetaView (Particle Metrix, Germany). Surface proteins on single EVs were analysed by high-resolution flow cytometry (FC), using an AMNIS ImageStreamX Mark II. All data was submitted to EV-TRACK (EV-TRACK ID: EV190112).

Results: Neratinib-resistant cell line variants were found to have reduced HER2 protein expression compared to their respective drug-sensitive counterparts. Neratinib-resistant cell line variants released fewer EVs, when normalised per number of secreting cells, compared to their-drug sensitive counterparts. Furthermore, EVs from drug-sensitive cells carried HER2, while those from drug-resistant cells lacked HER2 (similar to the EVs from the TNBC cells); reflecting the HER2 status of their cells of origin.

Summary/Conclusion: This study indicates that a reduction in HER2 protein expression is a mechanism by which cancer cells manifest resistance to HER2-targeted drugs (i.e. by making fewer HER2 receptors available on the cells surface to accommodate the drugs activity). Furthermore, EV-carried HER2 seems to reflect the HER2 status of their cells of origin. This suggest that analysis of HER2 on EVs in the peripheral circulation may help predict response to HER2-targeted drugs. Thus, analysis of EVs in appropriate cohorts of patients' specimens is warranted.

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PS09.15

Downregulation of Rab27a expression correlates with replication restriction of oncolytic herpes virus in tumour cells

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Introduction: Rab27a, a small GTPase involved in exosome biogenesis by regulating MVE docking at the plasma membrane, and its expression level highly correlated with oHSV replication ability in vitro. Oncolytic viruses is a newly promising therapeutic agent for cancer treatment. However, more than 50% of tumours naturally showed highly resistant to oncolytic viruses for unknown reasons. Uncovering the underlying mechanisms of resistance to oHSV can offer potential therapeutic targets to enhance oHSV activity to kill tumour cells. In addition, it will give new insights into the identification of therapeutic biomarkers, which can be used to predict patient response to oHSV in clinical.
Methods: Deep-sequencing data showed that lower expression level of Rab27a is present in oHSV resistant tumour cells compared to that in sensitive tumour cells. Then an oHSV resistant MC38 tumour cell line was established by repeated injections with oHSV in MC38 tumour-bear mouse model. Lastly, it was verified that oHSV resistance is associated with a downregulation of Rab27a and overexpression of Rab27a can rescue oHSV replication.
Results: 1) The lower expression level of Rab27a is shown in oHSV resistant tumour cells compared to that in sensitive tumour cells shows in Deep-sequencing data. 2) Furthermore, we established an oHSV resistant MC38 tumour cell line by repeated injections with oHSV in MC38 tumour-bear mouse model. Similarly, in oHSV resistant MC38 cell line, Rab27a expression was lower than parental MC38 cells. And the release of exosomes and virus was decreased in oHSV resistant MC38 cell line. These results were confirmed by Rab27a siRNA knockdown. 3) We verified that in oHSV naturally resistant human cancer cell lines, oHSV resistance is associated with a downregulation of Rab27a and overexpression of Rab27a can rescue oHSV replication.
Summary/Conclusion: Downregulation of Rab27a expression restricts the efficiency of oHSV replication and the spreading ability of the released progeny virus which also provide Rab27a as a potential target and biomarker for oHSV cancer therapy.
Funding: These studies were supported by grants from Shenzhen Overseas High-Calibre Peacock Foundation KQTD2015071414385495, Shenzhen Science and Innovation Commission Project Grants JCYJ201 70411094933148, JCYJ20180306173333907 to Shenzhen International Institute for Biomedical Research.

PS09.16

Inactivation of EMILIN-1 by proteolysis and secretion in extracellular vesicles favours melanoma progression and metastasis

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Introduction: Studies have demonstrated that melanoma-derived extracellular vesicles (EVs) home in distal organs and sentinel lymph nodes favouring metastasis. Although lymph node metastases are themselves rarely life threatening, they could be considered as one of the first step of metastasis in many cancer types. Therefore, defining the mechanisms involved in lymph node metastasis and pre-metastatic niche formation in lymph nodes could bring the clue to block the metastatic process from the beginning.
Methods: We have characterized secreted exosomes from a panel of mouse melanoma models representative of low metastatic potential (B16-F1), high metastatic potential (B16-F10) and lymph node metastasis (B16-F1 R2). We analysed the RNA expression in cells and protein content of exosomes derived from mouse melanoma lymph node metastatic models by RNA sequencing and mass spectrometry respectively. We validated expression by Western-Blot, qPCR and immunofluorescence. To define the mechanism of EMILIN-1 secretion cells were treated with the EV secretion inhibitor (non-competitive inhibitor of sphingomyelinase), GW4869. Cell viability and cell cycle assays with an overexpression model (B16-F1-EMILIN-1) were also performed. In vivo experiments based on subcutaneous and intrafootpad injection for studying the role of this protein during melanoma progression were performed to define the relevance of our findings in vivo. Human paraffin samples were analysed for EMILIN-1 expression by immunohistochemistry.
Results: We found a signature of over-expressed genes and hyper-secreted proteins in exosomes related to lymph node metastasis in the B16 mouse melanoma model. Among them, we found that EMILIN-1, a protein with an important function in lymph node physiology,

was hyper-secreted in exosomes. Interestingly, we found that EMILIN-1 is degraded and secreted in exosomes as a mechanism favouring metastasis. Further, we found that EMILIN-1 has a tumour suppressive-like role regulating negatively cell migration. Importantly, our in vivo studies demonstrate that EMILIN-1 overexpression reduced primary tumour growth and metastasis in mouse melanoma models. Analysis in human melanoma samples showed that cells expressing high levels of EMILIN-1 are reduced in metastatic lesions.

Summary/Conclusion: Overall, our analysis suggests that the inactivation of EMILIN-1 by proteolysis and secretion in exosomes reduce its intrinsic tumour suppressive activities in melanoma favouring tumour progression and metastasis.

Funding: This work was supported by grants from MINECO (SAF2014 54541 R), Asociación Española Contra el Cáncer, Fundación de Investigación Oncológica FERO and MINECO-Severo Ochoa predoctoral program.

PS10: Diverse EV Biomarkers

Chair: Pia Siljander – Faculty of Biological and Environmental Sciences, University of Helsinki, Finland

Chair: Angela Zivkovic – Department of Nutrition, UC Davis

PS10.01

Raman spectral signature of urinary extracellular vesicles as a diagnostic biomarker for diabetic kidney disease

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Introduction: Diabetic kidney disease (DKD) is a leading cause of chronic kidney disease. This complication of diabetes develops in about 10% of type 2 diabetes mellitus (T2DM) patients as a result of chronic hyperglycaemia. Urine is a reach reservoir of extracellular vesicles (EVs) released by glomerular epithelial cells present in the urinary tract. The alterations in molecular content of EVs related to the disease state make them promising biomarkers for DKD.
Methods: T2DM patients with different stage of diabetic kidney disease (n = 24) and healthy subjects (n = 7) were enrolled to the study. Patients were classified into 4 groups according to the estimated glomerular filtration rate (eGFR CKD-EPI) level: G2 eGFR = 60–89, G3 eGFR = 30–59, G4 eGFR = 15–29, G5 eGFR <15 ml/min/1.73cm2. The study protocol was approved by the Jagiellonian University Bioethics Committee (permission no. 1072.6120.268.2018 from 25 October 2018). Urinary EVs were isolated using low vacuum filtration method followed by ultracentrifugation. Raman spectra of urinary EVs were recorded using a Renishaw InVia Raman spectrometer. Data analysis was performed using Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). The size distribution and morphology of EVs were analysed by Transmission Electron Microscopy and Nanoparticle Tracking Analysis methods.
Results: Average Raman spectra obtained for urinary EVs from studied groups showed differences in intensities of specific bands in the region of 400–1800 cm-1. We found significant correlations between mean area under curve (AUC) calculated for Raman bands (phenylalanine, DNA, Proteins, Lipids and Amide I) and selected clinical parameters such as: eGFR, serum creatinine, glucose, urine creatinine. Chemometric methods showed spectral pattern responsible for separation between studied groups. NTA measurements visualized EVs with size of 204.7 ± 96.5 nm.
Summary/Conclusion: Our results showed that characteristic Raman spectra of urinary EVs are promising candidates for new, non-invasive biomarkers for DKD. Raman spectra of urinary EVs can be used to differentiate DKD patients with different stage of kidney damage.
Funding: This work was supported by the National Centre for Research and Development (Grant No. LIDER/9/0031/L-9/NCBR/2018 to A. Kamińska).

PS10.02

Isolation of circulating extracellular vesicles and cfDNA allows for ERBB2 detection in a single aliquot of breast cancer patients plasma

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Introduction: The amplification of ERBB2 gene and the consequent overexpression of the encoded protein HER2 have an important role in breast cancer classification at diagnosis and subsequent treatment decision with the anti-HER2 monoclonal antibody Trastuzumab. FISH and IHC have been used so far to detect ERBB2 gene amplification and HER2 protein overexpression respectively in tissue biopsies. In this context, a major goal for liquid biopsies is to take advantage of the information carried by circulating tumour – derived materials (such as extracellular

vesicles (EVs) and cell free DNA (cfDNA)) to non-invasively detect ERBB2 gene status in the blood. However, the isolation of diverse tumour–derived materials from a single aliquot of patients' plasma and the accurate detection of cancer biomarkers is still challenging.

Methods: By adopting a recently published nickel – based EVs isolation (NBI) protocol1 that allows for recovery of cfDNA after EVs isolation, we generated a high-sensitivity molecular assay to accurately detect ERBB2 amplification and consequent HER2 overexpression on a limited volume of plasma (1.5 ml) collected from breast cancer patients (stage I, II and III) at diagnosis.

Results: 1) we detected ERBB2 amplifications by droplet digital PCR (ddPCR) on the cfDNA isolated from the plasma of ERBB2 positive patients; 2) we confirmed HER2 overexpression on a subset of patients by setting up an antibody–based affinity reaction designed to detect HER2 protein on the surface of the isolated EVs; 3) we succeeded in the quantification of HER2 transcripts enclosed within EVs by performing ddPCR in samples of patients showing a range of circulating tumour – derived material. The specificity and sensitivity of these novel methodological assays were tested on a cohort of healthy individuals (n = 20) and on a cohort of HER2 positive (n = 20) or HER2 negative breast cancer patients (TNBC; n = 20).

Summary/Conclusion: Here we report a pilot study on a novel multimodal method for ERBB2 detection from a minimal amount of plasma. This approach integrates information from cfDNA with EVs-derived RNA and proteins analysis. This proof of concept may ultimately translate into relevant clinical applications for disease diagnosis as well as for therapy selection and monitoring of disease progression.

Reference: [1] Notarangelo, M. et al. EBioMedicine 43, 114–126 (2019).

PS11: Single EV Analysis

Chair: M. Selim Ünlü – Boston University, Department of Electrical Engineering

Chair: Randy Carney, PhD – Department of Biomedical Engineering, University of California

PS11.01

Fluorescence-based strategies for EVs quantification, characterization and assay development

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Introduction: The biological and medical importance of exosomes recognized over the last decade has given rise to a crucial need for the discrimination between true EVs and co-purified nanoparticles, such as lipoproteins, protein aggregates or debris. Additionally, it is imperative to develop methods to identify and characterize EV sub-populations. Considering EV biology and the reliability of labelled biomolecules, we developed both exogenous and endogenous labelling protocols, taking advantage of different dye properties which can target a multitude of compartments. This approach reveals key aspects of EV structure and integrity.
Methods: Nanosized EVs/exosomes were purified by size exclusion chromatography (SEC) from model cell lines and human plasma. Diverse dyes were orthogonally evaluated through different single particle and bulk analysis technologies, such as high-resolution cytometry, Nanoparticle Tracking Analysis and plate fluorimeter. Concomitant profiling of specific EV subpopulations was optimized using antibodies (Abs) against tetraspanins and cell type specific targets and assessed by single particle analysis and ELISA. To assess specificity of labelling protocols we used specific controls such as recombinant RFP expressing vesicles and purified lipoproteins.
Results: Our EV staining protocols allowed for high labelling efficiency and unprecedent EV discrimination, quantification and characterization by combining single particle analysis and bulk measurements in simple matrices (saline buffers) and in complex biofluids (i.e. plasma). Different approaches have diverse and complementary advantages (costs, capacity, sensitivity, informative readout) for implementation in research and diagnostic development flows, directly feeding in-house R&D and QC pipeline.
Summary/Conclusion: Overall, fluorescent EVs are versatile and valuable tracers that can be applied in the optimization of pre-analytical and purification protocols, selection of target biomarkers and diagnostic assay calibration and validation.
Funding: EndeVor (POR-FSE 2014–2020) Region Tuscany and Exosomics R&D Program.

PS11.02

Subpopulations of tissue-derived extracellular vesicles – methodological evaluation for vesicle size measurement

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Introduction: Introduction: Subpopulations of extracellular vesicle (EVs) are commonly classified by their different size, however, the EV size cut off is still under discussion. The aim of the study was to evaluate size range of six EV subpopulations using three methods: electron microscopy (EM), nanoparticle tracking analysis (NTA) and ExoView™.
Methods: Methods: EV subpopulations were isolated from melanoma tissues by a centrifugation based protocol recently established in our lab. Large and small EVs (lEVs and sEVs) were isolated with differential ultracentrifugation and these were further separated into low and high-density fractions (LD and HD). Size of EV subpopulations was then evaluated by: EM

(N = 8, large EVs and small EVs; N = 2, large and small EVs LD and HD), NTA (N = 7) and ExoView™ (N = 1).
Results: Results: Tissue-derived large and small EVs showed difference in size (mean 142 nm vs 75 nm) when examined by EM, whereas NTA and ExoView™ were unable to show a clear difference between the populations (NTA: mean 125.7 nm vs 122 nm, ExoView™: mean 81.7 nm vs 69.2 nm). After iodixanol density gradients, lEVs-LD were significantly larger in size than the sEVs-LD as determined by EM (mean 128.1 nm vs. 67.3 nm) but no difference was observed by NTA and ExoView™ (NTA: mean 134.1 nm vs 124.5 nm, ExoView™: mean 71.1 nm vs 63 nm). HD vesicles from both large and small EVs were the smallest in size when analysed by EM (mean size 41 nm vs 34 nm) but no significant difference was observed by NTA and ExoView™ (NTA: mean 127.8 nm vs 116 nm, ExoView™: mean 98 nm vs 66.4 nm).
Summary/Conclusion: Conclusions: Different methods have different limitations: EM preparations can shrink vesicles, NTA can only detected vesicles above 70 nm and ExoView™ only measures vesicles between 50–200 nm. Of the three different methods, EM analysis of single vesicles visualized in a significant number of micrographs was the only one able to distinguish EV subpopulations by size.
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PS11.03

Imaging of human plasma-derived small-extracellular vesicles using Transmission Electron Microscopy and Structured Illumination Microscopy

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Introduction: Small-extracellular vesicles have an important role in cell metabolism and cell-to-cell communication. Moreover, sEVs when secreted from cells are capable to act as mediators of various neurological diseases. However, sEVs show heterogeneity and this may impact their functions. Therefore, to characterize sEVs at a single-particle level is important to better detail the associated biological activity. In this scenario, we innovatively propose the Structured Illumination Microscopy (SIM) as a technique able to complement the non-optical methods (Transmission Electron Microscopy, TEM) to analyse single sEVs and their markers.
Methods: Human plasma sEVs were separated from healthy cognitive control (ctrl), Mild Cognitive Impairment (MCI) and demented subjects. The sEVs-containing pellet was resuspended in 4% paraformaldehyde or 2% glutaraldehyde solutions. For SIM, sEVs-enriched preparations were washed with the blocking solution and incubated with the primary antibody (L1CAM). The secondary fluolabelled antibody was then added. Between the steps with the blocking solution, the primary and secondary antibodies, two ultracentrifuge steps were performed. The image acquisition was done on a Nikon SIM system with a 100x oil immersion objective. sEVs were imaged with a 3D-SIM acquisition protocol. TEM was performed on 300 mesh formvar copper-coated grids. sEVs-enriched preparations were incubated first with the blocking solution and then, immunogold-labelled for CD63. Samples were counterstained with uranyl acetate and observed under a Jeol 1010 EX electron microscope. Data were recorded with a MORADA digital camera system. Participation to the present study was approved by relevant local ethics committee of Mendrisio and Lugano Hospital and written informed consents were obtained from subjects.
Results: For SIM methodology, only vesicles in the range from 180 to 190 nm were detected, as the final resolution achieved was 160 nm. Instead, for TEM, sEVs under 100 nm were identified. None of these methods provided relevant information about possible difference in morphology of the ctrl-, MCI or demented subjects-derived sEVs.
Summary/Conclusion: Even if both methods identified CD63 or L1CAM-positive vesicles, SIM resolution and the complexity of the protocol represent some disadvantages respect to TEM, that may be the first choice screening technique for EVs analysis to be then completed by SIM for particular tasks.
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PS11.04

Fabrication of nanopore structures via conformal metal-film deposition for EV sensing

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Introduction: Electrofluidics is an emerging technology of combining electronics and nanofluidics. One important device in electrofluidics is an ion transistor in which the ionic current through a nanopore is regulated by gate voltage bias. Here, we suggest a fabrication method of nanopore by introducing focused ion beam (FIB) and atomic layer deposition (ALD) to sense extracellular vesicle (EV) via metal electrode structures.
Methods: We deposited 100 nm-thick silicon-nitrite layers on both sides of silicon wafer by low-pressure chemical vapour deposition (LPCVD). We fabricated rectangular patterns by photolithography followed by reactive ion etching (RIE) on the backside of the wafer. Anisotropic silicon etching by KOH was performed. The front side of the chip was patterned by photolithography followed by Ti/Au deposition for the fabrication of electrode structures. We drilled 50 ~ 100 nm pores in the Si3N4 membrane by FIB. By the ALD process, we deposited highly-conformal metal film, either Platinum (Pt) or Ruthenium (Ru) to shrink nanopores by a self limiting process.
Results: We expect that the ion current through the nanopore is efficiently controlled by the gate-surrounding structures. The nanopore ion transistor can be used to count the number of EVs.
Summary/Conclusion: we suggest a fabrication method of nanopore ion transistors by introducing focused ion beam (FIB) and atomic layer deposition (ALD). This device will be applicable for single EV sensing.
Funding: This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science & ICT (2017M3A9G8083382).

PS11.05

Dimensional reduction of multi-parametric flow cytometry data reveals existence of distinct extracellular vesicle subpopulations

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Introduction: Extracellular vesicles (EVs) are key players in cell-cell communication and increasing evidence has shown that EVs function in cancer by promoting cancer cell motility and metastasis. Analysing tumour-derived EVs in biofluids is attractive because it would be a novel approach to a non-invasive liquid biopsy. Unfortunately, EVs are highly heterogeneous. They vary greatly in size, lipid composition, and cargo and are difficult to distinguish from other small particles in complex biofluids. We have developed a novel flow cytometry method to generate a distinct EV fingerprint to profile biological specimens.
Methods: EVs from cell culture media (purified and unpurified) and biological fluids (plasma and urine) were detected by flow cytometry using features on individual EVs produced by intrinsic (CD63-pHLuorin) and extrinsic (lipophilic dye, Di-8-ANEPPS, and antibodies) fluorescent labels. EV subpopulations were visualized with dimensional reduction (t-SNE and UMAP) of 20–150 features that defined the vesicle size, shape, and fluorescent emission spectra associated with the fluorescent marker. Unsupervised density based clustering (HDBSCAN) in conjunction with supervised machine learning (XGBoost) was subsequently used to define subpopulations. We refer to this method as "EV Fingerprinting".
Results: EV Fingerprinting was successfully used to detect EVs in complex biological specimens and trace their differential enrichment through conventional purification methods. EVs were readily distinguished from protein complexes, lipoproteins and non-lipid particles. Calibration with externally validated purified EV, as well as size, lipid, and fluorescence standards enabled EV Fingerprinting as a rigorous and reproducible method for resolving heterogenous EV samples. EV Fingerprinting applied to conditioned medium from tumour cells and biological fluids from cancer patients reveals unique EV profiles generated by cancer, further supporting the potential of EV fingerprinting as a liquid biopsy.
Summary/Conclusion: Our single-EV analysis approach characterizes whole EV populations in complex biological fluids without the need for purification, reducing time intensive purification protocols and subsequent sample loss, permitting efficient analysis of liquid biopsy samples.

PS11.06

Detection and quantification of extracellular vesicles with cargo protein and RNA Using the Amnis® CellStream® Flow Cytometer

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Introduction: Extracellular vesicles (EVs) are membrane-derived structures that include exosomes, microvesicles, and apoptotic bodies. These EVs – released under normal physiological conditions as well as in the pathogenesis of neurological, vascular, haematological, and autoimmune diseases – have been shown to transfer biological molecules such as protein and RNA between cells, potentially transmitting signals. To understand more about these signalling mechanisms, there is a need for detecting and quantifying EVs with cargo protein and RNA in a reproducible and reliable manner. However, this has been challenging due to the small size of EVs (ranging from 30 to 100 nm in diameter), and the lack of specific staining reagents.
Methods: Here, we utilize the Amnis® CellStream® Flow Cytometer, which enables high-throughput flow cytometry with increased sensitivity for detecting small particles. We demonstrate that a charge-coupled device (CCD)-based, time-delay-integration image capturing system can be used to detect and quantify EVs and their cargo labelled with ExoGlow™-Protein or ExoGlow™-RNA.
Results: In this study, we show flow cytometry data quantifying EV samples that have been labelled with cargo markers for proteins and RNA. The EV cargo contents along with the appropriate control samples will be shown.
Summary/Conclusion: The CCD based detection of the CellStream Flow Cytometer has the sensitivity to quantify EVs and their cargo.

PS11.07

Single EV imaging reveals novel EV biomarkers and DNA cargo

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Introduction: Extracellular Vesicles (EVs) are cell-derived membrane-bound particles that range in size from 30–1000 nm and carry active molecules such as DNA, RNA and proteins. Upon secretion, EVs can execute many biological functions such as initiating intracellular communication or regulating immune responses. Depending on their origin EVs have different characteristics and cargo, making them attractive candidates for early diagnostic and therapeutic applications. However due to their small size and heterogeneity, direct visualization and characterization of the surface markers expressed remains a challenge since these vesicles are below the resolution limit of standard light microscopy.
Methods: Here, we describe a method that provides size analysis of single-EVs, which falls below the diffraction limit of light. This was done with purified EVs, immunostained using fluorescently labelled primary antibodies raised against EV surface markers (CD63, CD81, CD9), specific cargo such as DNA and probed for tissue specific cargo. Characterization of the molecular content and structural properties of surface-immobilized EVs was performed using single-molecule localisation microscopy (SMLM) on the Nanoimager platform.
Results: Multicolour SMLM was used to detect up to three EV biomarkers showing successful characterization of the molecular signature for different EV subpopulations. The distribution of novel components on urinary EVs were visualized for the first time using this approach. In addition, SMLM revealed the presence of DNA on both the surface and also as a cargo inside EVs isolated from tumour cell culture media, which was validated using complementary biochemical characterization.
Summary/Conclusion: SMLM is a powerful technique for single-EV analysis and characterization. Visualization of single-urinary EVs enabled accurate sizing and further insights into novel components expressed on the subpopulation's membrane surface. Together, the data demonstrates that the quantitative abilities of SMLM can significantly enhance our understanding of EVs, as structure, phenotypes, and cargoes can now be successfully resolved.

PS12: EVs in Musculoskeletal System- Bone, Muscle, Tendon

Chair: Aaron James

PS12.01

Extracellular vesicles, microRNA and muscle damage

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Introduction: Working skeletal muscle is a common site for injury due to unaccustomed exercise with or without underlying pathology. Direct analysis of SkM injury requires invasive tissue biopsies. Circulating extracellular vesicles (EVs) are abundant in blood and have been shown to be enriched in microRNA; profiles of which may reflect the state of tissues. EVs may therefore serve as a non-invasive indicator of muscle injury and regenerative processes in vivo.
Methods: Two consecutive bouts of muscle-damaging exercise (plyometric jumping and downhill running) were performed by 9 healthy male volunteers. Serum creatine kinase (CK) and plasma EVs were analysed at baseline, 2 and 24 hr post-exercise. Perceived muscle pain (PMP) was assessed at 2 and 24 hr post-exercise. Large EVs were isolated using a 10 000 G centrifugation step, and small EVs were isolated using qEV columns. EV-enriched isolates were visualized using TEM, and size and numbers were quantified using NTA. Based on NTA results the highest particle fractions (7–10) were pooled for RNA analysis. qPCR was done on plasma, large EVs and small EVs. A group of muscle and immune cell-important miRs were analysed by means of normalization to an exogenous control.
Results: CK and PMP increased post-exercise, providing evidence for muscle damage. TEM revealed an abundant and heterogeneous pool of EVs. A concomitant abundance of EVs was seen with NTA (mean = 1.17 x 1010 particles/ml plasma). Mean EV diameters were 194 ± 22 nm across all timepoints. No change in EV size nor number was seen over time, however, miR-31 decreased at 24 hr when compared to 2 hr in the small EV isolate only. Plasma displayed an immediate increase in myomiRs-1 and −206 at 2 hr, which returned to baseline at 24 hr. In contrast, myomiRs-208b and 486 remained elevated over the 24 hr period. MyomiR-133b and 486, as well as immune-miRs, did not change in EVs or plasma as a result of the intervention.
Summary/Conclusion: The decrease in miR-31 in small EVs at 24 hr is consistent with previous data. No decrease in miR-31 in large EVs suggests specific packaging and hence a specific response to the muscle damage in small EVs. More changes occurred in plasma myomiRs suggesting less specific passive leakage into circulation from damaged cell membranes.
Funding: South African National Research Foundation

PS12.02

Pulsed electromagnetic fields potentiate the paracrine function of mesenchymal stem cells for cartilage regeneration

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Introduction: The mesenchymal stem cell (MSC) secretome, via the combined actions of its plethora of biologically active factors, is capable of orchestrating the regenerative responses of numerous tissues by both eliciting and amplifying biological responses within recipient cells. MSCs are "environmentally-responsive" to local microenvironmental cues and biophysical perturbations, influencing their differentiation as well as secretion of bioactive factors. We have previously shown that exposures of MSCs to pulsed electromagnetic fields (PEMFs) enhanced MSC chondrogenesis. Here, we investigate the influence of PEMF exposure over the paracrine activity of MSCs and its significance to cartilage regeneration. Also, the subsequent extracellular vesicles analysis and isolation are processed for the understanding of how the PEMFs affect stem cell EVs and consequent differentiation induction.
Methods: Conditioned medium (CM) was generated from MSCs subjected to either 3D or 2D culturing platforms, with or without PEMF exposure. The paracrine effects of CM over chondrocytes and MSC chondrogenesis, migration and proliferation, as well as the inflammatory status and induced apoptosis in chondrocytes and MSCs was assessed. The CMs which have

significant effects during chondrogenesis will be analysed by protein and miRNA studies.

Results: We show that the benefits of magnetic field stimulation over MSC-derived chondrogenesis can be partly ascribed to its ability to modulate the MSC secretome. MSCs cultured on either 2D or 3D platforms displayed distinct magnetic sensitivities, whereby MSCs grown in 2D or 3D platforms responded most favourably to PEMF exposure at 2 mT and 3 mT amplitudes, respectively. Ten minutes of PEMF exposure was sufficient to substantially augment the chondrogenic potential of MSC-derived CM generated from either platform. Furthermore, PEMF-induced CM was capable of enhancing the migration of chondrocytes and MSCs as well as mitigating cellular inflammation and apoptosis.

The CMs protein results in the significant promotion chondrogenesis condition showed an increase in proliferation and anti-inflammatory cytokines.

Summary/Conclusion: The findings reported here demonstrate that PEMF-stimulation is capable of modulating the paracrine function of MSCs for the enhancement and re-establishment of cartilage regeneration in states of cellular stress. The PEMF-induced modulation of the MSC-derived paracrine function for directed biological responses 1 in recipient cells or tissues has broad clinical and practical ramifications with high translational value across numerous clinical application.

PS12.03

Effects of extracellular vesicles from blood derivatives on osteoarthritic chondrocytes within an inflammation model

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Introduction: The degenerative disease osteoarthritis (OA) is one of the leading causes of disability especially of elderly people. Besides various treatment options depending on the severity of the cartilage degradation, the application of blood derived products such as platelet rich plasma (PRP) are getting more and more popular in clinical practice due to its high concentration of platelets and the perceived high growth factor levels. Drawbacks of using PRP include high donor variability, discrepancies among preparation protocols and the presence of cells (platelets, leukocytes) which can evoke cellular processes, especially inflammation, when injected into the diseased tissue. One possibility is to isolate only extracellular vesicles (EVs) from blood derivatives to overcome these problems. In the current study the effects of EVs isolated from blood derivatives on OA chondrocytes within an inflammation model was investigated.

Methods: CD14 positive primary monocytes were isolated from citrate anticoagulated whole blood by magnetic bead sorting. Monocytes were differentiated into resting M0 macrophages and activated into M1 macrophages according to published protocols. ELISA measurements verified successful differentiation and activation as IL1β and TNFα levels increased. As control, THP1 monocytes were used. Patient-derived OA chondrocytes were grown in 6 well plates and co-cultivated with activated M1 macrophages which were seeded into thincerts and added to the 6 wells representing the inflammation model. Furthermore, cells were treated for 48 hours with media containing FCS, EV depleted FCS or EVs isolated from PRP or hypACT serum.

Results: Successful differentiation and activation of monocytes (THP1 and primary monocytes) into M1 macrophages was demonstrated by elevated levels of the inflammatory cytokines IL1β and TNFα. Within the inflammation model (co-culture of OA chondrocytes with M1 macrophages), addition of EVs isolated from PRP or hypACT serum resulted in decreased secretion levels of IL1β and TNFα compared to media supplemented with either FCS or EV depleted FCS.

Summary/Conclusion: Taken together, EVs from blood derived products might be chondroprotective and anti-inflammatory mediators which protect cartilage from being degraded during OA.

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PS12.04

1α,25(OH)2D3 regulates growth cartilage matrix vesicle microRNAs

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Introduction: Matrix vesicles (MVs) are small (50–150 nm in diameter) lipid bound extracellular organelles isolated from calcifying tissues including the growth zone (GC) of growth plate cartilage. 1α,25 (OH)2 vitamin D3 (1α,25) is a regulator of GC chondrocytes and the MVs they produce. These MVs are key players in the mineralization process and are selectively enriched with enzymes and growth factors. We found that MVs are also selectively enriched with

microRNAs (miR), including miR-22, miR-122 and miR-451. The aim of this study was to determine the regulatory role of 1α,25 in the packaging of miRNA in MVs by GC cells.

Methods: GC cells were isolated by enzymatic digestion from costochondral GC cartilage harvested from 5 wk-old male Sprague Dawley rats (IACUC approved). Confluent fourth passage GC cell cultures were treated with 10–8 M 1α,25 or vehicle for 24 h. Media were removed, cell monolayers digested with trypsin and cells and MVs isolated by differential ultracentrifugation. RNA was precipitated from cells and MVs. Small RNAseq data were trimmed, aligned and counted before undergoing differential expression analysis. Experimental groups had an n = 3 per variable. Significant differences ($p < 0.05$) were determined using R v 3.6.2.

Results: 1α,25 treatment altered expression of 30 MV miRs compared to control MVs, whereas 5 cell miRNAs were differentially expressed. 62.1% of significantly up or down regulated miR found in MVs overlapped between 1α,25 and vehicle groups with the remaining being uniquely differentially expressed. 1α,25 increased MV miR-150 and decreased miR-384-3p two miRs known to regulate osteoblast proliferation (150 increases, 384 decreases).

Summary/Conclusion: 1α,25 regulates GC chondrocyte and MV behaviour and this study demonstrates that it also impacts the miR packaging within MVs. MiR discovered in MVs have been demonstrated to impact chondrocyte behaviour and the present study indicates that 1α,25 regulates the growth plate through miR delivered by MVs.

Funding: NIH

PS13: Advances in Characterization of EV-Associated Molecules

Chair: John Nolan – Scintillon Institute

PS13.01

Evaluating the impact of culture conditions on human mesenchymal stromal cell-derived extracellular vesicles molecular fingerprint through FTIR spectroscopy

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Introduction: Increasing evidence has proposed extracellular vesicles (EVs) as mediators of many of the therapeutic features of mesenchymal stromal cells (MSC) that have been widely studied in clinical trials over the last years. These EVs have been recognized as nanocarriers of important biological information, which play a central role in cell-to-cell communication. In this context, EVs can be used as an alternative to a cell-based therapy, with reduced risks. The present work aimed to evaluate the impact of different culture conditions on the MSC-derived EVs molecular composition through Fourier-Transform InfraRed (FTIR) spectroscopy.
Methods: EVs derived from MSC from different sources, expanded in two different culture media ((xenogeneic -free (XF) vs serum-containing medium (FBS)) were characterized by FTIR spectroscopy, a highly sensitive, fast and high throughput technique. Moreover, principal component analysis (PCA) of pre-processed FTIR spectra of purified EVs was conducted, enabling the evaluation of the replica variance of the EVs chemical fingerprint in a reduced dimensionality space. For that, different pre-processing methods were studied as baseline correction, standard normal variation and first and second derivative.
Results: EVs secreted by MSCs cultured with serum-containing medium presented a more homogenous chemical fingerprint than EVs obtained with XF medium. The regression vector of the PCA enabled to identified relevant spectral bands that enabled the separation of samples in the score-plot of the previous analysis. Ratios between these spectral bands were determined, since these attenuate artefacts due to cell quantity and baseline distortions underneath each band. Statistically inference analysis of the ratios of spectral bands were conducted, by comparing the equality of the means of the populations using appropriate hypothesis tests and considering the significance level of 5%. It was possible to define ratios of spectral bands, that can be used as biomarkers, enabling the discrimination of EVs chemical fingerprint in function of the culture medium used for MSC expansion and the MSC donor.
Summary/Conclusion: This work is a step forward into understanding how different culture conditions affect MSC-derived EVs characteristics.
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PS13.02

Performance qualification for MicroFlow Cytometers: understanding technical limitations to improve your research

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Introduction: As microflow cytometry and other techniques mature as validated modalities for analysing extracellular vesicles (EV), there has been a concerted effort to improve reproducibility . In order for this reproducibility to occur there has to be a critical understanding of advantages and limitations for each technology. For microflow cytometry, several instruments are available to analyse EVs. Each platform has different limitations as well as advantages over other platforms. To provide the optimal data for your specific research, it is critical to understand the limitations of your platform. To accurately define these limitations, a performance qualification (PQ) of your instrument should be undertaken.
Methods: An Apogee A60 platform was used in these experiments. Experiments were designed with expected ranges and cut-offs for acceptance criteria.Initial tests included autosampling of a 96 well plate with either single or double aspiration, single sample reproducibility and

linearity proportional to flow rate. Other experiments designed to show machine performance included minimal time to achieve valid data, sample volume required for double aspiration, determination of coincidence; detection sensitivity using a spiked sample; flow rate stability for extended periods (5–30 minutes). Tests should also be performed to determine carryover at a range of sample concentrations. If present, the means to remove contaminating samples should be determined. Any performance tests should be applicable to any instrument in the field.

Results: Auto-sampling helped demonstrate consistent data; reproducibility of total events and biomarkers was 2–8% C.V. Detected bead concentrations were linear with flow rates between 0.75 and 10.5 uL/min. Double well aspirations provided similar data with aspirations between 60–80uL. Valid data was achieved for a low abundant target (~200-400 events/uL) after only 30 s, <10%C.V. Detection sensitivity was determined to be ~1/400,000. Carryover ranges were determined in the presence of nominal unstained serum. An optimal number of machine washes was determined. Some membrane stains, such as cell mask and CFSE require much more rigorous cleaning to remove stain carryover.

Summary/Conclusion: To improve data reproducibility, performance qualification of any instrument is key. Operational limitations help define optimal performance parameters of any technology. Understanding the types of experiments to perform for your particular type of characterization technology depends on the requirements you set for your research. A good performance test should be applicable to any related instrument in the field.

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PS13.03

Operating guidelines to determine the size and concentration of extracellular vesicles with microfluidic resistive pulse sensing

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Introduction: The particle size distribution (PSD) of extracellular vesicles (EVs) is commonly measured by tunable resistive pulse sensing (TRPS) and nanoparticle tracking analysis (NTA). Both TRPS and NTA have limitations that hamper the accurate measurement of the PSD of EVs, specifically in the size range from 50 to 100 nm. An alternative technique for measuring the PSD of EVs is micro-fluidic resistive pulse sensing (MRPS). Because a standard operating procedure (SOP) for characterizing EVs by MRPS is absent, we aim to establish a reliable SOP to ensure reproducible PSD measurements of EVs by MRPS.

Methods: Measurements (n = 3) of red-blood cell, prostate cancer cell line supernatant, and human urine and plasma EVs were acquired in 10 × 10 s acquisitions. Two microfluidic cartridges were used to study a dynamic range of 50–450 nm. Samples were diluted into phosphate buffered saline with different concentrations of Tween 20 or BSA. Because the excess of particles affects the detection limit, serial dilutions were performed to find the optimal dilution for each sample. Data were evaluated using Data Viewer software.

Results: The optimal dilution was determined for each sample by maximizing the particle rate and minimizing the measurement time while preserving a robust detection limit of 50 or 65 nm. Moreover, we developed a procedure to optimize the peak filter settings of Data Viewer by fitting data to normal distributions and identifying threshold values for signal-to-noise ratio, symmetry, and transit time within 99% confidence.

Summary/Conclusion: We recommend to use 0.1% w/v BSA in DPBS as sample diluent, because Tween 20 affects EVs as confirmed by flow cytometry. By using orthogonal techniques and well-characterized biological test samples, we developed and validated a SOP for EV detection by MRPS, thereby making MRPS a valuable tool for EV researchers.

PS13.04

Real-time measurements of extracellular vesicles binding kinetics achieved through interferometric imaging in a multiplexed microarray modality

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Introduction: Extracellular vesicles are very promising diagnostic biomarkers. As a matter of fact, the properties of these biological nanoparticles depend on the health conditions of each individual.

However, experiments that involve EVs phenotyping are time consuming, due to 12 h- or overnight incubations. In order to get accurate results, maximizing binding efficiency is a necessity; that normally involves ensuring the saturation of the capture reaction, which can result in an unnecessarily long incubation time. With the ability of label-free kinetic binding measurements using interferometric reflectance sensing in a microfluidic chamber, we perform an optimization of the incubation time in different flow conditions, while demonstrating a new way of multiplexing for real-time EVs specific capture and detection.

Methods: All the real-time binding measurements were performed with the Interferometric Reflectance Imaging Sensor (IRIS). IRIS chips were first coated with an organic polymer (MCP-2), which provides an active surface for probe immobilization. Then, antibodies against CD9, CD81, CD63 markers were spotted at different densities in a microarray modality. The chips were then encapsulated with a glass window to form a microfluidic chamber that allows for imaging the sensor surface.

Samples of HEK-derived extracellular vesicles were flowed across the sensor surface in the IRIS system and real-time images were acquired. Incubation was performed at different flow rates, and in static and stop-flow modalities.

Results: In this work, we focus on the specific capture of EVs under different flow conditions to achieve an optimization of the incubation time. Indeed, through the acquisition of real time binding data, we are able to precisely monitor the equilibrium point of the capture reaction. In this configuration of IRIS, low magnification optics allow for simultaneous detection of binding on hundreds of capture ligand spots. Therefore, surface probes (surface density and specificity) as well as assay conditions can be optimized. We report on the optimization of antibodies against CD9, CD81, and CD63 markers. Since the sensor chips are identical to the single-particle detection assays developed by Nanoview Biosciences, the optimization of binding assays will directly impact the phenotyping of individual exosomes.

Summary/Conclusion: Our method proved to be very efficient in optimizing the most crucial aspects concerning EVs capture – flow conditions, incubation time, surface density and sample concentration.

Funding: This work was partially funded by European Union's Horizon 2020 program (grant n° 766466 – INDEX).

PS13.05

Spectral methods for EV detection in diabetic renal complications

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Introduction: Diabetes is a life treating diseases extending its impairing influence on more than 500 billion of people around the world within upcoming 20 years. The most harmful complication generating high treatment and social costs is diabetic nephropathy, which develops in about 10% of patients suffering diabetes. Still we do not have an effective and direct prognostic biomarker to diagnose renal complications in the primary stage of renal disease.

Methods: Extracellular vesicles were concentrated from diabetic patients' urine and washed to perform spectral analysis: Fourier Transform Infrared Spectroscopy (FTIR), based on the molecular absorption of electromagnetic radiation in the infrared region of the spectrum in a range from 400 cm-1 to 4000 cm-1 and Raman spectroscopy (RS) as a technique based on inelastic scattering of monochromatic light. Both techniques provide information on the chemical structure of compounds by identifying functional groups with high molecular specificity.

Results: Average spectral signature obtained for EVs from urine samples of patients in the different stage of kidney damage allowed distinguishing specific bands, representative for Amide (I/II), lipids, cholesterol and nucleic acids. Spectral parameters correlated with a clinical stage and a commonly used indicator of renal function (creatinine) in diabetic patients.

Summary/Conclusion: Infrared and Raman spectroscopy are promising tools to diagnose and monitor renal function in diabetes.

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PS13.06

Charge detection mass spectrometry measurements of exosomes and other extracellular particles enriched from Bovine Milk

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Introduction: Several existing bioanalytical strategies for purifying and characterizing exosomes have allowed for fundamental progress to be made. Mixtures of EVs can be enriched for exosomes by techniques such as ultracentrifugation and size-exclusion chromatography. But, these processes require large amounts of material that are often difficult to obtain and many different types of particles have similar sizes and densities. It is likely that unique subfractions within enriched samples exist, particularly in complex biological matrices such as blood, urine or milk which remain difficult to characterize and isolate with existing analytical technologies.
Methods: Bovine milk exosomes were isolated via differential ultracentrifugation and resolubilized in 100 mM ammonium acetate. These data were recorded using charge detection mass spectrometry (CDMS). In CDMS, individual particles are reflected back and forth through an electrostatic ion trap where they pass through a sensitive charge detector. Each time a trapped particle enters and exits the detector, its charge (z) and mass-to-charge (m/z) ratio is measured. Mass distributions are generated by multiplying the m/z values by the charge measured for each ion and binning the resulting masses.
Results: The masses of particles in a bovine milk extracellular vesicle (EV) preparation enriched for exosomes were directly determined for the first time by CDMS. Particle masses and charges span a wide range from m ~ 2 to ~90 MDa and z ~ 50 to ~1300 e and are highly dependent upon the conditions used to extract and isolate the EVs. In total, 57,350 particles were detected from eight CDMS measurements. A simple two-dimensional Gaussian model suggests that eight unique subpopulations of particles may be resolvable based on charge and mass. Complementary EM and proteomics analyses confirm that samples are enriched for exosomes. Particles associated with the S1, S2, and S3 families that are centred at ~ 3.5, ~5.9, and ~8.3 MDa, respectively, appear too small to be ascribed to exosomes. The remaining 45,229 (79%) particles detected by CDMS are within the mass range expected for exosomes. While CDMS measurements are at an early stage of development, this approach appears to provide a new physical basis for separating and characterizing EV particles.
Summary/Conclusion: This work describes a novel biophysical approach for measuring and characterizing the masses and charges of the extremely heterogenous population of exosomes and other extracellular particles enriched in bovine milk. As new sample preparation methods, aimed at purifying specific types of exosomes from different cell lines, tissues, and other body fluids continue to evolve, rapid and sensitive CDMS measurements of the physical properties of mass and charge may become an important means of assessing the efficacy of different protocols.
Funding: NIH (R01 GM131100-01). BAB is supported by Indiana University Quantitative Chemical Biology Fellowship (T32GM131994).

PS13.07

In situ detection of exosomal microRNA-10b by fusion with liposome-encapsulated nanomotor

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Introduction: Breast cancer is the most common cause of cancer-associated death in women and has raised global health concerns. Early diagnosis and treatment are crucial to improve the prognosis and survival rate of breast cancer patients. Liquid biopsy is expected to provide a strategy for early diagnosis of breast cancer. Exosomes have been regarded as novel liquid biopsy biomarkers due to their stable cargo of RNAs, lipids, and proteins from their origin cells. Exosomal micro (mi)RNAs have recently been recognized as promising indicators of cancer occurrence and progression. However, most of the reported exosomal miRNA detection methods require the lysis or extraction process, which increases the possibility of sample loss. In situ detection strategies avoid interference from body fluid. In this study, we developed a gold nanomotor fluorescence platform based on liposome fusion for breast cancer exosomal miRNA in situ detection.
Methods: The exosomal miRNA detection platform was constructed using a gold nanomotor (detector) and liposomes (carrier). The DNAzyme amplification sequences which could be especially triggered by miRNA-10b were identified by SDS-PAGE before modified on gold nano-motor and the capacity of the nanomotor was assessed using synthetic target sequence, breast cancer cell MDA-MB-231, miRNA-10b-encapsulated anionic liposomes, and miRNA-10b-expressing exosomes. Three kinds of liposomes were synthesized, characterized, and assessed for loading ability. Membrane fusion effect was evaluated by confocal laser scanning microscopy (CLSM) and nanoflow cytometry. The performance of this method to discriminate between breast cancer patients and healthy individuals was investigated.

Results: The chosen DNAzyme amplification sequences transformed "locked" status to "cleavable" status on target addition, releasing a fluorescence signal. The modified gold nanomotor showed a ten times higher fluorescence signal in the presence of miRNA-10b than the background and no noticeable fluorescence changes from a single-base-mismatch sequence. Moreover, among the three different liposomes, cationic liposomes exhibited great stability, high loading efficiency, and excellent membrane fusion effect. Furthermore, the fluorescent experiments confirmed that cationic liposomes could load and transfer the nanomotors into exosomes for miRNA-10b detection. Finally, we were able to distinguish breast cancer patients and healthy individuals by sensing exosomal miRNA-10b directly from plasma samples without exosome isolation.

Summary/Conclusion: A separation-free and sensitive assay based on DNAzyme amplification technique and membrane fusion effect was established for breast cancer-derived exosomal miRNA-10b detection, which could be a promising tool for the liquid biopsy of breast cancer.

PS13.08

Isolation of exosomes by membrane affinity column increases non-exosomal RNA recovery in comparison to differential ultracentrifugation

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Introduction: Exosome-based liquid biopsy is a potential aid in the diagnosis and prognosis of cancer patients. However, in order to incorporate exosomes into clinical routine, there is a need to compare different isolation methods. Here we analysed the impact, in exosomal RNA yield, of two intermediate recovery/intermediate specificity methods: differential ultracentrifugation (UCD) and a membrane-affinity column (MAC) kit. Although MAC has a faster performance which is more suitable to the clinic, we found that UCD results in a higher recovery of exosomes and less contaminating non-exosomal RNA.

Methods: Exosomes were enriched by MAC and UCD from identical volumes of human plasma (12,000xg, 34 min/0.22 μm filtration/110,000xg, 5 h)(n = 7) and lymphoma conditioned medium(300xg, 10 min/200xg, 20 min/1000xg, 30 min/120,000xg 1.5 h/120,000xg, 1 h) (n = 3). All exosomes were characterized by nanoparticle tracking analysis (NTA), immunoblotting of CD63/CD9/Flotilin/Alix and electron microscopy (TEM). Exosome pellets were pre-treated with proteinase K (1 mg/ml/56°C/10 min) and RNAse A (2 mg/ml/37°C/20 min) before phenol-chloroform/glycogen RNA extraction. RNA yield was measured by both fluorometer and bioanalyzer.

Results: Isolation of exosomes by UCD, in both plasma and medium, resulted in a higher yield in comparison to MAC. This was shown by an augmented intensity of marker bands in the UCD samples ($p = 0.005$, $n = 4$) as well as by an increased number of exosomes in TEM. In contrast, MAC final exosomal fraction (from both plasma and medium), resulted in a 13-fold and 200-fold increase in RNA, respectively, in comparison to UCD when measured by fluorometer. This was confirmed by bioanalyzer. To further investigate if the isolated RNA was inside exosomes, MAC and UCD final exosomal fractions were treated with enzymes before RNA extraction. Importantly, no significant effect was observed in RNA yield.

Summary/Conclusion: Together this data shows that MAC isolates fewer exosomal structures than UCD; however, it shows a greater recovery of total RNA thus suggesting that non-exosomal RNA is getting trapped by MAC. The fact that RNA showed resistance to RNAse A cleavage might result in the inclusion of contaminating RNA into the sample.

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PS13.09

Fluorescent Extracellular Vesicle characterisation using a long lasting cell tracker probe

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Introduction: The visualisation of extracellular vesicles (EV) via fluorescent means presents a promising method of characterisation. There is potential to specifically label individual EV families in order to distinguish them in samples of multiple EV subgroups. Nevertheless, fluorescent EV labelling has proven difficult. Nanosight NTA© is the standard for visualising EV and does possess means of fluorescence capture.

Methods: This method utilised a cell division probe consisting of 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) and anhydrous DMSO, forming a non-specific long-lasting fluorescent probe typically used for in vivo cell tracking that is internalised by cells. Human vascular endothelial cells (HECV) at confluence were introduced to the cell

tracker at a concentration of 10uM, before cells were incubated in serum free media at 21% or 1% oxygen, respectively. This fluorescence was visualised using a 488 nm fluorescent Nanosight© NTA laser under light scatter and fluorescent means of capture.

Results: The use of a CFSE Cell Tracker Probe proved that EVs were able to take up the probe from their parent cells. EVs from the cells with the tracker probe internalised, also contained the probe and exhibited fluorescence.The probe was long enough lasting to be identified on Nanosight and has shown promise with no significant difference in EV number when compared to light scatter.

Summary/Conclusion: Identifying a potentially cost effective method for visualising EV and combating photo-bleaching, the cell tracker proves that the technique works on a functional level. Further investigation is needed to determine if this can be used to specifically label and identify individual EV families in a population. This could allow for the numbers of individual EV families to be shown in a given sample.

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PS13.10

Multiplex electrochemiluminescence immunoassays for phenotyping of intact extracellular vesicles (EVs)

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Introduction: There is a need for better techniques for characterizing EV populations. We developed a sensitive multiplexed electrochemiluminescence (ECL)-based assay format to characterize EVs in cell-conditioned medium (CCM) and human biofluids. Here we use the format to analyse EV samples for the presence of 66 EV surface proteins, and to identify changes in EV phenotype associated with different cell lines, purification methods and growth conditions.

Methods: Multiplex plates were prepared on MSD's U-PLEX® platform with antibodies for 66 putative EV-surface proteins. Each well displayed an array of nine specific capture antibodies and a negative control antibody. EVs from samples were captured on the arrays and then detected with a cocktail of anti-tetraspanin antibodies (CD9, CD63 and CD81) conjugated to an ECL label. Three distinct cell types were grown at two sites, MSD and ATCC. Resulting CCM were each purified by four common methods: tangential flow filtration, PEG-based precipitation, size-exclusion chromatography and centrifugal ultrafiltration. All samples were also assayed without purification.

Results: Fifty-five of the surface markers were detected on intact EVs from at least one evaluated cell type. Datasets were analysed using correlation matrices, hierarchical clustering, and machine learning. For each cell type, when comparing unpurified CCM grown at different sites or EVs prepared by different purification methods, we typically observed correlations above 0.9, indicating that the purification methods did not introduce bias to EV phenotypes, and that the assay format can provide robust phenotypic information without any purification of EVs. Two unsupervised clustering analyses – hierarchical clustering and t-distributed stochastic neighbour embedding – both generated well-separated clusters for each of the cell types, regardless of purification method or source.

Summary/Conclusion: We developed multiplex EV surface marker assays and demonstrated their use for multimarker EV phenotyping. This flexible format enables rapid assay development for new EV subpopulations with or without sample purification. These results also demonstrate EV surface marker phenotyping via multiplex ECL assays may be used to distinguish EV populations from various cell types, and characterize bias introduced by purification.

PS13.11

Detection of MISEV recommended EV protein-markers using automated western blotting

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Introduction: The limited amount of material and the diverse methods for isolation of extracellular vesicles (EV) pose unique challenges to proper characterization of experimental EV preparations. The "Minimal Information for Studies of Extracellular Vesicles" (MISEV) guidelines recommend characterizing preparations for both trans-membrane-, cytosolic- and contaminating non-EV proteins. However, compliance with these guidelines can be a considerable effort due to lack of easy and robust analytical protocols and the time consuming and user variable nature of standard western blotting protocols. Here we present a simple

method for isolation of EVs and a simple western blotting platform for automated protein separation and immunodetection of MISEV-recommended proteins.

Methods: Total EVs were isolated by affinity-membrane spin columns from pre-filtered 0.5–4 mL plasma or 2–20 mL urine, respectively. Intact vesicles were eluted and the EV-depleted biofluid fraction was collected from the flow-through. A small fraction (4 μL) was analysed by a simple western blot workflow providing automated capillary electrophoresis-based protein separation and immunodetection, characterizing each fraction for presence or absence of MISEV-recommended proteins.

Results: A range of specific antibodies were identified and the EV fractions were shown to be enriched in EV-proteins, whereas contaminating non-EV proteins were significantly reduced. Isolation of EVs was necessary to allow detection of the low abundant EV protein markers, whereas non-EV proteins were readily detectable both in the neat biofluids and in the EV-depleted flow-through. We characterized the effect of washing on the purity of EV isolates and defined the dynamic range of the workflow using titrations of input volume of both plasma and urine EV isolations.

Summary/Conclusion: Simple western blotting protocols were established for quality control of isolated EVs in accordance with MISEV-guidelines. EVs isolated using affinity-membrane spin columns were shown to be enriched in EV markers and depleted for non-EV proteins.

PS13.12

AL-PHA beads: a library of extracellular vesicle-associated metalloproteinase biosensors

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Introduction: Lung cancer is a leading cause of cancer-associated deaths and early detection could lead to improved patient outcomes. Extracellular vesicles (EVs) are readily accessible from patient bio-fluids and could be a source of early-stage lung cancer biomarkers. Recent studies indicate that EVs contain metalloproteinases. The matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase (ADAMs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) are highly promising cancer biomarker candidates that have complex roles in cancer pathogenesis and metastasis. Importantly, within the context of lung cancer, the detection of ADAM10 proteolytic activity might be more informative than the level of ADAM10 protein. Therefore, the development of low-cost metalloproteinase biosensors could serve as useful biomarker research tools.

Methods: To this end, we developed Advanced proteoLytic detector PolyHydroxyAlkanoates (AL-PHA) beads – a library of biodegradable, biopolymer-based protease biosensors. Broadly, these biosensors utilise PhaC-reporter fusion proteins that are bound to microbially manufactured bioplastic beads. These PhaC-fusions also incorporate specific protease cleavage sites. In the presence of a specific protease, reporter proteins are cleaved off of the AL-PHA beads – resulting in a loss of bead fluorescence that can be measured using flow cytometry. These biosensors were assayed using either metalloproteinases, conditioned media or EVs from in vitro cancer models.

Results: Human metalloproteinase recognition motifs were identified in the literature and a total of 70 different AL-PHA bead biosensors were designed. A control, TEV-specific biosensor detected 0.5 U of tobacco etch virus protease activity and the MMP14 biosensor successfully detected 0.033 mU of recombinant MMP14 activity. A panel of AL-PHA biosensors also detected an array of MMP, ADAM and ADAMTS proteases within cell conditioned media and isolated EV samples from in vitro cancer models.

Summary/Conclusion: AL-PHA biosensors successfully detected EV-associated metalloproteinases and in the longer-term, we envision that AL-PHA beads may lead to the development of low-cost cancer diagnostics for use in resource-limited settings.

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PS14: Scholarship and Funding Opportunities

Chair: Lucia Languino – Professor of Cancer Biology, Thomas Jefferson University

Chair: Alissa Weaver – Professor, Department of Cell and Developmental Biology, Vanderbilt University School of Medicine

PS14.01

Program to assess the rigour and reproducibility of extracellular vesicle-derived analytes for cancer detection

Matthew Young and Sudhir Srivastava

National Cancer Institute, Rockville, USA

Introduction: Cancer cells release more EVs than normal cells and EVs secreted from tumour cells can promote tumour progression, survival, invasion and angiogenesis. The EV cargo may mirror the altered molecular state of the cell of origin. Therefore, EVs have potential for the development of non-invasive markers for early detection of cancers. EVs and their cargo also have the potential to be multiplexed with other molecular markers or screening modalities (e.g., imaging) to develop integrated molecular-based computational tools for the early detection of cancer.

One challenge with using EVs as a biomarker is the lack of robust and reproducible methods for the isolation of a pure vesicular population. There is a lack of clear consensus for an optimal method of isolation of a pure EV population that is devoid of contamination with similar-sized vesicles of different origins. There is also a lack of standards to ensure rigour reproducibility.

Methods: The current funding opportunity announcement (FOA), PAR20-053, is promoting research on the isolation and characterization of extracellular vesicles (EVs) and their cargo for the discovery of biomarkers to predict cancer and cancer risk.

Results: The previous cycle of this FOA, PAR16-267/277, successfully funded 7 R01 and 4 R21 grants. These awards are focused on proteomics profiling of EVs, effect of methodological and biological variability, asymmetric-flow field-flow technology, therapeutic monitoring, LSS and SERS lab on a chip optical spectroscopic, EVs in obesity-driven hepatocellular carcinoma, nanoscale structure and bio-molecular heterogeneity, urinary EV DNA, and EV markers in paediatric cancers.

Progress from these awardees have shown separation of two discernible exosome subpopulations and identified a distinct nanoparticle, the exomere (Nature Cell Biology, 2018); and have shown that large-EVs contain the entire genome of the cell of origin, including cancer-specific genomic alterations (Journal of Extracellular Vesicles, 2019). Protocols that critically evaluate and refine the existing methodologies to improve utilization of EVs in clinical use have been shared (Nature Protocols, 2019).

Summary/Conclusion: Drs. Sudhir Srivastava and Matthew Young are the programme directors for the PAR which began accepting applications on 5 January 2020. This and other EV funding opportunities will be discussed.

Funding: This is a Funding Opportunity Announcement offered by the National Cancer Institute.

PS15 = OP3

Oral with Poster Session 3: Neurological & ID

Chair: Jereme Spiers – La Trobe University

Chair: Sophie Rome – INRAE, department of Human Nutrition

PS15.01 = OP3.01

Mitovesicles: a new extracellular vesicle of mitochondrial origin altered in ageing and neurodegeneration

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Introduction: Brain extracellular vesicles (EVs) are heterogenous and include previously described microvesicles and exosomes. Herein we characterized a formerly unappreciated population of mitochondria-derived EVs that we term "mitovesicles". Mitochondrial dysfunction is a well-established hallmark of ageing and neurodegenerative disorders as Down syndrome (DS). Hence, we examined mitovesicle levels and cargo under these conditions to characterize in vivo mitovesicle biology and responsiveness to mitochondrial stressors.

Methods: Employing a high-resolution density gradient, distinct and novel populations of EVs were isolated from murine and human DS and diploid control post-mortem brains or from cell media. Morphometric EV features were analysed by nanoparticle tracking analysis and cryogenic electron microscopy, while EV constituents were characterized by Western blotting, mass spectrometry, lipid profiling and mitochondrial RNA qPCR.

Results: We identified a population of double-membrane, electron-dense brain EVs containing multiple mitochondrial markers ("mitovesicles") that are highly distinct from microvesicles and exosomes. Proteomic data show that mitovesicles contain a unique subset of mitochondrial proteins while lacking others, such as Tom20. Mitovesicles have a lipid composition that is unlike that of previously described EVs and is consistent with mitochondrial origin. Functionally, the complex-III inhibitor antimycin-A stimulated in vitro mitovesicle release into the cell media, suggesting an interrelationship between mitochondrial dysfunction and mitovesicle biology. In mouse brains, mitovesicle levels increased with age and were found to be higher in DS compared to diploid controls. Mitochondrial RNA and protein levels were also altered in DS compared to diploid controls.

Summary/Conclusion: We describe a previously unidentified type of metabolically competent EVs of mitochondrial origin that we designate mitovesicles. Our data demonstrate that brain mitovesicle levels and cargo are tightly regulated in normal conditions and are modified during pathophysiological processes in which mitochondrial dysfunction occurs, suggesting that mitovesicles are a previously unrecognized player in mitochondria quality control and may have a role in the trans-cellular tissue response to oxidative stress.

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PS15.02 = OP3.02

Reducing extracellular vesicle release with a novel neutral sphingomyelinase 2 inhibitor for the treatment of Alzheimer's disease

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Introduction: Alzheimer's disease (AD) is a devastating neurodegenerative disease leading to progressive memory loss and ultimately death with limited therapeutic options. Growing evidence supports the theory that toxic proteins, like tau and amyloid, may propagate from diseased cells by packaging toxic proteins into extracellular vesicles (EVs) and releasing them to infect other cells. One enzyme involved in the

biogenesis of EVs is neutral sphingomyelinase 2 (nSMase2), which catalyzes the hydrolysis of sphingomyelin to produce phosphorylcholine and ceramide. Several groups have reported improved cognition and reduced tau propagation when nSMase2 is pharmacologically inhibited or genetically knocked down in AD mouse models. Unfortunately, current nSMase2 inhibitors are not suitable for clinical development due to poor solubility and inadequate pharmacokinetic profiles.

Methods: Our group carried out a high-throughput screening campaign followed by extensive medicinal chemistry efforts leading to the discovery of phenyl (R)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo [1,2-b] pyridazin-8-yl) pyrrolidin-3-yl) carbamate (PDDC), an orally active, nM potent inhibitor with excellent selectivity and brain penetration. We tested PDDC's ability to inhibit exosome release in cultured primary glial cells as well as an in vivo model of acute EV release. We then treated 5XFAD mice with 10 mg/kg of PDDC daily for six months and monitored their behaviour in the fear conditioning assay.

Results: PDDC dose dependently reduced EV release from cultured primary glial cells and significantly reduced plasma EV numbers in an in vivo model. Following chronic treatment with PDDC, 5XFAD mice demonstrated significantly improved cognitive function in the fear conditioning assay.

Summary/Conclusion: These promising findings are currently being expanded using mouse models of tau propagation. If successful, these data would support PDDC as a novel compound for targeting the pathological spread of tau as a therapeutic for AD.

PS15.03 = OP3.03

Profiling EVs in the anterior cingulate cortex of individuals with major depressive disorder

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Introduction: Major Depressive Disorder (MDD) is one of the leading causes of disability worldwide, affecting 20% of the population. The environment has been thought to play a role in the disease development, resulting in biological changes mediated by epigenetic mechanisms. MicroRNA's (miRNA) are well known epigenetic regulators that are disrupted in the depressed brain, and they are packaged into extracellular vesicles (EVs). EVs have emerged as means of intercellular communication, a process that is also disrupted in MDD. They are thought to transfer miRNA between cells, which can alter gene expression in recipient cells. Therefore, we hypothesize that EV cargo is altered in MDD subjects compared to healthy controls (HC). The aim is to extract EVs from human post-mortem anterior cingulate cortex, a region previously associated with depression, and profile the miRNA cargo and compare it between MDD subjects and HC.

Methods: Post-mortem human brain tissue from the anterior cingulate cortex of 20 MDD subjects and 20 HC was mildly dissociated in the presence of collagenase type III. Residual tissue, cells, and large vesicles were eliminated, and EVs were isolated using size exclusion chromatography. The quality was assessed by western blots and transmission electron microscopy (TEM). RNA was extracted and a small-RNA library was constructed and sequenced using the Illumina Platform. Differential expression analysis was then performed.

Results: Western blots showed little to no Endoplasmic Reticulum (Calnexin), Golgi (BiP), or mitochondrial (VDAC) contamination, along with enrichment of the exosomal marker CD9. TEM images showed the typical cup-shaped morphology with sizes mostly between 30 and 200 nm. Preliminary sequencing results revealed that miR-33a-5p, which is predicted to target glutamate receptors, is downregulated in EVs from MDD subjects.

Summary/Conclusion: High quality EV extractions can be obtained from post-mortem brain tissue using our method. This will be the first study to profile brain-derived EV miRNA in the context of depression. Future studies will be needed to determine the effect of the different levels of miR-33a-5p. This could provide novel mechanistic insights into the pathophysiology of MDD and will serve as a starting point to examine the potential role of EVs in MDD pathology.

Funding: Réseau québécois sur le suicide, les troubles de l'humeur et les troubles associés (RQSHA) Student Award

McGill Faculty of Medicine Internal Studentship Award (Max E. Binz Fellowship)

PS15.04 = OP3.04

Combining nanomagnetic isolation and artificial intelligence to guide the treatment of traumatic brain injury

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Introduction: Traumatic brain injury (TBI) is characterized by diverse primary mechanisms of injury that lead to the development of secondary pathological cascades that drive neurological deficit post-TBI. Inability to separate patients based on the presence of these different endophenotypes represents a major challenge for diagnosis and treatment of TBI.

Extracellular vesicles including exosomes isolated from patient plasma have emerged as promising potential biomarkers for TBI due to their ability to cross the BBB into systemic circulation with molecular cargo intact for analysis. We have developed a novel microfluidic platform for rapid isolation of brain-derived EVs providing a tool with which the biochemical state of neurons and glia can be directly assessed post-TBI. We used the ultra-sensitive, single molecule array (SIMOA) to quantify concentrations of 7 protein biomarkers from the plasma and brain derived EVs from mild TBI patients and controls. By combining multiple protein biomarkers, we could discriminate mTBI patients from controls in both the training and the blinded test set.

Building on this work, we are also characterizing single EV heterogeneity of neuron derived EVs by developing novel droplet based digital assay for single EV quantification at ultra-low concentration. Droplet based assay for single EV analysis would potentially be very informative for early disease diagnosis and therapy decision.

Methods: Our microfluidic platform for EV isolation consists of tracked-etched membranes with millions of nanopores (600 nm), coated with a magnetic film (NiFe) to precisely capture immunomagnetically labelled brain-specific EVs from plasma. Single molecule array (SIMOA) was used to quantify concentrations of the 7 protein biomarkers (Tau, UCHL-1, NFL, GFAP, IL6, IL10, and TNF) in the plasma and brain-derived exosomes of mild TBI (mTBI) patients and controls. To identify single EV, we applied droplet based enzyme-linked immunosorbent assay and encoded the fluorescent signal for single EV quantification within parallelized microfluidic platform.

Results: We report that concentrations of plasma and exosome GFAP, NFL, and UCHL1 were elevated in mTBI patients compared to controls ($p < 0.05$), and that each of these biomarkers are uncorrelated with one another. Discrimination of mTBI patients from controls was most accurate when machine learning algorithms on the panel of biomarkers. Specifically, combining plasma NFL, GFAP, IL6 and TNF- with Tau from GluR2+ EVs showed 88% accuracy with 80% sensitivity and 100% specificity.

Summary/Conclusion: This data suggests that neuron-derived exosomes contain information that characterizes the injured and recovering brain. It also suggests that analysis of a panel of biomarkers from a combination of both blood and exosomal compartments could lead to more accurate diagnosis of mTBIs.

PS15.05 = OP3.05

L1CAM is not associated with extracellular vesicles in cerebrospinal fluid or plasma

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Introduction: Neurons in living psychiatric and neurological patients are inaccessible for cell type specific analysis of RNA and protein. Our understanding of these diseases instead relies upon imperfect sources of biochemical information such as post-mortem brain tissue analysis and animal models. Furthermore, there is a paucity of biochemical assays available to diagnose and manage brain diseases. Extracellular vesicles (EVs) present an opportunity to noninvasively sample the contents of neurons in cerebrospinal fluid (CSF) and plasma. In order to isolate neuron-derived EVs (NDEVs), a cell type specific transmembrane protein is necessary for immunocapture. L1CAM, a protein abundant on the surface of neurons, has been used extensively in the literature for NDEV isolation. However, L1CAM exists in humans in several isoforms without a transmembrane domain, and as such it can be secreted as a free protein. Additionally, the ectodomain of L1CAM can be cleaved off of the cell surface in physiological processes. It remains to be demonstrated whether the L1CAM found in CSF and plasma is EV associated, or if it is instead a spliced or cleaved isoform behaving as a free protein.

Methods: Using Single Molecule Arrays (Simoa), a digital form of ELISA, as well as Western blotting, we quantify EV markers (CD9, CD63 and CD81) as well as L1CAM and Albumin. We use these assays to determine in which fractions of size exclusion chromatography (SEC) and density gradient the L1CAM appears. We also immunocapture L1CAM from CSF and plasma and perform Western blots for the internal and external domains of L1CAM.

Results: Simoa and Western blot analysis of SEC and density gradient fractions demonstrated that while the EV markers peaked all together, L1CAM eluted in the free protein fractions along with Albumin in both CSF and plasma. When immunoprecipitation was

performed, Western blotting revealed different isoforms of L1CAM in CSF and plasma.
Summary/Conclusion: Our data utilize a multitude of distinct techniques that converge to demonstrate that L1CAM is not associated with EVs in CSF or plasma. Furthermore, our data suggest that the isoforms present in CSF and plasma are distinct, which indicates that the L1CAM in plasma is likely not coming from the brain. This data call into question the utility of L1CAM as a NDEV marker and point to the need to find novel candidates for immunoprecipitation of NDEVs.
Funding: Chan Zuckerberg Initiative

PS15.06 = OP3.06

An in vitro and in vivo perspective on the role of erythrocyte-derived extracellular vesicles in Parkinson's disease pathology

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Introduction: In Parkinson's disease (PD), α-synuclein (α-Syn) aggregates known as Lewy bodies (LB) are present in both the central and peripheral nervous system. Furthermore, data showing that α-Syn can spread from PD patients to transplanted tissue has led to a new theory postulating that pathological forms of α-Syn can drive disease by "infecting" healthy cells and corrupting normal proteins. The exact routes and mechanisms involved in such spreading are yet to be fully understood but it is known that α-Syn can be secreted from cells and transported via extracellular vesicles (EV). EV derived from erythrocytes (EEV) are of particular interest in this regard as they have been shown to contain α-Syn.
Methods: We first optimized a protocol for the isolation of fluorescently labelled human EEV. The capacity of these EEV to cross the blood-brain barrier (BBB) was then evaluated in vitro using a Boyden chamber composed of primary human brain endothelial cells. Next, EEV were added to a more complex and physiologically relevant 3D human BBB model including iPSC-derived brain microvascular endothelial cells. In both in vitro protocols, flow cytometry was performed on media collect from each compartment to determine the number of EEV. Immunofluorescence was performed to assess the localization of fluorophore tagged EEV. We are also using an in vivo paradigm for the extraction and testing of EEV spread and an in situ cerebral perfusion (ISBP) model in WT mice to investigate if and how EEV cross the BBB using confocal microscopy.
Results: In both in vitro models, flow cytometry analyses showed that fluorescently tagged EEV added to the luminal side traversed the endothelial cell barrier. Confocal analysis revealed that some EEV could also be found within endothelial cells themselves. Ongoing experiments are being conducted in our newly developed 3D BBB to further confirm these results. Our preliminary in vivo experiments showed that fluorescently labelled beads, similar in size to EEV, used in the ISBP experiments are detectable in the brain parenchyma of injected WT mice using confocal microscopy. Preliminary work also includes ISBP injections of EEV in 6-month-old WT mice, (n = 6/groups) derived from PD patients (at different stage of the disease) and a healthy individual as a control.
Summary/Conclusion: Our preliminary data suggests that EEV can indeed move across the BBB in both in vitro and in vivo experimental setups. Ongoing experiments will determine the dynamics and processes involved in this transport and whether EEV can precipitate and/or exacerbate disease-related features.
Funding: FRQS

PS15.07 = OP3.07

Exosomes from N-Myc amplified neuroblastoma cells induce migration and confer chemoresistance to non-N-Myc amplified cells: implications of intra-tumour heterogeneity

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Introduction: Neuroblastoma accounts for 15% of childhood cancer mortality. Amplification of the oncogene N-Myc is a well-established poor prognostic marker for neuroblastoma. Whilst N-Myc amplification status strongly correlates with higher tumour aggression and resistance to treatment, the role of N-Myc in the aggressiveness of the disease is poorly understood. Exosomes are released by many cell types including cancer cells and are implicated as key mediators in cell-cell communication via the transfer of

molecular cargo. Hence, characterising the exosomal protein components from N-Myc amplified and non-amplified neuroblastoma cells will improve our understanding on their role in the progression of neuroblastoma.

Methods: In this study, comparative proteomic analysis, nanoparticle tracking analysis, transmission electron microscopy, RNAi-based knockdown, migration and cellular survivability assays were performed to understand the role of exosomes isolated from cells with varying N-Myc amplification status.

Results: Label-free quantitative proteomic profiling revealed 968 proteins that are differentially abundant in exosomes released by the N-Myc amplified and non-amplified neuroblastoma cells. Gene ontology-based analysis highlighted the enrichment of proteins involved in cell communication and signal transduction in N-Myc amplified exosomes. Treatment of less aggressive SH-SY5Y cells with N-Myc amplified SK-N-BE2 cell-derived exosomes increased the migratory potential, colony forming abilities and conferred resistance to doxorubicin induced apoptosis. Incubation of exosomes from N-Myc knocked down SK-N-BE2 cells abolished the transfer of resistance to doxorubicin induced apoptosis.

Summary/Conclusion: These findings suggest that exosomes could play a pivotal role in N-Myc-driven aggressive neuroblastoma and transfer of chemoresistance between cells.

PS15.08 = OP3.08

Dissecting the heterogeneity of extracellular vesicle sub-populations at single vesicle level

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Introduction: Quantification and characterization of single extracellular vesicles (sEVs) based on surface markers can aid in dissecting the heterogeneous landscape of EV subpopulations. We and others have demonstrated the potential of imaging flow cytometry (IFC) to perform sEV characterization. We recently showed release of protoporphyrin (PpIX) positive sEVs by 5-aminolevulinic acid (5-ALA) dosed glioma cells, in vitro and in vivo. Rickfels et al. also used IFC to demonstrate the enrichment of CD63 +/CD81+ EVs in the plasma of glioma patients. Herein, we performed in vitro studies to characterize EV subfractions using 5-ALA as well as EV and CNS specific surface markers.

Methods: We use IFC to characterize EVs released by glioma using 5-ALA, fluorescently labelled EV (CFDA-SE, CD81) and glioma specific (tenascin C and epidermal growth factor receptor vIII, EGFRvIII) markers. Furthermore, we characterized EVs released by EGFRvIII positive glioma cells treated with dexamethasone, a steroid commonly used in glioma patients, to determine the effect of steroids on EV release. EVs were quantified by IFC and results were confirmed by qPCR for the levels of EGFRvIII mRNA.

Results: Firstly, we optimized protocols to label glioma sEVs using fluorescently labelled EV markers (CFDA-SE, CD81) and tumour specific markers (tenascin C and EGFRvIII). Of the total EVs (CFDA-SE), we demonstrate that 58% are tenascin C positive, 2.7% are EGFRvIII positive and 1.6% are 5-ALA positive. There was only a minor overlap (<16%) between the sub-populations. Finally, we show that dexamethasone treated glioma cells release lower total EVs (2.5-fold), tumour specific EVs (2.8-fold; EGFRvIII), EGFRvIII mRNA compared to mock treated cells.

Summary/Conclusion: We demonstrate the potential of IFC to monitor sEVs released by glioma cells exposed to different stimuli. This allows the characterization of EV sub-populations providing a working model to understand the dynamics of tumour EVs at a single vesicle level.

Funding: This work is supported by grants U01 CA230697 (BSC, LB), UH3 TR000931 (BSC), P01 CA069246 (BSC).

PS15.09 = OP3.09

Proteomic analysis of EVs from the filamentous fungal plant pathogens Fusarium graminearum and Fusarium oxysporum f. sp. vasinfectum

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Introduction: F. graminearum (Fgr) and F. oxysporum f. sp. vasinfectum (Fov) are severe fungal pathogens of cereals and cotton, respectively. Fgr and Fov cause economic losses and threaten food and fibre supplies worldwide. Understanding host-pathogen interactions is crucial for developing new strategies for disease control. We are determining whether extracellular vesicles (EVs) have a role in the interaction between fungal pathogens and their host plant.

Methods: We isolated EVs from Fgr and Fov by size-exclusion chromatography and characterized them by NTA and TEM. EVs from Fgr and Fov are between

100–300 nm and have morphology similar to EVs reported for other fungi. We performed label-free quantitative proteomics to describe the protein cargo of EVs from Fgr and Fov, including a comparative study of EVs from Fov grown on different media: Czapek Dox (CD) and Saboraud's Dextrose Broth (SDB).

Results: A total of 658 proteins were detected in Fgr EVs and, according to prediction software EffectorP, 12.5% of these were potential effectors. Similarly, 70% of EV proteins do not contain signal peptide indicating that packaging into EVs is a novel mechanism of secretion for these proteins. Notable Fgr EV proteins include lipases, proteases and synthases for toxins and chitin. Fov produced EVs in similar quantities in both growth media tested, but EV protein cargo differed between them. There was a 39% overlap in proteins identified in the 465 CD and the 658 SBD EV proteins. In general, EV proteins were involved in metabolism, cell wall architecture and oxidoreduction, with 15.4% and 12.9% of potential effectors, respectively. Polyketide and toxin synthases, proteases and effectors were present in both types of Fov EVs.

Summary/Conclusion: This new fungal EV isolation method was rapid, yielded high-quality EVs, and did not submit particles to high centrifugal forces. Our data revealed that both Fgr and Fov produce EVs enriched with proteins that could alter host immune responses or facilitate fungal infection. Furthermore, the protein composition of Fov EVs was dependant on culture conditions. This supports a potential role for fungal EVs in disease progression in plants and provides the foundations to pursue the role of EVs in plant-fungal interactions with the potential to identify new targets for disease control.

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PS15.10 = OP3.10

Trypanosoma cruzi releases different types of extracellular vesicles that distinctly modulate host cells

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Introduction: Extracellular Vesicles (EV) released by infective forms of Trypanosoma cruzi, the agent of Chagas' disease, modulate inflammatory response of macrophages through the activation of Toll 2 receptor (TLR2) via mitogen-activated protein kinase pathway. This induces the production of nitric oxide (NO) and expression of the cytokines TNF-α, IL-12 and IL-6, which could explain the inflammation observed in experimental Chagas' disease, and eventually in the progression of human disease. EVs released by the parasite are heterogeneous and it is unknown which factor, or factors present in the different vesicle populations act during the interaction with host cells. Objectives. The goal of the present work was to characterize and isolate the different populations of EVs released by T. cruzi and test their effects on macrophages.

Methods: EV released by trypomastigotes forms of T. cruzi (Y strain) were purified by Asymmetric flow field-flow fractionation (AF4) and characterized by Nanoparticles tracking analysis (NTA). The different populations of EVs were incubated with host human monocytes cells (THP-1) and cytokines production determined by ELISA and qPCR. The different EV populations were also incubated with LLCMK-2 epithelial cells and the infection by T. cruzi determined.

Results: We found two distinct populations of EVs. A population with 50 to 50 nm (EV1) and another with 100 to 120 nm (EV2). EV1 induced more TNF-alpha, IL-6, IP-10 and CCL20 than EV2. It was also more effective in promoting T. cruzi infection in epithelial cells.

Summary/Conclusion: T. cruzi released two EV populations that affects differently host cells. Identification of these EVs composition might help to better understand the role of EVs in the modulation of T. cruzi infection.

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PS15.11 = OP3.11

Commensal bacterial extracellular vesicles act as carriers for norovirus

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Introduction: Human norovirus (HuNoV) are one of the most common causes of gastroenteritis and, along with inducing morbidity and mortality by diarrhoea, have a massive economic impact resulting in approximately 60 USD billion each year in healthcare costs and missed worker productivity. Development of anti-viral therapies for HuNoV has been hampered by the lack of robust in vitro cultivation systems. Several cell types support viral replication but only produce modest amounts of virus

due to unknown reasons, making these systems insufficient for use in drug development and infectivity assays.

Noroviruses are known to attach to gram-negative enteric bacteria and this facilitates infection in vitro. However, the microbiome- norovirus-host communication link is missing. Noroviruses infect immune cells present in lamina propria during acute infection, but bacteria themselves are large enough to cross the mucosal and the tight epithelial barrier which separates gut lumen from lamina propria. We hypothesized that binding of noroviruses to bacteria enhances extracellular vesicles (EV) production. Because commensal bacterial EVs by themselves do not have any detrimental effects on host cells, we believe using EVs in in vitro culture will enhance norovirus infection, thus producing higher titre of viruses for vaccine and anti-viral drug development.

Methods: Attachment assay: Purified norovirus was incubated with Enterobacter cloacae, Lactobacillus acidophilus and Bacteroides thethiotaomicron, and grown to produce EVs. The attachment was confirmed via qPCR.

Isolation of EVs: Clarified media supernatants were subjected to ultracentrifugation at varying speeds and 0.2um filtration. Co-purification of norovirus with the EVs was checked.

EV quantification and characterization: EV total protein content was measured by microBCA. The number of vesicles were quantified by Nanoparticle tracking analysis. Scanning and Transmission electron microscopy was performed to check quality of EV preparation and determine if virus was attached to the vesicles. Internal EV protein content was evaluated using MS-HPLC. The EVs were also check for infectivity via TCID50 assay.

Results: Incubation of noroviruses with commensal bacteria resulted in significant increases in production of EVs compared to uninfected controls. Murine norovirus (MNV), used as a surrogate, was found to be associated with EVs. EM analysis determine association of viruses with the bacteria as well as the MVs, while also showing certain surface structural changes in virus attached bacteria compared to mock bacteria. The EVs were found to cause infection in naive macrophages.

Summary/Conclusion: Changes in EV production and content by bacteria exposed to noroviruses will provide insight into its pathogenesis and possible solutions to the low viral output from HuNoV culture systems.

PS15.12 = OP3.12

Detection of bacterial extracellular vesicles in blood from healthy volunteers

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Introduction: Bacteria constitutively produce biologically active extracellular vesicles (EVs), which contain RNA, DNA, and/or proteins. Bacteria use these EVs for communication with other bacteria and recent research suggests bacterial EVs can also affect host cells. Given these findings, it is necessary to examine the role of bacterial EVs in human disease. Current methods of bacterial EV isolation from human specimens cannot distinguish between bacterial species. However, there is utility in examining EVs from specific species, as bacterial species and their EVs may have unique contributions to human disease. Our objective was to isolate circulating EVs specifically from Escherichia coli (EEVs) and Haemophilus influenzae (HEVs), two known colonizers and pathogens in the gut and airway, respectively.

Methods: Total EVs were isolated from the blood of six healthy volunteers via precipitation and size exclusion chromatography. EVs were then selected via a novel latex bead-based fluorescent antibody construct targeting species-specific outer membrane proteins. We used flow cytometry to evaluate the isolated EVs.

Results: The constructs were saturated with EEVs at an antibody concentration of 11.5 μg/mL of plasma, as geometric means ≥11.5 μg/mL were nearly equal. HEVs were detected at 48 μg/mL of plasma, but saturation is yet to be determined. EEVs were imaged by a FEI Talos F200X electron microscope and measured between 40–90 nm, and HEVs were between 60–160 nm. Both types of EVs were spherical.

Summary/Conclusion: Using this novel technique, we were able to isolate, detect, and visualize EEVs and HEVs. This technique enables the study of specific bacterial EVs. In the future, EV contents will be assayed. Furthermore, this technique will be modified so that specific bacterial EVs from body fluids can be used for downstream functional applications. This is the first time that bacterial EVs from targeted bacterial species have been detected in blood from healthy humans.

LBS01: Late Breaking: Cancer Biology and Biomarkers

Chair: Josep Domingo-Domenech – Thomas Jefferson University

Chair: Al Charest – Harvard University

LBS01.01

Nasopharyngeal carcinoma exosomes modify the metabolism status of human dendritic cells and favour their recruitment through the CCL20 chemokine

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Introduction: Nasopharyngeal Carcinoma (NPC) is characterized by a large presence of regulatory T cells (Tregs) and the production of tumour-derived exosomes with immunosuppressive properties. Our team showed that NPC-derived exosomes favour the suppressive activity and recruitment of human Tregs via CCL20 chemokine, thus contributing to NPC immune escape (Mrizak et al., JNCI, 2015). More recently, our team has shown that NPC-exosomes could induce Tregs by altering the maturation of dendritic cells (DCs) and promoting tolerogenic dendritic cells (tDCs) (Renaud et al., HerPas congress 2017). Our main objectives in this study are (i) to define and compare the metabolic status of mature dendritic cells (mDCs), control tDCs and tDCs generated in the presence of NPC-exosomes (ExoCNPtDC) and (ii) to evaluate the chemoattractive potential of NPC-exosomes on ExoCNPtDCs, and notably to investigate the involvement of CCL20 in this recruitment.
Methods: DCs are generated from human monocytes in the presence or absence of NPC-exosomes. The maturation status of DCs was evaluated at a phenotypic level by studying the expression of maturation markers using flow cytometry and at a functional level by analysing cytokines secretion using ELISA. This cytokine analyse has been performed in both conditions, on treated DCs and during co-culture assays of autologous CD3 T lymphocytes with treated DCs. In a second step, a mitochondrial metabolic and glycolytic study was performed using the Seahorse technology (OCR and ECAR measurement). Finally, the chemoattractive potential of NPC-derived exosomes on the different induced DCs was analysed (i) using Boyden chamber chemoattraction assays or real-time videomicroscopy (Chemotaxis μSlide IBIDI) and (ii) using RT-qPCR analysis of the receptor expression of CCL20 (CCR6).
Results: NPC-exosomes alter DC maturation, which gives rise to tolerogenic DCs that favour the induction of Tregs. In addition, the metabolic analysis of DCs seems to put foward a specific metabolic signature of the tDCs induced by NPC-exosomes. And finally, chemoattraction assay suggests that NPC-exosomes preferentially attract tDCs and ExoCNPtDCs in a CCL20-dependant manner.
Summary/Conclusion: Taken together our results should allow us to characterize the major role of NPC tumour exosomes on the maturation and the recruitment of DC and so identify them as anti-tumoural therapeutic targets.

LBS01.02

Cytotoxic T lymphocyte EV that prevents tumour metastasis by collapse of tumoural mesenchymal stroma is classified into exosome, but not microvesicle or apoptotic body.

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Introduction: Recently, instead of ultracentrifugation, development of new preparation protocol is demanded for research of reliable bioactivity and drug discovery of extracellular vesicles (EVs). In this study, we propose a novel method for large scale preparation of high-performance extracellular vesicles focusing on membrane negative charge.
Methods: Murine cytotoxic T lymphocyte (CTL) EVs in supernatant were concentrated more than 20 times at over 97% purity without leaking by 750 kDa MWCO ultrafiltration, and subjected to ion exchange DEAE column chromatography after replacing with PBS. After ion exchange, EVs were characterized by BCA assay, NTA assay, cryoTEM observation, proteome analysis, DNA content measurement, miRNA microarray analysis, zeta potential measurement, lectin array analysis, and target cell analysis.

Results: Murine CTL EVs were broadly divided into two populations that were eluted at low salt (L-s: 0.15 M-0.3 M NaCl) and high salt (H-s: 0.3 M-0.5 M NaCl) concentrations. L-s CTL EVs were abundant in late endosome-related proteins, integrins, Rabs, and effective miRNAs, indicating exosome characteristics, and had biological activity for preventing tumour metastasis after depletion of tumoural mesenchymal cell populations by intratumoral administration (See Seo et al., Nat. Commun. 9: 435, 2018). Contrary, H-s CTL EVs were rich in DNA, core histones, ribosomal proteins, cytoskeleton proteins, and housekeeping proteins, considering microvesicles and apoptotic bodies, and easily phagocytosed by a Kupffer cell line (KUP5: Kitani et al., Results Immunol. 4: 68–74. 2014). In addition, there were noticeable differences between L-s and H-s CTL EVs in the negative zeta potential width and membrane glycan structure.
Summary/Conclusion: Thus, ion exchange can be an optimal mass fractionation method for discriminating bioactive exosomes from cargos for nucleic acids in EVs.
Funding: CryoTEM was conducted in Nara Institute of Science and Technology (NAIST), supported by Nanotechnology Platform Program (Synthesis of Molecules and Materials: 2019 #04) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT). This work was supported by grants from the Japan Agency for Medical Research and Development (Translational Research Network Program (Nagoya Univ. Seeds A64)) and the Japan Science and Technology Agency (CREST [JPMJCR17H2]).

LBS01.03

CLIC4 is essential for breast cancer metastatic competence and predicts disease outcome

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Introduction: Metastatic breast cancer is a consequence of complex interactions between cancer cells and the host. CLIC4, a member of a conserved gene family in the glutathione-S-transferase superfamily, mediates crosstalk between tumour and host in breast cancer. TCGA and METABRIC data indicated that elevated CLIC4 expression was associated with breast cancers from young women, those with poor prognosis, and those with early stage metastatic disease.
Methods: Since bulk tumour analysis does not distinguish between cancer and host stromal cells, we used genetic modifications of established syngeneic breast cancer mouse models to evaluate the contributions of CLIC4 in the host or tumour cells to develop metastases.
Results: Experimentally, the essential Clic4 host contributions for metastatic competence were related to circulating levels of pro-metastatic soluble factors, neoangiogenesis, tumour cell attachment to lung tissue, myofibroblast differentiation, and leukocyte migration. CLIC4 was detected as cargo in circulating extracellular vesicles (EVs) from breast cancer patients. Similarly, circulating EVs from tumour-bearing mice have abundant CLIC4 in comparison to those from mice bearing tumours that lack CLIC4. Tumour cells released EVs that induced myofibroblast conversion of wildtype but not Clic4 ablated lung fibroblasts.
Summary/Conclusion: These results illuminate CLIC4 expression as a prognostic marker for breast cancer patients, and experimentally, CLIC4 is a critical host factor for metastatic competence and potential target within host tissues for anti-metastatic therapy.
Funding: This work was supported by the intramural program of the National Cancer Institute under Project ZIA BC 005445.

LBS01.04

The application of flow cytometry in an EV-based liquid biopsy for the detection of Cancer Multidrug Resistance in Myeloma

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Introduction: Multiple Myeloma (MM) is an incurable cancer of bone-marrow plasma cells. It is characterized by unpredictable and highly variable therapeutic response and poor survival, attributed to the development of multidrug resistance (MDR) to chemotherapy. Presently, no clinical procedures allow for a continuous, minimally invasive monitoring of MDR. We identified unique extracellular vesicle (EV) populations in the blood of myeloma patients, which serve as biomarkers of disease evolution and MDR to combination chemotherapy. We describe approaches used to optimise the use of flow cytometry (FCM) for EV

biomarker detection and analysis and detail strategies for cross-platform analytical validation.

Methods: We conducted a cross-platform analysis using two commercially available flow cytometers designed for EV detection. Scatter resolution, enumeration accuracy and precision were determined across both platforms by analysing submicron silica beads (ApogeeMix, 180–1300 nm) of known concentration. We detected large EVs, as established by reference size beads, electron microscopy, expression of phosphatidylserine and the presence of integral membrane proteins of cell of origin. We analysed EVs isolated from plasma by high-speed centrifugation (18,900 g) as well performing analysis by direct plasma labelling followed by validation by detergent lysis of vesicular constituents. A clinical operating range was defined which ensures linearity and avoids swarm detection.

Results: We observed comparable scatter resolution, enumeration accuracy (error ≤15%) and precision (CV ≤5%) across both platforms used. We defined two EV size gates: a "Latex" gate (300 to 1100 nm polystyrene latex beads), and a "Silica" gate (180 to 1300 nm silica beads) for EVs at the lower end of our size range of interest. To improve detection sensitivity, we identified common contributors to signal noise and applied workflow strategies to minimize these. Finally, we identified linear ranges which avoid swarm detection, and which ensures reproducible EV counts (CV <20%) across both instruments.

Summary/Conclusion: We present an optimised, standardised and cross-platform reproducible working protocol which supports the use of FCM in an EV-based liquid biopsy application.

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LBS01.05

Metabolomic profiling of serum and exosomes isolated from head and neck cancer patients after radiotherapy

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Introduction: Cancer radiotherapy (RT) induces the response of the whole body that could be detected at the blood level. Searching for new molecular signatures which could correlate with treatment response in cancer patients is of particular importance. Radiation-induced changes in proteome and transcriptome of serum have been widely described. However, metabolomic changes in serum, exosomes and other classes of small extracellular vesicles (EV) of cancer patients after RT have not been given as much attention. Metabolomics of serum and EV of cancer patients could provide a valuable insight into the response of both tumour and whole organism to the treatment. The aim of the study was to compare serum and EV metabolomic profiles in head and neck cancer (HNC) patients before and after RT.

Methods: Serum samples from 10 HNC patients were taken before (A) and after (B) RT. 10 healthy volunteers were used as a control group (C). EV were isolated from 1 ml of serum using size-exclusion chromatography (SEC). Selected SEC fractions were subjected to extraction of metabolites. A mixture of MeOH/H2O was used for extraction of metabolites from serum and EV samples. Samples were analysed by gas chromatography-mass spectrometry (GC-MS).

The study protocol adhered to the tenets of the Declaration of Helsinki and was approved by the Bioethical Committee of the Maria Skłodowska-Curie National Research Institute of Oncology, Branch Gliwice, Poland (permit nr. DO/DGP/493/4/06/1/2016/G).

Results: An untargeted GC-MS-based approach allowed the detection of 189 metabolites in serum samples and 50 exosomal small molecules, of which 32 joint. The identified compounds included amino acids, fatty acids, carboxylic acids, sugars, and others. There were 49 metabolites which levels discriminated compared groups (A,B,C) of serum samples and 12 compounds that discriminate the EV isolated from HNC serum before and after RT from HC.

Summary/Conclusion: RT caused significant changes in levels of serum and EV metabolites witch are involved in amino acid metabolism, lipids metabolism, energy metabolism and oxidative stress response.

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LBS01.06

Proteomic profiling of small extracellular vesicles secreted by human pancreatic cancer cells implicated in cellular transformation

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Introduction: Small extracellular vesicles (sEV) secreted from tumour cells are functional vehicles

capable of contributing to intercellular communication and metastasis. Numerous studies have focused on elucidating their role in cancer progression. We recently showed that sEVs isolated from pancreatic cancer cells can function as an initiator in malignant cell transformation. Here, using a mass spectrometry (MS)-based proteomics approach, we analysed the differences in the protein cargo of sEVs secreted from normal pancreatic and cancer cells to better understand their biological characteristics.

Methods: sEVs were isolated from 3 human pancreatic cancer cell lines (Capan-2, MIA PaCa-2, and Panc-1) and normal pancreatic epithelial cells (HPDE) using a combined ultrafiltration-ultracentrifugation method coupled with a sucrose density gradient purification. Proteomic profiling of sEVs was carried out using an LC-MS/MS method. Protein identification from resulting MS/MS spectra was conducted using proteome database search software followed by Gene Ontology (GO) enrichment and Reactome pathway analysis.

Results: A total of 4,907 unique proteins were identified confidently across the combined samples. The proteins present in all four sEV types (1,135 proteins) consist of general housekeeping proteins. 348 proteins were uniquely found in all cancer sEVs but not in the normal HPDE sEVs. This group contains an enrichment of proteins that function in the endosomal compartment of cells responsible for vesicle formation and secretion and suggest their important role in driving the increased production of sEVs from cancer cells relative to normal cells. Moreover, this group includes a set of proteins that have been implicated in malignant cell transformation, consistent with our previous work showing that each of the cancer sEVs analysed here could initiate malignant transformation of NIH/3 T3 cells. Conversely, there were 313 proteins uniquely found in normal HPDE sEVs. This group includes a number of immune response proteins that are not found in any of the pancreatic cancer cell sEVs.

Summary/Conclusion: The differences in the proteomes of cancer and normal sEVs may be indicative of their varying roles in cell transformation and helpful in delineating the types of EVs that are being produced. In addition, these differences point towards their potential value as cancer biomarkers.

LBS01.07

Proteomic profile of tumour-derived exosomes in plasma of melanoma patients

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Introduction: Exosomes released by cancer cells referred to as "tumor-derived exosomes" (TEX) play a key role in tumour-induced suppression of immune effector cells and in the promotion of tumour growth by autocrine or paracrine mechanisms. Molecular profiling of extracellular vesicles circulating in human body fluids, including plasma exosomes, is a promising non-invasive strategy to identify cancer biomarkers. However, specific targeting of TEX remains a real challenge because there is no antigens specific for exosomes produced by cancer cells in general. Here we took advantage of CSPG4 antigen frequently overexpressed in melanoma cells for the immunocapturing of melanoma-derived exosomes (MTEX) present in the plasma of melanoma patients and their separation from non-malignant cell-derived exosomes (non-MTEX) present in the specimen.

Methods: Blood samples were obtained from 15 patients with melanoma treated at the UPMC Hillman Cancer Center Melanoma Program Outpatient Clinic. Total exosomes were isolated by the mini-SEC method. Fraction #4 containing the majority of exosomes was separated into MTEX and non-MTEX fractions using the immunoaffinity capture method with biotin-labelled anti-CSPG4 mAb. The full MS/data-dependent acquisition was performed with the use of the Dionex UltiMate 3000 RSLC nanoLC system connected to the Q Exactive Plus Orbitrap mass spectrometer.

Results: An untargeted MS/data-dependent approach allowed the detection of about 650 proteins. Proteins detected in the material from at least 8 patients were considered, which resulted in 488 proteins included in the analysis. To identify proteins enriched in MTEX, the individual patient ratio between MTEX and non-MTEX fraction was analysed for each protein. We found 49 proteins specific/characteristic for MTEX. These included several enzymes exemplified by ribosomal protein S6 kinase alpha-3, D-3-phosphoglycerate dehydrogenase, phosphomannomutase 2, hypermethylated in cancer 2 protein, acidic mammalian chitinase. Moreover, among components enriched in the MTEX fraction there were several proteins associated with

immune-related functions exemplified by podocalyxin-like protein 2, transforming protein RhoA, tyrosine-protein kinase Yes.

Summary/Conclusion: Using full MS/dd-MS/MS mode we found specific MTEX proteomic profile of tumour-derived exosomes, which needs further evaluation by targeted methods.

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LBS01.08

Isolation and characterization of prostate-derived extracellular vesicles as a liquid biopsy strategy in cancer diagnosis

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Introduction: In the past years, extracellular vesicles (EVs) have attracted considerable interest due to their ability to provide valuable diagnostic information from liquid biopsies. The high abundance in all bodily fluids and their cargo stability confers EVs the potential as a powerful tool to not only obtain novel biomarkers from inaccessible tissues, therapy response and monitoring, but also to reduce infection risks of conventional highly invasive biopsies.

Virtually all cells continuously release vesicles into the extracellular environment, diverse in size, content and features depending on the biogenesis, origin and function. This heterogeneity adds a layer of complexity when attempting to isolate and characterize tissue-specific vesicles.

Methods: Hence, we aimed to use a immunomagnetic capture approach for prostate-derived EVs from cell culture supernatants, with further investigation into human plasma and urine samples. Analysis was performed by nanoparticle tracking analysis, western blotting and electron microscopy. Additionally, an in-house spotted antibody microarray is in development. Here, we intend to detect different EV sub-populations based on their surface markers.

Results: Isolated immunocaptured EV populations based on the classical EV marker CD9 show an increased signal for the luminal protein TSG101. EV populations targeting the tissue-specific marker prostate specific membrane antigen (PSMA), were found positive for TSG101 in a lower extent indicating a subpopulation of EVs. The microarray uses less than 100 µL of sample (concentrated cell culture supernatant, human plasma, urine) and leads to a faster characterization within 3 h for EV surface marker as compared to western blot.

Summary/Conclusion: Immunomagnetic isolation might be a promising approach for liquid biopsy and thereby the microarray could be valuable to identify potential capture targets. The current design for 6 different surface marker from 16 samples simultaneously could be easily extended for sample size and surface profiling allowing for a more economical way to multiplex samples.

LBS01.09

Paving the way for implementing a feasible and reliable technique for assessing urinary extracellular vesicles as biomarkers for bladder cancer in clinical practice

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Introduction: Extracellular vesicles (EV) in urine have been proposed as biomarkers for bladder cancer (BC). However, at present there are no standardized methods for EV isolation or urine sampling. Our goal was to evaluate the EV isolation performance between different methods, the effect of the sampling time and the importance of urinary creatinine (UCr) normalization.

Methods: Two urine samples of 120 mL were collected from 5 patients with non muscle-invasive BC: one from the first micturition and another from any time of the day. Twenty mL were used for UCr measurement and 100 mL were used for EV isolation by either precipitation with polyethylene glycol (PEG), concentration by filtration (UF, Centricon Plus-70, 10 k, Millipore), sepharose size exclusion column (SEC), or combinations of these methods. Additionally, the effect of protease inhibitors (PI) and DTT treatment after collection or during processing was analysed. Size and number of particles were evaluated by Nanosight and the presence of exosomal markers was evaluated by Western Blot.

Results: Among the methods evaluated, UF + SEC showed the best performance retrieving the highest number of particles in the range of 50–200 nm, and the highest protein expression of exosomal proteins. UF alone showed the highest concentration of EV, but with a tendency to isolate larger particles. Particle concentration was positively correlated with UCr, reflecting the importance of UCr normalization before

comparing between patients. Finally, no differences in the performance according to the time of collection, nor in the use of PI or DTT were observed.
Summary/Conclusion: UF + SEC gave the highest EV yield and was not affected by the time of urine collection. The use of PI and DTT can be avoided, and normalization to UCr should be considered when implementing this technique for assessing EVs as biomarkers for BC in clinical practice.
Funding: PIDA 2019.

LBS01.10

The role of small extracellular vesicles secreted by cells with extra centrosomes in PDAC microenvironment remodelling

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Introduction: Human tumours, including pancreatic ductal adenocarcinoma (PDAC), often harbour a subpopulation of cancer cells with extra centrosomes. We found, that these cells secrete an increased number of small extracellular vesicles (sEVs), within the 20–120 nm size range. SEVs play a role in cancer signalling and progression and are widely studied for their diagnostic potential. We aim to understand the role of sEVs secreted by cells with extra centrosomes in shaping PDAC-associated stroma, particularly fibrosis.
Methods: To study the sEV mediated changes in the PDAC microenvironment, we purified sEVs through serial ultracentrifugation and Size Exclusion Chromatography, characterised the content through SILAC-based proteomics, and assessed phenotypic changes in pancreatic stellate cells (PSCs) and extracellular matrix (ECM) production through immunofluorescence staining.
Results: Our data indicates, that the sEVs secreted by cells with extra centrosomes are exosomes due to their endocytic origin, and we found, that they can activate PSCs, key mediators of fibrosis in PDAC. Indeed, we observed an increased level of collagen I produced by PSCs activated by sEVs from cells with extra centrosomes as compared to cells without extra centrosomes. Interestingly, we found, that PSC activation through sEVs is not mediated by TGF-β, assessed by the level of nuclear SMAD2 accumulation downstream of TGF-β activation, suggesting a novel mechanism of PSCs activation.
Summary/Conclusion: PDAC cells with extra centrosomes contribute to a novel type of PSC reprogramming, which could alter their ECM deposition and contribute to the extensive fibrosis observed in PDAC. We are currently characterising the signalling pathways associated with sEV mediated PSC activation and how it impacts PADC progression to better understand the role of centrosome amplification in the cancer-stromal crosstalk.
Funding: Barry Reed Cancer Research Fund, The Lister Institute, Medical Research Council, Cancer Research UK.

LBS01.11

Exosomal Carboxypeptidase E confers and CPE-shRNA loaded exosomes inhibit growth and invasion of hepatocellular carcinoma cells.

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Introduction: Carboxypeptidase E (CPE) is associated with growth and metastasis of liver, pancreatic and colorectal cancers. Here, we examined if exosome-based CPE plays a role in promoting malignant properties of liver hepatocellular carcinoma (HCC) cells, and if CPE-shRNA loaded exosomes can be used to target growth and invasion of HCC cells.
Methods: Exosomes were isolated from the culture media of high metastic HCC97 H cells and incubated with low metastatic HCC97 L cells. In other experiments, CPE-shNA loaded exosomes from HEK293cells were incubated with HCC97 H cells. The recipient cells were analysed for proliferation using MTT assay, colony formation, and Matrigel invasion.
Results: Analysis of exosomes derived from HCC97 H cells revealed CPE-WT mRNA and protein. Exosomes released from HCC97 H cells were able to enhance proliferation and invasion of HCC97 L cells. When CPE expression was suppressed in the HCC97 H cells before exosome isolation, the exosomes had no effect on proliferation and invasion. These data demonstrate the ability of exosomes to confer growth and invasion in HCC cells and the role of exosomal CPE in driving the process. Previously it was shown that down-regulation of CPE expression by shRNA can reverse tumour growth and metastasis in an HCC mouse model. We therefore loaded CPE-shRNA into exosomes by infecting HEK293 (Human Embryonic Kidney) cells with adenovirus carrying CPE-shRNA-GFP. These modified

exosomes were used to transfer CPE-shRNA to HCC97 H cells, resulting in significant reduction in proliferation and colony-forming ability of these cells. CPE-shRNA loaded exosomes were found to down-regulate the expression of Cyclin D1 and c-MYC, two genes with high relavance to tumour growth and metastasis.

Summary/Conclusion: Our results demonstrate the ability of exosomal CPE to enhance proliferation and invasion in low metastatic HCC cells and the potential to use shRNA loaded exosomes to target CPE as a therapeutic strategy to treat liver cancer.

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LBS01.12

Stress hormones promote prostate cancer aggressiveness through modulation of mir-628-5p expression and exosome release

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Introduction: Despite proactive screening and steady declines in mortality, prostate cancer (PCa) remains one of the most prevalent cancers among men. Evidence suggests that chronic activation of stress signalling pathways can result in an altered miRNAs transcriptome and affect exosomal content and release. Here, we study the interaction between leptin and miR-628-5p expression, previously shown to be downregulated in PCa patients. In addition, explored the effect of stress hormones cortisol and leptin on exosomal release and content from PCa cells.

Methods: We utilized normal prostate cell line RWPE-1, and PCa cells PC3, LNCaP and MDA-PCa-2b. Proliferation of cells treated with leptin in the presence or absence of miR-628-5p mimic or negative control was assessed by MTT, colony formation, wound healing, and expression of targets affected by miR-628-5p was assessed by western blotting. Moreover, exosomes were isolated via differential centrifugation from PCa cells treated with leptin or cortisol and exosome number was determined by Nanotracking Analysis. Exosome content was determined by western blotting and proteomic analysis by mass spectrometry.

Results: We observed that leptin significantly decreased expression of miR-628-5p in RWPE-1 cells. Co-treatment with miR-628-5p mimic and leptin abrogated these effects in a cell dependent manner. We also observed that co-treatment with leptin affected miR-628-5p target JAG1 and other molecules involved in epithelial to mesenchymal transition. In parallel, we demonstrated that cortisol increases exosome secretion particularly in PC3 cell exosomes with a 2.6-fold increase at 5 nM Cortisol compared to untreated. Western blotting revealed the presence of GR in exosomes particularly at 5 nM Cortisol.

Summary/Conclusion: Understanding epigenetic regulation through miRNAs and exosomes may be the key to understand stress hormone influence in PCa progression. These findings suggest that stress hormones effectively affect miR-628-5p expression and exosomal release and signalling.

LBS02: Late Breaking: New Technologies and Methods

Chair: Alicia Llorente – Department of Molecular Cell Biology, Institute of Cancer Research, Oslo University Hospital, The Norwegian Radium Hospital

Chair: Wyatt Vreeland – National Institute of Standards and Technology

LBS02.01

Exogenous microRNA loading into extracellular vesicles via producer cell transfection

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Introduction: Extracellular vesicles (EVs) are promising drug delivery vehicles for therapeutic microRNA (miRNA). For the loading of exogenous cargo, researchers broadly seek to either manipulate the EVs directly or the cell that produce them. Electroporation, sonication, and direct EV transfection are common methods that work by physical disruption or irreversible chemical addition, which may irreparably damage the molecules intended for therapy. On the other hand, transfection into the producer cells is a simple option that does not imperil EV integrity.
Methods: There are multiple factors that contribute to EV loading efficiency, including transfection reagent used, timing, and dosage. Thus, we sought to establish a basic protocol and improve understanding of the underlying dynamics involved in a basic system consisting of HEK293 T cells and miR-146a-5p mimic.
Results: In this work, we examined how different reagents lead to variable EV loading. Then we looked at variable dosages, specifically the relationship between RNA amount added to reagent, amount present in cell, and amount exported to EVs.
Summary/Conclusion: These results will help future studies produce EVs with exogenously loaded small RNA, and suggest future optimizations.
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LBS02.02

Single extracellular vesicle trapping by Aptamer-Au Nanoparticle Mediated Au Superlattices

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Introduction: xtracellular vesicles (EVs), secretory vesicles of 30–100 nm size from cells, are carriers of proteins and miRNAs. Despite a variety of EV isolation tools, it is still challenging to separate individual EVs. We report a single EV trapping method via aptamer-mediated assembly between Au nanoparticle (AuNP) and Au superlattice template. We propose a chip-based EV trapping technique based on semiconductor processes.
Methods: We introduce aptamer coated Au nanoparticle (AuNP) and Au superlattices as a template to capture EVs. First, we fabricated poly(methyl methacrylate) (PMMA) hole pattern on Au-coated Si substrates by using electron beam lithography (EBL). We designed 200 nm-diameter hole patterns to capture one EV in each hole. To connect the AuNP and the Au superlattice template, we used an aptamer molecule as a linker strand. Also, to capture individual EVs, the aptamer molecule is designed to have a hairpin structure to specifically bind to CD63, a protein marker of EV. We modified 5′-terminal and 3′-terminal of the CD63 aptamer with thiol group for the formation of self-assembly monolayer (SAM) on both AuNP and Au superlattice surface.
Results: First, we coat the CD63 aptamer on the surface of AuNP. Afterwards, we load the aptamer-coated AuNP into Au superlattice template. EV solution is specifically bound to CD63 aptamer. After washing step, each EV is expected to locate within a single hole due to the size confinement of the hole. To separate the EVs from the aptamer, we use restriction enzyme, BamHI, to recognize specific DNA sequence and cleave them.
Summary/Conclusion: In this report, we propose a AuNP -linked Au superlattice chip by aptamer molecules for trapping EVs. We selected CD63 aptamer for specifically binding with CD63 in EVs. In addition, we designed CD63 aptamer as a linker strand to connect

AuNP to Au superlattice chip. Using this chip, we differentiate the captured single EV which includes various biological information such as proteins and miRNAs.

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LBS02.03

Edit the use of a DLS-based clinical platelet instrument beyond the blood transfusion laboratory

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Introduction: A hallmark of platelet activation is the release of internal granules as extracellular vesicles/microparticles. ThromboLUX is a dynamic-light-scattering-based (DLS) instrument that was developed for use in clinical setting to check for platelet activation before transfusion. Compared to traditional DLS, the ThromboLUX requires no cleaning (single-use capillary) and requires very little sample (70 µL). Hence the ThromboLUX may be a useful instrument beyond platelet pack test in blood transfusion laboratory. We have evaluated its use as an in-process monitoring tool for industrial EV manufacturing, for both quantifying cells (input) and EVs (output).

Methods: The ThromboLUX was used to test the activation status of expired platelet packs (donated by ARCBS for research purpose). The readout was compared with platelet swirling test and flow cytometry data (surface marker). Furthermore, the ThromboLUX was also tested for process development and EV manufacturing monitoring purposes at different stages of the process for its ability to rapidly obtain particle presence and size information on EVs. Time to result was also compared between different particle analysis methods.

Results: The ThromboLUX was a better predictor of platelet packs variability compared to the traditional platelet swirling method. However, we did not observe a strong correlation between the activation status and the flow cytometry-based activation marker data. The ThromboLUX was able to provide a useful estimation of particle presence and sizing of EVs in-process. Results are obtained rapidly, within minutes, with minimal sample prep.

Summary/Conclusion: Although we did not observe a significant direct correlation between flow cytometry activation data and the % microparticles (within a small sample size), the ThromboLUX has shown potential to become a useful tool for in-process monitoring for EV manufacturing and other EV research, in particular through its speed and ease of use.

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LBS02.04

Secreted protein of MSC: Adipose vs Umbilical cord tissue

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Introduction: Mesenchymal stem cells (MSC) has been widely used in both clinical and pre-clinical trials as an alternative teraphy for degenerative diseases1. The therapeutic effect of MSC derived through paracrine effects that secreted protein as signalling molecules that support the process of cell and tissue regeneration3. Secreted proteins from MSC can be different depends on source of the cells, such as umbilical cord, adipose, dental pulp, and other potential sources. Therefore, it is considered quite important to know the better source of MSC to produce the protein that can be used for cell and tissue regeneration.

Methods: This study compared the total protein produced by MSC from umbilical cord and adipose tissue from various passages. MSC were cultured using growth medium until reach 70–80% of confluency. Afterwards, growth medium were replace with serum free media for 24–48 haours to harvest the secreted protein.

Results: Total protein produced by adipose-derived MSC is ranged from 55.7–188.2 µg/ml, it is higher compared to the total protein from umbilical cord-derived MSC which ranged from 55.1–141.2 µg/ml. Total protein produced by MSC is increased along with the passage in both sources of MSC. MSC release signalling molecules for cells communication, where in vitro condition it can be found in their culture medium. Protein expression from the culture medium is increased along with the passage of the cells. This is related to the process of cell maturation, where the more mature cells become more active to metabolize and secrete proteins. Adipose-derived MSC secreted a higher concentration of protein compared to umbilical

cord-derived MSC. The more mature tissue, the more protein secreted by the cells.
Summary/Conclusion: Adipose-derived MSC secreted a higher concentration of protein compared to umbilical cord-derived MSC.

LBS02.05

Development of scalable monolith chromatography processes for the purification of exosomes from a clinically relevant stem cell product

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Introduction: A major manufacturing challenge related to exosome bioprocessing is that of robust and scalable purification. As efforts to translate exosomes into clinics grows, the more important the design of quality systems which can reproducibly purify the product becomes. The current gold-standard, ultracentrifugation, was adopted from the viral vaccine industry, but remains imperfect in terms of scale up and manufacturing due to labour and time intensive process requirements. In order to follow the preferential adoption of more standard bioprocesses, as previously achieved by the viral vaccine industry, we show the development of two monolith chromatography steps which can be used to purify exosomes from a clinically relevant, allogeneic stem cell product (CTX0E03).
Methods: T-flask expansion of CTX0E03 cells was performed to yield batches of 5–15 L of conditioned medium. The medium was subsequently clarified by benchtop centrifugation, and concentrated into a crude concentrate by tangential flow filtration [TFF], using a combination of 0.22 μm dead-end filtration prior to concentration in a 300kDa hollow-fibre TFF system. TFF retentate was loaded onto 1 mL HIC or AEx monoliths, for further purification. Potency was assessed by a fibroblast wound healing assay in vitro.
Results: Exosome presence was verified in the TFF material by detection of CD 81 and CD 63. Exosomes recovered in this manner could achieve full wound closure in vitro over 72 hours, when dosed at 20 μg. Further purification by monolith chromatography showed high levels of reduction of albumin, detected by western blot, as well as heightened ratios of particles to both total protein, and total DNA. The results indicate that neither AEx nor HIC steps cause detrimental loss to product function, either alone or in combination with one another.
Summary/Conclusion: Monolith chromatography can achieve scalable and reproducible purification of stem cell derived exosomes, whilst maintaining their functional capacity.
Funding: Engineering and Physical Science Research Council (EPSRC) Industrial Doctoral Training Centre in Bioprocess Engineering Leadership (EP/G034656/1).

LBS02.06

Using degron-tagged reporters to specifically label and track extracellular vesicles in vivo

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Introduction: To clarify the roles of extracellular vesicles (EV) in vivo, it is important to visualize and track EVs from the source cells to their destination. However, since most EV reporters are also present in the releasing cell, it is challenging to visualize EVs in vivo.
Methods: To tackle these problems, we developed a degradation-based technique to remove background fluorescence. We use degradation motifs called degrons to target proteins for ubiquitination and degradation in the cytosol, while leaving EVs labelled. We re-purposed an endogenous zinc finger (ZF1) degron in the nematode model organism Caenorhabditis elegans, but show that the technique can also be applied to mammalian cells with the auxin-inducible degron (AID). To specifically label MVs in C. elegans, we ZF1-tagged the PI4,5P2-binding PH domain of the cytosolic phospholipase PLC1∂1, which is primarily found at the plasma membrane.
Results: The ZF1-tagged plasma membrane reporter is released in MVs outside the cell and remains fluorescent because intervening membranes hinder the proteasomal degradation of the ZF1 reporter in MVs. In the end, MVs maintain fluorescence, while the reporter is removed from inside the source cell. This increased the visibility of MVs from the neighbouring cells, enabling the visualization of MVs with a normal light microscope. Using this ubiquitous plasma membrane reporter, we could visualize released MVs during C. elegans embryogenesis, which helped us to identify new MV release inhibitors. Additionally, we show that this technique can be used to determine MV cargo in vivo.
Summary/Conclusion: Ultimately, our approach boosts the visualization and tracking of EVs in vivo. Since degron-mediated degradation is a widely used

tool to analyse loss of function effects in cell culture and model organisms, we propose that this technique can be readily adapted to EV experiments and will help to visualize and track MVs in a broad range of experimental systems.

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LBS02.07

Heterogeneity and batch variation of HEK293 extracellular vesicles

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Introduction: Extracellular vesicles (EVs), are emerging as a potentially powerful new class of multimodal therapeutics and drug delivery vehicles. However, challenges remain – EVs are highly heterogeneous and differ in terms of their biogenesis, size (~30-1000 nm) and complex molecular compositions (lipid bilayers, ncRNAs, proteins and small molecules). Furthermore, EVs exist within complex cell secretomes and body fluids. Thus, navigating EV heterogeneity through the use of robust isolation and characterisation methods (metrology) is beneficial to several research applications – including, therapeutic EV manufacturing. To this end, we isolated and characterised batches of HEK293 EVs that were harvested from a hollow fibre bioreactor.

Methods: HEK293 cells are an industrially important mammalian cell line that have been previously used to manufacture therapeutic antibodies and EVs. To prepare EV batches, HEK293 cells were cultured for 30 days within a hollow fibre bioreactor that was configured with a 20 kDa cartridge to concentrate HEK293 cells (up to ~10e9 cells) and their secretomes (>20 kDa proteins and EVs) within ~20 ml harvest volumes. EVs were isolated, for comparative purposes, using ultracentrifugation (UC), tangential flow filtration (TFF) and immunocapture (IC)-based methods. Isolated EVs were characterised using DLS, high-throughput NTA, dot blot array and partially using nanoflow cytometry, ExoView and TRPS.

Results: EV batches harvested at different intervals were characterised. EV/particle sizes (NTA): UC 140 ± 10.7 nm, TFF 134 ± 13.3 nm and IC 126 ± 3.9 nm. EV/particle concentrations (NTA): UC 17 ± 25x10e11/ml, TFF 0.2 ± 0.1x10e11/ml and IC 49 ± 35x10e11/ml). Total protein (Qubit): UC 0.8 ± 0.6 mg/ml, TFF 0.2 ± 0.2 mg/ml and IC 4 ± 0.6 mg/ml. EV batch diversity is likely due to a number of interacting factors including changes in cell density, cell growth rate and EV isolation or characterisation methodology.

Summary/Conclusion: EV heterogeneity varies across different EV batches and must be carefully monitored and assessed in terms of any potential impacts on scalable EV manufacturing.

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LBS03: Late Breaking: Specific Cell Derived EVs

Chair: Dimitrios Kapogiannis – Laboratory of Clinical Investigation, National Institutes of Ageing

LBS03.01

Blunted postprandial suppression of phosphoenolpyruvate carboxykinase in kidney-derived urinary exosomes in early insulin resistance in humans

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Introduction: Renal resistance to insulin's action could be an independent risk for chronic kidney disease (CKD) and diabetes. The existing methods to estimate kidney – specific insulin resistance in humans are not feasible for clinical and epidemiological studies or use in routine clinical practice. Blunted action of insulin on insulin-responsive genes in the kidneys, such as Phosphoenolpyruvate carboxykinase (PEPCK), could be an important indicator for impaired kidney-specific insulin sensitivity.
Methods: PEPCK, Glucose 6-phosphatase (G6Pase) and Fructose 1,6-bisphosphatase (FBPase) were estimated in kidney-derived exosomes in human urine (UE). Fold mRNA expression were estimated using qRT-PCR and related to fasting serum insulin levels. Proteins were estimated by ELISA and normalized to urine creatinine. Urinary exosomal protein after overnight fast and at 2 hours of oral glucose tolerance test (2 h-OGTT, postprandial) was compared. Subjects were categorized based on HOMA-IR values for the analysis. In vivo and in vitro models with PEPCK induction was used to study PEPCK regulation in kidney and in kidney-derived exosomes.
Results: Human UE had detectable protein and mRNA levels of PEPCK, FBPase and G6Pase, with FBPase having the highest abundance. Fasting insulin levels strongly predicted PEPCK mRNA levels in human UE. Also, Pepck protein in UE showed a significant suppression in the fed state, relative to the fasted state. HOMA-IR values significantly predicted the expression of the three Gng enzymes in uE from the fed state. Subgroup analysis showed blunted Pepck suppression in subjects with lower insulin sensitivity relative to subjects with better insulin sensitivity respectively. Immuno-blotting showed significantly higher PEPCK protein band-density for PEPCK protein in UE from pre-diabetic and diabetic subjects relative to non-diabetic controls. Renal PEPCK induction in rat using short-term acidosis, or in human proximal tubule (hPT) by glucocorticoid stimulation, resulted in higher PEPCK levels in rat UE and hPT-derived exosomes, respectively.
Summary/Conclusion: The urine-based approach would ease regular screening of kidney-specific insulin sensitivity in humans.
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LBS03.02

Differential characterization of extracellular vesicles in neuroprotective human platelet lysate preparations

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Introduction: Custom-made Platelet Pellet Lysate (PPL) and Heat-treated PPL (HPPL) exert strong neuroprotective effects of neurotoxin-exposed dopaminergic LUHMES neuronal cell culture. This effect is significantly enhanced using HPPL, which was also highly protective of TH-expressing neurons in mice Parkinson's disease (PD) model. The role of their EVs in neuroprotection is unclear. Our NanoBioAnalytical (NBA) platform can help to characterize platelet EVs phenotype, size, and morphology, and unveil the influence to neuroprotective functions of platelet lysates.
Methods: PPL and HPPL have been prepared by freeze-thaw lysis of purified platelets. HPPL underwent an additional treatment at 56°C for 30 min. Proteins were analysed by Western blot (WB). HPPL neuroprotective function was studied by cell viability assays of LUHMES cells exposed to erastin. The concentration

and size of EVs were evaluated in solution by several complementary approaches. In the multiscale NBA system, EVs were captured onto specific antibodies grafted on the biochip, quantified by Surface Plasmon Resonance imaging (SPRi) and then studied in situ by Atomic Force Microscopy (AFM) to unveil EVs subsets.

Results: WB revealed higher expression of CD9, CD41 and CD61 markers in PPL versus HPPL. HPPL exerted increased viability of erastin-exposed LUHMES cells compared to PPL. EVs concentration was in the order of 10e11/mL. SPRi results were consistent with WB, with higher capture PPL EVs on aCD9, aCD41, and aCD61 immunoarrays compared to HPPL. Evidence of EVs was confirmed by AFM that revealed spherical EVs and dimensions of the objects captured on spots. The density of EVs was consistent with the differential capture in SPRi.

Summary/Conclusion: HPPL exerts superior neuroprotection in in vitro and in vivo PD models. Our work confirms the presence of EVs in HPPL and PPL, suggesting the applicability of NBA platform for complex sample analysis. CD41 and CD61 markers seemed affected by PPL heat treatment. NBA platform can be valuable in identification of the role played by EVs in the beneficial neuroprotective effects of HPPL that can be exerted by the EVs cargo (growth factors, mRNA, miRNA). The proteomic and genomic contents of EVs present in both platelet lysates remains to be studied.

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LBS03.03

GMP compatible angiogenic exosome processing towards therapeutic for treating stroke

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Introduction: There is a critical unmet medical need for new therapies to treat age-related diseases including cardiovascular diseases such as stroke. Exosome derived from stem cells have shown intrinsic therapeutic potential in a variety of animal models of ischaemic diseases. We have identified scalable exosome production cell lines (PureStem) as a source of angiogenic exosomes and are aiming to generate good manufacturing practice (GMP) grade therapeutic exosomes that can effectively mediate angiogenesis and tissue regeneration.

Methods: We are developing exosome production and purification protocols that combine methods of Tangential filtration flow (TFF) and size exclusion chromatography (SEC). The particle number and size were measured by both tunable resistive pulse sensing (TRPS) as well as nanoparticle tracking analysis (NTA) for comparison. Exosomes were characterized by detection of exosome surface markers and absence of cellular markers. Purity was assessed by measuring particles per ug of total protein content. The angiogenic activity of PureStem-exosomes was assessed using live-cell imaging to measure endothelial wound-healing and tube formation assays. We further investigated the molecular cargo of PureStem-exosomes by screening miRNAs targets, RNA-seq analysis, and mass spectrometry analysis.

Results: The isolated PureStem-exosomes using our developed protocols were highly purified, resulting purity in the range of 1E10-5E10 particles/ug. We selected angiogenic exosome-producing cell lines from our PureStem library by screening for functional activity and characterizing their molecular cargo. We found that PureStem progenitor-derived exosomes showed higher angiogenic potency than primary mesenchymal stem cell (MSC)-derived exosomes. Furthermore, angiogenic microRNAs such as miR-126 were enriched in PureStem-exosomes from certain producer cell lines.

Summary/Conclusion: These data demonstrate the potential for using PureStem lines as a highly scalable source of therapeutic exosomes. We were able to obtain highly pure exosomes that retain their angiogenic activity. We anticipate that PureStem-exosomes will be a valuable resource for developing EV therapies for stroke and other ischaemic diseases. We have developed purification methodologies aimed at achieving a robust and scalable exosome production compatible with GMP for clinical grade PureStem-exosomes. These developments have great potential as therapeutic agents for future preclinical in animal model of stroke and clinical trials.

LBS03.04

Neuronal-origin plasma EVs provide biomarkers for Parkinson's Disease

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Introduction: The hallmark of Parkinson's Disease (PD) is a-synuclein accumulation, predominantly in dopaminergic neurons, causing neurodegeneration. PD is also associated with insulin resistance, a condition characterized by phosphorylated insulin receptor substrate-1 (IRS-1). Besides motor symptoms, some PD patients develop Mild Cognitive Impairment (PD-MCI) or dementia (PD-D). Given the importance for prognosis, there is an urgent need to develop biomarkers for distinguishing PD with normal cognition (PD-N) from PD-MCI/D. Neuronal-origin Extracellular vesicles (NEVs) contain cell signalling and pathogenic proteins (including a-synuclein), which may serve as biomarkers for Alzheimer's disease, PD and other dementias.
Methods: From 0.5 ml of plasma from 104 PD-N, 83 PD-MCI, and 39 PD-D patients, we immunocaptured NEVs using anti-L1CAM antibody. Then, IRS-1pSer312 and IRS-1pTyr20 and a-synuclein were measured in NEVs using electrochemiluminescence immunoassays.
Results: A-synuclein was lower in PD-MCI and PD-D compared to PD-N ($p < 0.005$) and significantly decreased with increasing motor symptom severity measured by MDS-UPDRS III score ($p = 0.005$). IRS-1pSer312 was lower in PD-D than in PD-N. IRS-1pTyr20 significantly decreased with increasing MDS-UPDRS III score ($p < 0.005$). No biomarker was associated with disease duration.
Summary/Conclusion: PD patients with cognitive impairment exhibited lower NEV levels of a-synuclein than cognitively intact PD patients, whereas a-synuclein and IRS-1pTyr20 were inversely associated with PD motor symptom severity. Additional biomarkers and measurements will be available by the time of ISEV. Plasma NEVs is a valuable tool for discovering biomarkers in PD and investigating aspects of disease progression.
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LBS03.05

Urinary extracellular vesicles as biomarkers of kidney allograft injury: optimization of isolation protocol and characterization

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Introduction: Despite decades-long advancement in transplant medicine, there is a necessity for personalized approach regarding early kidney allograft injury recognition and immunosuppression therapy towards improved transplant outcomes. Biopsy, a gold standard for assessment of kidney allograft injury, cannot be serially used for the diagnosis of subclinical injury due to it's invasiveness and possible sampling errors. Instead, urine is easily obtainable and bearing extracellular vesicles (EVs), potential carriers of pathological signals related to kidney injury. Our aim was to set up a urinary EV (uEV) isolation protocol that would allow consistent and reliable identification of their characteristics and cargo.
Methods: Second morning urine sample (25 mL) was collected from 7 patients and processed within 4 hours. Oxalate precipitation, pH and dilution variability, uromodulin polymerization and high protein content were taken into account. Isolated EVs were defined by Transmission Electron Microscopy (TEM) and Nanoparticle Tracking Analysis (NTA). uEV specific proteins and miRNAs were analysed by Western blot and qPCR, respectively.
Results: The optimal protocol relied on low speed urine centrifugation (2.000 x g, RT) for cell removal and storage at −80°C prior to further analyses. After urine thawing at RT, added EDTA averted cryoprecipitate and uromodulin polymer formation, while concentrated PBS neutralized the pH. Filtration through 0.22 μm pores was used for large particle removal, while centrifugal 100 kDa membrane units (Amicon®, Milipore) served for sample concentration followed by particle separation on size-exclusion chromatography (SEC; qEVoriginal, Izon Q). Protein vacant SEC fractions (as rated at A280) were pooled and concentrated to a volume of 70 μl. TEM micrographs revealed high sample purity and cup-shaped morphology of uEVs. As per NTA results, the average mean size of EVs was 129,9 nm with concentration range of 1 × 109 particles/mL of starting urine. uEVs were positive for the tested marker proteins Hsc70, flotillin, tubulin, GADPH and CD63. qPCR verified miRNA presence in uEVs, with CT for miR let-7i at 20.
Summary/Conclusion: We successfully isolated pure uEVs. The set up protocol will be used to assess uEVs as non-invasive biomarkers of allograft injury in kidney transplant recipients.

LBS03.06

Astrocyte-derived extracellular vesicles regulate dendritic spine formation and neuronal network connectivity

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Introduction: Recent advancements in the biology of extracellular vesicles have begun to implicate glial released microvesicles as mediators of glia to neuron communication, suggesting that alterations in the release and/or composition of astrocyte microvesicles could impact neuronal function.

Methods: Astrocytes were allowed to constitutively release extracellular vesicles (ADEV-CR), or stimulated with ATP (ADEV-ATP). ADEVs were isolated by ultracentrifugation followed by proteomic analysis. We developed a normative whole transcriptome database using primary neurons exposed to ADEV-CR, and identified changes in neuronal gene expression produced by exposure of neurons to ADEV-ATP. We identified a number of pathways associated with the biological response of synapse, spine and neurite outgrowth that were regulated by ADEV-ATP.

The molecular cargo of ADEV-ATP responsible for regulating synaptic functions in neurons were characterized by biochemical, molecular, and functional assays.

Results: ADEV-ATP enhanced the maturation of dendritic spines and produced functional enhancements in neuronal activity and network connectivity. The mechanism for this effect involved the delivery of Integrin-✔ 1 and EphA2 that were enriched in ADEV-ATP. Integrin-✔ 1 facilitated binding of ADEVs to the neuronal surface, and EphA2-receptor signalled through Ephrin to the tyrosine kinase ERBB2/4 that regulated the phosphorylation and activation of TrkB without increasing expression of the natural ligands BDNF or NTF3. This direct activation of TrkB increased the expression of the synaptic scaffolding proteins Disc1, Arc, and Cplx3 to promote the maturation of dendritic spines. This increase in mature dendritic spines was associated with increased neuronal activity and network connectivity demonstrating a functional strengthening of synapses.

Summary/Conclusion: These data identify a molecular mechanism whereby modifications in ADEV protein cargo produced by the stimulation of astrocytes with ATP regulates synaptic maturation through activation of TrkB in a manner independent of growth factors.

LBS03.07

Enhancement of immunomodulatory functions of MSC-derived extracellular vesicles through modification of up-stream parameters

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Introduction: Mesenchymal stem cell extracellular vesicles (MSC-EVs) have been shown to have an immunosuppressive effect in both autoimmune and inflammatory disorders. Despite this, clinical translation of EV therapies is hindered by potentially low potency in vivo and the lack of a scalable biomanufacturing process. Cell culture parameters are critical in modulating both yield and bioactivity of EVs. Thus, we hypothesized that the combination of chemical priming and 3D dynamic culture would enhance the yield and potency of immunosuppressive MSC-EVs.

Methods: Bone marrow-derived MSCs cultured in flasks were chemically primed using ethanol or curcumin. MSCs were also cultured using a 3D-printed scaffold-perfusion bioreactor using a flow rate of 5 ml/min. Anti-inflammatory effects were assessed following application of MSC-EVs to lipopolysaccharide (LPS)-stimulated murine macrophages. Subsequent inhibition of the production of the pro-inflammatory cytokine IL-6, quantified using an ELISA, was used to characterize EVs as anti-inflammatory. In addition, both chemical priming and the bioreactor will be simultaneously utilized to potentially uncover any synergistic effects on EV immunomodulation abilities. Nanoparticle tracking analysis (NTA) was used to assess EV size and concentration while protein mass was measured via a BCA assay.

Results: Preliminary data suggests that priming MSCs with 100 μM ethanol for 24 hours prior to EV collection results in a strong inhibition of IL-6 production in stimulated murine macrophages. NTA revealed that MSC-EV yield increased by about two orders of magnitude in the bioreactor (1.40E12 ± 7.92E10) when compared with flasks (2.28E10 ± 2.81E9). Protein measurements also indicated that EV production in the bioreactor (~7600 μg) was much greater compared with production in the flasks (~2400 μg). Additionally, average protein content per EV was reduced in the bioreactor when compared with flask EVs.

Summary/Conclusion: Although further investigation is required, our results potentially promise an effective and inexpensive priming agent (i.e., ethanol) for the production of anti-inflammatory MSC-EVs. This, combined with the significant increase in yield via 3D dynamic culture, presents practical solutions to both EV manufacturing scalability and potency issues.

LBS03.08

Donor source affects potency of mesenchymal stem cell-derived extracellular vesicles

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Introduction: Mesenchymal stem cell (MSC) therapies have been heavily investigated for their utility in applications such as wound healing and regenerative medicine due to their angiogenic, immunomodulatory and anti-apoptotic effects. Recently, MSC-derived extracellular vesicles (EVs) have been implicated as primary effectors in MSC-based therapies via protein and nucleic acid cargo transfer to patient cells. MSC EVs represent a superior alternative to MSC-based therapies, as they lack the ability to replicate and are much smaller in size, circumventing related safety concerns such as immunogenicity, teratoma formation and blood vessel occlusion. However, a key drawback with MSC therapies in general is their variable therapeutic potency, which is dependent on donor source. As a cell derived therapeutic, this crucial limitation is hypothesized to exist in MSC EVs as well. Here, we demonstrate the varying bioactivities of isolated MSC EVs from differing donors and tissue sources.
Methods: Six separate MSC lines were obtained from different donors, with three MSC lines derived from donor adipose tissue, and the other three from the bone marrow of separate individuals. EVs were isolated from each MSC line at passage 3 via differential centrifugation and ultrafiltration. These isolated MSC EVs were then characterized for size/concentration via nanoparticle tracking analysis, and EV markers (TSG101, ALIX, CD63) via western blot. Pro-vascularization capacities of MSC EVs were determined by a gap closure assay using human umbilical cord vein endothelial cells (HUVECs).
Results: Characterization of MSC EVs revealed similar sizes and EV marker expression across donor groups, regardless of tissue source. Furthermore, comparison of adipose tissue-derived (AD) MSC EVs from three donors indicates varying pro-vascularization bioactivity between those donors evaluated in vitro via gap closure assay. Similar results were observed for the bone marrow-derived (BM) MSC EV donor groups.
Summary/Conclusion: This work highlights the need for screening of donor derived-MSCs before use for therapeutic EV production. Additionally, standardized criteria for MSC donor selection are needed before isolated MSC EVs can be used as a large-scale, repeatable therapeutic treatment.

LBS03.09

Analysis of extracellular vesicle populations from malaria-infected erythrocytes by field-flow fractionation reveal distinct sub-sets

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Introduction: Malaria is one the most devastating infectious disease in the world and Plasmodium falciparum (Pf) represents the deadliest species. This parasite invades human red blood cells (RBCs) and releases extracellular vesicles (EVs) carrying DNA, RNA and protein cargo components which are involved in the pathogenesis of the disease. Recently, it has been shown in mammalian systems that EVs are subdivided into different subpopulations, each with a distinct biological function. However, it is still unknown whether Pf-infected RBCs (Pf-EVs) release different EV subpopulations with distinct cargo.
Methods: We isolated EVs from Pf- infected and uninfected RBCs, Pf-EVs or ui-EVs, respectively, using differential centrifugation. The EV pellet was subjected to field flow fractionation (FFF). The different subpopulations were collected, concentrated with size-exclusion filters and evaluated by Nanoparticle Tracking Analysis. Additionally, the presence of EV markers (SR1 and HSP90) were examined by Western blot analysis.
Results: The FFF analysis showed four particle subpopulations derived from the Pf-EVs and five in the ui-EVs. The first three subpopulations were similar in their detection signals in both samples, but the fourth subpopulation was consistently higher in ui-EVs than in Pf-EVs. Moreover, HSP90 was detected in subpopulations 3 and 4 of both Pf-EVs and ui-EVs, whereas SR1 only in subpopulation 3.

Summary/Conclusion: Pf-EV and ui-EV have similar separation profiles and proteins markers in their subpopulations, consistent with the fact that both samples are derived from host RBCs. Additional data regarding the DNA and RNA cargo, as well as microscopic observations of the Pf-EV and ui-EV subpopulations is necessary. This will clarify how malaria parasites sort their components into EVs and which fractions are associated to immune evasion and pathogenesis.

LBS03.10

Evidences on microalgal extracellular vesicles: a morphological assessment

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Introduction: We have established a small size laboratory production of the microalgae culture in order to harvest the extracellular vesicles (EVs) for pharmaceutical and medical uses. In this work we report on globular particles in the isolates from media of microalgae of two types, that we recognize as EVs. We observed changes in their production at different temperatures and conditions.

Methods: Samples were fixed by various combinations of aldehyde fixatives and/or osmium tetroxide. They were dehydrated in a graded series of ethanol, hexamethyldisilazane, and air dried. They were Au/Pd coated for inspection with Scanning Electron Microscopes (SEM) Crossbeam 550 FIB-SEM Gemini II (ZEISS, Germany) and JSM-6500 F Field Emission Scanning Electron Microscope (JEOL Ltd., Tokyo, Japan).

Results: Microalgae were incubated overnight at 22°C and 37°C in growth medium and in growth medium supplemented with detergent. The samples obtained from the microalgae culture contained particles that we recognized as extracellular vesicles, however, these particles do not correspond to characteristic shapes of membrane enclosed entities without internal structure. Increased temperature and/or presence of surfactant (Triton X-100 and sodium dodecyl sulphate) stimulated formation of EVs of different shapes and sizes. The isolates of these samples were rich with EVs. In the presence of surfactant, the cell-walls detached from the cell and collapsed upon dehydration. This was documented by SEM.

Summary/Conclusion: Focused Ion Beam technique revealed complex internal structure of the algae. It seems from the shapes of the observed structures that the particles deposited on the surface of the microalgae do not derive from budding of the membrane surface, but are instead shed by the cells from the cell interior upon the rupture of the cell wall.

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