Extracellular vesicles from glioblastoma cells as potential drug-delivery vehicles to isotypic tumours

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Abstracts

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Scientific Program ISEV 2015 meeting
Thursday April 23, 2015
Oral Presentations

Registration	08:30-10:00
Welcome & networking coffee	08:30-10:00
Setting up posters (Poster sessions I, II, III, IV, V, VI, VII)	08:30-09:00
Sponsor exhibition	10:00-18:00

Ballroom D-E
Plenary Opening Session
Chairs: *Jan Lötvall and Kenneth W. Witwer* 10:00-12:00

James E. Rothman, Ph.D.
Wallace Professor of the Biomedical Sciences at Yale University
Principles of vesicle transport

Francis S. Collins, M.D., Ph.D.
Director, United States National Institutes of Health
Nurturing innovative science at NIH

Roundtable discussion
Moderator: Matthew Herper, *Forbes*

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Networking lunch	12:15-13:15
Poster viewing sessions I, II, III, IV, V, VI, VII Posters attended by authors	12:45-13:15
Poster walk by chairpersons, sessions I, II, III, IV, V, VI, VII Posters attended by authors	14:00-15:00

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Ballroom D
Symposium session 1A - EV biogenesis I
Chairs: *Clotilde Théry and Stephen J. Gould* 13:15-14:00

O-1A-1

ApoE regulates ESCRT-independent sorting on exosomes and endosomal amyloid formation

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Introduction: Exosomes are generated within secretory multivesicular endosomes (MVEs) as intraluminal vesicles (ILVs). To serve specific cellular functions, notably once secreted, ILVs are enriched with defined sets of proteins by various and still elusive sorting mechanisms within MVEs. Pigment cells have tuned their MVEs to produce amyloid fibrils derived from the protein PMEL. For this purpose, PMEL – the first protein reported as an ESCRT-independent cargo – is sorted in a CD63-dependent manner on ILVs that likely serve as potential seeding platforms for PMEL amyloidogenesis. Contrary to amyloids such as those associated with Alzheimer's disease, PMEL amyloids are non-toxic and are functional as they serve as a scaffolding structure for the synthesis of melanin. To better understand the mechanisms exploited on ILVs to avoid potential toxicity during PMEL amyloidogenesis, we have used exosomes as reporters of these endosomal processes. *Methods*: For this purpose, we have characterized exosomes derived from pigment cells by cryo-electron microscopy, mass spectrometry and western blot. We then investigated the role of the intracellular counterparts of exosomes, ILVS in cell lines and in vivo using siRNA, western blotting and morphological analysis by electron microscopy. *Results*: Characterization of exosomes derived from pigment cells revealed the association of exosomes and ILVs with apolipoprotein E (ApoE) and lipoparticles. We could show that ApoE is targeted to endosomes in a CD63-dependent/ESCRT-independent manner and facilitates the ESCRT-independent sorting of PMEL amyloidogenic fragments onto ILVs. At the surface of ILVs, ApoE regulates the formation of mature fibrils in melanocytic cell lines and in pigment cells in vivo. *Summary/conclusion*: These results established a clear molecular mechanism for ESCRT-independent sorting of PMEL. Moreover, the novel evidence that lipoparticles are associated to exosomes provides a breakthrough that might be exploited to reconsider the respective roles of each extracellular particle in pathologies. Finally our study establishes a paradigm for the mechanism by which ApoE, the first genetic risk for early onset Alzheimer's disease, regulates the assembly of mature amyloid fibrils under benign and pathological conditions.

O-1A-2

Prion protein regulates autophagy and endocytic trafficking with essential roles for the biogenesis of extracellular vesicles

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Introduction: Prion protein (PrP^C) plays important roles in neuronal physiology and in tumour biology. Primary cultures from PrP^C knockout astrocytes ($Prnp^{0/0}$) show a defect in the secretion of neurotropic factors, and the protein and lipids content in conditioned medium (CM) of these cells were reduced when compared to wild-type (WT) astrocytes (Lima et al., *J Neurochem*. 103(6): 2,164–76, 2007). Thus, suggesting that PrP^C regulates extracellular vesicles (EV) release. *Methods*: EVs were isolated using ultracentrifugation and their size and concentrations analyzed by NTA. Electron and confocal microscopies and biochemical analysis were performed to evaluate EV and endocytic components. siRNA was used to knock down beclin1. *Results*: $Prnp^{0/0}$ astrocytes secrete lower EVs in CM than WT cells whilst the reconstitution of PrP^C expression in $Prnp^{0/0}$ astrocytes restores the levels of EV secretion. In astrocytes overexpressing PrP^C, the number of secreted EVs is higher than those secreted by WT cells. The same differences were observed in CM from primary fibroblasts cultures and in the blood circulation of these mice. The absence of PrP^C causes a delay in caveolin-mediated endocytosis and in the traffic of EGF-EGFR to late endosomes/multivesicular bodies (LE/MVB). $Prnp^{0/0}$ astrocytes present altered MVB formation, LAMP1-stained lysosomal compartments were enlarged and shown an increased number of autophagosomes when compared with WT cells. Autophagy inhibition by beclin1 knockdown restored the levels of EVs released by $Prnp^{0/0}$ cells. Conversely, autophagy induction by serum starvation or rapamycin treatment inhibited exosome release in WT cells. *Summary/conclusion*: PrP^C modulates endocytic pathways and impairs autophagy. In the absence of PrP^C, endosomes can be directed to fuse with autophagosomes contributing to the impairment in EVs biogenesis/release. The cellular levels of PrP^C can regulate the amount of secreted vesicles with major roles in heath and disease.

O-1A-3

Rabs involved in extracellular vesicle biogenesis each affect multiple different steps in endolysosomal and secretory trafficking in Drosophila secondary cells

Clive Wilson[1], Siamak Redhai[1], Ben Kroeger[1], Laura Corrigan[1], Shih-Jung Fan[1], Mark Wainwright[1], Ian Dobbie[2], Aaron Leiblich[1], Carina Gandy[1], John Morris[1], Freddie Hamdy[3] and Deborah Goberdhan[1]
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Introduction: We have recently characterized a new in vivo system to dissect the role of the endolysosomal and secretory pathways in regulating the biogenesis of extracellular vesicles. It takes advantage of the Drosophila male accessory gland (AG), which shares similarities with the human prostate. A small subset of epithelial cells within the AG called secondary cells (SCs) specifically produces vesicles, which can be marked by a GFP-tagged form of the putative exosome marker, human CD63. CD63-positive vesicles are made inside giant ($\sim$5 μm diameter) endolysosomal compartments and secreted into the AG lumen in a process that requires the function of ESCRTs like ALiX and Hrs, and of different Rabs associated with mammalian exosome secretion, such as Rab11, Rab27 and Rab35. We investigated how these Rabs alter membrane trafficking within SCs to affect extracellular vesicle secretion. *Methods*: We have used molecular genetic approaches in the fly combined with both confocal and super-resolution (3D-SIM) microscopy to determine how knockdown of specific Rabs alters trafficking and vesicle biogenesis pathways in SCs. *Results*: Knockdown of Rab11, Rab27 or Rab35 with at least two independent RNAi molecules affects EV secretion. However, each Rab has very different effects on SC intracellular compartments. Importantly, overexpression of dominant negative Rabs does not produce these specific defects, suggesting that their effects are not

the result of simple loss-of-function. Super-resolution microscopy of living glands reveals previously unappreciated complexity in endolysosomal trafficking events. For example, highly dynamic intraluminal vesicles and membranes are found inside endolysosomal and secretory compartments, suggesting that EV trafficking to the cell surface may not just involve late endosomal compartments. Although neither Rab11 nor Rab35 appear to be expressed on the surface of large acidic endolysosomes, when either is knocked down, these large compartments do not appear to mature properly, and vesicles accumulate inside. The numbers of non-acidic secretory compartments are also affected by these two treatments, but in completely opposite ways, with the secretory compartments being almost entirely lost after Rab11 knockdown. *Summary/conclusion*: Our data reveal that knockdown of specific Rabs alters several different membrane-bound compartments in SCs. Therefore, though at least one key effect of knockdown may be on a specific endosomal trafficking pathway to the plasma membrane, the defect in EV secretion may primarily be explained by indirect effects on other parts of the secretory or endolysosomal systems. The interpretation of the effects of Rab knockdown on EV secretion, therefore, requires careful analysis of trafficking in compartments in addition to endolysosomes.

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Ballroom E

Symposium session 1B - EVs and stem cells

Chairs: *Susmita Sahoo and Weian Zhao* 13:15-14:00

Introductory Lecture: Stefano Pluchino, M.D., Ph.D.

University of Cambridge

O-1B-1

Exosomes for heart failure associated with Duchenne muscular dystrophy in *mdx* mice and in human iPS cells

Mark Aminzadeh[1], Prasanthi Durvasula[1], Rachel Tobin[1], Rachel Smith[1], Xuan Guan[2], Martin Childers[2], Linda Marbán[1] and Eduardo Marbán[1]
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Introduction: Cardiosphere-derived cells (CDCs) promote cardiomyogenesis and angiogenesis, while inhibiting oxidative stress, inflammation and fibrosis, in ischemic and non-ischemic cardiomyopathy. Recent mechanistic investigations implicate exosomes as central mediators of CDCs' beneficial effects. The *mdx* mouse model of Duchenne muscular dystrophy develops cardiomyopathy due to dystrophin deficiency and the resultant intense oxidative stress, inflammation and apoptosis. We have shown that CDCs are salutary in this model; here we tested the hypothesis that transplantation of CDC exosomes (CDC-XO) may generate similar beneficial effects. *Methods*: A total of 78 mice, including the mice recruited for assessment of CDC efficacy, were studied at a point when global cardiac dysfunction was already evident by echocardiography. CDC-XO (70 μg) or vehicle-only were injected intramyocardially in 4 left ventricular (LV) sites in 10-month-old *mdx* mice. *Results*: Similar to CDC-treated *mdx* mice, LV ejection fraction markedly improved over 3 months after treatment with CDC-XO compared to vehicle-treated mice (68.73 ± 2.82 vs. 57 ± 3.05; $p = 0.011$). The functional improvement was associated with reduced collagen I and III deposition and enhanced cardiomyocyte proliferation in the CDC-XO-treated mouse hearts. To assess the role of CDC-exosomes on mitochondrial function, which is impaired in Duchenne cardiomyopathy, cardiomyocytes derived from human Duchenne iPS cells (hDMD-CM) were primed with CDC-XO and assessed for mitochondrial respiratory capacity 1 week after. Oxygen consumption rate was impaired in control hDMD-CM, but normalized in hDMD-CM that had been treated with CDC-XO ($p < 0.05$). *Summary/conclusion*: Cardiac function improved in *mdx* mice treated with CDC-XO, accompanied by reduced cardiac collagen content and fibrosis, and augmented cardiomyogenesis. Priming hDMD-CM with CDC-XO markedly enhanced their respiratory capacity. Thus, CDC-exosomes mimic CDCs' beneficial effects in the heart failure associated with Duchenne muscular dystrophy. CDCs themselves, and their exosomes, are viable therapeutic candidates for Duchenne cardiomyopathy.

O-1B-2

Activation and reprogramming of hematopoietic stem and progenitor cell fate by MSC exosomes

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Introduction: Bone marrow mesenchymal stem cells (MSC) provide unique environmental cues to support homeostatic and regenerative function in the hematopoietic stem/progenitor cell (HSPC) pool. MSC are potent producers of vesicles, but their role in regulating hematopoiesis has not been elucidated. Here, we hypothesized that MSC exosome trafficking impacts cell fate decision making and proliferation of HSPCs. *Methods*: Murine bone marrow-derived MSC (*plastic adherent, osteocyte/adipocyte differentiation, CD45 −, lineage −, CD44 +, Sca-1 +, CD105 +, CD29 +),* HSPC (*C-kit +/ Sca-1 +/ Lineage −*), CFU-c assay, qRT-PCR, inflammatory cytokine PCR microarray, flow cytometry, and Ki67 cell cycle. *Results*: After 48 hours of in vitro exposure to MSC exosomes, we observed a 2-fold increase among C-kit + / Sca-1 + / Lineage- (KSL) progenitors, with an 80% increase in the KSL/CD48-/CD150 + (SLAM) stem cell populations, compared to the vesicle-free media control. Consistent with expansion of the multipotent progenitor cell pool, combined Hoechst/Ki-67 cell cycle analysis showed that exosome-exposed cells were more actively dividing compared to control. That loss of quiescence upon MSC exosome exposure was reflected in the downregulation of critical self-renewal genes (*Bmi-1, C-myc, Mef/Elf4*) and a significant increase ($p = 0.01$) in the number of progenitor CFU colonies. Furthermore, colony formation of pluripotent progenitors (CFU-GM) was decreased by 26%, against an increase in committed myeloid progenitors (CFU-G, + 74%; BFU-E, + 92%) in exosome-exposed cells. Indeed, extended exosome exposures resulted in comparative exhaustion of the colony-forming progenitors. Correlative experiments are now ongoing. To understand the mechanistic basis of our observations, we performed a survey of cytokine activity in exosome-exposed HSPC. Our experiments revealed the activation of inflammatory regulation of HSPC fate, and qRT-PCR analysis confirmed the upregulation of type I interferon signaling pathway members (*Stat1, Irf1, Irf9*) in exosome exposed HSPCs. Finally, molecular analysis of MSC exosomal content revealed an enrichment of a panel of microRNA, which we are currently investigating for dysregulation of interferon signaling. *Summary/conclusion*: Taken in aggregate, these data suggest that HSPC activation occurs via MSC exosome trafficking of microRNA and involves the upregulation of type I interferon-signaling pathways.

Ballroom F-H

Symposium session 1C - Standardization and organization of EV research

Chairs: *Alain Brisson and Louise Laurent* 13:15-14:00

Introductory Lecture: Rienk Nieuwland, Ph.D.

Medical Centre of the University of Amsterdam

O-1C-1

Bioinformatics analysis of extracellular vesicles using FunRich and Vesiclepedia

Suresh Mathivanan, Shivakumar Keerthikumar and Mohashin Pathan
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Introduction: In the last decade, extracellular vesicles (EVs) have attracted significant interest among scientists for their proposed role in intercellular communication, as reservoirs for disease biomarkers and as targeted drug delivery vehicles. Since its initial discovery, multiple groups have reported the secretion of EVs and characterized the transcriptomic, proteomic and lipidomic content of EVs. As high-throughput techniques including proteomics become more accessible to individual laboratories, there is an urgent need for a user-friendly bioinformatics analysis system. Here, we describe FunRich, an open access, standalone functional enrichment and network analysis tool. FunRich is designed to be used by biologists with minimal or no support from computational and database experts. *Methods*: FunRich tool was developed using Visual C#. Hypergeometric distribution test was performed to check the statistical significance of enriched and depleted terms. In addition, Bonferroni and Benjamini-Hochberg (BH) aka FDR (false discovery rate) method was also implemented to correct for multiple testing. *Results*: Using FunRich, users can perform functional enrichment analysis on background databases that are integrated from heterogeneous genomic and proteomic resources ($>$1.5 million annotations). Besides default human-specific FunRich database, users can download data from the UniProt database which currently supports 20 different taxonomies against which enrichment analysis can be performed. Moreover, the users can build their own custom databases and perform the enrichment analysis irrespective of organism. In addition to proteomics datasets, the custom database allows the tool to be used for genomics, lipidomics and metabolomics datasets. Thus, FunRich allows complete database customization and thereby permits the tool to be exploited as a skeleton for enrichment analysis irrespective of the data type or organism used. FunRich is user-friendly and provides graphical representation (Venn, pie charts, bar graphs, column, heatmap and doughnuts) of the data with customizable font, scale and colour (publication quality). *Summary/conclusion*: FunRich is designed to be used by biologists, biochemists and geneticists with minimal or no support from computational and database experts. Data sets from EV studies can be analysed using this software to identify signaling pathways that may underpin the pathophysiological role of EVs.

O-1C-2

ExRNA Biospecimen virtual repository: the CSF working group demonstration project

Sai Lakshmi Subramanian[1], William E. Butler[2], Bob Carter[3], Matthew Huentelman[4], Kendall Van Keuren-Jensen[4], Aleksandar Milosavljevic[1], Joseph F. Quinn[5], Julie Saugstad[5] and Fred H. Hochberg[2]
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Introduction: The development of minimally invasive biomarkers to assess disease risk, direct treatment or prevent secondary medical complications has the ability to profoundly change patient care in the coming decade. To develop, replicate and validate these biomarkers, we envision the necessary coordination of multiple steps to optimize yields of mRNAs and miRNAs emerging from SOPs for specimen acquisition and processing as well as seamless sharing of collected biospecimens to power these efforts. Each step in this process has the potential to exhaust scarce disease-specific biofluids. Equally problematic is the comparative analysis of these biomarkers from patients with specific medical conditions and normative populations. We hope to use these comparative analyses to identify optimal ways to enrich exRNAs of particular interest for the central nervous system. Ultimately this can be best facilitated through the sharing of IRB consent language, the use of universal MTAs and intellectual property agreements and biofluids with appropriate acknowledgement of the authors and funding agencies. We have piloted the database specimen acquisition and distribution of this shared NIH Common Fund biorepository within centers, whose aims involve the evaluation of cerebrospinal fluid for the diagnosis of primary tumours of brain (UCSD, MGH); subarachnoid hemorrhage (BNI, TGen); Alzheimer's disease and Parkinsonian syndromes (OHSU). *Methods*: To implement this collaboration we have proposed: (a) comparative studies of RNA yields resulting from 4 preparative approaches applied to pooled CSF specimens; (b) comparisons of 3 analytic approaches (RNA Seq; Qiagen qPCR; Nanostring) to detect disease-specific amplifications or mutations of genes of interest. Refinements to the database and repository will then be made after which, it will be offered as a resource to the Data Management and Resource Repository (DMRR) of the NIH Director's Office Extracellular RNA Communication Consortium. *Results*: The exRNA biospecimen virtual repository is a collective effort of the Resource Sharing Working Group and the DMRR to facilitate tracking of biospecimens. The repository is developed on the existing Genboree KnowledgeBase (GenboreeKB), a distributed and extensible infrastructure. Genboree REST APIs provide extensibility and enable controlled data sharing across physically distributed nodes using Linked Data technologies. The pilot biorepository provides inventory tracking of various biospecimens from the CSF group. Users see a Dashboard with a grid of specimens from specific diseases and samples from fluids of donors. *Summary/conclusion*: During an oral presentation we will provide the rationale and structure of the biorepository and patient safety standards and then demonstrate file-encoding, acquisition, bulk file acquisition on a "real-time basis" as a model for synergies within the ISEV community.

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Ballroom D
Oral with poster A
Chairs: *Dae-Kyum Kim and Fatemeh Momen-Heravi* 15:00-16:00

P-IV-16

Differential detergent sensitivity of extracellular vesicle subpopulations

Xabier Osteikoetxea[1], Barbara Sódar[1], Andrea Németh[1], Krisztina Pálóczi[1], Katalin Szabó-Taylor[1], Krisztina Vukman[1], Ágnes Kittel[2], Éva Pállinger[1] and Edit I. Buzas[1]
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Introduction: Earlier work from our research group provided evidence that extracellular vesicles can be differentiated from protein aggregates (e.g. immune complexes) by low concentration detergent treatment in flow cytometric experiments. *Methods*: In the present study, we investigated the sensitivity of different subpopulations of extracellular vesicles (including exosomes, microvesicles and apoptotic bodies) to detergent treatment using tunable resistive pulse sensing (qNano), flow cytometry and transmission electron microscopy. *Results*: We found that tunable resistive pulse sensing detection of extracellular vesicle subpopulations was compatible with differential detergent lysis. We determined the detergent concentrations that lysed each of the different vesicle subpopulations. Our qNano data show that microvesicles and apoptotic bodies are more sensitive to detergent lysis, while the signals of exosomes disappear only at higher detergent concentrations. The qNano findings are also in line with the disappearance of fluorescent labelling of the extracellular vesicles in flow cytometry. *Summary/conclusion*: Taken together, our data suggest that the combination of differential detergent lysis with tunable resistive pulse sensing or flow cytometry may provide useful for simple and fast validation of the vesicular nature of the detected particles. Furthermore, this approach may also provide information about the vesicle subpopulation being studied.

P-IV-15

A novel method to validate fluorescence nanoparticle tracking analysis for phenotyping extracellular vesicles

Rebecca Dragovic[1], Gavin Collett[1], Patrick Hole[2], Christopher Redman[1], Ian Sargent[1] and Dionne Tannetta[1]
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Introduction: Fluorescence Nanoparticle Tracking Analysis (fl-NTA) has the potential to enable phenotyping of extracellular vesicles (EV) down to ~50 nm in size, which is not possible using conventional flow cytometry. However, unlike flow cytometry, fl-NTA cannot currently measure EV in scatter and fluorescence modes simultaneously. To accurately determine the number of fluorescence positive EV, the operator must establish optimal camera levels for each mode. To do this requires a ''standard'' containing a known amount of EV positive for the antigen being studied. We aimed to prepare an EV standard to enable optimization of fl-NTA settings for quantification of placental alkaline phosphatase (PLAP – a trophoblast marker) positive EV in preparations of syncytiotrophoblast (STB) microvesicles (STBMV) and exosomes (STBEX) from human placentas. *Methods*: STBMV and STBEX were prepared using a dual placental perfusion model and differential centrifugation. Pools of STBMV and STBEX were prepared (4 samples/pool) and the percentage of PLAP+ve EV determined using anti-PLAP antibody Dynabead depletion. Pools were also labelled with anti-PLAP-Qdot605 or IgG1-Qdot605 control and measured using fl-NTA in scatter and fluorescence modes using camera levels 12–14. Scatter and fluorescence concentration measurements were used to determine the percentage of PLAP+ve EV and compared to the percentage of PLAP+ve EV removed by immunodepletion. Optimal camera settings were defined and used to analyse individual preparations (n = 8). *Results*: Using anti-PLAP Dynabeads, 59.8 ± 5.2% and 51.6 ± 3.4% of EV in STBMV and STBEX pools respectively were PLAP+ve. fl-NTA of STBMV and STBEX pools showed 48.5 ± 3.0% and 17.9 ± 3.3% PLAP+ve respectively. PLAP+ve EV in individuals ranged from 18.8 to 67.0% (STBMV) and 3.2–51.7% (STBEX). A positive correlation ($r^2 = 0.737$, $p < 0.01$) was found between STBEX size and PLAP positivity (%). *Summary/conclusion*: The use of anti-PLAP immunobead depletion provided the necessary standard to set fl-NTA settings. We show that fl-NTA is a robust method for determining the percentage of PLAP+ve EV in STBMV. However, fl-NTA is not as sensitive as Dynabead capture for STBEX detection using this particular marker, possibly due to the smaller STBEX having less surface PLAP. This same method could be used to validate settings for all fl-NTA immunolabelling.

P-IV-14

A new fluorescent probe for identifying extracellular vesicles

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Introduction: Flow cytometry methods for sub-micron particle quantification require higher precision in identifying extracellular vesicles from various sources of background noise. Currently many extracellular vesicles, particularly microvesicles (100–1,000 nm), are commonly identified with procoagulant markers annexin V or lactadherin but both have limitations. Whilst membrane loading techniques are possible, long incubation periods and wash steps are required making their practicality in large studies is questionable. They do however benefit from being able to use a fluorescent threshold making higher precision acquisition possible. They also have the benefit in distinguishing vesicles from noise. Here, we describe the use of a new generation of fluorescent markers capable of loading extracellular vesicles with the advantages of a short incubation period, no wash steps and the unique property of providing a ratiometric indication of negative and neutral charged phospholipids simultaneously. *Methods*: Twenty microlitres of extracellular vesicle-rich samples from 8 ml whole blood isolated in citrated tubes, and then centrifuged at 1,500g × 2, were suspended in 50 μl HBS or HBS + 2 mM Ca^{2+} and then stained with either 1 μl of 200 μM VRD and 5 μl of 25 μg/ml Annexin V or 10 μg/ml Lactadherin. Within these samples, microvesicles were identified with platelet markers CD41 and CD42, whilst lipoproteins were identified using ApoE antibodies. *Results*: Preliminary data show that lactadherin stains a higher number of microvesicles when compared to annexin V. There are, however, multiple lactadherin positive populations, most likely due to the fact that lactadherin binds to integrins such as CD61. As well as not having phosphatidylserine specificity, lactadherin staining shows a higher amount of noise when compared to VRD, which has little/no observable background noise at the 160 nm calibration. The VRD has a distinct linear correlation of size to fluorescent intensity, unlike lactadherin. Staining of microvesicles is equally as effective as lactadherin. We have shown that the VRD not only stains microvesicles but also lipoproteins identified as ApoE positive

events. The VRD, due to having dual emission peaks at 530 nm or 580 nm depending on lipid exposure, can indicate whether vesicles are primarily negatively charged phospholipids or neutrally charged phospholipids. *Summary/conclusion*: From the results above, VRD proves to be superior as a new generation dye for identifying extracellular vesicles. We believe that VRD has huge potential for future microvesicle studies due to its short incubation time, little/no background noise, and membrane specificity. It matches lactadherin for staining of microvesicles but without the draw backs of unspecific binding and blocking of integrins. Using VRD in future microvesicle studies will improve detection accuracy and throughput by allowing use of fluorescent thresholds over SSC/FSC thresholds, which to date have shown the highest accuracy and allow lower sizes of extracellular vesicles to be detected.

P-IX-7

Systematic comparative lipidomic and proteomic analysis of exosomes: a path toward reverse engineering and artificial exosome development

Reka Haraszti[1], Marie Didiot[1], Andrew Coles[1], Matthew Hassler[1], Julia Alterman[1], John Leszyk[2], Scott Shaffer[2], Hannah Rockwell[3], Michael Kiebish[3], Neil Aronin[4] and Anastasia Khvorova[1]
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Introduction: Extracellular Vehicles (EVs) are believed to be involved in native small RNA cell-to-cell transfer and signalling and are being explored as a potential novel formulation for therapeutic oligonucleotide delivery. The mechanistic underpinning of how EVs traffic to target cells and which components of the vesicles are essential for trafficking is unknown. EVs are usually derived from cell lines, and their potential mass production for therapeutic application is limited by cell-based manufacturing methods, with no quantitative QC parameters established. To realize the full potential of exosomes as a therapeutic oligonucleotide formulation, it is necessary to define the functional characteristics essential and responsible for efficient cellular delivery. *Methods*: The exosomes and corresponding cells were purified ($n = 3$) from different origins, and their lipidomic and proteomic content was evaluated using LC-MS/MS. Artificial lipid vesicles based on the exosome lipidomic profile were engineered, and their cellular trafficking and tissue distribution profiles were evaluated. *Results*: Systematic lipidomics detected 19 structural lipids. Surprisingly, exosomes were characterized by a specific lipidomic profile and were enriched in sphyngomyelin, glycolipids, ceramide and cholesterol-esters and depleted in triacyl-glycerol and coenzyme Q. Alteration of the lipid profile affected the cellular trafficking and tissue distribution profiles of the artificial exosomes engineered based on native exosome profiles. Proteomics identified 2,394 proteins, with 223 being present only in cells, 85 in exosomes and 19 in microvesicles. Further studies will be focused on defining the protein exosome components essential for trafficking. *Summary/conclusion*: Preliminary analysis showed distinct protein and lipid profiles of exosomes. This information may help engineer artificial exosomes as delivery vesicles of therapeutic oligonucleotides and help define a QC matrix for exosome manufacturing.

P-III-1

Specific isolation of tumour-derived extracellular vesicles using microfluidic technologies

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Introduction: Extracellular vesicles (EVs), lipid vesicles present in blood and other biofluids, have sizes that vary from 100 nm to a few microns and carry rich biological cargo that includes proteins, mRNA, microRNA and DNA. Through their content, EVs preserve the transcriptome information of their parent cells. Thus, they serve as a unique nich of opportunities for the development of cancer diagnostic tools. However, EVs have not seen widespread clinical testing due to limitations in isolation technologies that rely on ultracentrifugation and precipitations processing that yield low-throughput outcomes, and a lack of tumour-EV specificity. Here we present a microfluidic platform with a temperature responsive nanointerface for tumour-specific recovery of exosomes through immunoaffinity immobilization. *Methods*: The microfluidic device consists of a ridged geometry that induces mixing and promoting collisions between exosomes and the antibody functionalized walls of the device. The nanointerface consisted of an ultra-thin (135 nm) gelatin membrane functionalized with nanoparticles that created a nanotexture to maximize EV surface interactions. To mimic conditions present in patient samples, we used engineered exosomes derived from human Gli36 glioblastoma cells that stably express the fluorescent (PalmGFP/tdTomato) and bioluminescent EV reporter (EV-GlucB), and subsequently spiked them in healthy human serum. *Results*: Using confocal microscopy, we identified tumour-specific exosomes at the surface of the device that were capture using anti-cetuximab, an antibody for EGFR. Our system was able to isolate 21% of tumour-derived spiked exosomes at processing flow rates of 1 ml/h. Moreover, the temperature sensitivity of the nanocoating was used for the recovery of EVs on demand from the surface of the microfluidic device after heating at 37°C for 10 minutes. We also performed molecular analysis to determine the total amount of RNA present in the isolated EVs. We detected RNA from the isolated EVs that indicated the effectiveness and sensitivity of our method. From this amplified RNA, deep sequencing or transcriptome analysis is currently being performed to identify tumour-specific microRNAs. *Summary/conclusion*: We have developed a microfluidic technology that can be used for the specific isolation of tumour-derived EVs, that can be applied to different type of cancers. The responsive nanocoating, allowed the recovery of EVs on demand to perform additional characterizations like imaging or size characterization. This unique system also will allow the identification of mRNAs or microRNAs as biomarkers due to the purity of the EVs population.

P-XVI-4

Diversity of extracellular vesicles in human ejaculates identified by cryo-electron microscopy and tomography

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Introduction: Extra-cellular vesicles in ejaculate are important for male fertility as the vesicles capacitates the sperm, increases sperm motility and modulates female genital tract immune cell response. This population of extra-cellular vesicles found in ejaculate are mostly of prostate (prostasomes) and epididymal (epididymosomes) origin but vesicular contributions of other tissues are also present. *Methods*: Ejaculates were plunge-frozen and visualized using cryo-electron microscopy and tomography. *Results*: Here, we show that when EVs in ejaculates are studied in unaltered plunge-frozen ejaculates using cryo-EM and electron tomography, a high number of morphologically distinct subgroups of extra-cellular vesicles are revealed. Some vesicles have a smooth surface, whereas others show spiky membrane protrusions. Cryo-electron tomographic 3D reconstructions show that vesicles may contain one or more smaller vesicles and large sacs filled with extra-cellular vesicles are also detected. A large population of tubular structures, sometimes branched and up to 1–2 μm in length, was also found. Frozen events of vesicular fusion (or budding) with the spermatozoa were imaged, showing which vesicle sub-populations can interact or originate from the spermatozoa membrane. *Summary/conclusion*: Identification of these EV sub-populations provides an inventory of the complex composition of extra-cellular vesicles found in human ejaculate, their morphologies and prevalence.

This inventory is important to quality assure future extracellular vesicle isolates. We are now faced with many new questions about the role of the individual extracellular vesicle subgroups in human fertility.

P-XVI-12

Extracellular vesicles and small RNA of the cervicovaginal compartment in Macaques

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Introduction: Macaques are an excellent model for human disease. Comparatively little is known about extracellular vesicles (EV) and their RNA cargo in the cervicovaginal compartment, especially, in macaques. In this study, we compare EV and RNA in cervicovaginal secretions and cervicovaginal lavage (CVL) of rhesus and pigtailed macaques and report on a case study of a macaque with endometriosis. *Methods*: Four total cervicovaginal lavage (CVL) and vaginal swab (VS) samples from rhesus and pigtailed macaques were utilized. Nanoparticle tracking analysis was performed on whole CVL and EV fractions enriched by stepped ultracentrifugation. Total RNA was obtained with an optimized method for biofluids RNA extraction. miRNAs were profiled with a medium-throughput stem-loop/hydrolysis probe qPCR platform in CVL, VS, and matched vaginal tissue samples. *Results*: As in our work with archived human CVL, miRs-223 and -186 were among the most abundant in CVL of rhesus and pigtailed macaques. Of more than 60 miRNAs detected consistently in all samples, only miR-29b and miR-184 were approximately as abundant in the EV as in total CVL. U6 was present almost exclusively in whole CVL. On average, 1% of CVL miRNA copies were found in EV but 2% of VS, likely due to better liberation of EV from mucus during VS swab processing. No differences were apparent in RNA expression in CVL or VS fractions from the 2 macaque species, although minor expression differences were observed at the tissue level, interestingly in levels of numerous let-7 family members. Comparison of particle numbers in the various reproductively healthy samples and in an individual rhesus with severe endometrial disease revealed lower particle count in the diseased animal. *Summary/conclusion*: EV analysis may be useful as a diagnostic tool in the cervicovaginal compartment, especially in the case of reproductive tract disease. Controlled studies of cervicovaginal EV and their RNA cargo are merited to characterize the potential role of EV and specific small RNAs as markers for any manifestation of reproductive tract disease.

P-IV-8

Pre-preparation of extracellular vesicles by electro-dialysis and adsorption-elusion methods

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Introduction: Currently existing extracellular vesicle (EV) pre-preparation methods have been challenged. Ultracentrifuge has low yield, consumes time and is laborious. Precipitating methods have coagulation problem on blood plasma and nano-particle contamination. In immune-affinity-based separation, elimination of attached EV antibody is difficult, and this elimination causes irreversible damages to whole EV or surface protein. In this study, we introduce electrodialysis and elution system, which can achieve high yield, low buffer and nano-particle contamination, and no coagulation. *Methods*: Separation chamber is composed of 2 poles of electrode at both the ends and thin planar sandwich partitioned channels between the electrodes. Polycarbonate track-etched membranes partition the chamber, and sample flow goes into the channel covered by the membranes. In the channels outside of the membranes, where electrodes are located, waste buffer runs tangentially to membranes. As original sample flow also crosses the channel tangentially, electric field is applied to the direction of membranes. Particle streamline is deflected and particles approach the membrane surface. Because the membranes have specific pore diameter, 30 nm, particles bigger than 30 nm get adhered on the membrane, but smaller particles get through the pores. By this process, larger particles, EVs, remain until the dialysis process gets over. After the dialysis, remaining sample is collected and membranes are disassembled. Adhered EV on the membranes is eluted by pipetting. *Results*: With protein solution, it was checked that lower sample flow and higher voltage show higher protein removal rate, but higher voltage causes heat damage on the sample. The damage was detected where the average power dissipation is higher than 0.5 W, so following tests were set to have power limit and 0.04 ml/min sample flowrate. Comparison of EVs yield and purity from mouse blood plasma was done for existing methods: ExoQuick, ultracentrifuge and this system. According to RNA recovery rate, yield of this system is similar to ExoQuick, but far higher than ultracentrifugation. This system's protein removal rate is slightly better than ExoQuick. By dynamic light scattering test, ExoQuick showed no clear peak on size distribution, but this system showed clear size peak at the range of typical EVs, from 50–200 nm. Also, CD9 western blotting and RT-PCR of beta-actin were done. All methods preserve surface proteins and RNA contents well. *Summary/conclusion*: This electrodialysis-elution system showed fast and high yield milliliter scale EV pre-preparation methods. Because of size exclusive membrane dialysis, this system showed minimal nanoparticle contamination than existing methods. Also, the buffer used in this test is a sucrose-based buffer in which cells can be cultured without immune reaction or pH disturbance. Because of it, this method may preserve surface property of EV than other chemical based preparation methods.

P-XX-1

Role of extracellular vesicles and miRNA cargo in the control of epididymal functions important to sperm maturation

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Introduction: Among male infertility cases, 30% are idiopathic and may result from impairment of sperm maturation in the epididymis. This organ is a single tubule of the male reproductive tract in which spermatozoa transit to acquire their motility and capacity to bind and fertilize the oocyte. Because of its unique region-specific gene expression pattern, the epididymis is a model of choice to study the influence of regulatory factors on gene expression. Among these factors, we assessed the contribution of extracellular vesicles and their miRNA cargo from the male reproductive system in the control of target cell functions via a mechanism of intercellular communication. *Methods*: Our multidisciplinary research combines the use of microarray as well as electron microscopy and cytometry technologies on human epididymal samples and transgenic mouse models, which display severe male infertility phenotype due to impairment of miRNA maturation or production. *Results*: We identified a strong correlation between the expression of miRNAs and their target transcripts all along the human epididymis, suggesting the involvement of miRNAs in the regionalized pattern of expression in this organ. Among these targets we identified several genes, including Claudin 10, that encode proteins involved in the blood-epididymis barrier integrity. Furthermore, we explored the possibility that miRNAs could be involved in a mechanism of extracellular communication between epididymal epithelial cells via extracellular vesicles, referred to as epididymosomes. We identified 1,645 extracellular miRNAs associated with extracellular vesicles from the proximal and distal regions of the epididymidis. Importantly, we showed that extracellular miRNA repertoires from these two epididymal regions are distinct and differ from the miRNA signature identified in epididymal epithelial cells,

suggesting that miRNA populations released from the cells are selectively sorted in a region-specific manner. Furthermore, *in vitro* assays performed on primary cultured epididymal cells indicated that extracellular vesicles from the proximal region interact with distal epithelial cells and may transfer their miRNA content to recipient cells, as evidenced by our recent findings on transgenic mouse models. Since the population of extracellular vesicles from the epididymis are highly heterogeneous, we recently optimized the detection and characterization of these extracellular vesicles by cytometry and cryo-electron microscopy. *Summary/Conclusion*: Altogether, our findings reveal that extracellular miRNAs conveyed by extracellular vesicles throughout the male reproductive tract could be efficient regulators of epididymal functions and male fertility, and may be used for the non-invasive diagnostics of idiopathic male infertility cases.

P-XIII-1

Isolation and characterization of *Citrus limon* L. derived nanovesicles: potential use as antineoplastic agent

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Introduction: A large amount of data has been produced on the release of extracellular vesicles from mammalians, but only a few information is available on the presence of nanosize vesicles in plant species. Here we focused on the isolation and characterization of nanovesicles released from lemon juice in order to test their antitumor efficacy. *Methods*: Nanovesicles were isolated from *Citrus limon* L. juice by differential centrifugation and filtration steps. Vesicles were characterized through electron microscopy, dynamic light scattering analysis and proteomic approaches. Viability assays and real-time PCR analysis were performed in cancer (colon, lung, chronic myeloid leukaemia, multiple myeloma, liver) and normal cells following treatment with nanovesicles. A tumour xenograft model was used to test the *in vivo* effect of isolated nanovesicles. *Results*: Isolated nanovesicles were identified as a homogenous population (50–70 nm) of exosomes-like particles and the analysis of vesicles protein composition revealed the presence of proteins that were homologous to mammalian exosomal proteins. PKH26-labelled nanovesicles were internalized in a dose dependent way by cancer cell lines. Furthermore, different cancer cell lines treated with citrus nanovesicles showed a dose and time dependent decrease of cell viability and a simultaneous increase of cell death pathways. These results were also confirmed in an *in vivo* tumour xenograft model. *Summary/Conclusion*: We isolated and characterized nanovesicles from edible *Citrus limon* with size and composition similar to mammalian-derived exosomes. Furthermore we show an *in vitro* and *in vivo* anti-proliferative and pro-apoptotic effect of these vesicles. This study opens the possibility of using this natural plant-derived nanovesicles as antineoplastic agents.

P-XX-5

Acoustic microfluidic system for microvesicle purification

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Introduction: Circulating microvesicles (MVs) have emerged as a promising surrogate for tissue-based markers, enabling non-invasive and real-time disease monitoring. Purifying MVs for analyses, however, remains a challenging task, which often involves time-consuming and extensive procedures (e.g., ultracentrifugation, multiple filtration). *Methods*: We herein present a new microfluidic platform for MV isolation and enrichment from clinical samples. The system utilizes acoustophoresis to size-selectively separate MVs. Interdigitated electrodes, patterned on piezoelectric substrate, were used to generate standing surface acoustic wave (SSAW) inside a microfluidic channel, and the resulting acoustic radiation force separated MVs according to their size and density. The design and operation of the device was optimized through numerical simulation. *Results*: The system achieved $>90\%$ sorting yields, and the size cut-off could be tuned *in situ* through controlling the acoustic power. We used the system to collect MVs from pRBC (packed red blood cell) samples as well as from cell culture media. The microfluidic-SSAW device successfully isolated and enriched pure MV population, which was confirmed by downstream molecular analyses. *Summary/Conclusion*: Enabling label-free and continuous in-flow separation, the developed platform could be an ideal tool for fast preparation of intact MVs.

P-XIII-17

Label-free single exosome detection using frequency-locked microtoroid optical resonators

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Introduction: Recently exosomes have attracted interest due to their potential as cancer biomarkers. We report the real time, label-free sensing of single exosomes in serum using microtoroid optical resonators. We use this approach to assay the progression of tumours implanted in mice by specifically detecting low concentrations of tumour-derived exosomes. *Methods*: Our approach measures the adsorption of individual exosomes onto a functionalized silica microtoroid by tracking changes in resonant frequency of the microtoroid. When exosomes land on the microtoroid, they perturb its refractive index in the evanescent field and thus shift its resonance frequency. *Results*: Through digital frequency locking, we are able to rapidly track these shifts with accuracies of better than 10 attometers (one part in 10^{11}). Samples taken from tumour-implanted mice from later weeks generated larger frequency shifts than those from earlier weeks. Analysis of these shifts shows a distribution of unitary steps, with the maximum step having a height of 1.2 fm, corresponding to an exosome size of 44 nm. *Summary/Conclusion*: Our results demonstrate the development of a minimally invasive tumour "biopsy" that eliminates the need to find and access a tumour.

Ballroom E
Oral with poster B
Chairs: *Shivani Sharma and Hakho Lee* 15:00-16:00

P-IX-17

Proteome-wide profiling of circulating exosomes for identification of scirrhous gastric cancer biomarkers

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Introduction: Recently biological significance and clinical utility of exosomes have been extensively investigated. Particularly, in the field of cancer diagnosis, exosomes are considered as ideal targets for biomarker discovery due to their molecular characteristics. Indeed, a set of molecules expressed in original tumour cells can be detectable from exosomes in blood circulation. However, despite enormous interest in exosomes, difficulties in exosome isolation from biological fluids have significantly hindered effective discovery of biomarker components. In the present study, we developed EV-Second (Extracellular Vesicle isolation by Size Exclusion Chromatography ON Drip column) technology allowing rapid collection of highly pure exosomes in flow-through fraction by simple gravity drip in order to identify early detection biomarkers for scirrhous gastric cancer. *Methods*: We employed EV-Second columns to perform quantitative proteome profiling of serum exosomes from 58 individuals (10 normal controls, 17 early-stage gastric cancer patients, 17 advanced-stage gastric cancer patients, and 14 scirrhous gastric cancer patients) using LC-MS/MS shotgun analysis. The proteome-wide datasets of exosomes were quantitatively compared and statistically evaluated on the Expressionist proteome server system to extract exosomal biomarker candidate proteins. The identified biomarker candidates were biologically assessed in vitro and in vivo. *Results*: Among 822 identified proteins by LC-MS/MS analysis, 299 serum-derived proteins were removed based on GO. Student's t-test (normal group vs. gastric cancer group) using 523 exosomal proteins revealed that 13 proteins were significantly up-regulated in gastric cancer-derived exosomes ($p < 0.05$, fold change > 2.0, and valid value $> 80\%$). We furthermore focused on 3 of them and investigated for cancer-associated functions. These experiments strongly suggested the existence of an exosome-driven positive feedback loop regulating tumour microenviroments. *Summary/conclusion*: Our EV-Second technology (commercially available from GL Science company) can provide the best way for high-throughput exosomal biomarker screening studies using multiple clinical specimens. The 13 biomarker candidate proteins in cancer-associated exosomes would be able to explain more new insights concerning tumour progression or metastasis.

P-XII-7

Characterization of the functional role of rhabdomyosarcoma-derived exosomes in tumour cell biology and investigation of their miRNA cargo

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Introduction: Rhabdomyosarcoma (RMS) is an aggressive childhood soft tissue tumour, with two distinct subtypes, alveolar (ARMS) and embryonal (ERMS) histologies. ARMS is characterized by a more aggressive clinical phenotype and a specific translocation PAX-FOXO1, thought to contribute to its aggressive and metastatic behaviour. Exosomes are small membranous vesicles secreted into body fluids by multiple cell types, including tumour cells. Tumour exosomes contain intact and functional proteins, mRNA and miRNA that may alter the cellular environment to favour tumour growth. *Methods*: We characterized exosomes from a panel of 5 RMS cell lines and examined their functional effects on cell migration, invasion, proliferation and viability. We characterized the miRNA cargo of ERMS- and ARMS-derived exosomes using microarray profiling. *Results*: RMS-derived exosomes significantly increased the cellular migration and invasion of normal fibroblasts and had a positive effect on cell viability of both fibroblasts and tumour cells. Expression array analysis showed that exosomal miRNA clustered together well, and to a higher extent than cellular miRNA, in both ARMS and ERMS cell lines. Commonly enriched miRNA in exosomes derived from ERMS cell lines were different from those in exosomes derived from ARMS cell lines. There were only 2 miRNA in common among both ERMS and ARMS cell lines; putative targets were found to be implicated in cancer and inflammation. *Summary/conclusion*: RMS exosomal cargo results in specific effects on cell biology, enhancing invasive potential of recipient cells. Commonly enriched miRNA in exosomes of ERMS cells are separate from those of ARMS cells, possibly defining potential biomarkers for ERMS and ARMS, respectively. Moreover, these enriched miRNA may contribute to the paracrine signaling specific to each tumour subtype. Current work is focused on analysis of the effects of the identified miRNA on paracrine signaling in RMS, and on their validation as possible biomarkers.

P-XII-8

Identification of optimal culturing conditions for studying exosomes from normoxic and hypoxic colorectal cancer cell lines

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Introduction: Tumour hypoxia (oxygenation deficiency) contributes significantly to treatment resistance, metastatic progression and poor survival in colorectal cancer (CRC) and evidence supports a central role of exosomes in the aggravated biology caused by tumour hypoxia. In this study, we aimed to identify the optimal culturing conditions for the study of exosomes in normoxic and hypoxic CRC cell lines. *Methods*: Growth characteristics were investigated in the CRC cell lines HCT 116, RKO, LoVo, and HT-29 under normoxia (21% O2) and hypoxia (0.2% O2), using 4 different cell culture media: RPMI-1640 with 10% fetal bovine serum (FBS), RPMI-1640 with 10% exosome-deprived FBS, RPMI-1640 with bovine serum albumin (BSA), and serum-free RPMI-1640. Adherent cells were washed thrice with phosphate-buffered saline before media were added and cells were exposed to normoxia or hypoxia for 24–48 hours. Cell confluency was determined by visual inspection; cell viability by a proliferation assay (MTS); cell doubling-time by cell count; and cell cycle progression by flow cytometry. *Results*: After 24 hours, the viability of normoxic and hypoxic HCT 116, RKO and LoVo cells was increased at all conditions. In contrast, for normoxic and hypoxic HT-29 cells, both serum-free and BSA-containing medium caused a decrease in cell viability. The doubling time of normoxic and hypoxic RKO cells was 16–23 hours and for LoVo cells 19–35 hours depending on the condition. For normoxic HCT 116 and HT-29 cells the doubling time was 17–34 hours depending on the condition. For hypoxic HCT 116 and HT-29 cells cultured in serum-free medium, estimated doubling time was 96 and 66 hours. Cell cycle analysis revealed that normoxic and hypoxic RKO and LoVo cells in serum-free medium arrested in G1 phase. *Summary/conclusion*: Bovine exosomes (in FBS) and exosomes from cells arrested in the G1 phase of the cell cycle may impinge on

downstream analyses. Hence, our data suggested that RPMI-1640 with BSA is the optimal culturing medium for studying exosomes in normoxic and hypoxic CRC cells. The HT-29 cell line was found ineligible for exosome studies.

P-XII-11

Tumour cells and macrophages: can mutant p53 be the matchmaker using exosomes?

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Introduction: Both epithelial mutations in p53 as well as the presence of tumour-associated macrophages (TAMs) are hallmarks of solid tumours. We hypothesized that an epithelial cell harboring mutant p53 (mutp53) can educate the adjacent macrophages to be polarized towards a specific macrophage subset promoting tumorigenesis, eventually resulting with a TAMs population inside a tumour. *Methods*: By designing a co-culture system, we incubated human primary monocytes together with colorectal cancer (CRC) cells differing in their p53 status. Relevant macrophages markers were evaluated on RNA level (qPCR) and protein level (ELISA, Flow cytometry). In addition, co-cultured macrophages were subjected to various functional assays (phagocytosis, migration, invasion). In an attempt to confirm clinical relevance, samples from a cohort of human CRC patients were analyzed using genomic and immunohistochemical methods. To identify the interaction between the tumour cells and the macrophages, we isolated exosomes from the CRC cells and subjected them to a Nanostring analysis to learn about their microRNAs composition. *Results*: When monocytes were matured to become macrophages while exposed to tumour cells harbouring mutp53, they became polarized towards a distinguished subset of macrophages characterized by TAMs-related markers. These findings were in high correlation with samples taken from CRC patients sequenced with mutp53 tumours but not wild-type (WT) p53 tumours. Investigating the exchange of messages between the 2 entities via exosomes revealed a mutp53-specific miRs signature led by miR-1246 which could be incorporated into adjacent macrophages promoting the TAM phenotype and creating an invasive front together with tumour cells. *Summary/conclusion*: This study is the first to show a non-cell-autonomous role played by mutant p53 – the most common form of mutation found in human cancers. Deciphering the intricate microenvironmental regulation shared by the tumour cell and its surrounding macrophages may lead to breakthroughs in prognosis, diagnosis (bio-markers) and therapeutics aiming to target the key molecules involved.

P-XII-15

Functional analysis of extracellular vesicles as a novel regulatory agent of scirrhous type gastric cancer microenvironment

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Introduction: Scirrhous type gastric cancer (GC) is highly metastatic and characterized clinically by rapid disease progression and poor prognosis. Therefore, better understanding of the pathological and biological basis of scirrhous type GC is necessary to improve diagnosis and treatment. Extracellular vesicles (EVs) are small membrane vesicles that are secreted by various cell types including cancer cells and surrounding stromal cells. Evidence support that some components of EVs derived from cancer stroma, including microRNAs (miRNAs), function to promote the progression, growth and spread of cancer. However, the precise mechanisms controlling cancer microenvironment are not well understood. In the present study, we aimed to investigate the difference of miRNA expression in EVs between cancer stromal fibroblasts and normal fibroblasts (NFs) and identify the specific extracellular miRNAs of cancer stromal fibroblasts (CaFs). *Methods*: To check the differences of amounts and characters between NF derived EVs and CaF derived EVs, we performed nanoparticle tracking analysis (NTA) and western blot analysis of EV markers. Furthermore, we also investigated the effect of EV derived from NF and CaF on the proliferative activity of GC cell lines by MTS assay. *Results*: NTA showed that the amount of EVs was not changed between NF and CaF. CD9 and CD63 expression, as markers of EVs, were detected on each fibroblasts derived EVs. There was no difference of these marker expression between NF derived EVs and CaF derived EVs. However, CaF derived EVs could affect on GC cell proliferation, but not NF derived EVs. *Summary/conclusion*: These data suggested that CaF-derived EVs might be effective on cancer progression. Now, we will perform miRNA microarray analysis and the results of expression analysis of miRNAs in each EVs will be discussed on the basis of function on the intercellular communication.

P-XII-18

Role of extracellular vesicles derived from mesenchymal stem/stromal cells in breast cancer progression and metastasis

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Introduction: Recent studies have demonstrated that extracellular vesicles (EVs) play a supportive role in cancer initiation, progression, angiogenesis and metastasis. Human mesenchymal stem cells (hMSCs) are shown to act as stromal cells in solid tumours and promote tumour progression, partly through their secretome. Our lab has shown that hMSCs are resilient to serum deprivation and have the ability to survive for longer periods using autophagy. This in vitro model is used to study solid tumour core. The secretome of the stressed cells is tumour supportive, demonstrating that this model mimics solid tumour core. EVs are secreted by various types of cells and are made up of a double membrane of phospholipids that contain proteins, mRNA and microRNAs (miRNA). miRNAs act as post-transcriptional regulators and affect the regulation of protein-coding mRNA. In our previous studies, we characterized the EV cargo from serum deprived hMSCs (SD-hMSCs) and their roles in breast tumour progression. In this study, we have developed an invivo model to investigate the role of EVs in breast tumour metastasis. *Methods*: hMSCs were cultured in α-MEM without serum for a period of 15–30 days. Culture supernatant was centrifuged to remove large floating debris and was concentrated 120 times using an ultrafiltration cellulose membrane (cutoff 1 kDa) mounted on a N_2 positive pressure system (Amicon). EVs were purified from concentrated supernatant after a series of ultra-centrifugation steps and were quantified by protein content. For internalization studies, EVs were labelled with PKH26 red fluorescence cell linker. miRNA silencing in hMSCs was performed using locked nucleic acids (LNAs) from Exiqon. In vivo orthotopic tumour progression modelling was performed on nude mice and tumour size was measured by vernier calipers. For invivo metastatic mouse model, nude mice were administered with luciferase expressing breast tumour cells with or without EVs into their left ventricle. Different organ metastasis was monitored by luciferase signal detected by IVIS imaging station. *Results*: Next-Gen sequencing assays for non-coding RNAs in EVs from SD-MSCs indicated the presence of tumour supportive miRNAs. As a proof of concept, miRNA silencing studies were performed which confirmed the role of miRNA-21 and -34a as tumour supportive miRNAs. The orthotopic xenograft mouse model using breast cancer cells (MCF-7) demonstrated the tumour supportive function of SD-MSC derived EVs. On the contrary, in vivo metastatic mouse model demonstrated that EVs from SD-MSCs

suppresses breast cancer (MDA-MB-231) metastasis. *Summary/conclusion*: These findings suggests that EVs transfered miRNAs from hMSCs may promote breast cancer progression but inhibit metastasis. Further studies to identify-specific factors responsible are in progress.

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P-VII-8

Newly designed size exclusion chromatography columns for isolation and purification of extracellular vesicles in clinical samples

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Introduction: Isolation of pure extracellular vesicles (EV) from body fluids is crucial for the development of novel diagnostic and therapeutic tools. Ultracentrifugation and EV isolation kits are currently the methods of choice but recently Size Exclusion Chromatography (SEC) has shown to be a simple and efficient method for isolating EVs. In this study, we have designed 2 SEC columns to improve reproducibility, separation efficiency and purity of EVs in plasma. *Methods*: Platelet free plasma (PFP) from healthy donors and patients was applied to 3 different sepharose CL-2B columns; a 10 ml commercially available and an in-house 31 ml column, both separating by gravity, and an in-house 130 ml column separating by Fast Protein Liquid Chromatography (FPLC). The fractions were analyzed by: electrophoresis and spectrophotometer to verify the columns efficiency and vesicle purity, Nanoparticle Tracking Analysis (NTA) for total vesicle concentration and size distribution, Transmission Electron Microscopy (TEM) for morphology and quantitative Mass Spectrometry (MS) to annotate the identified proteins associated to specific EVs according to JEV guidelines. *Results*: NTA, spectrophotometry and electrophoresis data show a distinct separation of particles and proteins for all columns. Furthermore, all columns separate particles by size. TEM and MS data demonstrated that SEC holds potential in isolating EVs from lipoproteins. EV isolation on the commercial available, and the in-house 31 ml column showed analogous separation and purification profiles. However, the in-house FPL-connected column improved the purity and isolation of EVs in plasma substantially with excellent reproducibility. *Summary/conclusion*: Our results accentuate that our newly designed SEC columns hold potential in the research field of EVs, and a 130 ml column using FPLC clearly improves separation. SEC may add to the study of EVs in diseased states enabling recognition of novel biomarkers for diagnosis and treatment.

P-XI-9

Astrocyte-derived exosomes regulate dendritic complexity and synaptic protein expression in neurons

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Introduction: Bi-directional communication between astrocytes and neurons regulates synaptic formation, synaptic strength and participates in the regulation of neural circuitry by coordinating activity. To date, relatively few mediators of this network have been identified. Recent advancements in the biology of extracellular vesicles suggest that microparticles may function as mediators of glia to neuron communication. *Methods*: To address this question, we developed a method that selectively isolates exosomes from astrocytes stimulated with ATP (10 μM, 2 hours). To evaluate the functional effect on neurons, a dose response of exosomes (1−100 particles/cell) were applied to a primary rat neuronal culture. *Results*: These isolated particles were 70 ± 30 nM in diameter, were enriched in CD63 and contained a ceramide content that was characteristic of endosomal origin. When applied onto neurons, these astrocyte-derived exosomes produced a dose dependent increase in dendritic complexity, increased the expression of the postsynaptic marker PSD95 and the requisite NMDA receptor subunit NR1. To determine the molecular mechanisms for these effects, we performed whole genome shotgun sequencing in neurons treated with exosomes for 6 and 12 hours. A total of 11,194 genes that contained a minimum of 4-hits per transcript were analyzed, and we identified 546 transcripts showing expression changes ≥ 2 SD (192 increased and 354 decreased). *Summary/conclusion*: A number of these transcriptional products are known to regulate PSD95, receptor trafficking or function as scaffolding proteins that regulate the localization and function of receptors to post synaptic specifications. These data suggest that exosomes released from astrocytes may regulate neuronal function through the modulation of synaptic structures.

P-VI-11

Altered compartmentalization of KIT enhances death receptor 5 and KIT-enriched exosome release by imatinib-treated gastrointestinal stromal tumours

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Introduction: Gastrointestinal stromal tumours (GISTs) are oncogene addicted tumours that are driven by activating mutations in the receptor tyrosine kinase KIT. The use of imatinib mesylate, a tyrosine kinase inhibitor, as first line therapy in metastatic GIST patients has been shown to delay tumour progression. However, most patients who initially respond to the drug eventually develop resistance through activation of autophagy and/or quiescence. The lack of curative strategies, emergence of secondary resistance and ultimately tumour progression underscore the need for additional therapeutic targets other than inhibition of KIT. To address the role of autophagy mediated survival on KIT receptor expression and its subcellular localization during imatinib treatment, we identify the molecular events involved in this process and the qualitative/quantitative impact on exosomes release and composition. *Methods*: Exosomes were isolated by differential ultracentrifugation from the conditioned medium (CM) of untreated or vehicle (DMSO), imatinib mesylate (IM), balfilomycin A1 or the drug combination-treated GIST882 and GIST-T1 and analyzed by Nanoparticle tracking analysis and electron microscopy. Cell viability after treatment and combination index calculation were performed using CellTiter-Blue reagent according to the manufacturer's protocol (Promega). Apoptosis and autophagy-related proteins and transcript were analyzed by protein array, western blotting, flow cytometry and qRT-PCR. Effects of the drugs on KIT compartmentalization was performed using both cellular fractionation and confocal microscopy. All experiments were performed in triplicate. *Results*: Treatment of GIST cells with bafilomycin A1 alone impaired recycling and significantly increased KIT at the plasma membrane (PM) and in the late endosomal compartment. Although, the combination of bafilomycin A1 and IM enhanced KIT expression at the PM it synergistically inhibited GIST cells viability, impaired AKT activation and sensitized GIST cells to imatinib-induced apoptosis. In addition, we observed a significant increase in the expression of death receptor 5 (DR5) and phosphorylation of p53 in GIST cells after combination treatment using bafilomycin A1 and IM. Finally, analysis of exosomes released during this dual therapy showed copious amount of KIT, DR5 and autophagy-related proteins, suggesting the possibility to follow these markers systemically on circulating exosomes. *Summary/conclusion*: Our findings provide novel insights on how endocytic receptor trafficking regulates

activation of downstream signaling pathways and exosomes composition. Our study provides new evidence to prove that targeting molecules involved in KIT compartmentalization could be used therapeutically to augment the cytotoxicity of imatinib in GIST. Finally, since combination treatment enhances expression of several autophagy-related proteins on exosomes, this finding further suggests the possibility to use exosomes as markers of response.

P-VII-10

Exosome fractions of conditioned media from normal and pre-eclamptic placental villi are selectively anti-angiogenic and reversed by low molecular weight heparin

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Introduction: Severe pre-eclampsia (sPE) is a placenta-mediated disorder characterized by systemic endothelial cell (ET) dysfunction. The syncytiotrophoblast (SCT) layer, which is the outer surface of the human placenta, communicates with the maternal circulation via the secretion of soluble proteins, microvesicles and exosomes. Media conditioned by sPE or first trimester placental villi are anti-angiogenic. This effect is reversed by low molecular weight heparin (LMWH). Clinical trials demonstrate that LMWH significantly reduces the recurrence risk of sPE via unknown mechanisms. We hypothesize that LMWH treatment will modulate the intracellular signalling mechanisms and block the uptake of placenta-derived vesicles by maternal endothelium. *Methods*: Conditioned media was obtained from floating human villus explant (across gestation and in sPE; $n = 5$/category) over 72 hours; the exosome-enriched fraction (ExF) was derived using differential centrifugation and characterized using Nanoparticle tracking analysis, Western blotting and electron microscopy. ExF was labelled using PHK67 to track entry into target ET cells using confocal microscopy. Angiogenic properties of ExF were assessed using the endothelial (ET) tube formation assay (branch point/capillary tube length) in human umbilical vein (HUVEC) and uterine microvascular (UTMEC) cell lines in the presence/absence of LMWH (enoxaparin; 0.125–50 IU/ml). *Results*: Exosome release increased throughout gestation with a further increase in sPE as compared to first trimester ($3.51e+009 \pm 1.2e+009$ vs $2.98e+008 \pm 1.11e+008$ particles, $p = 0.02$). ExF derived from first trimester (tube length 19063 $\pm$235.1 vs 23837 $\pm$246.2 pixels, $p = 0.001$) and sPE (17293 $\pm$541 vs 23837 $\pm$246.2 pixels, $p = 0.0001$) villi significantly inhibited angiogenesis. Cytoplasmic uptake of labelled ExF, from normal and sPE villi, was observed for 24 hours in both ET cell types. Pre-incubation of ExF with LMWH (2.5 IU/ml, 30 minutes at 37°C) blocked labelled ExF entry into both ET cell lines and significantly reversed the anti-angiogenic effect of first trimester (20124 $\pm$318.2 vs 19063 $\pm$235.1 pixels, $p = 0.04$) and sPE exosomes (24680 $\pm$630.7 vs 17293 $\pm$541 pixels, $p = 0.0009$). *Summary/Conclusion*: The anti-angiogenic effect of first trimester and sPE media is concentrated in the ExF and is reversed by therapeutic LMWH via blockade of exosome entry. This may explain the beneficial effect of LMWH to prevent recurrent pre-eclampsia via a non-anticoagulant mechanism. Evaluation of their composition may prove useful in the development of more specific exosomes derived biomarkers to identify women at risk of developing sPE.

P-VII-11

Cancer-associated urinary extracellular vesicles as novel bladder cancer biomarker

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Introduction: Small membranous extracellular vesicles (EVs) are naturally secreted by almost all cell types including cancer cells and have been found in various body fluids. Recently, EVs attract much attention as potential biomarker because tumour cells have been shown to release EVs into circulation which mirror their cellular origin. Therefore, detection of tumour-associated EVs in body fluids from cancer patients could serve as a non-invasive liquid biopsy for diagnosis and monitoring of cancer. The main objective of this study is to investigate the potential use of urinary EVs from bladder cancer patients. *Methods*: We have developed a bead-based proximity assay named ExoScreen, which is based on AlphaLISA technique (Yoshioka et al., 2014 Nat Commun). In this assay, EVs are captured by two antibodies modified in distinct ways. One is a biotinylated antibody, and the other is an antibody conjugated with AlphaLISA acceptor beads. To characterize the membrane components of tumour-associated EVs, we performed proteomic analysis using urinary EVs of bladder cancer patients and those of healthy donors. Using ExoScreen system, we then explored the feasibility of the identified membrane proteins as biomarker for bladder cancer patients. *Results*: We first confirmed that ExoScreen using anti-CD9 and -CD63 antibodies enabled us to detect EVs present in 5 μl of healthy donor urine. Our proteomic analysis using clinical samples identified several transmembrane proteins. Of these, we selected 3 proteins as candidate biomarkers, and performed ExoScreen using antibodies against these urinary EV proteins. Urine from bladder cancer patients contained EVs that are double positive for one of these three proteins and a general EV marker, CD9 or CD63, more abundantly than that from healthy donors. *Summary/Conclusion*: ExoScreen propose a novel liquid biopsy technique to detect bladder cancer-specific urinary EVs.

P-XI-4

Lysosome status modulates exosome function in intercellular signalling and intracellular protein disposal

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Introduction: Exosomes are extracellular nanoscale vesicles derived from multivesicular bodies (MVBs) fusion with the plasma membrane. In the endosomal pathway, MVBs fuse with lysosomes, resulting in degradation of their content. Fusion of MVBs with autophagosomes has also been reported. The mechanisms that regulate the distinct fates of MVBs are unknown. Here, we asked how lysosome inhibition affects MVB sorting, exosome release and functions. *Methods*: Sequential centrifugations were used to isolate exosomes from culture media and ExoQuick® followed by immunoaffinity from plasma samples. Exosomes were quantified using NanoSight® and the content was measured by immunoblot or ELISA. *Results*: Etoposide/temozolomide increased exosome concentration in U87 glioblastoma culture medium by 20–30%. Naïve U87 cells, treated with exosomes released from treated cells, exhibited resistance to the cytotoxicity of these drugs. Intriguingly, lysosome inhibition with bafilomycin further increased exosomes concentration and the level of Lysosomal-associated membrane protein 1 (LAMP1) and ubiquitinated proteins in them, but abolished their cytoprotective effect. Lysosome inhibition in primary cortical neurons increased exosome-mediated transfer of neurotoxic aggregated forms of α-synclein between neurons. When human neuroglioma cells that express mutated forms of presenilin 1 that cause familial Alzheimer's disease

were treated with bafilomycin, they released exosomes containing higher amounts of pathogenic Aβ42, tau, cathepsin D and ubiquitinated proteins. We found these proteins increased in neuronal-enriched exosomes isolated from plasma of 56 Alzheimer's patients compared to age-matched controls. *Summary/conclusion*: Exposure to cytotoxic agents, lysosome functional status and genetic factors (presenilin 1 mutations) alter the protein content of exosomes released by neural cells, impacting the vulnerability of other neurons to cytotoxicity related to Alzheimer's and Parkinson's disease.

P-XX-4

Source cell microenvironment impacts extracellular vesicle cargo composition

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Introduction: Extracellular vesicles (EVs) have been implicated in vascular intercellular communication and remodelling and thus have potential as biotherapeutics and/or drug delivery vehicles for therapeutic vascular regeneration applications. However, before this potential can be fully exploited, an increased understanding of the influence of cell source and cargo composition on EV bioactivity is necessary. Here, we investigated how EVs isolated from endothelial cells (ECs) derived from distinct microenvironments – human umbilical vein EC (HUVEC) and human dermal microvascular EC (HDMEC) – differ with regard to their cargo composition, which may be determinant of their bioactivity. In particular, we analyzed microRNA (miRNA) profiles of EVs, as miRNA has been identified as critical in EV-mediated stimulation of vascularization. *Methods*: EVs were isolated from cultured ECs via differential ultracentrifugation and analyzed by NanoTracking Analysis using a Nanosight LM10. The expression of the 1,066 most abundantly expressed miRNAs in the human transcriptome were analyzed in EVs derived from HDMECs or HUVECs using a qPCR-based whole miRNome array approach. *Results*: Human miRNome array results revealed differential expression of more than 50 miRNAs between EVs from HUVECs and HDMECs. Interestingly, our results indicate that HUVEC-derived EVs contain higher amounts of miR-328, miR-137 and miR-573, which are associated with an anti-angiogenic phenotype, than EVs from HDMECs. The average difference in Ct values of miR-328, miR-137 and miR-573 between HDMEC and HUVEC EV miRNAs was 11.6, 16.0 and 18.3, respectively. In addition, HDMEC-derived EVs contained significantly higher amounts (delta Ct values >10) of miR-9, miR-135a and miR-135b, all associated with pro-angiogenic gene regulation activity, than in EVs obtained from HUVECs. *Summary/Conclusion*: Our results show that HDMEC-derived EVs contain higher amounts of known pro-angiogenic miRNAs and lower amounts of known anti-angiogenic miRNAs relative to HUVEC-derived EVs, indicative of relatively greater pro-vascularization potential. The disparity in miRNA cargo between these EV subsets from similar cell types may be due to the fact that HDMECs experience a microenvironment that typically undergoes continuous remodelling and thus may play an active role in the formation of new blood vessels via EV-mediated communication. Further *in vitro* and *in vivo* analysis of the therapeutic potential of EVs from ECs is ongoing in order to better understand the effect of source cell phenotype-specific differences on the potential of EVs to stimulate vascularization.

Ballroom F-H
Oral with poster C
Chairs: *John Nolan and Matias Ostrowski* 15:00-16:00

P-XIV-15

Isolation, characterization and procoagulant role of platelet-derived extracellular vesicles

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Introduction: We aimed to study the procoagulant activity of microvesicle (MV) and exosome (EX) fractions enriched from platelet concentrates. *Methods*: Platelet concentrates were produced using a Trima Accel blood collection system (Version 5.0, Gambro BCT) and stored for a maximum of 2 hours at RT before use. After removal of platelets by centrifugation (1,500 g, 15 minutes, RT), MVs were obtained by centrifugation at 20,000 g (2×30 minutes, 4°C). To enrich exosomes (EX), the supernatant after MV removal was centrifuged at 1,00,000 g (2×60 minutes, 4°C). Alternatively, a commercial exosome isolation kit (Invitrogen) was used. Size distribution of isolated EVs was assessed by nanoparticle tracking analysis (Nanosight, Malvern). Flow cytometry was performed using CytoFLEX (Beckman Coulter, detection limit 100 nm) with Annexin V (AV) as MV marker, CD41 as platelet marker and CD63 as EX marker. EV preparations were standardized with respect to protein content (DC assay, Biorad). Tissue factor (TF) expression was assessed by western blotting (TF9-10H10 antibody). EV-induced thrombin generation was studied using a thrombin generation assay (Technoclone). *Results*: Mean particle sizes were 156 nm for MVs versus 135 nm for EX (centrifugation) and 80 nm for EX (kit). Flow cytometry of the MV preparation showed 55% $CD41^+AV^+$ and 0.5% $CD63^+$ events in the MV gate. For the EX preparations, the distribution of markers depended on the isolation protocol with 4% $CD41^+AV^+$ and 2% $CD63^+$ events in the MV gate for EX (centrifugation) versus 0.5% $CD41^+AV^+$ and 10% $CD63^+$ events for EX (kit). Western blotting revealed the presence of TF in all preparations with an approximate signal ratio of 1:3:10 for MVs versus EX(centrifugation) versus EX(kit). All EV preparations induced thrombin generation in MV depleted plasma with the strongest effect for MVs. *Summary/conclusion*: Our data support the procoagulant role of EVs and reveal differences between MV and EX preparations with respect to TF content and thrombin generation.

P-X-1

Role of miRNA-132 and extracellular vesicles in liver fibrosis

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Introduction: miRNAs are small regulatory RNAs and they are frequently deregulated in liver disease. Extracellular vesicles (EVs) are small vesicles shed from almost all types of cells. Circulating EVs and miRNAs have promising potentials for biomarker discovery and treatment monitoring. miRNAs are also associated with EVs in circulation. Liver fibrosis is characterized by excessive scarring caused by chronic inflammatory processes. In this study, we determined the role of miR-132 using LNA-anti-miR approach in liver fibrosis. *Methods*: For in vivo study C57BL/6 mice ($n = 8$) were injected either with scrambled (control) or miR-132- lock nucleic acid (LNA) inhibitor. Mice received either corn oil or CCl_4 for 2 weeks. Total liver, hepatocytes and Kupffer cells (KCs) were isolated. EVs were characterized from plasma using Nanosight, electron microscopy and western blot analyses. *Results*: Total number of EVs in plasma was increased after CCl_4 treatment. Size and morphology characterization of EVs revealed increased number of exosomes than microvesicles. A significant induction of miR-132 was found in the livers of mice treated with CCl_4. Inhibition of miR-132 function in mice with LNA-anti-miR-132 caused a decrease in CCl_4-induced collagen deposition and α smooth muscle actin and induction of macrophage metalloelastase (MMP12). CCl_4 treatment increased caspase-3 activity in anti-control but not in anti-miR-132 treated mice. At cellular level, miR-132 was increased in hepatocytes and KCs isolated after CCl_4 treatment. Inhibition of miR-132 in KCs and not in hepatocytes was associated with augmentation of MMP12, suggesting a potential role of miR-132 in KCs. Mice that received anti-miR-132 did not show any increase in EVs indicating miR-132 inhibition was able to prevent CCl_4-induced EVs release. Plasma levels of miR-122, miR-21 and miR-132 were increased in CCl_4 treated mice. Sorting of miRNAs into the EVs showed a specific pattern as miR-122 levels were increased while miR-21 was decreased in exosomes. The data suggest that anti-miR-132 treated mice are protected from CCl_4-induced liver fibrosis. *Summary/conclusion*: Results from this study suggest a functional role of miR-132 in liver fibrosis. Therapeutic inhibition of miR-132 might be a new approach to alleviate liver fibrosis and efficacy of treatment can be monitored with EVs count.

P-X-3

High-resolution flow cytometric analysis of synovial fluid-derived extracellular vesicle populations during joint inflammation

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Introduction: Extracellular vesicles (EVs) in the synovial fluid are likely to play a role in the communication between articular cells and tissues during health and disease. Gaining insight into this form of intercellular communication may be of great benefit for the progress of cartilage regenerative medicine and treatment of joint disease. In this study, synovial fluid-derived EVs from equine joints with and without LPS-induced inflammation were isolated and analyzed using high-resolution flow cytometry. *Methods*: Synovitis was induced in middle carpal joints of Warmblood horses by injection of 0.5 ng lipopolysaccharide (LPS) from *E. coli* into each joint. Synovial fluid samples were collected at 0-, 8-, 24- and 168-hour post LPS injection. Synovial fluid EVs were isolated using an optimized protocol and pelleted at 10,000 *g* and 1,00,000 *g* respectively, labelled with PKH67 and separated according to buoyant density by iodixanol gradien-tultracentrifugation. Concentrations of PKH67-labelled EVs were analyzed by high-resolution flow cytometry (BD Influx). To further characterize the different EV subsets, lipidomics using HPLC/LCMS is currently performed. *Results*: Using high-resolution flow cytometry, differences between EVs present in the 10,000 *g* and 1,00,000 *g* pellets were readily observed based on the light scattering patterns of individual EVs. Quantitative EV analysis revealed that the highest concentration of EVs, derived from both the 10,000 *g* and 1,00,000 *g* pellets were found at 8-hour post LPS injection while concentrations gradually returned to baseline at 168 hours. These findings are in line with previous measurements of inflammation markers (prostaglandin E2, substance P, bradykinin, MMP activity) and leukocyte and neutrophil infiltration in the same samples, which also showed a peak at 8-hour post LPS injection (De Grauw *et al*. Arthritis Res Ther

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2009;11:R35). Differences in kinetics were observed between the 10,000 *g* and 1,00,000 *g* EVs, suggesting subset-specific characteristics for production/infiltration or clearance of these vesicles in the joint after an acute inflammatory insult. *Summary/conclusion*: These data show that EV concentrations in synovial fluid increase during inflammatory responses in the joint, suggesting a role for EV-mediated signalling in this process. To characterize the different EV subsets, comprehensive lipid analysis is currently performed to define specific lipid profiles for EV subpopulations. Further studies will be undertaken to examine the cellular origin of these EVs and their specific function during inflammation.

P-XI-6

Stress-induced changes in exosomal histone secretion

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Introduction: Histones are often isolated as a protein component of purified exosomes from a wide range of sources. However, they are thought to represent a contaminant of extracellular chromatin or apoptotic bodies. Conversely, histones also have a number of non-chromatin, extranuclear or extracellular functions such as antimicrobial proteins and neurodegenerative signaling factors. The linker histone H1, but not the core histones (H2A, H2B, H3 and H4), is toxic to cortical neurons in vitro at low nM concentrations. Furthermore, H1 promotes glial reactivity suggesting a specific role for secreted, extracellular histone H1, in at least two conserved aspects of the neurodegenerative process: neuronal death and gliosis. *Methods*: We have investigated the exosome pathway to understand how histones are secreted. Exosomes from the oligodendroglial progenitor cell line OLN-93 have been isolated to a high degree of purity using OptiPrep and sucrose equilibrium density, as well as sucrose step, gradient centrifugation and verified by nanoparticle tracking analysis and transmission electron microscopy (EM). *Results*: Western blotting and immuno-EM verified the presence of all histones in purified exosome preparations. Exosomal histones do not appear to be associated with exosomal DNA in a chromatin-like configuration. Surprisingly, histones are strongly associated with the exosomal membrane, and trypsin mapping and western blotting confirms that H3 and H4 are integrated into the exosome membrane. To understand this in the context of neurodegeneration, we have investigated exosomal histones in response to both oxidative and heat stresses by western blotting and proteomic analyses. *Summary/conclusion*: Histones are exosomal proteins and their membrane association, together with the fact that they are specifically up-regulated in response to cellular stress, indicates a novel function for exosomal histones.

P-XVI-2

Exosomes isolated from trophoblast cells modulate the response of skeletal muscle cells to insulin under diabetic conditions

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Introduction: Gestational diabetes mellitus (GDM) is a pregnancy complication associated with hyperglycaemia and insulin resistance in maternal peripheral tissues, including skeletal muscle cells (SKMC). Previously, we established that the release of exosomes from placental cells into maternal plasma is higher in women with GDM than normoglycaemic women. The aim of this study was to determine the effect of trophoblast-derived exosomes (exo-CT) on SKMC migration and glucose uptake. *Methods*: Exosomes were isolated by differential and buoyant density centrifugation from conditioned media of trophoblast cells cultured with normal (5 mM, EXO-CT5) and high (25 mM, EXO-CT25) D-glucose concentration under 8% O_2 for 48 hours. Exosomes were enriched by sucrose-continuous-gradient and characterized by western blot (CD63), size distribution (NanoSight) and electron microscopy. Primary SKMC from normal and diabetes type 2 (D-SKMC) patients were obtained from LONZA (Lonza Pharma & Biotec). The effect of exosomes on cell migration and glucose uptake (2-NBDG, a fluorescent glucose analog) were quantified using a real-time, live-cell imaging system (Incucyte™). *Results*: Trophoblast exosomes significantly increased the migration of skeletal muscle cells from diabetic patients ($p < 0.05$, $\sim$1.2 for EXO-CT5 and $\sim$1.5-fold, EXO-CT25) but did not affect the migration of skeletal muscle cells from normoglycemic patients. Insulin (10 nM) alone increased SKMC migration ($p < 0.05$, $\sim$1.3-fold) was inhibited by high D-glucose and independent of trophoblast-derived exosomes. Insulin also increases D-SKMC migration ($p < 0.05$) but was not independent of exo-CT (EXO-CT5 increased and EXO-CT25 decreased insulin-induced migration). Insulin increased glucose uptake in SKMC, an effect inhibited in the presence of EXO-CT25. Insulin-induced glucose uptake was significantly higher in D-SKMC in the presence of EXO-CT5 but inhibited in the presence of EXO-CT25. Sonication completely abolished the effect of exosomes on SKMC migration and glucose uptake. *Summary/conclusion*: Skeletal muscle cells migration and glucose uptake is regulated by exosomes released from the placenta. Thus, under diabetic conditions a placental exosomal signaling pathway may contribute to the skeletal muscle insulin resistance, an event associated with GDM.

P-XVI-3

Follicular fluid extracellular vesicles regulate cumulus-oocyte-complex expansion through preferential uptake by cumulus cells

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Introduction: Cumulus-oocyte-complex (COC) expansion is critical for ovulation and female fertility. It is achieved by well-coordinated signals within the ovarian antral follicle. The antral follicle houses a rich and complex fluid which contains numerous proteins, nucleic acids and other macromolecules, thus generating an environment for storing and exchanging cell communication signals. Recent studies have shown that extracellular vesicles (EVs) are abundant within antral fluid. The importance of EVs in female health arose because microRNA contents in follicular fluid EVs were different in polycystic ovarian syndrome. To date, no functions of EVs have been demonstrated in the ovary. In our study, the effect of follicular fluid EVs on COC expansion was assayed. *Methods*: First, EVs were isolated using ultracentrifugation from follicular fluid of early antral (3–5 mm diameter) and late antral ($>$9 mm) bovine follicles ($n > 3$) and then analysed by nanoparticle tracking analysis (NTA), electron microscopy and western blot analysis. RNA isolated from EVs were made into libraries by TruSeq Small RNA kit and sequenced on an Illumina HiSeq 2500. To test EV bioactivity, mouse COC were cultured with or without EVs (100 μg/ml) from different sized follicles and expansion was measured 16 hours later. Uptake of EVs was evaluated by observing PKH67 labelled EVs in intact COC, cumulus-free (zona intact) and denuded (zona removed) oocytes. *Results*: Electron microscopy indicated that the ultra-centrifuged pellet contained numerous bilipid membrane enclosed vesicles (50–200 nm) and negligible amounts of protein aggregates. The concentration of EVs decreased from 19.4×10^{12} in early follicle to 2.8×10^{12} in late antral follicles ($\sim$7 fold) as determined by NTA. Exosomal markers, CD81 and Alix were enriched in EV preparations while gp96 (endoplasmic reticulum

marker) was only evident in cell lysates. Small RNAseq indicated that 52 or 32 miRNAs were over-represented ($p < 0.05$) in early or late antral follicle EVs, with differences ranging from 1.8–883-fold. EVs from early or late antral follicles induced COC to expand 20% or 15% in diameter (compared with 0 hour) ($p < 0.05$). Uptake of PKH67 labelled EVs was observed in cumulus cells as numerous green punctate spots. In contrast, the oocyte had no detectable EV uptake in COC intact, cumulus free or denuded oocytes. *Summary/conclusion*: The number of EVs changed as follicular development proceeded as did their miRNA contents. Uptake of EVs by cumulus cells was dramatic, yet no uptake was observed in oocytes. For the first time our study demonstrates a biological effect of a highly purified follicular fluid EV preparation on COC expansion, an important event in the process of ovulation.

P-V-1

Powering the production of extracellular vesicles as drug delivery vectors using microfluidics

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Introduction: Cell-released microvesicles and exosomes are recently considered as the nature's own carrier system to deliver biological informations in an horizontal way. In order to take advantage from this cell communication pathway for therapeutic purposes, we engineered extracellular vesicles (EVs) to encapsulate a set of multifunctional nanoparticles and drugs. Cell microvesicles encapsulating iron oxide nanoparticles were magnetically responsive, readily manipulated and isolated by magnetic forces, and they could be monitored by MRI in vivo. We also demonstrated that vesicles could be loaded with different therapeutic agents such as doxorubicin, t-PA, TPCS2a and mTHPC in addition to nanoparticles. Anti-tumoral action of photosensitizer-loaded EVs was demonstrated in vitro as well as in vivo. Importantly, EVs uptake by cancer cells could be spatially controlled with magnetic field and cancer cell death was enhanced by magnetic targeting. In studies above, EVs were released from serum-starved cells previously labelled with drug and nanoparticles. Here, we aim to optimize the production of extracellular drug-delivery vectors using a microfluidic method. *Methods*: Microfluidic channels were used to induce a mechanical stress on cells loaded with drug and iron oxide nanoparticles. The passage of cells through microchannels for a few seconds induced the release of EVs. EVs were characterized and quantified by nanoparticle tracking analysis (NTA), diffusion light scattering, imaging flow cytometry. Parent cells were also analysed before and after passage in microchannels. *Results*: Microfluidic device successfully triggered vesiculation of loaded cells. Loaded vesicles of a controlled size (100 nm) were produced in large quantity (100 times more efficient than serum starvation method) and in a very short time. *Summary/conclusion*: This microfluidic strategy may be very promising for increasing the availability and standardization of cell-derived drug delivery vectors.

P-XV-10

The role of platelet-derived microvesicles in neutrophil recruitment to endothelium during inflammation

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Introduction: Platelet microvesicles (PMV) account for >80% of circulating MVs and have been suggested to promote leukocyte recruitment to the vessel wall. The rate of PMV binding to endothelial cells (EC) and its influence on neutrophil recruitment is not well understood. We aimed to determine the binding kinetics of PMV to EC and the resultant effect on neutrophil recruitment flow. *Methods*: PMV were generated from stimulated CD41 labelled platelets stimulated with collagen-related peptide (CRP-XL, 1 μg/ml) and were incubated with EC. PMV mediated stimulation of EC was assessed by flow cytometry of adhesion receptors. Flow based adhesion assay assessed neutrophil recruitment on PMV-coated on glass capillaries or on EC grown in flow chambers and treated with combinations of PMV and different concentrations of TNF-α. *Results*: PMV binding to EC was detected within 1 h and maximal by 4 hours with >60% dual positivity for CD41 and VE-cadherin on EC. The PMV uptake resulted in upregulation of endothelial activation markers (ICAM-1, VCAM-1 and E-selectin). Neutrophils bound directly to PMV enabling frequent inflow capture and low levels of stable adhesion to a PMV-coated surface. Similar effects of PMV were observed on unstimulated or minimally stimulated (1U/ml TNF-α) EC, where capture was significantly enhanced by 3.6 and 1.8-fold, respectively, compared to untreated EC. An additive effect of PMV was not observed at higher concentrations of TNF-α. *Summary/conclusion*: Surface-bound PMV can directly capture flowing neutrophils and also activate endothelial cells. Thus PMV may promote neutrophil recruitment in inflammation, by potentiating effects of low levels of cytokines acting on EC.

P-XIII-9

Vesicle flow cytometry of extracellular vesicles in cerebral spinal fluid

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Introduction: We present data on the enumeration, sizing and surface marker characterization of individual extracellular vesicles (EVs) in cerebrospinal fluid (CSF) using vesicle flow cytometry (VFC). VFC employs a fluorogenic membrane probe and fluorescent antibodies to stain EVs for analysis with a high sensitivity flow cytometer (HSFC). We analyzed CSF from patients diagnosed with five different neurological disorders and from normal subjects. *Methods*: CSF was obtained and pooled from patients with high grade glioma (HGG) and low-grade glioma (LGG), Alzheimer's disease (AD), Parkinson's disease (PD), or subarachnoid haemorrhage (SAH) as well as normal relatives of patients. Aliquots were analyzed by nanoparticle tracking analysis (NTA) or stained with a fluorogenic membrane probe (di-8-ANEPPS) and DyLight488-labeled surface markers (annexin V or anti-CD41), and then analyzed by a custom HSFC. *Results*: In the pool of normal samples, NTA reported a total nanoparticle concentration of $4.32 \times 10^6/\mu l$, while VFC reported a membranous nanoparticle concentration of $2.16 \times 10^6/\mu l$. NTA distribution ranged from <50 nm to >250 nm, with a mean of 118 nm. VFC size distribution was from ~75 nm to > 400 nm, with a mean of 192 nm. In general, particle concentrations by VFC were lower than NTA, and the sizes were larger, perhaps due to VFC's selectivity for membranous nanoparticles. Pools of patient-derived samples showed 2- to 7-fold increases in EVs compared to normals, with no notable differences in size, indicating that EV concentrations in CSF may have diagnostic value. Staining EVs with fluorescently labelled surface !markers revealed a variable fraction annexin V+ vesicles in all samples, with some showing a significant fraction of CD41+ EVs. *Summary/Conclusion*: EVs in CSF can be characterized with high sensitivity compared to NTA, while VFC providing fluorescent antibody based speciation of EVs. Funded by NIH EB003824 (JPN), NIH TR000931 (BSC, FHH), TR000891 (KJ, MH) and TR000903 (JAS, JFQ).

P-XX-9

Engineered glycosylation stabilizes exosome targeting peptides and enables targeted exosome delivery

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Introduction: Extracellular vesicles (EVs) show great promise as therapeutic delivery vehicles with cargo versatility, immune compatibility and, in some cases, inherent therapeutic activity. Several reports indicate that targeting EVs to specific recipient cells can enhance functional cargo delivery, yet in general, targeting EVs has proven challenging. This work investigated one potential explanation – that in the case of exosomes, which are endosomally derived vesicles, targeting peptides expressed on the vesicle surface are degraded during exosome biogenesis. This work also explored a strategy for achieving robust display of targeting peptides on exosomes. *Methods*: Peptides were fused to the N-terminus of the endosomal membrane protein, Lamp2b. Presence of N-terminal peptides on exosomes was analyzed by western blotting, and targeting peptide functionality was assessed by uptake of fluorescently labelled exosomes by target cells. *Results*: Peptides fused to the N-terminus of Lamp2b were not displayed robustly on the surface of exosomes. This loss of N-terminal peptides was mediated by acid-dependent proteolysis, which can occur during exosome biogenesis. Engineered glycosylation motifs were added at various positions with respect to the targeting peptide, and protection of targeting peptides was observed for several constructs. Moreover, engineered glycosylation was required to achieve targeting peptide-enhanced delivery of exosomes to neuroblastoma cells. Delivery of targeted exosomes to prostate cancer cells was also investigated. *Summary/Conclusion*: Engineered glycosylation is a robust method for protecting targeting peptides on the surface of exosomes from degradation, and glycosylation strategies that did not impair targeted uptake of vesicles by recipient cells were identified. This strategy could be applied generally to improve both safety and efficacy of therapeutic vesicles by enabling targeted uptake to specific recipient cells.

P-XX-11

Characterization of extracellular nucleic acids inside and outside exosomes

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Introduction: Exosomes contain a variety of RNAs, including both protein-coding messenger RNAs (mRNAs) and non-coding RNAs. There have also been reports of DNA in exosomes. Previous reports have found about extracellular microRNAs that some exist inside vesicles whereas others are contained outside the vesicles in protein complexes. It is unclear that in what proportion other extracellular RNA or DNA resides inside versus outside of vesicles. *Methods*: We used differential ultracentrifugation to isolate exosomes from the K562 Leukemia cell line. We then performed various enzymatic treatments (with proteinase, DNase or RNase) to get rid of nucleic acids not protected by intact lipid membranes, and analyzed the resulting nucleic acids. *Results*: We found that double stranded DNA is present in the exosome pellet. However, upon treatment with DNase, this DNA is all digested, suggesting that it is outside of vesicles as opposed to inside. We also treated the exosome pellet with RNase or Proteinase and then RNase. We are analysing the resulting RNA by a variety of *Methods*, including qRT-PCR and RNA-Seq. *Summary/Conclusion*: The exosome pellet contains both DNA and RNA, but some of these nucleic acids (particularly double-stranded DNA) are not inside the exosome but rather appear to be stuck to the outside. Identifying nucleic acids that are truly inside vesicle has important implications for studying the role of exosome cargo in intercellular communication.

Networking coffee 16:00-16:30

Poster sessions I, II, III, IV, V, VI, VII continued
Posters not attended by authors 16:00-16:30

Ballroom D
Biotechnology: sponsored session A
Chairs: *Yong Song Gho and Joanne Lannigan* 16:30-17:00

O-SSA-1

Measurement and analysis of vesicles for clinical use
Hans van der Voorn
Izon Science Ltd, Christchurch, New Zealand

Extracellular vesicles (EVs) are the subjects of wide ranging research leading to many new medical and biological insights. There is a high level of excitement and anticipation around the potential medical benefits of improved diagnostic capabilities, therapeutics and understanding of medical biology. The EV research may or may not prove to be the start of a revolution in medicine, but it is clear that moving the field from a research base to routine clinical use will require substantial improvements in separation, measurement and analysis of EVs. The requirements for measurement include basic accuracy, but also data detail or resolution, repeatability and calibration back to known physical standards. Objective measurements that can be reliably compared across different research groups and ultimately, different patients, are fundamental to progress. A primary thesis is that the medical understanding around EVs cannot develop effectively unless measurement and analysis improves. That means continual development of the core measurement tools and close working relationships between EV researchers, clinical practitioners and technology providers including Izon Science. TRPS offers a reliable measurement solution for the field, but uptake has been hampered by unfamiliarity and difficulty with learning how to use it well. That means a step in change in practicality and time to measurement has been required. The use of SEC columns helps to address accuracy, repeatability and practicality. The time taken to separate and measure EVs has reduced by about 75% in the last 12 months, partly through the use of SEC columns for EV separation. Phenotyping of the EVs is a key problem that does not have a workable solution as yet. A development road map that attempts to cover all of these requirements will be proposed.

O-SSA-2

Exploring sub-populations in EV samples with transient nanoparticle tracking analysis (t-NTA)
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Introduction: In conventional Nanoparticle Tracking Analysis (NTA) small particles such as EVs are visualized by an ultra-microscopy set up and analyzed for size distribution from their Brownian motion (translational diffusion). Generally, sub-populations with different behaviour and appearance can be distinguished by the naked eye but are difficult to quantify and to discriminate. *Methods*: We present the transient NTA (t-NTA) method, which is derived from the statistical learning theory t-SNE [1] and projects a multidimensional solution into a two-dimensional scatter plot. *Results*: From analyzing image features in a series of images such as intensity, area, size or fluctuation characteristics of the visualized objects, similar sample appearances are grouped in clusters. The discrimination and quantification of sub-populations of a sample with the t-NTA method will be discussed on exosome examples. *Summary/conclusion*: The clustering of visually similar particles by t-SNE helps to gain a deeper understanding of the sample composition, that is, the particle size distribution of several sub-populations can be calculated. In addition, this algorithm is capable of identifying artifacts like rotational diffusion making the NTA particle size analysis more robust.

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Ballroom E

Biotechnology: sponsored session B

Chairs: *Sai Kiang Lim and Steven M. Jay* 16:30-17:00

O-SSB-1

Beads for your extracellular vesicles needs

Kenneth Henry[1], Tetsuji Yamaguchi[2], Hiroaki Sagawa[1], Marybeth George[1], Dennie Magcase[1], Hiroya Fujii[3], Motoaki Mizuuchi[3], Kimiko Hasegawa[3], Hiroki Abe[3], Manami Matsukawa[4], Yoshihiro Fujii[4] and Satoshi Katayose[1]
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Extracellular vesicle (EV) area is no longer the domain for just the membrane traffic and vesicle biogenesis researchers. Today, translational, clinical and basic researchers recognize the importance of EVs and their growing potential in vaccine development, diagnostics and therapeutics. As origins, roles, destinations and vast utilizations of EVs are discovered, improvements in isolation techniques, downstream applications and standards are needed. Innovative solutions and scientists are working to make these improvements. We have beads to help you with your innovation. We have latex and magnetic particle product lines with various surface chemistries for your specific needs. Immutex, our latex beads ranging from 50 nm to 1 μm in size, have been used in the immunoassay development and fungal adherence. Oligotex™-dT30 Super Latex beads are being used for mRNA isolation. Magnosphere™, our magnetic beads, have been used for immunoassay development, bioseparations, circulating tumor cells (CTC) isolation, cell separation, immunoprecipitation, nucleic acid isolation and most recently EV isolation. ExoCap™, an immuno-affinity magnetic beads based exosome isolation kit, was developed to ease the complications of exosome isolation. ExoCap™ Isolation and Enrichment Kits consist of magnetic particles coupled to antibodies that recognize antigens on the exosome surface, a diluent/washing solution and a reagent that releases the captured intact exosomes for analysis. Antibodies against 3 tetraspanins (CD9, CD63 and CD81) and EpCAM (an adherent protein associated with various cancer derived exosomes), are preferentially chosen for the kits. In addition, the Composite ExoCap™ Kit, composed of the 4 selected antibodies, may better serve your research needs. ExoCap™ can easily separate antigen-specific exosomes, without an ultracentrifuge or any special equipment. In exosome rich samples, 100–1000 μL will be sufficient for the exosome isolation. Exosomes isolated from various body fluids (such as human serum, plasma, urine) and cell culture media using ExoCap™ ensures compatibility with downstream applications such as western blot, particle size distribution, RNA analysis and electron microscopy. We have beads for your innovative EV needs.

O-SSB-2

From basic research to clinical setting: adapting methods for EV culturing, enrichment and analysis

Brian Paszkiet and Axl Neurauter
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The isolation and characterization of exosomes from body fluids and cell culture systems will provide important information that may be useful for early disease detection monitoring of disease status and the development of effective treatments for cancer, inflammation and autoimmune disease. Further insight into exosome biology may also accelerate the use of these microvesicles in regenerative medicine and vaccination research and increases the efficacy of therapeutic antibodies. The aim of this presentation is to provide information on how to optimize experimental conditions for studying cell culture exosomes by (a) introducing a Gibco® Exosome-Depleted FBS and (b) giving an overview over a direct method for fast, efficient and selective isolation of exosomes from cell culture supernatant that is compatible with a wide range of downstream applications. Currently, researchers using cell culture to study exosomes still incorporate fetal bovine serum (FBS) into their culture medium, even though FBS possesses extremely high levels of bovine exosomes that can confound downstream analyses. To circumvent the problems arising from contaminating bovine exosomes in FBS, researchers typically deplete FBS of exosomes by ultra-centrifugation. However, this process is not very efficient, generally achieving only about 50% depletion of exosomes, while adding considerable time and effort to the cell culture workflow. To address these issues, we have developed an exosome-depleted FBS to achieve a more consistent cell culture reagent for exosome research as well as improve the exosome workflows. Pilot and manufacturing-scale depletion studies have exhibited >90% exosome depletion from FBS while maintaining cell culture performance at >90% of the parental FBS lot for multiple relevant cell lines. Further, we describe the use of magnetic beads coated with antibodies against the tetraspanins CD9 and CD81 – common exosomal markers – to isolate and characterize pre-enriched exosomes (pre-enriched by ultracentrifugation and/or precipitation) derived from adherent and suspension cells. Critical factors such as volume, time and exosome concentrations are addressed in order to establish optimal and comparable isolation conditions. In addition, we have developed a method for isolating exosomes directly from cell culture medium using magnetic beads. Direct isolation methods have the potential not only to shorten the workflow, but also to reduce any artifacts or contamination that can result from the enrichment procedure.

Ballroom D
Symposium session 2A - EV isolation and characterization
Chairs: *Yong Song Gho and Joanne Lannigan* 17:00-18:30

O-2A-1

Quantitative light scattering of extracellular vesicles for flow cytometry standardization

Edwin van der Pol[1], Rienk Nieuwland[2], Auguste Sturk[2], Ton G. van Leeuwen[1] and Frank A. Coumans[1]
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Introduction: Although flow cytometry is the most widely used method to study single vesicles, comparison of flow cytometry results between laboratories remains challenging. At present, most laboratories select vesicles by setting an inclusion gate based on the scatter signal from 2 polystyrene bead sizes, resulting in a coefficient of variation (CV) of the vesicle concentration of 91%. This moderate reproducibility is attributed to the variety of optical configurations in flow cytometers and the refractive index difference between vesicles and polystyrene. Due to this refractive index difference, polystyrene beads scatter light >10-fold more efficiently than vesicles. *Methods*: A well-defined polystyrene beads mixture (metves.eu) and 2 vesicle standards are measured on 45 flow cytometers in 31 laboratories worldwide. The relation between particle size, refractive index and scatter is obtained for the specific optical configuration of each instrument by describing the data from beads with Mie theory. This relation is used to set 3 vesicle size gates, taking into account the latest insights on the refractive index of vesicles. *Results*: Preliminary data show that our calibration procedure reduces the CV of the vesicle concentration with 21% compared to the current standard. Circa 30% of all instruments is unable to detect 400 nm FITC-labelled polystyrene beads. For comparison, a 400 nm polystyrene bead scatters light more efficiently than a 1 μm urinary vesicle. In addition, the size of the smallest detectable vesicle differs 2-fold between instruments of the same type. Final data and results are expected in February and June 2015, respectively. *Summary/conclusion*: Well-defined beads and Mie theory are used to standardize the size range detected by flow cytometry, thereby, improving the CV of the vesicle concentration with 21% compared to the current standard. This unique approach provides a profound understanding of vesicle detection by flow cytometry, which is essential to inter-laboratory data comparison.

O-2A-2

Fluorescence-activated vesicle sorting (FAVS): a novel method for analysis of individual extracellular vesicles and their EGF receptor (EGFR) activation state

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Introduction: Various methods exist for analyzing the size, constituents and number of secreted extracellular vesicles (ECVs). However, analysis of small 30–120 nm ECVs, termed exosomes, by flow cytometry represents a formidable challenge. We previously developed FAVS to isolate a subset of exocytic vesicles (*MCP 7: 1,651–1,667, 2008*) and subsequently adapted this method to isolate exosomes from MDCK cells individually overexpressing the EGFR ligands AREG, TGFA and HBEGF (*Curr Biol 21: 779–786, 2011). Methods*: In the present study, exosomes were isolated by sequential ultracentrifugation. We employed FAVS to analyze endogenous human EGFR on exosomes released from a human colorectal cancer cell line, DiFi, both in vitro and from the plasma of mice bearing DiFi xenografts. *Results*: Using human-specific antibodies to EGFR and the exosomal marker CD9, we isolated DiFi EGFR/CD9 double-positive exosomes mixed with mouse exosomes purified from mouse plasma. The presence of individual double-positive 100 nm vesicles was confirmed by super-resolution microscopy. Using antibodies that recognize total and activated EGFR, respectively, we employed FAVS to assess EGFR activation in individual exosomes from DiFi and A431 epidermoid carcinoma cells. Although both cell lines express high levels of total EGFR, DiFi-derived exosomes were selectively enriched for activated EGFR compared to exosomes derived from A431 cells; these results were confirmed by immunoblotting with the relevant antibodies. *Summary/conclusion*: FAVS is a novel method for isolation and characterization of individual exosomes.

O-2A-3

nanoFACS for EV isolation, purification and functional studies

Jennifer Jones[1], Thomas Musich[2], Aizea Morales Kastresana[1], William Telford[2], Kathy McKinnon[2], Roy Overton[3], Aparna Kesarwala[2], Ionita Ghiran[4], John Tigges[4], Vasilis Toxavidis[4], Marty Bigos[5] and Jay Berzofsky[2]
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Introduction: Cytometric separation of individual biological submicron particles is limited both by the small and overlapping sizes of these biological particles and by the overlap of particle-associated signals with background instrument noise. In order to identify, sort and study distinct subsets of submicron extracellular vesicles (EVs) and other submicron particles, we developed nanoscale Fluorescence Activated Cell Sorting (nanoFACS) methods. Whereas, other groups have pioneered methods for nanoparticle flow cytometric analysis that rely on coupling EVs to beads (PMID 23925291) and require the use of a fluorescent label with a corresponding threshold/trigger (PMID 22722367) or use cytometric image analysis (PMID 24903900). Our nanoFACS method uses a scatter threshold parameter and fluorescent labels to enable the analysis and sorting of EVs or virions as single, functional particles. *Methods*: We configured jet-in-air sorters with high-resolution optics and high-speed digital electronics with 2 side scatter (SSc) detectors: one as the trigger and the other as a detector. With this configuration, we defined and used two informative regions of interest in the data – the "parallel sub-threshold" events and the background instrument noise, or "reference noise," events. We used a side scatter threshold and fluorescent labels to discriminate and sort distinct EV and viral preparations. Because there are no well-defined EV subset standards for demonstrating the biological activity of sorted particle (EV) subsets, we used 2 HIV ~100 nm viral preparations (each with distinct co-receptor tropism) and matched reporter cell lines to quantify preservation of biological activity (infection) and purity (specific tropism) among the sorted nanoparticles. *Results*: The nanoFACS method detected EVs, liposomes and other particles as small as 20–40 nm and sorted particles as small as 80 nm. We were able to sort 10–100 million particles per hour, as individual particles without swarming or coincidence, in two-way sorts. nanoFACS sorting achieved purity (>96%, based on flow cytometric re-analysis) and preserved the biological activity of sorted particles. Crossover testing of sorted viroins with reporter cell lines demonstrated preservation of co-receptor integrity and clean separation of the 2 virion populations. *Summary/conclusion*: With precise configuration and close attention to the

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reference noise distribution and experimental controls, nanoFACS can extend the range of high-speed particle sorting to an order-of-magnitude smaller than standard flow cytometry. Our goal is to use nanoFACS to identify, sort and study relevant subsets of EVs arising from tumours and the immune system. Between ISEV 2013 and 2014, the focus of many basic science studies shifted from isolation/purification to characterization. To our knowledge, nanoFACS is the first method that can be used to analyze, identify and isolate specific EV subsets for further functional studies.

O-2A-4

Definition of extracellular vesicle subtypes released simultaneously by dendritic cells

Joanna Kowal[1], Guillaume Arras[2], Marina Colombo[1], Mabel Jouve[3], Jakob Paul Morath[1], Florent Dingli[2], Damarys Loew[2], Mercedes Tkach[1] and Clotilde Théry[1]
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Introduction: The heterogeneous nature of extracellular vesicles (EVs) is now clearly documented in the literature where EVs of various sizes and origins are frequently reported. However, the field of EVs still lacks tools to fully separate or characterize different EV subpopulation. In this study, we define diverse subtypes of EVs based on their protein composition and we propose to use a set of markers to estimate the heterogeneity of any bulk preparation of EVs. *Methods*: We applied differential ultracentrifugation with or no subsequent floatation into density gradients to purify EVs from conditioned medium of human primary monocyte-derived dendritic cells or of 7 widely used cell lines. EVs recovered at each step of the process were analyzed by western blotting, electron microscopy and NTA. Quantitative LC-MS/MS analyses were performed in 4 different fractions recovered after iodixanol separation. We also analyzed the effect of GW4869, an inhibitor of ceramide formation, in secretion of all EV subtypes. *Results*: DCs secrete a wide range of EVs recovered at each step of differential ultracentrifugation. HSC/P70, MHC class I/II, flotillin-1and CD63, often used as ''exosome-markers,'' were equally present in all of them. GW4869 decreased secretion of exosomes but also of larger microvesicles. Our quantitative proteomic comparison of 4 subfractions separated by iodixanol gradient, identified new proteins uniquely present in large – pelleting at 2 and 10,000 g, and small – pelleting at 1,00,000 g, vesicles, and showed that the latter contain at least 2 distinct populations: endosome-derived and extracellular matrix-containing EVs. *Summary/conclusion*: Our data unravel new specific protein markers, whose systematic analysis in a bulk pellet could reveal the proportion of different types of EVs present in the preparation.

O-2A-5

Biofluid-derived exosomes: a challenge for proteomics

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Introduction: There is much interest in biofluid exosomes as a biomarker source, however, a major challenge for proteomics is isolating sufficient high purity exosomes for analysis. When using mass spectrometry (MS), removal of abundant non-exosomal protein is imperative to limit genuine exosomal protein masking. The aims were to develop biofluid exosome isolation methods, assess sample purity and perform preliminary proteomics analysis. *Methods*: Healthy donor plasma and urine was used as an exosome source to test isolation methods, including ultracentrifugation (UC), filtration, gradient UC and size exclusion chromatography (SEC). Purity was tested using: ELISA-like assays, cryo-electron microscopy, western-blot and nanoparticle tracking. We used LC/MALDI MS or a novel aptamer based protein array (SOMAscan™) for proteomics analysis. *Results*: Isolating exosomes from plasma using UC and gradient UC insufficiently removed plasma proteins resulting in inadequate purity when measured by particle/protein (P/P) ratio. The use of SEC, however, removed >95% of contaminating protein and effectively separated EV associated tetraspanins from serum albumin. Unfortunately, even with an increase in sample purity this was still insufficient to generate high quality datasets by LC/MALDI MS analysis (21 identifications, mostly plasma proteins). However, with SOMAscan™ hundreds of proteins were identified from urine- and plasma-exosome samples. Some abundant non-vesicular proteins were still identified, particularly in plasma-exosome samples but this did not impede the identification of many vesicle associated proteins. *Summary/conclusion*: SEC removed >95% of the most abundant biofluid proteins. Urine exosome preparations exhibited higher purity and increased numbers of protein identifications by SOMAscan™. This workflow will now be used to examine the protein profiles of matched patient plasma and urine to identify potential biomarkers associated with prostate cancer.

O-2A-6

Monoclonal antibodies with specificity for subfractions of extracellular vesicles derived from human mast cells

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Introduction: Despite the widespread use of proteins such as TSG101, Rab-5b, annexin 2, flotillin 2, CD63, CD9 and CD 81 as markers of extracellular vesicles (EVs), these proteins are not consistently detected in all EVs of different origin. Also, even though some (CD9 and CD81) are more universally present and enriched in EVs of different origin they are not absent from the parent cells or from non-vesicular cell fragments. This hampers their use as specific tools, for example, when monitoring purification procedures and characterizing EV depletion or yield from cell culture supernatants or biofluids. Finally, there are a number of distinct EV subfractions in biofluids and even in cell line-derived EVs that have physical characteristics that allow them to be subfractionated but where little is known about specific markers characterizing these EV subsets. Thus, specific immunological reagents are needed for EV research. *Methods*: We hypothesize: (a) that subsets of EVs display subset-specific markers and that (b) some EV markers will be common for groups of EV representing more than one subset, that is, there are EV-specific and EV-subset-specific epitopes that may be targeted by immunological reagents and thus be highly valuable tools for EV characterization. Using EV subfractions (EV1 and EV2) purified by differential centrifugation of the supernatant of a human mast cell line (HMC-1), we here report on the establishment and characterization of a panel of monoclonal antibodies against these EVs. *Results*: We have developed monoclonal antibodies that bind specifically to either EV1 or EV2 or both EV1 and EV2 in a screening assay. We present results on the epitope characterization of these monoclonal antibodies using immunoblotting, mass spectrometry, flow cytometry and electron microscopy. *Summary/conclusion*: We have developed monoclonal antibodies specific for EVs and EV subfractions. The antibodies represent the first group of immunological reagents that have been specifically developed for EV characterization based on primary immunization with highly purified EV preparations.

Ballroom E

Symposium session 2B - EV therapeutics I: protein and drug delivery

Chairs: *Sai Kiang Lim and Steven M. Jay* 17:00-18:30

O-2B-1

HEK293 cell toolbox for exosome engineering: small-scale screening and bioreactor scale-up of exosomes carrying additional functional proteins

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Introduction: Exosomes have significant attractive therapeutic potential, given their effect on cellular function via their cargo and surface proteins. In addition, they can be engineered and modified with molecular biology methods. This study soughts to establish a simplified toolbox for screening of DNA constructs expressing exosome-associated proteins with therapeutic potential, and to scale-up production of bioactive exosomes, enabling in vitro and in vivo studies. Interleukin-15 (IL-15), based on its ability to stimulate NK and cytotoxic T-cells and its potential as an anti-cancer immunotherapeutic agent, was used as a model protein in this study. *Methods*: We designed a DNA construct encoding murine IL15 (muIL15) fused via a flexible linker to the exosome-associated protein human lactadherin. To assess the presence of muIL15/lactadherin on exosomes, HEK293 cells were transfected with the muIL15/lactadherin-expressing plasmid, and cultured in 500 kDa ultrafiltered (exosome-free) DMEM + 10%FBS + antibiotics for 48 hours. For development of scale-up protocol, a stable HEK293 cell line overexpressing heterodimeric IL-15 (IL-15 + IL-15 Receptor α [hetIL15]) was grown in a hollow fiber bioreactor, using protein-free medium. Exosomes were purified from supernatants by filtration and differential centrifugation, and their bioactivity was assessed in vitro by measuring IFNγ production from NK92 cells stimulated with several concentrations of purified hetIL-15 exosomes. *Results*: Triplicate dynamic light scattering of purified exosomes showed a monomorphic particle population, with a mean diameter of 119 nm (standard deviation 4–10 nm). Enrichment of the exosome markers CD63 and Alix was confirmed by western blot as compared to cell lysates. muIL-15/lactadherin fusion protein and hetIL-15 were also detected on the purified exosomes. Molecular weight of exosomal muIL-15/lactadherin was slightly higher than that of cell-associated protein, perhaps, as the result of additional glycosylation within the endosomal pathway. The presence of hetIL-15 on exosomal outer surface was confirmed on exosomes immobilized on CD63-binding beads by flow cytometry. Finally, hetIL-15 exosomes showed comparable IL-15 bioactivity versus purified protein. *Summary/conclusion*: The novel muIL-15/lactadherin construct was well expressed on exosomes, despite previous findings that mammalian expression of IL-15 without its receptor α results in an unstable protein. Thus, this linker-fusion approach may be useful to express even unstable proteins on exosomes. Transfection with engineered constructs and culture of HEK293 in 500kDa ultrafiltered media allows for rapid screening of protein exosome-localization. Finally, the protein-free hollow fiber bioreactor system comprises a platform for continuous scalable production of engineered exosomes by HEK293 cells stably expressing exosome-associating proteins, completing the HEK293 exosome engineering toolbox.

O-2B-2

Comparison of two novel strategies for (re)targeting of extracellular vesicles with anti-EGFR nanobodies

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Introduction: Extracellular vesicles (EVs) facilitate cargo transfer between cells and could potentially be exploited for the delivery of therapeutics. Their applicability could be improved with universal methods to target them to cells other than those reached through their native tropism. In this work, 2 novel strategies to decorate the EV surface with targeting ligands are presented, and their effects on EV-cell interactions are compared. *Methods*: EVs from EGFR-negative Neuro2A cells or platelets were isolated by differential (ultra)centrifugation. Anti-EGFR nanobodies, which serve as targeting ligands, were either covalently conjugated to EV membrane proteins using chemical crosslinkers or inserted into EV membranes through the use of nanobody-polyethylene glycol(PEG)-phospholipid micelles (post-insertion). After purification of EVs, modification efficiency was analyzed by western blotting and immuno-EM. Cell uptake by EGFR-positive tumour cells was assessed using flow cytometry. *Results*: Regardless of EV source, covalent conjugation resulted in high ligand density and impaired functions of EV proteins responsible for cell interactions, whereas, post-insertion was more subtle and yielded EVs with decreased unspecific interactions with cells due to PEGylation. In both methods, EV size and morphology was maintained as determined by NTA and immuno-EM. Importantly, cell association and uptake of (re)targeted EVs by EGFR-positive tumour cells was significantly increased when compared to unmodified EVs or EVs modified with control nanobodies. In addition, EV uptake became largely EGFR-dependent after decoration with nanobodies. *Summary/conclusion*: In order to employ EVs as drug delivery systems, it is essential that they can be (re)targeted to specific cell types or tissues. Covalent conjugation and post-insertion techniques can be used to modify the tropism of EVs while maintaining their integrity. Currently, biodistribution studies are ongoing to assess the effect of both methods on EV behaviour in vivo.

O-2B-3

Recombinant exosomes for immunotherapy of chronic lymphocytic leukemia (CLL)

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Introduction: CLL continues to be an incurable disease that is characterized by progressive accumulation of malignant B cells and abnormalities of T-cell functions. CLL cells are poor antigen-presenting cells because they lack costimulatory molecules. However, immunogenicity of the tumour cells is increased upon CD40 stimulation with its ligand, CD40L. Our work aims at the development of a novel immunotherapeutic approach for CLL based on the transfer of CD40L together with immunodominant Epstein-Barr-Virus (EBV) and Cytomegalovirus (CMV) proteins to malignant CLL cells by means of

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engineered exosomes, re-targeting the strong herpesviral immunity to CLL cells. *Methods*: Recombinant exosomes were generated in HEK293 cells overexpressing the proteins of interest and isolated from cell culture supernatants by centrifugation and density gradient fractionation. Exosome preparations were characterized by western blot. Primary CLL cells were loaded with exosomes and binding was analyzed by flow cytometry. Functionality of exosomal CD40L was validated by measuring induction of costimulatory molecules on CLL cells. CLL cells were loaded with exosomes overnight and incubated with specific T-cell clones for 24 hours. T-cell activation was analyzed by IFNγ ELISA. *Results*: We showed that CD40L, the EBV protein gp350 and the CMV protein pp65 are efficiently packed into exosomes released by HEK293 cells and retain their functionality. Gp350 increased the exosomal binding capacity to $CD21^+$ CLL cells, while exosomal CD40L induced the expression of the costimulatory molecules CD54, CD80, CD86 and CD95. Loading of CLL cells with $CD40L^+$/$gp350^+$ or $CD40L^+$/$pp65^+$ exosomes also led to efficient restimulation of gp350-specific T cells or pp65-specific T cells, respectively. *Summary/conclusion*: Our findings suggest that modified exosomes that transfer functional proteins to specific target cells and further trigger virus-and tumour-specific immune responses are powerful tools for various immunotherapeutic approaches and might facilitate new strategies for treatment of CLL and other B-cell malignancies.

O-2B-4

Exosomes as drug delivery vehicles for Parkinson's disease therapy

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Introduction: Exosomes are naturally occurring nanosized vesicles that have recently attracted significant attention as drug delivery vehicles. We posit that exosomes secreted by monocytes and macrophages can provide an unprecedented opportunity to avoid entrapment in mononuclear phagocytes (as a part of a host immune system) and at the same time enhance delivery of incorporated drugs to target cells ultimately increasing drug therapeutic efficacy. In light of this, we developed a new exosomal-based delivery system of a potent antioxidant, catalase, to treat Parkinson's disease (PD). *Methods*: Catalase was loaded to exosomes ex vivo using different methods: permeabilization with saponin, freeze-thaw cycles, sonication and extrusion. A reformation of exosomes upon sonication and extrusion or permeabilization with saponin resulted in high loading efficiency, sustained release and catalase preservation against proteases degradation. *Results*: The size of the obtained catalase-loaded exosomes (exoCAT) was in the range of 100–200 nm. Exosomes were readily taken up by neuronal cells in vitro. A considerable amount of exosomes was detected in PD mouse brain following intranasal administration. ExoCAT provided significant neuroprotective effects in in vitro and in vivo models of oxidative stress. *Summary/conclusion*: Overall, exosome-based catalase formulations have a potential to be a versatile strategy to treat inflammatory and neurodegenerative disorders.

O-2B-5

Targeted therapeutic extracellular vesicles produced by liposome-based cellular engineering

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Introduction: Extacellular vesicles (EVs), such as exosomes anc microvesicles, are secreted from most cell types and play a critical role in intercellular communications involved in various pathophysiological processes. However, efficient engineering of these EVs for their functional applications remains a great challenge. Current methods for EV engineering are divided into largely 2 approaches: loading of exogenous agents directly to isolated EVs and genetic engineering of parental cells to produce functional EVs. However, these methods have shown limitations such as membrane damage, aggregation, low yield of EVs, laborious procedures and restriction of loaded cargo. Additionally, none of these previous methods allows simultaneous loading of compounds with disparate characteristics. *Methods*: Various liposomal formulations loaded with hydrophilic or hydrophobic/lipophilic cargo were prepared using a film hydration/extrusion method. Functional (cargo-loaded) EVs were isolated from the supernatant collected from the liposome-engineered cells by a series of centrifugations. For transwell experiments, the cells on the transwell filter were treated with cargo-loaded liposomes for 30 minutes and then placed on the lower chamber onto which fresh cancer cells were plated and incubated for 12 hours to allow production of functional EVs and their subsequent internalization into the cells in the lower chamber. To visualize the cellular uptake of functional EVs, the cells in the lower chamber were stained with Hoechst 33342 and imaged using confocal microscopy. To evaluate the therapeutic effect of functional EVs, the cell viability in the lower chamber was evaluated using MTT assay. *Results*: We report here a liposome-based EV engineering method, inspired by the biogenesis of EVs, to equip EVs with an arsenal of functional agents. This method allows the individual or simultaneous packaging of EVs with functional agents including, but not limited to fluorophores, drugs, lipids and bio-orthogonal chemicals in an efficient and controlled manner by delivering the agents to parental cells via synthetic membrane fusogenic liposomes. We further utilized this method to produce versatile EVs that can be customized with any desired functional agent using copper-free click chemistry. We demonstrated that the EVs co-loaded with anti-cancer drugs and azide-lipids using this method enhanced targeting efficacy to cancer cells after in situ conjugation of EVs with targeting peptides. Importantly, the EVs engineered using this method preserve their native transmembrane proteins and lipids, thus likely maintaining their biological functions. *Summary/conclusion*: We believe that our method has great potential to help elucidate the biological roles of EVs, utilize them as self-originated drug carriers, and engineer site-specific EVs in vivo through delivery of synthetic liposomes to desired sites.

O-2B-6

Therapeutic potentials of mesenchymal stem cell-derived extracellular vesicles

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Introduction: MSCs have been used to treat a variety of different diseases such as myocardial infarction, stroke and graft-versus-host disease (GvHD). Despite contrary reports regarding the outcome of MSC treatments, increasing evidence suggests that MSCs exert their beneficial effects via small extracellular vesicles (EVs, 80–160 nm), such as exosomes and microvesicles, rather than by intercalating into affected tissues. Indeed, we observed beneficial therapeutic effects following MSC-EV administration in a steroid-refractory GvHD patient (Kordelas et al., 2014) and in animal models for stroke and preterm brain injury. At the functional level MSC-EVs were shown to exert immunosuppressive functions in vivo and in vitro. Due to the contrary reports regarding the outcome of MSC treatments and the fact that MSCs are a very heterogeneous, ill-defined class of fibroblast-like cells, we consider that not all human MSCs release therapeutic effective EVs. *Methods*: To compare the immunomodulatory features of different MSC lineages, MSCs were raised from BM samples of 20 different stem cell donors. Their cell surface phenotype was analyzed by flow cytometry and their differentiation potential in conventional differentiation assays. EVs were harvested from MSC conditioned media using the PEG method followed by ultracentrifugation. Obtained MSC-EV fractions were characterized by western blot and NTA. The MSC-EVs' immunomodulatory properties were studied in

T-cell proliferation and activation assays in which T cells were stimulated with the lectin phytohemagglutinin (PHA). *Results*: *Bona fide* MSCs were obtained from all 20 donor samples. All of them expressed the cell surface antigens CD44, CD73, CD90, CD105, CD146 and CD166 and were negative for CD14, CD31, CD34 and CD45. They revealed osteogenic and adipogenic and as far as tested chondrogenic differentiation potentials. Huge differences regarding the averages cell size of the different MSC lineages were observed. All MSC-EV fractions were positive for the tetraspanins CD9, CD63 and CD81 as well as for Tsg101 and HSP70 and revealed average size distributions ranging between 110 and 150 nm. However, only a proportion of the MSC-EVs revealed immunosuppressive features in the T-cell readouts. *Summary/conclusion*: MSC-EV preparations of different MSC lineages differ in their in vitro immunomodulatory capabilities, suggesting that only a proportion of MSCs produce therapeutically active EVs. This might explain the controversy reports of MSC therapies in a variety of clinical settings. Even though, all MSC-EV fractions which we had used in in vivo studies were selected according to their capabilities to exert immunosuppressive functions in vitro, it will be important to also study the therapeutic impact of MSC-EV fractions lacking in vitro immunosuppressive functions. Currently, we are improving the platform to produce MSC-EVs for the clinical setting and search for surrogates to discriminate therapeutic effective and non-effective MSC-EV samples.

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Ballroom F-H

Symposium session 2C - EVs in immune regulation

Chairs: *Adrian Morelli and Huang-Ge Zhang* 16:30-18:30

O-2C-1

Passage of clusters of donor-derived exosomes promotes T-cell immunity against donor MHC molecules after heart transplantation

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Introduction: It is assumed that, after heart transplantation, T cells are primed against donor intact MHC molecules by donor dendritic cells (DCs) mobilized from the graft to the graft-draining secondary lymphoid tissues. Nevertheless, growing evidence suggests that donor DCs from the allografts home in relatively low numbers in recipient secondary lymphoid tissues, they are short-lived and they are killed by recipient NK cells and CTLs. Thus, an old question in transplantation is how so few donor DCs can efficiently prime recipient T-cells against intact donor MHC molecules. One possibility is that donor DCs, once homed in lymphoid tissues, transfer donor MHC molecules to recipient Ag-presenting cells, which in turn prime donor-reactive T cells. Interestingly, the mechanism of transfer of MHC molecules and the in vivo relevance in transplantation is unknown. Thus, we investigated the role of extracellular vesicles (EVs) in such phenomenon. *Methods*: Heterotopic (abdomen) heart allograft transplantation in mice, quantitative RT-PCR, FACS, cytoline ELISPOTs, in vitro generation of DCs, and electron and high-resolution confocal microscopy. *Results*: After transplantation of CD45.2 BALB/c hearts in CD45.1 C57Bl/6 (B6) mice, the few donor DCs that migrated to the recipient spleen (1127 ± 372 cells per spleen on post-operative day 3) transferred donor MHC molecules to a higher number recipient splenic conventional DCs (cDCs). Transfer of donor MHC molecules was mediated through clusters of exosome-like EVs of 75 ± 32 nm in size. The transferred MHC molecules were functional, since FACS-sorted recipient cDCs induced proliferation and effector cell differentiation of 2C CD8 T cells, specific for the BALB/c intact $H2L^d$ molecule. Cultures of CFSE-labelled BALB/c splenic DCs with acceptor B6 splenic DCs in presence of inhibitors of release of exosomes (GW4869 or Rab27a siRNA), microparticles shed from the cell surface (imipramine), or apoptotic cell-derived EVs (DEVD), confirmed that the passage of BALB/c MHC molecules occurred via exosomes transferred in clusters. The passage of MHC molecules did not occur when donor BALB/c DC were separated from the acceptor B6 DCs by 0.4 μm pore transwells. By high resolution confocal microscopy, BALB/c DCs, genetically engineered to release RFP (red fluorescent protein)-tagged exosomes and injected (s.c. or i.v.) in CD11c-YFP (yellow fluorescent protein) B6 mice, transferred RFP^+ exosomes in clusters (which were above the limit of resolution of the imaging system) to recipient YFP^+ DCs, both in lymph nodes and spleen. Accordingly, depletion of recipient DCs in diphtheria toxin (DT)-treated CD11c-DTR recipients prevented presentation of donor MHC molecules to T cells and acute rejection of BALB/c cardiac allografts. *Summary/conclusion*: Transfer of exosome clusters bearing donor MHC molecules explains an old enigma in transplantation, which is, how a few donor migratory DCs promote such a strong anti-donor T-cell response against donor MHC molecules after transplantation?

O-2C-2

Differential effect of microparticles and exosomes isolated from mesenchymal stem cells on T-cell proliferation and experimental arthritis

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Introduction: Mesenchymal stem cells (MSC) are multipotent cells that possess regenerative and immunomodulatory functions that are of high interest for therapeutic purposes in osteoarticular diseases such as rheumatoid arthritis (RA). These functions are primarily mediated by soluble mediators that are released in the extracellular milieu or within extracellular vesicles (EV). EVs consist of 3 main populations: exosomes, microparticles and apoptotic bodies that mirror the effect of parental cells. But little is known about the respective role of the various subsets of EVs secreted by a specific cell type. The aim of this study was to compare in vitro and in vivo the immunomodulatory effects of exosomes and microparticles secreted by MSCs. *Methods*: Exosomes and microparticles were isolated from culture supernatants of murine primary MSCs by differential ultracentrifugation. Size and structure of exosomes and microparticles were evaluated by Dynamic Light Scattering and/or electron microscopy. Expression of membrane and endosomal markers was tested by flow cytometry or western blot. Proliferation of freshly isolated murine splenocytes or isolated $CD4^+$ T cells, activated with concanavalin A was quantified after 72 hours of incubation with different quantities of EVs, using the Cell TiterGlo assay. In vivo, EVs (10 μg) were injected systemically in the collagen-induced arthritis (CIA) model. *Results*: We first confirmed that MSC-derived exosomes were less than 100 nm in diameter and expressed CD9, CD81 and TSG101 while microparticles were around 400 nm in diameter and expressed CD29, CD44 and Sca1 MSC membrane markers. In vitro functional analysis of the 2 EV subsets indicated that addition of microparticles in proliferative assays significantly inhibited the proliferation of total splenocytes and $CD4^+$ T cells in a dose-dependent manner, whereas exosomes were not able to exert a suppressive effect. This immunomodulatory function of microparticles was also observed in vivo in the CIA model of inflammatory arthritis with a reduced incidence and significant reduction of clinical symptoms: paw swelling and inflammation. *Summary/conclusion*: Our in vitro and in vivo data indicated that the immunosuppressive effect of MSCs is, at least in part, mediated by EVs and microparticles seemed to play a major role in T-cell proliferation inhibition.

O-2C-3

Extracellular Vesicles released from primary mucosal and connective tissue type mast cells differ in composition and are capable of modulating adaptive immunity

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Introduction: Murine mast cells can be divided into 2 main subtypes that differ in tissue distribution, protein content, mediator release and function: mucosal mast cells (MMC) and connective tissue mast cells (CTMC). We here characterized EV release from both MC phenotypes at resting and activated conditions and analyzed their capacity to modulate the induction of adaptive immune responses. *Methods*: Primary murine MMC and CTMC were obtained from spleen cell or peritoneal cell cultures, respectively. EVs were collected from culture supernatants by differential centrifugation and subsequently floated into a density gradient and analyzed by western blotting. For multiparameter analysis by high-resolution flow cytometry, which allows for single nano-sized particle analysis, EVs were stained with antibodies and CFDA-SE prior to floatation. For functional analysis, isolated EVs were added in a primary T-cell stimulation system using SEA superantigen. *Results*: Both MMC and CTMC released distinct EV populations after stimulation as indicated by changes in buoyant

density, light scattering and CD9 and CD63 contents. Unstimulated cells released primarily CD9+ EVs with buoyant densities of 1.13–1.19 g/ml, whereas IgE-mediated activation induced a massive and rapid release of CD9+CD63+ and CD63+CD9- EVs from MMC and CTMC, respectively. EV released by the activated CTMC were characterized by lower buoyant densities (1.21–1.23 g/ml). Functional analysis indicated that MC-derived EVs had a modulatory effect on dendritic cells and on cytokine production during antigen-driven $CD4^+$ T-cell priming. *Summary/conclusion*: The two major mast cell phenotypes MMC and CTMC release extracellular vesicles that differ in phenotypic characteristics after IgE-mediated activation and which show the ability to shape adaptive immune responses.

O-2C-4

Exosomes coated with antibody light chains bind antigen peptides on APC to antigen specifically suppress effector T-cells by delivery of miRNA-150

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Introduction: We previously described antigen (Ag)-specific suppressive exosomes derived from T & B cells in hapten-induced contact sensitivity (CS). We studied similar regulation of ovalbumin (OVA) protein-induced delayed-type hypersensitivity (DTH), focusing on the mechanism of suppression. In CS, the hapten-self peptide complexes on the Ag presenting cells (APC) cannot be analyzed. In contrast, OVA protein DTH allows analysis of the APC controlling the targeted effector T cells. *Methods*: According to Bryniarski et al. (J Allergy Clin Immunol 2013;132:170–181) inhibitory T-cell exosomes produced by CD8+ suppressor T (non-Treg) cells were induced by Ag high dose tolerance. They had a coating of B1a cell-derived Ag-specific antibody free light chains (Ab FLC) and a cargo of inhibitory miRNA-150. Suppressor B cell exosomes, that already expressed surface anti-OVA Ab FLC, were produced by B1a cells induced by intradermal Ag immunization and associated with miRNA-150. *Results*: Both suppressor T-cell exosomes containing miRNA-150 and B cell exosomes associated with miRNA-150 strongly inhibited DTH when injected systemically into mice actively sensitized with OVA. When the T or B cell exosomes were injected at the 24-hour peak of DTH, subsequent responses at 48–120 hours were inhibited by 60–80%. Importantly, and shown for the first time, orally administered T-cell exosomes caused even stronger inhibition, with 67–99% suppression of 48–120 hours DTH. To determine the nature of the Ag on the APC that Ab FLC coated exosomes binds, we studied 4 anti-OVA monoclonal Ab IgG antibodies that bound native OVA protein with markedly different strengths (strong, medium and weak) as did, but to a lesser extent, their FLC. Then we tested the suppressive ability of originally non-suppressive T-cell exosomes induced by Ag high dose tolerance in Ab deficient JH-/- mice. Separate groups of JH-/- tolerized exosomes were rendered Ag-specific by coating in vitro with FLC from each anti-OVA mAb. Resulting suppression of DTH via Ag-specific targeting likely of the Ag peptides of OVA on the APC surface was the inverse of their ability to bind native Ag. Two mAb FLC that hardly bound native OVA were the strongest mediators of exosome suppression. This suggested that the FLC coated exosomes may suppress by binding OVA peptides on the APC. *Summary/conclusion*: These results showed that Ag-specific T and B cell exosomes coated with Ab FLC can suppress OVA-induced DTH. Further, they were remarkably inhibitory orally. Interestingly, the mechanism of suppression seems to involve exosome-surface Ab FLC binding to Ag peptides complexed with MHC molecules on the targeted APC in an inverse manner to mAb ability to bind native OVA protein. Thus, AB FLC-coated exosomes carrying and delivering inhibitory miRNA-150 seem to bind OVA peptides on the APC surface and then subsequently to Ag to specifically suppress responses of their companion OVA/MHC-specific DTH-effector T cells.

O-2C-5

Defined breast milk EV subsets boost the immune response and skew the T-cell balance towards a regulatory phenotype

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Introduction: In the past years it has become clear that cell-derived extracellular vesicles (EV) are present in human breast milk and that these EV can play a role in the instruction of the immune system. Since breast milk impacts the development of the neonatal immune system by conveying environmental and maternal information to the child, we investigated the EV composition of human breast milk and evaluated the potential of these EV to modulate immune responses. *Methods*: For efficient and reliable recovery of naturally occurring EV from human breast milk, we applied a recently developed protocol, based on differential centrifugation and density gradient separation. Isolated EV were characterized by western blotting, EM, high-resolution flow cytometry, and lipidomics. For functional analysis of breast milk EV, removal of density gradient medium by column filtration was essential to avoid gradient medium-induced side-effects. EV-induced modulation of immune responses were analyzed in a T-cell stimulation assay with PBMC by profiling T-cell activation and cytokine release. *Results*: EV subsets were identified that differed in protein composition, light scattering, size and lipid composition. Furthermore, breast milk EV were also found to be highly enriched for several immune modulatory molecules, such as MHC class II, HSC/HSP-70, MFG-E8, butyrophilin 1A1 and MUC-1. Addition of breast milk EV to a T-cell stimulation assay revealed that milk EV skew T cells towards a regulatory phenotype, while boosting the release of the pro- and anti-inflammatory cytokines, such as IFN-g, TNF-a, IL-17, IL-10 and IL-6. *Summary/conclusion*: These data indicate that breast milk EV have a broad potential to steer immune responses, and could be transporters of (antigen-specific) immune information. As such, these EV may be involved in inducing immunogenicity, as well as oral tolerance in the neonate via the gastrointestinal tract.

O-2C-6

CD47 modulates T-cell micro-RNA expression and sorting into extracellular vesicles

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Introduction: Extracellular vesicles (EVs) mediate cell to cell communication in part by transferring mRNAs, miRNAs and other non-coding RNAs into recipient cells. The thrombospondin-1 receptor CD47 plays an important role in regulating communication between T cells and endothelial cells. EVs released by T cells alter gene expression and angiogenic signaling of recipient human umbilical vein endothelial cells (HUVEC) in a CD47-dependent manner (Matrix Biol 2014;37:49). Gene enrichment analysis of HUVEC treated with Jurkat T-cell-derived EVs also showed induction of T-cell signaling genes. *Methods*: To further examine the role of transferred RNAs, we performed global mRNA expression profiling of WT parental and CD47-deficient (JinB8) Jurkat T cells and their EVs and performed proteomic profiling of EVs. *Results*: Comparing Jurkat and JinB8 cells and EVs, 590 and 178 transcripts, respectively, were differentially expressed. EVs contained less mRNA than their cells of origin but

were enriched in miRNAs and non-coding RNAs. miRNA microarray analysis revealed 257 differentially expressed miRNAs between Jurkat and JinB8 cells. Of these, 95 were confirmed to be CD47 dependent by treating Jurkat cells with CD47 siRNA. Notably, miRNA expression profiles of EVs derived from WT and JinB8 T cells were distinct from those of their parental cells. Recognition of specific miRNA sequence motifs by heterogeneous nuclear ribonucleoproteins (HNRNPs) can mediate selective sorting of miRNA into EVs (Nat Commun. 2011;2:282). MEME and JASPAR Bioinformatics and computational analysis of the miRNA data identified 3 motifs that are enriched in JinB8 EV miRNAs (AGAAAA, GGGG and GGGAAGG) and 1 motif (AGGCAG) that is enriched in Jurkat EV miRNAs. Differential expression of HNRNPD and HNRNPAB between EVs derived from WT and JinB8 cells revealed 2 RNA binding proteins that could mediate CD47-dependent miRNA sorting into EVs. *Summary/conclusion*: We currently are validating our miRNA and proteomics results to define the role of specific HNRNPs and sequence motifs in CD47-dependent miRNA sorting but the present data demonstrate that the presence of CD47 at the surface of T cells alters their cellular miRNA expression as well as the specific sorting of those miRNAs into EVs that mediate communication between T cells and endothelial cells.

O-2C-7

Immunosuppressive exosomes present in human ovarian tumour microenvironments rapidly and reversibly arrest T-cell activation

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Introduction: One of the conundrums in tumour biology is that tumours grow despite the presence of infiltrating anti-tumour T cells. T cells present within tumour microenvironments have been found to be hyporesponsive to activation via the T cell receptor. Both cellular and acellular checkpoints have been believed to contribute to the loss of function of tumour-associated T cells, and some of them have been identified. The identification of these immunosuppressive factors within human tumour microenvironments and the ability to block these factors would be expected to enhance patients' anti-tumour immune responses by re-activating hypo-responsive tumour-associated T cells. *Methods*: Ovarian tumour ascites fluids were received from Roswell Park Cancer Institute. The ascites fluids were delipidated by the addition of methanol:diethyl ether (20:80 v/v) in an extraction funnel. Exosomes were isolated by ultracentrifuged at 200,000 $\times$ g for 90 minutes. Human NDPBL or sorted T cells were activated with immobilized anti-huCD3/CD28 with or without exosomes and the percentage of activated T cells was determined by monitoring the translocation of NFkB from the cytosol into the nucleus using fluorescence confocal microscopy. PS expressing exosomes were depleted magnetically using anti-PS antibody conjugated magnetic beads. Immunophenotyping of exosomes was carried out using an 18-parameter LSR Fortessa that was mechanically and electronically adapted in order to optimize the detection of submicron particles. *Results*: We have identified that one of the inhibitory factors present in ovarian tumour ascites fluids that reversibly inhibits the activation of T cells is a phospholipid, phosphatidylserine (PS). We report here that the inhibitory activity is mediated by small, 50–120 nm, extracellular vesicles (exosomes) that are present in ovarian tumour ascites fluids and in solid tumour tissues. Tumour ascites fluids act directly upon T cells to induce a rapid and reversible arrest in their activation. The exosomes present in the ascites fluids are surrounded by a lipid bilayer in which PS is expressed in its outer leaflet. Phenotyping by flow cytometry reveals that these tumour-associated exosomes are heterogeneous and express surface markers associated with both tumour and tumour-associated non-malignant cells, indicating that they are derived from multiple cellular sources. We report that the tumour-associated exosome-induced T cell signalling arrest is dependent upon PS, as a blockade of PS with anti-PS antibodies significantly reverses the inhibitory activity of the exosomes. The inhibitory activity is overcome by the addition of diacylglycerol kinase inhibitors, suggesting a possible mechanism for the T cell inhibition and also potential molecules to be targeted in developing approaches to reverse it. *Summary/conclusion*: Exosomes present within ovarian tumour microenvironments represent a novel T cell checkpoint and are a potentially viable target to block and thereby enhance anti-tumour immune responses in ovarian cancer patients.

O-2C-8

Modulation of monocyte activation by retinal pigment epithelium-derived exosomes

Jared Knickelbein[1], Anush Arakelyan[2], Susan Hannes[1], Baoying Liu[1], Jean-Charles Grivel[2], Sonia Zicari[2], Arvydas Maminishkis[3], Sheldon S. Miller[3], Leonid Margolis[2] and Robert B. Nussenblatt[1]
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Introduction: Exosomes are important mediators of intercellular communication and have been implicated in modulation of the immune system. We sought to investigate if exosomes released from retinal pigment epithelial (RPE) cells, which are critically important to the normal function of the retina, alter the activation status of immune cells in vitro. *Methods*: ARPE-19 cells were stimulated or not with the inflammatory cytokines IFN-γ, TNF-α, and IL-1β for 48 hours. Exosomes were isolated from the culture supernantant using ExoQuick TC isolation kit, and ARPE-19 cells were assayed by flow cytometry for the expression of surface and intracellular CD81 and CD107b (LAMP-2). Isolated exosomes were quantified with a NanoSight NS300 nanoparticle analyzer and incubated for 24 hours with either THP-1 or enriched human donor monocytes, which were then stained for the expression of ICAM-1 (THP-1) or CD14 and CD16 (human monocytes) and analyzed with flow cytometry. *Results*: Stimulation of ARPE-19 cells with IFN-γ, TNF-α, and IL-1β reduced the expression of surface and intracellular CD81, while levels of intracellular CD107b were unaltered, compared to non-stimulated controls. There was no difference in the number of exosomes secreted from ARPE-19 between stimulated and non-stimulated cultures. THP-1 monocytes upregulated ICAM-1 expression upon exposure to exosomes isolated from both non-stimulated and cytokine-stimulated ARPE-19 cells, however those from cytokine-stimulated ARPE-19 cells caused significantly higher ICAM-1 expression per THP-1 cell. Exposure to exosomes from non-stimulated ARPE-19 cells induced undifferentiated human monocytes into a more regulatory phenotype with a significantly higher percentage of $CD14^{++}CD16^{+}$ cells compared to human monocytes exposed to exosomes from ARPE-19 cells stimulated with cytokines. *Summary/conclusion*: RPE constitutively secrete exosomes. The quality but not the quantity of exosomes secreted from ARPE-19 cells is altered by cytokine stimulation. Exosomes from cytokine-stimulated ARPE-19 cells promoted expression of ICAM-1 in THP-1 cells, while exosomes from non-stimulated ARPE-19 cells skewed human monocytes toward a regulatory phenotype. These findings suggest that the normal RPE milieu may skew monocyte differentiation towards a regulatory phenotype and the inflammatory milieu of the RPE reverses this regulatory phenotype in particular through exosomes.

Evening poster viewing 19:00-21:00

A cash bar will be available for drinks and snacks

Bioinformatics Workshop: Exploring exRNAs using Web-based RNA-Seq pipelines/tools and public data resources of the exRNA Communication Consortium (ERCC) 19:30-21:00

Ballroom D

This bioinformatics workshop will introduce the Genboree Workbench, a Web and cloud-based platform for collaborative genome-centric research, virtual integration of data, bioinformatics tools, and other resources across the Web. Demonstrations will focus on the exRNA-seq analysis pipelines ('RSEQTools' and 'exceRpt') developed by members of the NIH Common Fund ExRNA Communication Consortium (ERCC), in addition to the incorporation of pathway visualization/ analysis tools such as Cytoscape, Wikipathways, etc for exRNA analysis. ERCC researchers will demonstrate through use cases how to upload data and run the RNA-seq analysis tools, which will include explanation of the available parameters and how to interpret results.

Presenters: Joel Rozowsky (intro/overview), Roger Alexander (use case), Sai Lakshmi Subramanian (exRNA Atlas), Rob Kitchen (RNA-Seq pipelines/WikiPathways/Cytoscape).

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Poster Presentations

Poster session I - EV biogenesis
Chairs: *Paola de Candia and Valentina Minciacchi*

P-I-1

Polarized epithelial cells secrete exosomes through distinct membrane domains that carry different exosomal cargo

Leslie Morton
Internal Medicine, Division of Gastroenterology and Hepatology, Mayo Clinic, Rochester, NY, USA

Introduction: Recent data from our lab have shown that: (a) cholangiocytes, the polarized epithelia lining bile ducts in the liver, secrete exosomes from their apical surface into the bile duct lumen; and (b) these exosomes influence signalling pathways and functional behaviour of target, neighbouring cholangiocytes via interactions with primary cilia. Based on these observations, and recognizing that polarized epithelia, including cholangiocytes, also have a basolateral domain facing a complex, extracellular matrix that includes many cell types, we began to test the hypothesis that cholangiocytes and other polarized epithelia release exosomes from their apical and basolateral domains that differ both in their content and in the target cells with which they communicate. *Methods*: Culture media from above (apical) and below (basolateral) the polarized epithelia was collected and exosomes isolated by differential ultracentrifugation. Transmission electron microscopy, nanoparticle tracking, western blots and flow cytometry confirmed exosomal shape, particle size and the presence of the exosomal membrane protein marker, CD63, on exosomes collected from both apical and basolateral cell poles. *Results*: Normal human cholangiocytes (NHCs) were grown on membrane inserts. Their confluency was confirmed by microscopy, and the integrity of their tight junctions supported by the increase in trans-epithelial electronic resistance measurements over the course of 5–6 days. While exosomes from both domains were similar in size, the 2 exosome populations differed in number (apical > basolateral) as well as protein and miRNA content. *Summary/conclusion*: The data support the concept that polarized epithelia release exosomes from both apical and basolateral membrane domains that differ in number and content. Studies underway are focusing on the (a) mechanisms of biogenesis and secretion of exosomes originating from both domains of polarized epithelia, (b) the target cells that these subpopulations of exosomes interact with and (c) the functional consequences of these forms of intercellular communication.

P-I-2

Live-visualization of tumour exosome release dynamics with a pH-sensitive fluorescent reporter

Frederik Verweij[1,2], Anoek Zomer[3], Serena R. Baglio[1], Juan Garcia-Vallejo[4], Tamara Sequeiros Fontán[1], Marc Coppolino[5], Florence De Groen[1], Hans Janssen[6], Jacques Neefjes[6], Matthijs Verhage[7], Jaap Middeldorp[1], Guillaume Van Niel[2], Jacco Van Rheenen[3], Ruud Toonen[7] and Dirk M. Pegtel[1]
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Introduction: Individual cancer cells may secrete thousands of 40–100 nm membrane vesicles a day many of which are presumed to originate from multivesicular bodies (MVBs). However, what drives MVBs to fuse with the plasma membrane (PM) leading to exosome release is not understood. *Methods*: We labelled intraluminal vesicles of acidic MBVs with a pH-sensitive optical reporter (CD63-pHluorin) to reveal fusion dynamics of peripheral MVBs with the PM using live-cell imaging. *Results*: In contrast to rapid transport vesicle-PM fusion, MVB-PM fusion events are rare and under control by endosomal cholesterol trafficking. MVB-PM fusion increases upon stimulation with cAMP-dependent protein kinase (PKA) and histamine treatment, supporting a direct role for G protein-coupled receptor (GPCR) signalling in MVB-fusion and exosome release. We identified the non-neuronal t-SNARE SNAP23 and Syntaxin-4 as mediators of MVB-PM fusion, and inactivation of SNAP23 in invasive cancer cells reduced extracellular matrix remodelling and invasion. Moreover, increased SNAP23 expression was observed in tumour tissues and correlated with increased incidence of metastasis. *Summary/conclusion*: Together, our findings demonstrate that MVB fusion with the PM is a dynamic, physiologically relevant process in cancer cells that can be modulated by soluble factors present in the tumour microenvironment.

P-I-3

Alix regulates epithelial cell polarity by bridging the actin cytoskeleton with tight junction proteins

Yvan Campos[1], Xiaohui Qiu[1], Simon Moshiach[1], Elida Gomero[1], Randall Wakefield[2], Wojciech Brutkowski[3], Antonella Bongiovanni[4] and Alessandra d'Azzo[1]
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Introduction: The multivalent scaffold protein Alix, first identified as a binding partner of ALG-2, has since been implicated in multiple cellular processes, due to its ability to interact with proteins in different cell compartments, which are themselves components of large complexes. For example, by interacting with ESCRT I and III, Alix synergistically coordinates endocytosis and recycling of membrane receptors, extracellular vesicle/exosome biogenesis, viral budding and cytokinesis. By binding to F-actin Alix can also influence the maintenance and remodelling of the actin cytoskeleton. These multi-tasking properties of Alix have largely been inferred from the functions of proteins with which it interacts. The aim of this study was to elucidate the role(s) of Alix in vivo by investigating the consequences of Alix loss of function in a novel knockout model. *Methods*: We generated the first *Alix*-KO mouse and characterized the phenotype by: (a) MRI scans; (b) 3D-reconstruction of scanning electron microscopy (3D-SEM) images, and transmission electron microscopy (TEM); (c) immunofluorescence and co-immunoprecipitation of protein complexes; (d) purification of actomyosin; (e) tunnel assays. These experiments were performed on whole mount preparations of the choroid plexus (CP) and lateral wall from WT and *Alix*-KO brains; ex vivo cultures of tracheal epithelial cells; cultured CP cells. *Results*: *Alix*-KO mice were viable and had a normal lifespan, but were

often identifiable at young age by the dome-shape of their head. Sequential MRI scans of WT and mutant crania starting at P8 and ending at 44 weeks of age revealed enlargement of the lateral ventricles, resulting in progressive hydrocephalus. This phenotype is linked to 2 major defects: abnormal production/absorption of the cerebral spinal fluid by the CP; abnormal development and function of the cilia. SEM of the *Alix*-KO CP identified overt morphological changes, that is, irregular cell shape and size, abnormal positioning of the primary cilia, blebbing of the microvilli, and incorrect alignment and organization of the epithelial cell layer. By IF analyses, we found that these defects were accompanied by increased extrusion of apoptotic cells and aberrant structural organization of the tight junctions. We further demonstrate that loss of Alix in the CP affected the correct assembly and maintenance of the actomyosin and tight junction complexes. Together these phenotypic alterations were indicative of loss of epithelial cell polarity in the *Alix*-KO CP. *Summary/conclusion*: We demonstrate for the first time in an in vivo model that Alix plays a central role in the maintenance of epithelial cell polarity, by bridging the F-actin actomyosin with tight junction protein complexes. Combined the CP defects in the *Alix*-KO mice justify the occurrence of the progressive hydrocephalus phenotype in this mouse model.

P-I-4

Role of cytoskeletal organization in the biogenesis of matrix vesicles during odontoblast-supported mineralization

Sandeep C. Chaudhary[1], Maria Kuzynski[1], Morgan Goss[1], Callie G. Mobley[1], Anne Poliard[2], Odile Kellermann[3], Jose-Luis Millan[4] and Dobrawa Napierala[1]
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Please see Symposium Session 5C

P-I-5

Regulation of a metal transporter via membrane budding

Kimberly Mackenzie[1], Natalie Foot[1], Hazel Dalton[1], Brett Collins[2] and Sharad Kumar[1]
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Introduction: The regulation of plasma membrane proteins is crucial for maintaining optimal functional responses. Ubiquitination often acts as a major regulatory mechanism of internalization, intracellular sorting and degradation of these proteins. Here we present evidence that an iron transporter, highly expressed in duodenal enterocytes, is regulated in an unexpected manner that involves membrane shedding as microvesicles (MVs) involving a ubiquitin ligase and 2 α-arrestin family members. *Methods*: We investigated the role of α-arrestins in the regulation of an iron transporter through functional and biochemical assays using cell lines transiently expressing tagged constructs. MVs were purified by differential centrifugations from culture media of cell lines and gut explants cultured under normal and low iron conditions. RT-PCR assessed α-arrestin expression in the duodenum of mice fed a standard iron versus high iron rodent diet. *Results*: Two of the α-arrestins act as ubiquitin ligase adaptors to negatively regulate the activity of the iron transporter. Surprisingly, instead of leading to endocytosis, the transporter is released from the plasma membrane in α-arrestin-positive MVs. Both α-arrestins are similarly dependent on the ubiquitin ligase to efficiently target the transporter into MVs; however, they bud through different mechanisms. The release of the endogenous transporter in MVs is significantly increased from mouse gut explants cultured under high iron conditions, and the expression of an α-arrestin is significantly upregulated in the duodenum of mice fed a high iron diet. *Summary/conclusion*: The release of an iron transporter in MVs may represent a novel mechanism for the maintenance of iron homeostasis which may also be important for the regulation of other membrane proteins.

P-I-6

Proteomic characterization of outer membrane vesicles derived from antimicrobial resistant and susceptible Escherichia coli

Jung-Seok Lee, Si-Won Kim, Seong-Bin Park, Jae-Sung Kim, Young-Shin Chon, Se-Pyeong Im, Jassy Marry Lazarte and Tae-Sung Jung
Laboratory of Aquatic Animal Diseases, College of Veterinary Medicine, Gyeongsang National University, Jinju-si, Republic of Korea

Introduction: Outer membrane vesicles (OMVs) have been observed to be released from gram-negative bacteria. These released OMVs help in defending cells against outer membrane-acting antibiotics based on the nearly identical surface ingredients of the OMVs and the bacterial outer membrane. In this way, they are suspected to be involved in antimicrobial resistance, thus antimicrobial sensitive bacteria would survive in antimicrobial media by adding the OMVs released from antimicrobial resistant bacteria. *Methods*: Antimicrobial resistant bacteria (RC85+) were established by conjugation assay using multidrug resistant bacteria (Sal45) and antimicrobial sensitive bacteria (RC85). The OMVs from RC85 or RC85+ were purified by ultracentrifugation followed by QuixStand. The morphology of their OMVs was monitored by transmission electron microscopy. To evaluate the effects of the OMVs, the growth rates of RC85 treated with the OMVs from RC85 or RC85+ were determined. The OMVs from RC85 or RC85+ were analyzed using LC-ESI-MS/MS to compare their respective protein compositions. *Results*: As a result of the antibiotic resistance test, we found that the OMVs from RC85+ diminished the activity of the antibiotics to inhibit the growth rate of RC85 and guess that the OMVs from RC85+ could consume the antibiotics in the media, thus let RC85 keep growing. From the result of the protein analysis by LC-ESI-MS/MS, total 453 proteins were detected in the OMVs from both RC85 and RC85+. Among them, 103 and 163 proteins were uniquely found in antibiotic-susceptible *E.coli* (RC85) and -resistant *E.coli* (RC85+), respectively. The OMVs released from RC85 solely possessed chain O and chain I proteins, which are part of structural proteins of bacterial ribosome. On the other hand, only the OMVs released from RC85+ possessed long-chain-fatty-acid-CoA ligase and fimbrial protein prsG. *Summary/conclusion*: We demonstrated that the survival rate of RC85 in the antibiotic media was improved with the treatment of the purified OMVs released from RC85+. Furthermore, we compared the protein compositions of the OMVs from RC85 or RC85+ using gel free LC-ESI-MS/MS in order to evaluate the proteomes involved in the antimicrobial resistance. With the information, we suggest that the presence of these proteins found in the OMVs from RC85+ is essential for the bacterial growth and survival in an environment with antibiotics.

Poster session II - EVs and stem cells
Chairs: *Susmita Sahoo and Thomas Würdinger*

P-II-1

Mesenchymal stem cell-derived exosomes mediate angiogenesis

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Introduction: Elucidating the mechanisms of new blood vessel formation (angiogenesis) has important implications for numerous diseases including cardiovascular disease and cancer. Bone marrow-derived mesenchymal stem cells (MSC) have been well characterized for their immunomodulatory, tissue healing and pro-angiogenic capabilities. Studies have shown MSCs mediate angiogenesis through the secretion of pro-angiogenic factors. Studies to date have focused on canonical secretory proteins such as VEGF as the mediators of MSC's ability to induce angiogenesis. However, recent studies have shown MSC also secrete significant amounts of secreted vesicles called exosomes, which can transport biologically active non-secretory proteins and miRNA from their cell of origin to target cells. We aimed to investigate the potential role of MSC exosomes in MSC induced angiogenesis. *Methods*: Exosomes were isolated from MSC conditioned media. MSC-exosomes were used to stimulate endothelial cells [human umbilical vein endothelial cell (HUVEC)] in vitro. miRNA expression in MSC-exosomes was quantified via qPCR. *Results*: We show that MSC exosomes induce angiogenesis-like tubule formation in endothelial (HUVEC) cells in vitro. We show that MSC-derived exosomes contain pro-angiogenic miRNAs. *Summary/conclusion*: These findings indicate that MSC exosomes have the potential to deliver miRNA payloads and suggest that this capability may be further enhanced through genetic engineering, perhaps leading to new therapeutic avenues involving the delivery of extracellular RNAs.

P-II-2

A GMP-grade standard protocol for exclusively human mesenchymal stromal cell-derived extracellular vesicles

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Introduction: Production and application of extracellular vesicles (EVs) for regenerative, immune-modulatory or neuroprotective therapies requires stringent control of identity, purity and potency. Vesicles present in bovine serum (FBS) containing growth media interfere with the purification of pure cell-derived EVs and lead to heterogeneous EV preparations. As an alternative to FBS, human platelet lysate (HPL) can be used as a growth medium supplement for the propagation of human mesenchymal stromal cells (MSCs). However, fibrinogen that is present in HPL negatively affects the immune-modulatory functions of MSCs and high Ca^{2+} concentrations, commonly used for fibrinogen clotting and removal negatively influence osteogenic differentiation. Moreover, heparin (used to prevent fibrin clotting) impairs the uptake of EVs into recipient cells and the trilineage differentiation of MSCs under HPL conditions. We have addressed the apparent need for heparin and fibrinogen-reduced growth media devoid of serum-derived EVs and present a method for the production of such growth media. *Methods*: We have developed a novel method to avoid heparin and to physically remove fibrin without addition of Ca^{2+} that results in a clear and stable growth medium which supports MSC growth comparable to formulations containing heparin. Prior to use HPL-derived vesicles are depleted from the medium in a single ultracentrifugation step. EVs are purified from the conditioned growth medium by sequential ultracentrifugation and filtration. *Results*: Applying minimal physical interventions, we were able to generate a growth medium suitable for human MSCs in vitro culture. MSCs retain their in vitro potential for adipogenic and osteogenic differentiation, and surface marker expression, cell morphology, migration, contractility and cell death rate are unaltered compared with cells grown in heparin-containing medium. The proliferation rate is decreased by $15 \pm 0.3\%$, and consequently the doubling time is increased by $15 \pm 1.1\%$. Bioanalyzer-based miRNA profiling revealed significant differences between EVs from cells grown in heparin-free and fibrinogen-reduced growth media compared to EVs isolated from cells propagated in standard HPL- or FBS-containing media. *Summary/conclusion*: We present a GMP-grade protocol for the purification of exclusively human MSC-derived EVs from cells propagated in heparin-free and fibrinogen-reduced growth medium avoiding FBS and lacking contaminating serum-derived EVs. With this protocol, a thorough characterization and establishment of protein and miRNA profiles from MSC-derived EVs can now be achieved to identify the active components of therapeutic EVs for future clinical application.

P-II-3

Mesenchymal stem cell-derived microvesicle therapy for stroke: neurogenic/angiogenic effects and biodistribution in a rat stroke model

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Samsung Medical Center, Seoul, Republic of Korea

Introduction: We hypothesized that mesenchymal stem/stromal cells (MSCs) exert their action via microvesicles in the ischemic brain and that MSC-derived microvesicles could minimize cell trapping within organs that filter the bloodstream of systemically introduced stem cells. Thus, we evaluated the neurogenic and angiogenic potential of microvesicles and their biodistribution in a rat stroke model. *Methods*: Microvesicles were obtained from supernatants of MSC cultures after treatment with ischemic brain extracts. MSC-derived microvesicles were injected stereotaxically or intravenously in a rat stroke model. *Results*: When neural stem cells and endothelial cells were treated with 100 μg/ml microvesicles, neurogenesis and angiogenesis increased in a dose-dependent manner ($p < 0.01$). MSC-derived microvesicles stimulated neurogenesis and angiogenesis. To test microvesicle biodistribution, microvesicles and MSCs were labelled with either fluorescent dyes carboxyfluorescein succinimidyl ester (CFSE) or green fluorescent protein (GFP) transfection and intravenously injected. On western blots, higher GFP levels were observed in the infarcted brain of microvesicle-treated rats than MSC-treated rats, whereas GFP levels in the lung and liver were higher in MSC-treated rats than microvesicle-treated rats. Mortality rates were higher in MSC-treated rats than microvesicle-treated rats (50% vs. 5%, $p < 0.0001$). Microvesicle-treated rats exhibited greater behavioural improvements than control rats ($p < 0.05$). Cargo protein analysis showed that microvesicles included VEGF/VEGFR-2, HGF/c-Met, SDF-1/CXCR-4, synaptophysin and TGF-beta. Additionally, microvesicles contained various miRNAs associated with neurogenesis and angiogenesis. *Summary/conclusion*: MSC-derived microvesicles promote neurogenesis and angiogenesis in the injured brain. Stem cell-derived microvesicle therapy could be a novel, feasible and safe strategy that avoids cell-associated problems.

P-II-4

Experimental conditions of exosomes derived from adipose-derived mesenchymal stem cells effectively protect glutamate-induced PC-12 cell injury

Xiaofei Ji[1], Jing Liu[1], Yu Sun[1], Chao Li[1], Rutao Liu[2], Jingjing Zhang[1], Yan Ren[1] and Wenjuan Wei[1]

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Introduction: Exogenous exosomes delivered from stem cells are considered to have cytoprotection in cell injury. However the optimal exosomal concentration and co-culture time target to injury cells are still not clear. Our previous research indicated exosomes enriched adipose-derived mesenchymal stem cells conditioned medium could reduce the glutamate induced cell injury, which prompted adipose-derived mesenchymal stem cells exosomes might involved in cytoprotection. *Methods*: In this study, we co-cultured exosomes delivered from adipose-derived mesenchymal stem cells with glutamate-treated PC12 cells to confirm the exosomal cytoprotection by LDH detection and Trypan blue staining, and then determined the optimal experimental conditions by comparing different exosomal concentration and co-culture time. Finally, we dynamically observed the process of PKH67 dyeing exosomes target to PC12 cells in the Live Cell Imaging System. *Results*: The results showed that exosomes delivered from adipose-derived mesenchymal stem cells significantly decreased LDH level and improved cell survive in glutamate-treated cells, especially in 40 ng/μl exosomal concentration and 12 hours co-cultured. Furthermore, PKH67 dyeing exosomes were most obvious in PC12 cells after 10–12 hours co-cultured in the Live Cell Imaging System. *Summary/conclusion*: Our study indicated exosomes delivered from adipose-derived mesenchymal stem cells had cytoprotection to glutamate induced cell injury. The optimal exosomal concentration was 40 ng/μl, and co-culture time was 12 hours.

P-II-6

Extracellular vesicles from mesenchymal stem/stromal cells contain a novel miRNA that inhibits polycomb protein SUZ12

Patrice Penfornis[1], Joseph D. Fernandes[1] and Radhika R. Pochampally[2]
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Introduction: Current knowledge in cancer biology shows that human mesenchymal stem/stromal cells (hMSCs) play a role in cancer progression through their secretome. Our lab has shown that hMSCs are resilient to serum deprivation using autophagy. The secretome of these stressed cells is tumour supportive, demonstrating that this model mimics solid tumour core. It has been demonstrated that extracellular vesicles (EVs) secreted by cells contain microRNAs (miRNAs), which in turn affect a recipient cell by modifying its protein translation, thus inducing a cascade of signalling events. In this study, we focused on a new and unknown miRNA contained in EVs, its potential RNA targets in recipient cells and its regulation in parent cells. *Methods*: EVs were isolated from serum free hMSCs using concentration followed by serial ultracentrifugation. EVs small RNA were isolated using the miRVANA kit, and a small RNA library was generated. The purified cDNA library was used for cluster generation, then sequenced on Illumina GAIIx. Human cancer cells (osteosarcoma and breast cancer) miRNA knockdown models were obtained using agomirs and shRNA strategies. Cell proliferation assays were performed using trypan blue and Cyquant DNA detection. In vivo tumour progression in nude mice was tracked by caliper measurement and relative GFP signal was detected by in vivo imaging station. miRNA targets were revealed by Affimetrix Human gene 2.0 ST array analysis and confirmed by 3'UTR luciferase assays. *Results*: Next generation sequencing of EVs from hMSCs identified an unknown microRNA and here we report the characterization of this miRNA provisionally named miR-G665A. miR-G665A is highly conserved among mammals and its expression is confirmed in most cell types. Both in vivo and in vitro knockdown studies using agomirs and shRNA strategies demonstrate that miR-G665A plays a role in cell proliferation and tumour progression. Microarray data from these knockdown studies revealed potential end targets of miR-G665A (e.g. interleukins, TNF, MMPs), some are confirmed using 3'UTR luciferase assays. One of these targets, SUZ12, is a component of the polycomb repressive complex PRC2, which play a role in conferring the neoplastic phenotype to adult cells. *Summary/conclusion*: The role of this novel miRNA contained in EVs on the regulation of an essential transcription factor could lead to a better understanding of the crosstalk mechanisms between mesenchymal stem/stromal and cancer cells.

This work was supported in part by NIH grant CA1515851 and the UMMC Cancer Institute startup funds.

P-II-7

Mesenchymal stem cell exosomes are enriched in complement and coagulation cascade proteins

Luis Ortiz, George Leikauf, Michelangelo DiGiuseppe, James P. Fabisiak, Joel Njah and Bryan Brockway
EOH, University of Pittsburgh, Pittsburgh, PA, USA

Introduction: Complement is a highly conversed innate immune response that becomes activated during tissue injury and remodelling. Although mesenchymal stem cell (MSC) exosomes are currently being employed in several clinical trials, the ultimate utility of this therapy may dependent a full characterization of the transferred exosomal cargo of proteins, mRNA and microRNA. *Methods*: We examined the proteome of exosomes from human MSCs procured by the PACT program at the University of MN and currently being used in clinical trials. *Results*: We identified 845 proteins, which was comparable to the 856 identified by Lai et al. (PMID: 22852084) with 346 proteins present in both analyses. Typical exosome-associated cytoskeletal proteins (actins, moesin and tubulin) co-purified with MSC exosomes (1.10–1.18 g/ml fraction). Pathway enrichment analysis highlighted cytosolic and mitochondrial proteins with enzymatic activity. Among these enzymes the entire 20S proteasome were detected, which was accompanied by several chaperons (heat shock 40, 70 and 90 kDa proteins) confirming possible proteolytic enhancement and maintenance of protein homeostasis. Another predominant pathway was ''complement and coagulation cascades'' that contain proteins in the classic (C1Q, C1R, C1S, C2-6, C8 and C9), lectin (MASP2) and alternative (C3, CFB) complement activation cascades that also can promote phagocytosis (C1Q and C3) or anaphylatoxn responses (C3, C4A and C5). Coagulation factors included F2, F3, F5, F8, F10 and F12, and F2R and kallirein-kinin system proteins included plasminogen, kininogen 1, plasminogen activators (PLAT, PLAU) and plasminogen receptor (PLAUR). In addition, inhibitory proteins that can modulate complement (CFH, CFI, SERPING1 and CD59), coagulation (SERPINA1, C1 and A2M) and plasminogen (SERPINF2) pathways were identified. *Summary/conclusion*: This study indicates that complement proteins are enriched in MSC-derived exosomes. Recently, an emerging role of complement proteins in promoting tissue reprogramming, pattern formation and regeneration has gain support. Future investigations of the functional consequences of complement proteins in MSC-derived exosomes are warranted.

P-II-8

Impact of purification methodologies on the characterization of extracellular vesicles from pluripotent stem cell sources

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Introduction: Differential centrifugation (UC) is the standard method used for purifying extracellular vesicles (EVs) from all biological sources. However, there have been some recent debates regarding the low yields, purity and biophysical properties of these purified EVs. We have recently demonstrated in a systematic comparison study that an alternative strategy – size-exclusion liquid chromatography (LC), is more efficient than UC in deriving higher yields of pure EVs

from standard cell culture media. Here, we extend this comparison study on complex media types from pluripotent stem cells and discuss how different purification methodologies can have a profound impact on the characterization of these EVs. *Methods*: Conditioned stem cell media was collected from mouse embryonic (ES) and induced pluripotent stem (iPS) cells grown on gelatin-coated plates. EVs were then purified, either by UC or LC. Purified EVs were subsequently quantified and characterized with nanoparticle tracking analysis, transmission electron microscopy, sucrose gradient separation, western blotting, RNA bioanalyzer and proteomics. *Results*: Generally, EVs isolated by both UC and LC had similar size distribution with a modal size of 80–90 nm. Interestingly, more particles were isolated by UC than LC, opposite to our previous findings with conventional conditioned media. Further analysis showed that the general protein expression of vesicle markers and pluripotency factors as well as the RNA profiles differed greatly between the UC and LC-purified EV sample, which we speculate is due to the presence of protein contaminations pelleted with the UC technique. *Summary/conclusion*: EVs are reported to be potent source of communication between cells through the transfer of their vesicular content of proteins and RNA. Depending on the type of purification method used, the resultant deduction of vesicular phenotype differs greatly. Selecting the least biased methodology for purification is crucial for more accurate characterization of vesicles from complex biological fluids.

P-II-9

Mast cell-derived exosomes induce migration and matrix metalloproteinase expression in human bone marrow mesenchymal stem cells

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Introduction: Mesenchymal stem cells (MSCs) have been shown to play important roles in inflammatory lung diseases, such as acute lung injury, asthma and chronic obstructive pulmonary disease. Many regulatory factors released during the process of these diseases, can induce migration of MSC to the target tissue. However, many of the mechanism underlying MSCs trafficking still remains to be understood. When mast cells get activated during inflammation they release several soluble factors as well as extracellular vesicles such as exosomes which are intricately involved in communication between cells. The aim of this study was to determine whether mast cell exosomes can induce migration of human bone marrow-derived MSCs. *Methods*: Human bone marrow-derived MSCs were expanded in vitro and used for experiments in early passages (less than passage 4). Mast cell exosomes were isolated from 3 days conditional media using differential centrifugation protocols which include a long centrifugation (3 hours). In vitro migration and invasion assays toward exosomes were performed with a Boyden chamber. The expression of matrix metalloproteinase (MMP) was analyzed by gelatin zymography. Levels of TGF-beta in supernatants were detected by ELISA assay. Gene expression in MSC with and without treatment of exosomes was analyzed by real time PCR. *Results*: MSCs migrated toward mast cell-derived exosomes in a dose dependent manner. The release of MMPs and TGF-beta into the supernatant by the MSC was increased after treatment with exosomes compared with untreated cell. Furthermore, the stimulation of MSC by mast cell exosomes influenced the TGF beta-mRNA expression in the MSCs. *Summary/conclusion*: Mast cell-derived exosomes can induce migration of MSC and induce release of MMPs involved in the invasion process. The TGF-beta production in MSC was up-regulated by treatment of mast cell exosomes, which might be associated with the immune regulatory function of MSC in inflammatory lung diseases.

P-II-10

Melanoma cell-derived exosomes alter the microenviroment of malignant tumours via re-education of mesenchymal stem cells by miRNAs

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Please see Symposium Session 4A

P-II-11

Exosomes expressing the CIC marker tandem EpCAM and Claudin-7 promote epithelial-mesenchymal transition in non-metastatic tumour cells

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Introduction: In colorectal cancer (CoCa) EpCAM is frequently associated with claudin-7. There is evidence that tumour-promoting EpCAM activities are modulated by the association with claudin-7. *Methods*: To support this hypothesis, claudin-7 was knocked-down (kd) in HT29 and SW948 cells. *Results*: HT29-cld^{kd} and SW948-cld7^{kd} cells display decreased anchorage-independent growth and the capacity for holoclone-, respectively, sphere-formation is reduced. Tumour growth is delayed and cld7^{kd} cells poorly metastasize. In line with this, migratory and invasive potential of cld7^{kd} clones is strongly impaired, migration being inhibited by anti-CD49c, but not anti-EpCAM, although motility is reduced in EpCAM siRNA-treated cells. This is due to claudin-7 recruiting EpCAM in glycolipid-enriched membrane fractions towards claudin-7-associated TACE and presenilin2, which cleave EpCAM. The cleaved intracellular domain, EpIC, promotes epithelial-mesenchymal transition (EMT-associated transcription factor expression, which together with fibronectin and vimentin are reduced in claudin-7^{kd} cells. Importantly, uptake of HT29^{wt} and SW948^{wt} exosomes by non-metastatic, non-CIC sufficed for EMT-related transcription factor and mesenchymal protein upregulation, which promoted metastatic capacity. We are currently controlling whether cld7 facilitates recruitment of miRNA, which regulate EMT genes, into exosomes. *Summary/conclusion*: Taken together, claudin-7 contributes to motility and invasion and is required for recruiting EpCAM towards TACE/presenilin2. EpIC generation further supports motility by promoting a shift towards EMT, where EMT features of claudin-7-competent metastatic CoCa cells can be transferred via exosomes to poorly metastatic cells. We hypothesize, that cancer-initiating cell exosomes reprogram neighbouring non-CIC towards the EMT phenotype. This could well explain the MET reversion after reprogrammed non-CIC leave the primary tumour and settle in distant organs.

P-II-12

Approaching the functionality of CIC markers in pancreatic and colorectal cancer via exosomes

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Introduction: Pancreatic and colorectal cancer initiating cells express CD44v6, EpCAM, claudin7 and Tspan8, which are recovered in exosomes. Downregulation of either of these markers sufficed for a striking downregulation of circulating immune complexes (CIC)

features like anchorage independent growth, drug resistance, invasiveness and metastatic capacity, while exosomes expressing this set of CIC markers sufficed to restore motility, invasiveness, drug resistance, reprogramming of distinct host cells and EMT induction in non-CIC. Thus, the question arose, how a cell-matrix adhesion molecule that signals via associating kinases (CD44v6), a homophilic cell-cell adhesion molecule, whose intracellular domain acts as a cotranscription factor (EpCAM), a tight junction protein that looses contact to other tight junction components upon palmitoylation (cld7) and a tetraspanin acting via associating laminin-binding integrins (Tspan8) display indispensable converging activities in CIC exosomes? *Methods*: Exosome composition and activity of CIC was evaluated in dependence on the presence of CD44v6, Tspan8, EpCAM and claudin7. *Results*: (a) All 5 proteins associate, where the cld7-EpCAM association is the only direct protein-protein association that depends on cld7 palmitoylation, which is accompanied be recruitment into GEM/TEM (glycolipid/tetraspanin enriched membrane microdomains). (b) Within GEM/TEM the 5 CIC markers associate with additional molecules that are constitutive GEM/TEM components or become recruited upon activation by individual CIC markers like activated ERM proteins binding to cld7 or CD44. (c) TEM are prone for internalization, recruitment into MVB and release in exosomes, where tetraspanins are associated with fission, scission and transporter complexes. Target cell structures are also located in internalization prone GEM/TEM. By recognizing protein complex rather than individual molecule, some selectivity is conferred to exosome-binding. (d) Target modulation can be a direct consequence of exosome binding as ECM degradation by exosomal proteases or T-cell activation by exosomal peptide-loaded MHC complexes, where the T cell receptor complex and exosomal MHC complexes are GEM located. Alternatively exosome uptake promotes reprogramming by signal transduction like EMT induction via targeting Notch. Reprogramming also relies on exosomal miRNA, our data pointing towards a major contribution of Tspan8 and CD151 in miRNA recruitment. *Summary/conclusion*: Though CIC exosomes are suggested to drive tumour progression, the functionality of CIC protein markers is still disputed, and their contribution to exosome assembly and activity is largely neglected. We provide evidence that CIC markers working in concert in CIC exosomes guarantees tumour progression; Notably all CIC markers described so far are GEM-located or can be driven into GEM and are recovered on exosomes. According to our studies, distorting the CIC marker network is sufficient to break the vicious circle of tumour progression.

P-II-13

Leukaemia cell microvesicles promote survival in umbilical cord blood hematopoietic stem cells

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Introduction: Microvesicles can transfer their contents, proteins and RNA, to target cells and thereby transform them. This may induce apoptosis or survival depending on cell origin and the target cell. In this study, we investigate the effect of leukemic cell microvesicles on umbilical cord blood hematopoietic stem cells to seek evidence of apoptosis or cell survival. *Methods*: Microvesicles were isolated from both healthy donor bone marrow samples (written consent form was obtained) and Jurkat cells by ultra-centrifugation and were added to hematopoietic stem cells sorted from umbilical cord blood samples (written consent form was obtained) by magnetic associated cell sorting (MACS) technique. After 7 days, cell count, cell viability test, flow cytometry analysis for hematopoietic stem cell marker (CD34) and qPCR for P53 gene expression were performed. *Results*: Our Data showed higher cell number, higher cell viability rate and lower P53 gene expression in leukaemia group in comparison with normal and control groups ($p < 0.05$). Also, CD34 expression as the most important hematopoietic stem cell marker, did not change during the treatment and lineage differentiation was not observed. *Summary/conclusion*: This study showed anti-apoptotic effect of leukaemia cell-derived microvesicles on umbilical cord blood hematopoietic stem cells.

P-II-14

Isolation of extracellular vesicles from murine induced pluripotent stem cells and their potential role as carriers of complex molecular information

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Introduction: Cells release nano-sized vesicles into their milieu either by shedding from the plasma membrane or through secretion from late endosomal compartments. Extracellular vesicles (EVs) contain bioactive molecules and are present in cell culture-conditioned medium where they function as vehicles for intercellular communication. EVs participate in physiological and pathological processes and have potential applications in diagnostics or therapeutics. Although EVs from stem cell populations have been already studied, the phenotypical and biological profile of EVs derived from murine induced pluripotent stem cells (miPSCs) has not been investigated yet. Thus, we characterized EVs collected by differential centrifugation of supernatant harvested from the serum- and feeder-free culture of miPSCs obtained in our laboratory. *Methods*: Atomic force microscopy (AFM) in liquid environment was used to reveal the morphology and size of EVs. The level of transcripts related to pluripotency, angio- and cardiomyogenesis (e.g. Oct4, Nanog, Gata4) in miPSCs and their EVs was compared by real-time RT-PCR. Further analysis of miPSC-EV molecular composition was performed by Western blot, high-sensitivity flow cytometry as well as mass spectroscopy. *Results*: AFM analysis showed that miPSC-EV samples contain homogenous spherical vesicles with a diameter smaller than 100 nm. We established that miPS-EVs are rich in mRNAs, miRNAs and proteins carried by donor cells. We also evaluated the expression of selected exosomal markers in iPSC-EVs, including CD9, CD63 and CD81. Our preliminary data demonstrated that miPSC-EVs may represent natural nanocarriers of genetic material to target cells. *Summary/conclusion*: This study brings novel insights on iPSC-derived EVs and provides a reference for their analysis as therapeutic tools for tissue regeneration. Moreover, we optimized harvesting of EVs from serum- and feeder-free cultures of murine iPSCs that may be further used for studying their potential regenerative capacity in models of ischemic tissue injury that is currently on-going.

P-II-16

Isolation and characterization of extracellular vesicles of different cellular origins

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Introduction: Extracellular vesicles (EVs) including exosomes, secreted by numerous cells in the body, carry different kinds of functional molecules, including proteins, mRNAs and miRNAs. Research on

EVs is a rapidly growing scientific field, but the characterization of EVs derived from human cells is not sufficiently explored. For the applications of EVs in therapeutics, the yield of EVs produced by cells is a great concern. In this study, we semi-quantitate and characterize EVs that are isolated from different cell types, including mesenchymal stem cells, breast cancer cells (primary patient xenograft cells and cell lines), acute monocytic leukaemia cells (THP-1), embryonic kidney cells HEK293FT) and others. *Methods*: The cells were cultured in medium with foetal bovine serum depleted of EVs (18 hours, 100,000 × g) for 48 hours under an atmosphere of 5% CO_2 at 37°C. After 48 hours with a cell-confluency of approximately 80%, EVs were isolated by differential centrifugation. The isolated vesicles were characterized based on their size, shape and the exosome-specific surface markers (e.g. CD63 and Lamp2B). The size and shape of the vesicles were evaluated using a Particle Size Analyzer (90plus, Brookhaven Instruments Corporation, USA), and Electron microscopy. Yields of vesicles were determined by measuring the total amount of vesicular protein and RNA content using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). *Results*: A wide range (50–500 nm in diameter) in the distribution of size was observed in vesicles derived from the cultured cells, where a population of vesicles produced by the primary patient xenograft cells was found comparatively larger. The isolated vesicles were found positive for CD63 and Lamp2B irrespective of their cellular origins. Based on the analysis of the protein, the THP-1 cells produce about 4 folds higher amount of vesicles as compared to the HEK293FT cells. The mesenchymal stem cells produce significantly lower quantity of vesicles. *Summary/conclusion*: Tumour xenografts produced larger EVs than cell lines, but the total yield was higher with the THP-1 cells. Extended, this study will provide insight into the degree of EVs production by different cells and their expression of different surface proteins.

P-II-18

Microparticles produced by wound-healing myofibroblasts cells stimulate endothelial cells

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Introduction: Myofibroblasts (Wmyo) are differentiated cells appearing during wound healing process that play a central role into the extracellular matrix reconstitution. Production of microparticles (MPs) by Wmyo is a new finding in the field of cellular communication. We have determined the proteome of MPs produced by Wmyo and selected only highly expressed and extremely conserved proteins between 6 different populations of WMyo's MPs. Using mass spectrometry, 292 different proteins were identified with a 95% probability of being peptides unique to one protein. *Methods*: Using DAVID software, we have identified clusters of proteins involved in extracellular matrix production (19 proteins, enrichment score: 3; $p < 2.3 \times 10^{-6}$) but also into blood vessel development/angiogenesis (19 proteins, enrichment score: 2.83, $p < 6.7 \times 10^{-5}$). WMyo were cultured during 48 hours in DMEM + 20% FBS. MPs were collected and washed using differential centrifugations. MPs were quantified using protein assay before to be added on microvascular skin endothelial cells during 6 days before being trypsinized and counted. Migration of endothelial cells in presence of MPs was also evaluated using scratch test. *Results*: Presence of MPs produced by Wmyo in culture medium induced a significant increase of MVEC growth as well as migration rate. Both mechanisms are involved into capillary formation during wound healing. In addition of the proteins detected by mass spectrometry, ELISA performed on MPs preparation showed that they contained VEGF and FGF2, 2 cytokines that have been described as highly pro-angiogenic. *Summary/conclusion*: MPs produced by myofibroblasts could stimulate angiogenesis during skin healing enhancing the potential role of these cells during wound healing process.

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P-II-19

Extracellular vesicles as mediators of immune-mesenchymal stem/stromal cells crosstalk

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Introduction: Bone repair depends on interactions between cells, like immune cells and Mesenchymal Stem/Stromal Cells (MSC) and mediators in injury microenvironments. Previous results show that immune cells recruit MSC via paracrine factors. The hypothesis underlying this work is that extracellular vesicles (EVs) can be major players in this crosstalk. *Methods*: Human monocyte-derived macrophages (MO) and dendritic cells (DC) were obtained from buffy coats, and MSC isolated from bone marrow of patients undergoing hip arthroplasty. EVs were isolated from cell culture media (using EV-depleted FBS) by differential (ultra)-centrifugation (2,000, 10,000, 100,000 × g), and characterized by TEM, flow cytometry and DLS. For internalization assays, PKH26-labelled EVs were added to cell cultures for 6 hours. Cells were then fixed and stained, and internalization was evaluated by confocal microscopy. For cell migration assays, MSC were cultured on transwell inserts, with DC-EVs in bottom wells. After 8 hours, cells on the outer surface of insert membranes were fixed, stained and counted. For MSC-EVs content characterization, RNA was isolated and miRNA expression analyzed by quantitative real-time PCR with Taqman-based assays. *Results*: DC-EVs are internalized by MSC and able to specifically promote their migration in a dose-dependent manner. Conversely, MSC-EVs contain microRNAs like miR-29b and miR-20a, and can be internalized by MO. *Summary/conclusion*: These results show that immune cells produce EVs capable of recruiting MSC, while MSC-EVs contain microRNAs known to mediate MO inflammatory responses. Also, MSC internalize immune-EVs, and immune cells internalize MSC-EVs. Thus, EVs can mediate immune cells-MSC bidirectional communication and are promising targets for bone repair therapies.

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P-II-20

Replicative senescence impacts mesenchymal stem cell-derived extracellular vesicle bioactivity for therapeutic vascularization

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Introduction: Mesenchymal stem cells (MSCs) have demonstrated efficacy in inducing vascularization in a variety of models, with substantial evidence supporting secretion of paracrine factors and extracellular vesicles (EVs) as a primary mechanism. Moreover, MSC-derived EVs have been specifically identified as potential mediators of therapeutic vascularization in numerous studies. Thus, MSC-derived EVs may represent an alternative to MSC transplantation for therapeutic

vascular regeneration. However, critical biological parameters of MSC-derived EVs remain undefined. For example, replicative senescence can have dramatic effects on MSC protein and microRNA (miRNA) expression, yet it is unknown how MSC-derived EV cargo and bioactivity might be affected by this phenomenon. Better understanding these effects might yield design parameters to enable scalable biomanufacturing of MSC-derived EVs for therapeutic vascularization and myriad other therapeutic applications. *Methods*: We isolated EVs via differential ultracentrifugation and their sizes and concentrations were determined by nanoparticle tracking analysis (NTA) with a NanoSight LM10. EVs were isolated from passages 2 to 5 (P2 to P5). We analyzed the effect of EVs on tubule formation in human umbilical vein endothelial cells (HUVECs) using a Matrigel angiogenesis assay. EV-associated microRNA was analyzed via quantitative RT-PCR. *Results*: In 3 independent experiments, we observed a decrease in EV production per cell in MSCs after P3. Vessel formation parameters in HUVECs were analyzed as a function of EV passage number (P2 to P5) as well as EV concentration (1, 5, 10 and 25 μg/ml). Overall, MSC-derived EVs of all passages showed an increase in activity over the positive control (EGM media with growth factors). However, pro-vascularization bioactivity significantly decreased after P2, suggesting a change in EV composition or cargo. *Summary/conclusion*: The phenomenon of replicative senescence in MSCs correlates with changes in MSC-derived EV vascularization bioactivity. The mechanism of this change is currently under investigation. These data have significant implications for potential production of MSC-derived EVs for therapeutic applications.

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Poster session III - EV isolation
Chairs: *Casey Maguire and Cecilia Lässer*

P-III-1

Specific isolation of tumour-derived extracellular vesicles using microfluidic technologies

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Please see Oral with Poster A

P-III-3

In situ loading, labelling and manipulation of exosome content via HDL-like gold nanoparticles

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Introduction: Exosomes are 30–100 nm diameter lipid vesicles produced by most cells and contain a diverse variety of cargo including DNA, RNA, proteins and lipids. In particular, primary tumour cells produce an abundant amount of exosomes, which are released into the tumour microenvironment and the circulation. These exosomes may be intrinsically targeted to certain cell types and tissues to influence cancer progression and metastasis. It is not currently possible to specifically identify tumour-derived exosomes in complex mixtures such as serum. The ability to load exosomes at time of synthesis for tracking and isolation would provide a better understanding of their contribution to tumourigenesis and cancer progression and potentially provide a means of specifically delivering therapeutic cargo. Our group previously developed a synthetic high-density lipoprotein-like gold particle (HDL NP), which we utlized for this purpose. *Methods*: Cultured CWR22Rv1 prostate cancer cells were treated with HDL NPs containing a lipid conjugated to a rhodamine fluorophore. Exosomes were isolated using ultracentfugation or ExoQuick-TC and characterized via spectroscopy, transmission electron microsopy, dynamic light scattering, western blot and flow cytometry using the ExoFlow kit. Exosomes from cells treated with HDL NPs were incubated with naive CWR22Rv1 cells and the cells then analyzed via flow cytometry. *Results*: Exosomes from prostate cancer cells treated with HDL NPs are associated with gold particles. HDL NP treatment does not affect exosome production or morphology. Additionally, exosomes from cells treated with rhodamine HDL NPs are fluorescent for rhodamine, and a portion of the rhodamine is on the exterior of the exosome and can be used for antibody or fluorescence based isolation. Finally, naive CWR22Rv1 cells treated with exosomes containing rhodamine HDL NPs become fluorescent for rhodamine. *Summary/conclusion*: Cells treated with rhodamine HDL NPs produce exosomes with a functionalized fluorophore which can then be used for isolation and tracking of exosomes from a specific cell type of origin in complex matrices such as serum using antibody- or fluorescence-based isolation methods. Exosomes incorporate the HDL NP with a minimum of processing or effects on cells or exosomes and has the potential for use in both in vitro and in vivo applications. Furthermore, lipids associated with the HDL NPs are delivered back to the cell type of origin via exosomes, suggesting that HDL NPs may be able to deliver cargo conjugated to them using exosomes as a delivery vehicle. Thus, this methodology may provide a unique means to manipulate exosome content for a variety of diagnostic and therapeutic uses.

P-III-4

Exosomes in the blood plasma: characterization, isolation and stability

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Introduction: Although exosomes are emerging targets for novel diagnostic and therapeutic approaches, isolation and storage of blood plasma-derived exosomes have not yet been optimized. Therefore, we investigated the effect of duration of ultracentrifugation (UC) and different storage conditions on qualitative and quantitative properties of exosomes isolated from rat and human blood. *Methods*: Exosomes were isolated from rat and human blood plasma by differential centrifugation, filtration and UC at 120,000 $\times$ g for different time periods (1, 3, 6 or 14 hours). Size distribution of exosomes was assessed by dynamic light scattering (DLS) and transmission electron microscopy. Isolated exosomes were stored at 4°C and −80°C in PBS for 4 and 8 weeks. The amount of intact isolated exosomes was estimated by Western blot for flotillin-1 and CD63. *Results*: DLS showed that exosomes after 1 hour UC had a mode diameter of 98.49 nm. Although longer UC time increased exosome yield, it also altered exosome size distribution and increased contamination, as assessed by transmission electron microscopy and immunoblotting. Using 1 hour UC, exosomes were also successfully isolated from human blood plasma. Morphology of exosomes was preserved after 8 weeks at −80°C, but not at 4°C. However, CD63 levels were decreased in exosome preparations after storage at −80°C for 4 or 8 weeks indicating compromised exosome quality. *Summary/conclusion*: We conclude that the optimal duration of UC is 1 hour for the isolation of pure blood plasma-derived exosomes, and that the exosomes should be analyzed immediately after isolation to minimize artefacts and avoid deterioration.

P-III-5

A practical comparison of exosome isolation kits for primary endothelial and monocytic THP-1 cell samples with low working volumes

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Introduction: Use of proprietary exosome isolation kits may be driven by lack of an ultracentrifuge, limited sample volume, high sample number or even unfamiliarity with the field of exosome research. Using commonly available commercial kits, we examined the relative recovery of product from low volume samples of cell culture supernatant media from primary human endothelial cells (HEC) and monocytic THP-1 cells. Samples of 4 ml and under are realistic in vitro working volumes and are within the advertized ranges of all kits,

indeed 4 ml is the maximum suggested volume for one tested kit. *Methods*: Cells were grown to subconfluency then washed and exposed to serum-free media for 48 hours. Conditioned media was collected by low speed centrifugation, cell number and viability was determined. To standardize samples, media from several flasks was pooled for each cell line. Samples were processed according to manufacturer guidelines. Relative recovery was determined by protein content, using a standard colorimetric assay. Protein content is a primary sample determinant for downstream studies such as western blot. T-test was performed, $n = 5$ for all samples. *Results*: Mean sample protein content was highest for the Invitrogen kit in both HEC, 11.3 μg/sample and THP-1 cells, 26.28 μg/sample. Recovery from each kit was significantly different from the others ($p \leq 0.005$) for both HEC and THP-1, following the order Total Exosome Isolation (Invitrogen) > Exoquick (System Biosciences) > PureExo (101Bio). Notably mean total recovery from a 4 ml sample was mostly lower than the normal limit for downstream protein assays such as western blot. To compare relative recoveries from both cell lines, protein mass per million cells was calculated; HEC sample levels were markedly higher than THP-1 levels. *Summary/conclusion*: The decision to use a commercial kit for exosome recovery may have many influences but low protein recoveries for all kits, from sample volumes common to cell culture studies, lends caution to that choice.

P-III-7

Analyzing the ultracentrifugation productivity in extracellular vesicle isolation from HEK293T cells grown in suspension culture

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Introduction: Due to their ability to transport cargo in vivo, extracellular vesicles (EVs) have been proposed as useful drug delivery vehicles. Our long term plan are to genetic modify HEK293 cells to produce therapeutically loaded EVs. *Methods*: In this investigation, we adapted HEK293T cells to grow in suspension cultures and characterized the exosome yield following the standard sequential ultracentrifugation protocol. Using 10 different 400 ml suspension cultures/batches, we measured the yield of EVs using nanoparticle tracking analysis at 3 different steps in the isolation protocol; (a) after 10 K × g spin, (b) the EV cleared supernatant after first 110 K × g ultracentrifugation spin, and (c) PBS wash step. Following the isolation, we compared the EV yield isolated from 10 K × g spin using Invitrogen isolation kit. *Results*: We found that following a 110 K × g spin (supposedly cleared of exosomes) the supernatant still contained a measurable concentration of EVs within the size range of exosomes (mode size < 100 nm), and that the recovery of the whole ultracentrifugation process is on average 40%. The recovery of exosomes by the commercial kit appeared to increase the recovery to 80%. In some samples the kit appeared to have added measureable quantities of EVs to the system. *Summary/conclusion*: In conclusion, the implications of ultracentrifugation on recovery and productivity of EV isolation is evaluated for optimizing a protocol for large scale production of therapeutic exosomes.

P-III-8

Costs of manufacturing extracellular vesicles

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Introduction: As interest in extracellular vesicles (EVs) as a therapeutic or research tool continues to rise, there is a growing demand for large-scale production of EVs. To academia and industry alike, minimizing costs of scaling up is imperative. In this study, we modelled EV manufacturing based on existing protocols and estimated the associated costs. Key cost determinants were identified. *Methods*: Using mesenchymal stem cells as a model cell type, an equation describing the kinetics of EV accumulation was developed after tracking cell and EV numbers over time in serum-free media, permitting prediction of total EV output as cells are expanded. A range of manufacturing processes were simulated in MATLAB by combining 15 distinct technologies for cell expansion with 2 distinct technologies for EV harvest, namely ultracentrifugation and precipitation. Costs were obtained from literature and commercial suppliers. *Results*: 3D culture (e.g. in stirred-tank bioreactors) as opposed to 2D culture (e.g. in T-flasks and cell factories) can reduce overall costs by an order of magnitude. The choice of harvest technology does not significantly affect overall costs but changes the cost structure: labour and equipment costs dominate when EVs are harvested by ultracentifugation, while costs of consumables dominate when EVs are harvested by precipitation. *Summary/conclusion*: Cost modelling is a swift and affordable evaluation of the economic feasibility of an envisioned manufacturing process as well as the commercial value of a new expansion or harvest technology. Our model provides an initial framework for academic and industrial parties seeking to develop or invest in EV products.

P-III-9

Hyaluronic acid-based enrichment of extracellular vesicles: lessons from the extracellular matrix

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Introduction: Extracellular vesicles (EVs) are a group of nano-scale membrane-bound compartments that actively transport cargoes of molecules, including genetic materials, to proximal and distal cells. EVs have emerged as an organelle-based intercellular communication network and represent real-time physiological and pathological status of their cells of origin. Thus EVs have the potential to be an important source of materials for minimally-invasive diagnostics and other clinical applications. Most cells release EVs into their surrounding extracellular matrix (ECM) where they make their way to neighbouring cells, distal cells or body fluids. Similarly, circulating EVs encounter the ECM of recipient cells. *Methods*: During the course of our research, we found that long-chain Hyaluronic acid (HA), a major component of ECM, has specific affinity for a subpopulation of EVs and can be used as a method for the enrichment of HA-specific EVs (HA-EVs) from cell culture-conditioned media and human body fluids using standard laboratory set-ups. *Results*: We profiled and validated the above-described methodology using electron microscopy, atomic force microscopy, proteome-based cellular component ontology analysis, nanoparticle tracking analysis, immunoblotting and cellular uptake assays. Furthermore, our results also indicate that HA-EVs are biologically active and capable of inducing transformation when the source of HA-EVs is aggressive cancer cells. *Summary/conclusion*: HA can be used to enrich and profile EVs from cell culture-conditioned media and human body fluids.

P-III-10

Detection of cancer-derived extracellular vesicles from body fluid by aqueous two-phase system

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Introduction: Extracelluar vesicles are released by various cell types, particularly tumour cells, and may be potential targets for body fluid-based cancer diagnosis. However, studies of extracellular vesicles from body fluid have been relatively undermined by time consuming process of isolating extracellular vesicles and lack of effective purification strategies. In this study, we isolated extracellular vesicles by a new method on specific time intervals and detected tumour-derived extracellular vesicles from 2 body fluids, blood and saliva. *Methods*: Six-week old C57BL6 mice had been grown after subcutaneous injection of $10^6 \times$B16BL6 melanoma cells into their bodies. Blood and saliva were collected on specific time intervals from each subject. Extracellular vesicles were isolated from the fluids by ultracentrifugation or aqueous two phase system, a new method which uses a mixed solution of polyethylene glycol and dextran. Isolated melanoma exosome was identified by western blots, and expression of tumour-specific marker was compared. *Results*: In the case of ultracentrifugation, tumour-specific marker was not detected from the great part of body fluid samples because of low isolation efficiency. On the other hand, aqueous two phase system efficiently isolated extracellular vesicles, thus tumour-specific maker was detected with high probability. Especially, aqueous two phase system showed about 10 times higher cancer detection chances than ultracentrifugation when extracellular vesicles was isolated from the body fluids in 8-week old melanoma injected mice. *Summary/conclusion*: To date, it was impossible to use extracellular vesicles for practical cancer diagnosis system because of small amount of extracellular vesicles. We overcame the restriction by using aqueous two phase system. High isolation efficiency of aqueous two phase system gave a new direction for practical cancer diagnosis system.

P-III-11

Comparison of different extraction methods for exosome and exoRNA from cell culture supernatants and body fluid

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Introduction: Exosomes are cell-derived vesicles presenting in many biological fluids. RNAs contained in exosomes could serve as a novel platform for diagnosis. To obtain reliable results, efficient and reproducible methods to isolated exoRNA are mandatory requirements. *Methods*: In our study, nanoparticle tracking analysis was used to measure and characterize exosomes from cell culture supernatants and body fluid isolated by ultracentrifugation, ExoQuick and Total Exosome Isolation Reagent. The quantity and quality of the exoRNA isolated by Trizol-LS, SeraMir ExoRNA Amplification Kit and HiPure Liquid RNA Kit were determined by Bioanalyzer 2100, Nanodrop and Qubit. *Results*: Both ExoQuick (6.94E+10 particles/ml serum) and Exosome Isolation Reagent (4.56E+10) had higher extraction efficiency than ultracentrifugation (1.23E+09). Particles isolated by ExoQuick were more homogeneous distributed than other methods. Total Exosome Isolation Reagent and SeraMir had highest extraction yield of exoRNA (Qubit result: 273 ng/10 ml supernatants) than other combinations of exosome and exoRNA methods. Though ExoRNA concentrations of 500 ul serum were below the detection limits of Quibit (20 ng/ml), Bioanalyzer 2100 result showed that HiPure Liquid RNA Kit had higher extraction efficiency (112.5 ng/500 μl serum) than SeraMir (88.56 ng) and Trizol-Ls (37.38 ng). Supernatants exosome isolated by ultracentrifugation had better RNA size distributions than kits, which may have cellular 18sRNA contamination. However, no contamination was found in exoRNA from serum isolated by kits. *Summary/conclusion*: For cell culture supernatants, 2 new nanomaterial exosome Isolation kits have higher extraction quantity than ultracentrifugation, but exoRNA extracted by kits have lower stability and quality. With respect to serum, the convenient and efficient exosome and exoRNA kits maybe best choice for exosome research.

P-III-12

Scalable isolation and purification of a therapeutic stem cell line exosome product by tangential flow filtration

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Introduction: The ability to produce a commercially valuable therapeutic product from stem cell-derived exosomes demands a standardized stem cell producer line appropriately sourced and isolated, manufactured at scale under GMP and grown in serum-free conditions. ReNeuron's proprietary CTX neural stem cell line fulfils all of these requirements and is a highly efficient producer of therapeutically active exosomes. Current manufacturing scale produces in excess of 25 l of GMP produced conditioned medium from which exosomes can be harvested. However, the use of conventional methods used to isolate and purify exosomes is restricted due to limitations in scalability. We have therefore developed a completely scalable process based on tangential flow filtration (TFF) which enables the isolation and purification of exosomes from large scale production of conditioned medium. *Methods*: A TFF system, comprising of an initial 0.1 μm filter step to separate extracellular vesicles on the basis of size followed by a 300 kDa filter step to remove contaminating protein, was used to isolate and purify an exosome population. Shear stress was maintained at a level of 3,000 s^{-1} or less in order to minimize exosome damage. The concentration and size of the exosome population were measured using nanoparticle tracking analysis, and the protein, DNA and RNA concentrations were quantified. An in vitro model of wound healing and a xenograph model of glioblastoma were used to assess the efficacy of TFF sourced exosomes. *Results*: A yield in excess of 3.0×10^{13} particles per litre of starting material has been achieved with an exosome purity of up to 3.6×10^9 particles/μg protein and an average modal size of 98 nm. TFF isolated exosomes maintained efficacy in both in vitro and in vivo models of disease. *Summary/conclusion*: A scalable TFF based process has been developed to isolate and purify an exosome product as part of the GMP manufacture of a standardized stem cell line with demonstrated patient safety. The resultant exosome product shows efficacy as a therapeutic product in a number of disease models, both in vitro and in vivo.

P-III-13

Comparative analysis of physical-chemical precipitation methods of circulating exosome isolation from human biofluids

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Introduction: Exosome isolation from complex biofluids, such as plasma/serum or urine, is the critical step for downstream applications in diagnostic and therapeutic areas. Differential ultracentrifugation is still the most appreciated method for obtaining an enriched exosome pellet, although is time consuming, requires capital equipment and large sample handling. Numerous commercially reagents have been produced in order to separate exosomes via chemical precipitation. This technique allows a fast and easy microvesicle isolation and is especially useful when small volumes of samples are available. *Methods*: Ultracentrifugation and commercial reagents were used to precipitate exosomes from different small volumes of human biofluids (plasma, serum, urine). The exosome isolation efficiency of chemical reagents was compared with the ultracentrifugation yield, and isolated exosomes were tested for common protein or nucleic acid exosome markers with various techniques (WB, ELISA, qRT-PCR). *Results*: Chemical precipitation revealed consistent advantages compared to ultracentrifugation in terms of time and exosome yield from small volumes of samples. Main disadvantage has been reported in exosome pellet solubilization, very hard for some reagent tested. Remarkable, a chemical reagent showed high efficiency in isolating

exosomes from only 100 μl of plasma/serum, easy pellet solubilization and isolated exosomes were suitable for all downstream performed analyses. *Summary/conclusion*: In this study, we compared the efficiency of different commercial reagents in isolating exosomes from small volumes of complex human biofluids (plasma, serum, urine). Exosome chemical precipitation is an efficient method for isolating exosomes from small volumes of samples, when pellet solubilization is an easy step. This method is really useful as a source of exosome biomarkers for proteomic and trascriptomic with potential development to diagnostic and therapeutic area.

P-III-14

Newly designed size exclusion chromatography columns for isolation and purification of extracellular vesicles in clinical samples

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P-III-15

Exosomes isolation by differential centrifugation: theoretical analysis and the experiment

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Introduction: Differential centrifugation though recognized as ''gold standard'' method of exosomes isolation may give dissimilar and improper results. It is rather common practice to apply the same centrifugation protocols for different types of rotors, which leads to significant differences in yield and purity of exosome preparations. In present work, we perform the detailed theoretical consideration of the sedimentation process in the 2 types of rotors – swinging bucket (SW) and fixed-angle (FA) and apply the theory to the sedimentation behaviour of extracellular vesicles (EV). *Methods*: HT29 cell culture supernatant was centrifuged at 500 g for 5 minutes, at 2,000 g for 10 minutes, at 10,000 g for 30 minutes and at 100,000 g for 70 minutes. Vesicles sizes and concentrations were measured by NTA. *Results*: General equation, describing the velocity of a particle under centrifugal acceleration was adapted to the form convenient for calculation of EV sedimentation profile. The proportion of pelleted vesicles of a given size and the ''cut-off'' size of completely sedimented vesicles were presented as dependent on centrifugation force and duration and sedimentation path length for both SW and FA rotors. The theoretical analysis shows that the application of common centrifugation protocol without the account of rotor's sedimentation pathlength may be misleading. The usage of K-factors for adjustment of the centrifugation duration at a change of rotor is reasonable in case of SW rotors, but for FA rotors K-factor makes no sense. Experimentally obtained NTA particle size distributions rather well coincide with theoretically predicted vesicles size distributions. *Summary/conclusion*: We demonstrate for a number of commonly used rotors how the proper centrifugation conditions can be selected using rather simple theoretical estimates of ''cut-off'' sizes of vesicles. To make easy the adjustment of centrifugation protocol for any rotors in use we present a specially designed web-calculator.

P-III-16

Effective and gentle isolation of extracellular vesicles in human and bovine milk without ultracentrifugation

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Introduction: Milk has over millions of years evolved to provide offspring with crucial developmental components adapted for survival under extreme aspects of early life. A multitude of health promoting milk components supports infant development. Extracellular vesicles in milk represent most likely an additional health promoting component though with a less understood diverse and complex nature. In understanding the molecular- and bio-functional diversity of extracellular vesicles in milk, and their physiological function, gentle isolation procedures and well isolated vesicle fractions are crucial. We have identified and validated a novel and effective milk-EV isolation procedure applicable on both human and bovine milk with focus on purity, and gentle treatment, therefore steps including ultracentrifugation and sedimentation/resuspension are omitted. *Methods*: Untreated fresh human or bovine milk is centrifuged to remove milk fat globules. The resulting skim milk is subjected to centrifugation at 30,000 × g. Resulting supernatant is subjected to size exclusion chromatography to remove remaining casein and whey proteins from the vesicles. The isolated EV fraction, as well as control fractions, are investigated by; SDS-PAGE for primary protein components, EV markers by western blotting and mass spectrometry, phospholipid- and neutral lipid profiles by thin layer chromatography, particle size by nanoparticle tracking analysis and total RNA profiles using a Bioanalyzer. *Results*: Using this purification method extracellular vesicles in milk can be gently and effectively isolated from all major milk proteins without the use of ultracentrifugation. Isolated vesicle fractions are highly enriched in lactadherin, CD63, CD9, MUC1 and Hsc70, compared to control fractions. Isolated milk-EV fractions contain moreover a phospholipid composition similar to the plasma membrane and do not contain triglycerides as opposed to milk fat globules. Particles in the vesicle fraction show a mean diameter of 200 nm. Finally, vesicle fractions are also associated with various RNAs and low amounts of ribosomal RNA. *Summary/conclusion*: The described method enables a successful and gentle isolation of extracellular vesicles from both human and bovine milk without the use of potential detrimental methods. The obtained vesicle fraction shows several extracellular vesicle characteristics and represents a good starting material for further analysis of the bio-diversity and – function of milk vesicles.

P-III-17

From basic research to clinical setting: adapting methods for EV enrichment and analysis

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Introduction: Exosomes are small (30–100 nm) vesicles secreted by all cell types in culture and found in most body fluids such as urine. Depending on the disease and state of progression, the number of exosomes may vary significantly. Urine represents an easy and accessible exosome source for downstream analysis. The main purpose of this study was to establish a workflow for exosome enrichment and characterization applicable to a clinical lab setting. *Methods*: A platform for pre-enrichment and analysis of cell culture exosomes has previously been developed in collaboration with Dr. Oksvold (University of Oslo) and Dr. Vlassov (ThermoFisher, Austin, Texas). This platform has here been optimized for direct capture and analysis of exosomes from cell culture media and clinical urine sample including flow cytometry, western blotting, qRT-PCR. *Results*: Here we demonstrate parameters important for efficient capture kinetics of

exosomes from cell culture includes pre-clearing by centrifugation, incubation time, capture volume, exosome sample input, nature and concentration of solid support. The flexibility built into the method allows for reduced incubation time, increased sample volume and omitting pre-enrichment. Reduced incubation time (down to 1 hour) was associated with reduced signal to noise level but well within acceptable levels. This optimized method also applies to clinical urine samples. *Summary/conclusion*: We demonstrate efficient direct capture and analysis of exosomes from 25 to 800 μl of cell culture media and up to 400 μl from urine with linear correlation between sample input and output signal in flow analysis. Direct capture ensures no loss of exosomes during sample preparation which may occur during the different pre-enrichment methods commonly used.

P-III-18

Impact of trehalose on isolation, storage and biological activities of extracellular vesicles released from beta-cells

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Introduction: Pancreatic beta-cells release extracellular vesicles (ß-EVs) carrying autoantigens and microRNAs among which miR-29b. We have previously shown that microRNA-29b modulates innate and antigen-specific immune responses in mouse models of autoimmunity suggesting a possible role for ß-EVs in autoimmune diabetes. Trehalose is a natural non-toxic sugar known to stabilize protein structures and to prevent exosome aggregation. In addition, trehalose may suppress inflammatory responses and reduce insulin resistance. Here we studied the influence of trehalose for isolation and storage on ß-EV stability, cryoconservation and biological activity. *Methods*: ß-EVs were harvested from murine insulinoma MIN6 cells through differential centrifugation and ultrafiltration using either PBS or PBS 25 mM trehalose (TRE) for washing, recovery and storage. EVs were analyzed by nanoparticle tracking analysis, automated electrophoresis, western blot and assessed for cryoconservation and biological activity on murine immune cells. *Results*: Both ß-EVs expressed $CD63^+$ $CD81^+$ tetraspanins and were approximately 100 nm in size. Compared to PBS, TRE isolated ß-EVs showed a narrowed unimodal distribution and a higher number of particles per microgram of protein indicative of increased colloidal stability. PBS and TRE purification of ß-EVs yielded 0.4 ± 0.2 and 0.7 ± 0.2 μg/ml of culture supernatant, respectively. We are currently investigating whether use of trehalose affects EV uptake, activation and cytokine secretion by murine immune cells. *Summary/conclusion*: This study assessed the utility of intrinsic properties of trehalose for the development of EV-based approaches for immune therapy.

P-III-19

Comparison of different preanalytical workflows for isolation of intact exosomes and other extracellular vesicles

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Introduction: The scientific literature on exosomes and other extracellular vesicles (EVs) continues to be characterized by a wide variety of vesicle isolation and characterization procedures, as well as nomenclature, resulting in considerable difficulty comparing results between independent studies. An additional layer of variability is added by sample handling and pretreatment. This may include the type of blood collection tube, time between blood draw and generation of plasma or serum, as well as measures taken to remove residual cells and cell fragments, such as pre-centrifugation or filtration steps. In this study, we compare different handling and pretreatments, and how they affect physical characteristics, as well as RNA content of vesicle preparations resulting from a spin column-based purification approach. *Methods*: Blood from healthy donors was collected in different collection tubes. After generation of plasma and removal of residual cells and fragments, vesicular RNA was generated and relative abundance of selected RNAs compared by qPCR. In addition, effects of the time between blood collection and generation of plasma were tested on the RNA level. Finally, we compared different approaches to remove residual cells and cell fragments by additional centrifugation and filtration steps prior to vesicle isolation and analyzed the resulting vesicle size distribution and RNA content. *Results*: In all tested preprocessing workflows, intact vesicles and vesicular RNA could be isolated. However, the choice of collection tube, anticoagulant, etc. does have an influence on RNA representation, so it is strongly recommended to not switch between different collection tubes within the same study. *Summary/conclusion*: Storage of blood prior to plasma generation can result in release of additional vesicular RNA from blood cells, which in most cases represent unwanted background. Centrifugation and filtration steps affect representation of different classes of vesicles.

P-III-20

Immuno-magnetic separation assay: a new tool for the measurement of microparticle-associated plasmin generation capacity

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Introduction: Blood cells-derived microparticles (MPs) may become clinically relevant biomarkers in several processes such as coagulation, inflammation and angiogenesis. Plasmin generation capacity (PGC) was recently assigned to MPs as an additional, pro-fibrinolytic, function. A new Immuno-Magnetic Separation (IMS) assay is described here. Circulating MPs are directly captured from plasma samples, and their PGC is then measured in a purified system. *Methods*: Plasma models were prepared by spiking different amounts of purified MP from various origins into first MP-free and later platelet-free plasma (PFP). MP subsets were selectively extracted using MAb-coated magnetic Dynabeads and incubated overnight at 37°C in the presence of plasminogen to generate plasmin whose activity was measured using its chromogenic substrate. PGC was also measured on MPs extracted by high-speed centrifugation (24,000 g, 1 hour). Efficacy and specificity of IMS was monitored by flow cytometry using MP-optimized settings standardized with Megamix-Plus. *Results*: MP-PGC was solely linked to leukocyte- and not to platelet- nor erythrocyte-derived MPs since $>90\%$ PGC was specifically associated with CD15+ MP after IMS with various MAb-coated beads. This IMS-based MP-PGC assay displayed superior sensitivity and reproducibility when compared to using centrifugation. Samples from SSK patients displayed highly variable MP-PGC values, from sub-normal to $100 \times$ normal levels, indicating heterogeneity in the fibrinolytic capacity of their MPs. A significant association was found between MP-PGC and the risk of premature death in a small cohort of SSK patients ($n = 34$). *Summary/conclusion*: This new assay measuring MP-PGC in human plasma samples in a specific, sensitive and reproducible manner showed encouraging prognostic value in a small-sized pilot study of sepsis. These preliminary results warrant extensive clinical studies in Septic Shock and other pathologies where outcome may be linked to the fine balance between procoagulant and profibrinolytic functions of MPs.

P-III-21

Optimization of extracellular vesicle isolation directly from melanoma metastases

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Introduction: Recent studies argue that melanoma-derived extracellular vesicles (EVs) promote tumour metastasis, and EV cargo may function as biomarkers. Studies focusing on tumour tissue-EVs are limited, because EV isolation protocols are not validated. The aim of this study was therefore to optimize the isolation protocol of human melanoma metastasis tissue -EVs. *Methods*: Subpopulations of EVs were isolated from melanoma metastasis acquired by surgery ($n = 5$) using a centrifugation-based protocol. Tumours were chopped into approximately 2 mm pieces and were incubated in medium for 30 minutes at 37°C. After a 70 μm filtration, cells and tissue debris were eliminated by centrifugation at $300 \times g$. Subsets of vesicles were collected from the supernatant with validated differential centrifugation steps. Tumour pieces or EV pellets showing entrapment in fibrotic material were treated with collagenase and DNase. Vesicles were characterized by protein quantity, electron microscopy and RNA profiles. *Results*: Melanoma metastasis-derived EVs showed typical morphology and RNA profiles compatible with extracellular vesicle subpopulations. Smaller vesicles (40–100 nm) exhibited RNA profiles similar to exosomal RNA profiles, without prominent ribosomal RNA peaks. Larger vesicles (200–800 nm) showed a RNA profile comparable to microvesicles with the presence of 18S and 28S ribosomal RNA peaks. Collagenase and DNase treatment did not affect EV morphology or RNA profiles. *Summary/conclusion*: We here optimized a centrifugation based protocol to isolate vesicles directly from tumour tissue. Collagenase and DNase treatments are useful to isolate EVs from dense fibrotic tumours, without any apparent effect on characteristics.

P-III-22

A new method to capture exosomes for diagnosis of glomerular diseases

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Introduction: Many efforts have been made to discover novel exosomal biomarkers from plasma/serum, but it is sometimes very difficult to detect target cell's small difference from tremendous amount of exosomes, which are originated from various types of cells. In cancer research field, to obtain exosomes from cancer cells, epithelial adhesion molecule (EpCAM) antibody is used to narrow down the target exosomes. The aberrant miRNA expressions in mesangial cells are reported in diabetic nephropathy, IgA nephropathy and lupus nephritis. If we could collect exosomes form mesangium cells, we might be able to diagnose kidney diseases without biopsy. The aim of this study is to establish the method for capturing exosomes in plasma from kidney mesangial cells and to use captured exosomes for diagnosis of kidney diseases. *Methods*: We employed 2-step-method. First, pre-enrich the exosomes by standard ultracentrifugation. Then we incubated the sample with magnetic beads that are coated with alpha integrin antibody beforehand. Finally, beads-bound exosomes were isolated with magnetic separator and analyzed. Using this method, we purified exosomes from culture medium of human primary mesangial cells, and human plasma. *Results*: Immunofluorescent staining of human kidney biopsy specimen revealed that alpha integrin was exclusively positive in mesangium area. According to the immuno-electron microscopy, captured exosomes had alpha integrin on them. Using magnetic beads that were coated by alpha integrin antibody, we could confirm captured exosomes from human plasma by electron microscopy. miRNAs could be extracted from these plasma exosomes. Bioanalyzer result indicated these miRNAs were in good condition, and we could amplify miRNA by PCR technique using appropriate primers. *Summary/conclusion*: We could successfully collect exosomes from cultured human mesangial cells and from human plasma using alpha integrin antibody. To date, there is no specific surface marker for kidney mesangium cells. But instead of collecting all exosomes from plasma, it is a better way to discard the disinterested exosomes and narrow down the target.

P-III-23

To the standardization of exosome isolation and characterization workflow

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Introduction: Standardization of exosome isolation and characterization methods is critical to the advancement of this exciting, emerging field. Currently, the most popular approach for exosome isolation uses ultracentrifugation, which generates highly pure exosomes; however, the workflow is lengthy and often not reproducible. The most tedious step involves layering and fractionating from a density gradient and often contributes the most disparity. Despite new, commercially available isolation kits, depending on your downstream needs, the classical approach of ultracentrifugation for exosome isolation offers significant advantages. *Methods*: Here, we present an automated method for exosome isolation that works towards standardizing the workflow to reduce discrepancy in results. An automated workflow that begins with measuring cell number and viability with a Vi-CELL then proceeds into benchtop pelleting and later ultracentrifugation for sample cleanup. Higher purity is achieved by density gradient separation in which both layering and fractionation is achieved by automation. Finally, size characterization is achieved by dynamic light scattering. *Results*: Automation of the layering and fractionation technique for density gradient separation generated a more reproducible isolation than manual preparation. Furthermore, the pervasive Théry protocol for preparation was improved upon by time and efficiency using shortened pathlengths. *Summary/conclusion*: Exosomes are becoming a major research area in science as both diagnostic and therapeutic biomarkers. Additionally, exosomes have been shown to be part of intercellular communication networks and implied in tumor regulation. However, the field is still in its infancy, and often times, the size range and especially exosome RNA content are misreported or vary from publication to publication. In order to advance this field, it is essential to standardize the workflow to purify and better characterize the entire exosome population for a more representative sample.

Sponsored poster

P-III-24

Optimized solutions for isolation, extraction and analysis of nucleic acids shuttled by extracellular vesicles in body fluids

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Introduction: Extracellular vesicles (EVs) carry a plethora of nucleic acids with potential roles in health and disease and are emerging as a novel platform for biomarker discovery and therapeutics. Currently, EV-derived nucleic acids are extracted with commercial kits following EV isolation with physical and/or chemical methods. These methods are time-consuming and co-isolate contaminants such as protein aggregates and lipoproteins that may affect downstream analyses. Additionally, physical-chemical methods do not allow for analyses of nucleic acids from cell/tissue-specific EV subpopulations. HansaBioMed has developed kits that couple isolation of either total or specific EV subpopulations with an efficient extraction of EV-carried nucleic acids. *Methods*: EVs were isolated from cell supernants or body fluids with the appropriate HBM kit and isolation methods optimized according the nucleic acid type. miRNA and messenger

RNAs (mRNAs) were retro-transcribed, and expression of selected targets was evaluated through qRT-PCR. Wild type and mutated copies of the BRAF gene from EV-derived DNA were amplified by allele-specific locked nucleic acid-(LNA)-PCR. *Results*: Following EV immunocapture with HBM beads, vesicle-derived miRNAs and mRNA were successfully amplified from 100 and 500 μl of plasma or serum respectively. HBM beads for tumor-derived EVs capture enriched for miR-21 in cancer patients samples, while beads for overall capture did not. EV-derived DNA was extracted from 1 ml of serum or plasma with ultracentrifugation, chemical precipitation or immunoprecipitation. LNA-PCR confirmed that extracted DNA is suitable for analysis of point mutations in cancer patients. Finally, EV-derived nucleic acids were successfully extracted and amplified by RT-qPCR from diluted matrices such as urine and cell supernatants. *Conclusions*: HBM kits for isolation and extraction of EV-derived nucleic acids are optimized to yield EV-derived mRNA, miRNA and DNA from cell supernatants and complex body fluids. The kits isolate high-yield/high-quality nucleic acids from low volumes of starting sample and the extracted nucleic acids can be used for all the most common molecular analyses.

Sponsored poster

Poster session IV - EV characterization
Chairs: *Martijn van Herwijnen and Cherie Blenkiron*

P-IV-2

Tumour-derived vesicle identification by Raman microspectroscopy combined with resistive pulse sensing

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Introduction: The cellular origin of extracellular vesicles is usually established by fluorescent antibody labelling, which is laborious, expensive and involves practical problems. We are developing an ultra-modern setup capable of determining (a) size, (b) refractive index (RI) and (c) chemical composition of single vesicles without labelling. *Methods*: Resistive pulse sensing (RPS) determines the size from the increase in resistance caused by the passage of a vesicle through a sub micrometre orifice. A laser beam is focused on top of the orifice to optically trap the vesicles. The elastically scattered light of an optically trapped vesicle is measured to derive the RI from Mie theory. The Stokes shift of Raman scattered light is measured by a spectrograph to obtain chemical information. *Results*: We have studied the performance of each task of the setup separately. (a) RPS was capable of sizing vesicles >60 nm with an uncertainty $<5\%$. (b) The RI of vesicles >100 nm was determined with an uncertainty $<3\%$ using nanoparticle tracking analysis. (c) We have applied Raman microspectroscopy to distinguish tumour-derived vesicles from normal vesicles. The Raman spectra of single optically trapped vesicles showed spectral transitions characteristic of phospholipids. Moreover, optically trapped tumour-derived vesicles showed unique Raman transitions compared to normal vesicles. *Summary/conclusion*: (a) The sensitivity and accuracy of RPS are sufficient to distinguish vesicles from small cells, such as platelets. (b) The accuracy of determining the RI with elastic light scattering is sufficient to distinguish vesicles (RI <1.38) from lipoproteins (RI >1.42). (c) For the first time, single tumour-derived vesicles were distinguished from normal vesicles without fluorescent antibody labelling using Raman microspectroscopy. Thus, Raman microspectroscopy combined with RPS has great potential for tumour-derived vesicle identification in plasma.

P-IV-3

Detection of specific exosomes using in situ proximity ligation with flow cytometry as a read-out

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Introduction: Capturing, detecting and identifying microvesicles and exosomes using conventional flow cytometry are challenging due to the small size of the particles, and the correspondingly low amounts of proteins present. To identify and distinguish the true signals over any background is one of the major limitations. The need to specify different subpopulations in heterogeneous populations of microvesicles raises the bar even higher. *Methods*: We have developed a method that can be used to detect and distinguish different exosomes in a sample, based on the in situ proximity ligation assay (PLA) (1). By using a multiplex version of the in situ PLA, we can simultaneously detect 5 different antigens on the exosome surfaces. *Results*: The in situ PLA utilizes DNA oligonucleotides that give rise to a local DNA amplification reaction via rolling circle amplification (RCA). Our multiplex assay produces 3 classes of RCA products each of which is labelled with a distinct fluorophore. The RCA products, when bound to the surfaces of the microparticles, increase their apparent size. *Summary/conclusion*: This enhances side scatter in the flow cytometer and serves to separate true exosome signals from background. The different colours can be used to distinguish subpopulations of exosomes according to the presence of target molecules on their surfaces.

Reference

1. Söderberg O, Gullberg M, Jarvius M, Ridderstråle K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods. 2006;3: 995–1000.

P-IV-4

Microchip-based exosome analysis system: 2D-histogram of size and protein expression

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Introduction: Surface protein profiling using antibodies is the most desirable approach for characterizing extracellular vesicles (EVs) due to their heterogeneity. Although recent improvements of high-resolution fluorescent flow cytometry have enabled the detection and analysis of fluorescence-labelled EVs of $\sim$100 nm, the requirement for an experienced operator and expensive apparatus will limit their widespread use. We have proposed an analysis method for profiling surface proteins of individual EVs employing the principle of on-chip particle immunoelectrophoresis and have been developing a prototype analysis system. In this paper, we will present the observation of exosomes with a diameter of 50 nm or smaller. *Methods*: The system for on-chip immunoelectrophoresis comprises a microcapillary electrophoresis (μCE) chip and a laser dark-field microscope with a CMOS camera. To achieve more sensitive detection, a chip design and laser beam irradiation were improved for reducing background noise. Exosome samples were extracted from supernatant of human fibrosarcoma cell line (H1080) after cultivation with a serum-free MEM medium for 24 hours. After the differential centrifugation and filtration using a 220 nm-pore-size filter, the samples were further purified using Opti-prep density-gradient ultracentrifugation. The fractions were collected and ultracentrifuged, and the final pellet was suspended in PBS. *Results*: To examine the detection limit of our system, artificial polymeric vesicles (average diameter of 100 nm) were used. After introduced into the flow channel of a μCE chip, motion of the vesicles was analyzed, and its hydrodynamic diameter was calculated using the Stokes-Einstein equation. The vesicles with diameter between 55 and 233 nm could be observed. For obtaining images of smaller vesicles, optimization of the optics of the system is now under intense study. To examine the applicability of our system to exosome analysis, exosomes secreted from H1080 cells were analyzed. A fraction of 1,065 g/ml Opti-prep solution was determined for the experiment by immunoblotting to compare the abundance of 2 exosome markers of CD81 and CD63 for the fractions. After introduced into the μCE chip, both diameter and zeta potential of each exosome were measured simultaneously. The exosomes with the diameter between 53 and 404 nm and with the zeta potential between -3 and -17 mV were detected. Since surface protein expression level of exosomes can be estimated from the zeta potential values before and after immunoreaction, 2-dimensional-histogram of size and protein expression level of each exosome can be obtained. *Summary/conclusion*: A microchip-based

exosome analysis system has been developed to provide a 2D-histogram of size and protein expression level of each exosome. Using a prototype system, small exosomes with a diameter up to 53 nm are reliably detected. This system is expected to make a substantial contribution to the ever-growing exosomal biomarker research.

P-IV-6

Development of high-sensitivity flow cytometry for rapid and high-resolution characterization of single exosomes

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Introduction: Exosomes are cell-secreted nanometre-sized vesicles (30–100 nm) that hold great potential in disease diagnosis and therapeutics. Rapid and high-resolution analysis of single exosomes is critical to illustrating their biological functions and to promoting applications. Flow cytometry has been widely used for vesicle analysis in clinical and research laboratories. However, the small size, low refractive index and extreme heterogeneity of exosomes render it challenging to measure single exosomes by conventional flow cytometry. Adopting strategies for single-molecule fluorescence detection in a sheathed flow, our laboratory has developed high-sensitivity flow cytometry (HSFCM) that allows light scattering detection of single silica and gold nanoparticles as small as 24 and 7 nm in diameter, respectively. In this report, we will demonstrate how HSFCM can be applied to the analysis of single exosomes. *Methods*: Exosomes were extracted from human platelet free plasma (PFP) through a 2-step ultracentrifugation process (100,000 g for 3 and 2 hours, respectively). Employing a set of silica nanoparticles with known diameters as the size references, high-resolution size distribution analysis of exosomes can be rapidly achieved. By using fluorescent nanoparticles of known concentration as the internal standard, particle concentration of exosomes was determined via single particle enumeration at a rate up to 10,000 particles per minute. *Results*: For exosomes isolated from human PFP, it was found that particle size fell in the range of 32–110 nm. The median size of exosomes was measured to be 44 nm, with more than 90% of them distributed between 32 and 70 nm, which agrees well with TEM data. Meanwhile, the concentration was determined to be 1.7×10^{11} particles/ml, higher than the values reported by other approaches such as scanning ion occlusion sensing ($\sim 2.0 \times 10^{8}$ particles/ml) and nanoparticle tracking analysis ($0.5 \sim 5 \times 10^{10}$ particles/ml). It's worth noting that besides the sensitivity issue of instruments, different exosomes extraction methods also contributes to the variance. *Summary/conclusion*: We demonstrate that with a sensitivity 4–5 orders of magnitude higher than that of conventional FCM in light scattering detection, the laboratory-built HSFCM is capable of providing much needed analysis of single exosomes. Moreover, HSFCM offers concurrent assessment of biochemical properties via sensitive fluorescence detection. Thus, quantitative and multiparameter analysis can be obtained to reveal more detailed information of exosome populations.

P-IV-7

Capturing extracellular vesicles on solid surfaces

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Introduction: Extracellular vesicles (EVs) carry mRNAs, microRNAs, lncRNAs, proteins, metabolites and lipids originating in the parental cells and are involved in epigenetic communication between cells. For these reasons, the development of the EV-based diagnostic and therapeutic devices have been attracting much attention. The successful development of these types of devices requires an in-depth understanding of the interactions between EVs and surfaces of inorganic materials; however, our knowledge of these interactions remains limited. Here, we use atomic force microscopy (AFM) to examine the adsorption behaviour of EVs from various sources onto surfaces of different materials (including peptide modified surfaces). *Methods*: EVs were prepared from several cell lines by a density-gradient centrifugation method. The EVs adsorbed onto the surfaces of various materials were observed in aqua by MFP-3D (Asylum Research) with an AC (tapping) mode. *Results*: Adsorption behaviours of EVs onto the surfaces varied substantially depending on the substrate used. The coating of surfaces with zwitterionic polymers such as 2-methacryloyloxyethyl phosphorylcholine (MPC) copolymer substantially suppressed the adsorption of EVs. The presence of some EV markers on the vesicles was confirmed by gold particle-modified antibodies and AFM observation. *Summary/conclusion*: AFM revealed distinct adsorption behaviours of EVs on different materials.

P-IV-8

Pre-preparation of extracellular vesicles by electro-dialysis and adsorption-elusion methods

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P-IV-9

Characterization and functional evaluation of distinct subpopulations of extracellular vesicles released by melanoma cells

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Introduction: Extracellular vesicles (EVs) are heterogeneous populations of nano-sized cell-derived membrane vesicles involved in intercellular communication by transferring biological information between cells. However, it remains to be determined whether subpopulations of EVs serve different physiological functions. Here, we sought to characterize and evaluate the biological effects of distinct subpopulations of melanoma cell-derived EVs. *Methods*: EVs were isolated from conditioned medium of B16F10 melanoma cells using differential ultracentrifugation followed by sucrose gradient separation. Vesicles were analyzed by Nanosight analysis, Western Blotting and electron microscopy. EV subpopulations were further characterized for protein and RNA composition using LC-MS/MS and Bioanalyzer analysis. Effects on gene expression in recipient H5V endothelial cells were assessed using an Affymetrix gene array. *Results*: Using Nanosight analysis, Western Blotting and electron microscopy, 3 EV subpopulations were identified with different sedimentation and floatation characteristics. EV biogenesis was found to be ceramide-dependent. LC-MS/MS analysis allowed quantification of 1,884–2,893 proteins (1% FDR), of which 533, 354 and 110 proteins were exclusively found in 1 of the 3 subpopulations. Exposure of H5V cells to EVs induced changes in gene expression, some of which were specific to 1 EV subpopulation. *Summary/conclusion*: Cells release different subpopulations of EVs that differ in composition and biological effects on recipient cells. Discrimination between subpopulations may be important for studies on EV biology and function, and the development of EV-based diagnostics and therapeutics.

P-IV-11

Detection of epithelial antigens on tumour vesicles by flow cytometry: selection of antibodies

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Introduction: Most tumours are of epithelial origin. To identify tumour-derived vesicles in plasma of cancer patients by flow cytometry, we compared several antibodies for their ability to identify such vesicles. *Methods*: We isolated total extracellular vesicles (EV) populations from human cell-free urine containing vesicles originating from normal epithelial cells and from conditioned culture media of a pancreatic cell line (BXPC3). Antibodies tested against epithelial antigens were (anti) EpCAM (Dako, ThermoFisher and Miltenyi), MUC-1 (Pharmingen and BioMeda) and CD24 (MACS and BioConnect). Stained samples were analyzed by flow cytometry on a FACS Calibur (BD) and an A50 micro (Apogee), and data were analyzed by Cellquest Pro version 4.02 and FlowJo, respectively. *Results*: In all experiments, the A50 micro detected 30–200 fold higher numbers of positive events than the Calibur. In urine-derived EV, only large vesicles stained positive for EpCAM (8.0×10^5 vesicle/ml; A50 micro) with all 3 tested antibodies. In contrast, large as well as small vesicles stained positive for MUC-1 with both antibodies (9.0×10^6/ml). In BxPC3-derived EV, small and large vesicles both stained positive for EpCAM (1.0×10^6/ml) with all tested antibodies, whereas lower numbers of both populations stained positive for MUC-1 (2×10^5/ml) with both tested antibodies. In urine and BxPC3, low numbers of small as well as large vesicles stained for CD24 (1.2×10^5/ml) with both antibodies. *Summary/conclusion*: Due to the detection of smaller vesicles, the A50 micro detects more than 30 fold higher numbers of epithelial-derived EV. The exposure of epithelial antigens of small and large vesicles differs and seems to be dependent on the source of the material. Therefore, to study tumour-cell-derived vesicles, we advise to use both anti-EpCAM and anti-MUC-1.

P-IV-13

Size exclusion chromatography of extracellular vesicles: comparison of different stationary phases

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Introduction: The purity of vesicle samples is a key issue in basic research in the EV field but also in clinical investigations. As it was shown previously, the commonly used isolation techniques often co-isolate non-vesicular materials like lipoproteins and protein aggregates. Size exclusion chromatography (SEC) is a powerful tool for the separation of different macromolecules and biological nanoparticles, and it was found to be very useful for the isolation of EVs. Despite the wide variety of macroporous stationary phases in SEC, only limited number of them was applied in the EV research. The aim of our study was to compare different stationary phases in SEC regarding their ability of separation and size characterization of EVs. *Methods*: EV samples isolated by differential centrifugation from Jurkat cell line and from erythrocyte concentrates were investigated in this study. Separation and size characterization of the studied EV samples was performed by HPLC-SEC with the following pre-packed columns: TSK G6000PW (Tosoh Corp.), PL aquagel-OH 60 (Agilent Technologies), Nucleogel GFC 4000-8 (Macherey-Nagel). Additionally, a Tricorn 5/200 glass column filled with Sepharose CL-2B (GE Healthcare) cross-linked agarose gel was also investigated. SEC was performed on a Jasco HPLC system consisting of a PU-2089 pump with UV-2075 UV/Vis detector supplemented with an on-line coupled W130i DLS instrument (Avid Nano Ltd.) for the identification of the vesicle fraction. Synthetic liposomes, lipoprotein particles and different soluble proteins were also measured for reference. *Results*: Based on the fractionation ranges of the used columns (TSK G6000PW: up to 8,000 kDa for polyethylene glycol, PL aquagel-OH 60: 200–10,000 kDa for polyethylene glycol, Nucleogel GFC 4000-8: 100–20,000 kDa for dextrans and Sepharose CL-2B: 70–40,000 kDa for dextrans) all of them was found to be suitable for the separation of EVs from soluble proteins and lipoprotein particles. On the other hand, only slight differences were found in the elution profiles of synthetic liposomes and EVs with different sizes, hence the applicability of SEC for discrimination of different vesicle fractions by size is limited. However, liposomes with different surface characteristics and EVs from different origin resulted slightly different elution times and profiles, which indicates that not only steric interactions governs the retention of these biological nanoparticles in SEC. *Summary/conclusion*: SEC using different macroporous stationary phases was found to be suitable for the characterization of the purity of different EV preparations. The use of SEC is limited for the size determination of the vesicles, although, more investigations are needed to reveal the physicochemical background of the observed elution profiles.

P-IV-14

A new fluorescent probe for identifying extracellular vesicles

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P-IV-15

A novel method to validate fluorescence nanoparticle tracking analysis for phenotyping extracellular vesicles

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P-IV-16

Differential detergent sensitivity of extracellular vesicle subpopulations

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P-IV-17

Simplified protocol for flow cytometry analysis of fluorescently labelled microvesicles and exosomes using dedicated flow cytometer

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Introduction: Flow cytometry is a powerful method, which is widely used for high-throughput quantitative and qualitative analysis of cells. However, its straightforward applicability for extracellular vesicles and

mainly exosomes is hampered by several challenges reflecting mostly small size of these vesicles (exosomes: ~80–200 nm, microvesicles: ~200–1,000 nm), their polydispersity and low refractive index. *Methods*: The currently best and widely used protocol employs ultracentrifugation coupled to floatation in sucrose gradient for isolation of exosomes, labelling with lipophilic dye PKH67 and optimized version of commercial high-end cytometer. However, this approach requires experienced flow cytometer operator capable of manual hardware adjustments and calibration of cytometer. Here we provide novel and fast approach for quantification and characterization of both microvesicles and exosomes suitable for multiuser labs by utilizing flow cytometer especially designed for small particles, which can be used without adjustments prior to data acquisition. *Results*: Extracellular vesicles can be fluorescently labelled with protein- (CFSE) and/or lipid- (FM) specific dyes, without the necessity to remove unbound fluorescent dye by ultracentrifugation, which further facilitates and speeds up characterization of microvesicles and exosomes using dedicated flow cytometry. Additionally, double labelling with protein- and lipid- specific dyes enables to separate extracellular vesicles from common contaminants of sample preparations, such as protein aggregates or micelles formed by unbound lipophilic styryl dyes, thus not leading to overestimation of their numbers. Moreover, our protocol is compatible with antibody labelling using fluorescently conjugated primary antibodies. *Summary/conclusion*: Our methodology opens possibility for routine quantification and characterization of extracellular vesicles from various sources. Finally, it has the potential to bring desired level of control into routine experiments and non-specialized labs.

P-IV-18

Immunocapture and analysis of tumour-derived extracellular vesicles

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Introduction: Analysis of extracellular vesicles (EVs) in biological fluids offers exciting possibilities for the development of diagnostic tests. The use of antibody-coated magnetic beads is a straightforward method for isolation and subsequent analysis of EVs amenable to routine laboratory analysis. This approach was applied to analyze specific subpopulations of tumour-derived EVs in cell culture medium and in platelet-free plasma (PFP). *Methods*: EV size distribution and concentration were determined by NTA. Antibody-coated (CD9, CD63, CD81, EpCam) magnetic beads were used to capture tumour cell-derived EVs in cell-free culture medium, in PFP containing spiked-in EVs and in PFP obtained from tumour-bearing mice. EV-bead complexes were incubated with fluorescently-labelled antibodies directed at tetraspanins and tumour markers, followed by 96-well plate-based flow cytometry analysis. Alternatively, EV tetraspanins and tumour-markers in culture medium and PFP were analyzed by ELISA. *Results*: Analysis of cell-free tumour cell culture medium and PFP spiked with EVs revealed linear correlations between number of EVs and mean fluorescent intensity. Semi-quantitative flow cytometry analysis of tetraspanins on tumour cell-derived EVs showed distinct profiles for various tumour cell lines. ELISA development and analysis of EVs in PFP derived from tumour-bearing mice are currently ongoing, and our latest results will be presented. *Summary/conclusion*: Immunocapture is a promising start for parallel diagnostic analysis of EV surface markers, proteins and RNA, which can be performed in a high-throughput manner using the same sample.

P-IV-19

The importance of selecting the right EV markers for analytical detection in the discovery of biomarkers

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Introduction: Currently, no proteins are known to be constitutively sorted into extracellular vesicles (EVs) independently of the subcellular origin and the producing cells' activation status. This lack of invariant ''housekeeping'' markers hampers the quantitative analysis of these EVs, and all antibody-based EV detection assays suffer from this. In the discovery of, for example, cancerous biomarkers, selecting the right EV markers for detection is essential. The Extracellular Vesicle (EV) Array (Jørgensen et al., 2013, JEV) facilitates the ability to detect and profile EVs for the presence of multiple surface-exposed antigens simultaneously. The aim of the study was to illustrate the impact of changing the EV markers for cancerous biomarker discovery by using the EV Array. *Methods*: The novel, multiplexed platform of the EV Array was used for capturing, detecting and profiling EVs in plasma from 10 healthy donors and 10 lung cancer patients (NSCLC). The assay is based on the antibody capture of EVs and subsequent detection by biotin-labelled antibodies. Antibodies against 30 different EV biomarkers were used to capture the EVs. Subsequently, 9 different antibodies (including CD9, CD63, CD81, EGFR and EpCAM) were used as detection. *Results*: In general, the known exosomal markers CD9, CD63 and CD81 showed great variation in their ability to distinguish healthy from cancerous patients. Unexpectedly, the healthy donors tend to have a higher protein load of CD9 and CD81 in relation to the cancerous patients. Illustrated by the use of the EV Array this study clearly shows that the phenotype is affected by the choice of detection marker. *Summary/conclusion*: The use of various detectors and antibodies clearly revealed the importance of selecting the right EV markers when exploring novel markers in NCSLC.

P-IV-20

Zeta potential measurement of different sub-populations of extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are secreted from most cell types and are involved in the intercellular communication. Our group has identified several different sub-population of EVs from a single cell type, based on the EV density. However, the physiochemical properties of each EV sub-population are unknown. In this study, we measured the zeta potential of different sub-population of EVs with/without proteinase K treatment using ZetaView analyzer. *Methods*: Two different sub-populations of EVs (based on density) were isolated from HMC-1 cells, and both sub-populations were treated with proteinase K to remove surface proteins. Each sample was diluted with phosphate-buffered saline with appropriated concentration and measured the zeta potential using the ZetaView analyzer. *Results*: The zeta potential of lower density EVs showed a greater negative value than higher density EVs. The zeta potential of higher density EVs is shifted to a greater negative value after proteinase K treatment, but lower density EVs are not affected by proteinase K treatment, even though surface proteins are removed. *Summary/conclusion*: Our results show that zeta potential differ between sub-population of EVs. Importantly, 2 different sub-populations of EVs show different patterns of zeta potential shift after proteinase K treatment, implying that these EVs may have different surface proteins or lipids, which can influence functions and behaviours.

P-IV-21

Violet SSC: an alternative to FSC PMT or fluorescence in the detection of extracellular vesicles

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Introduction: Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). Although advances in various fields, including microscopy, have addressed some of the preliminary hindrances, flow cytometry remains the dominant approach for the characterization of submicron cell-derived particles. In this independent study, several of those technologies are evaluated and compared. As most of the hardware adjustments are accomplished by enhancements to the FSC parameter, the study will also evaluate the use of Violet SSC on Beckman Coulter's Cytoflex as a novel approach to small particle detection. *Methods*: For this flow cytometric assay, particles were chosen at specific sizes for later cell tracking. By using beads of differing sizes and fluorescent intensities, one can optimize the flow cytometer for cellular analysis. Bangs Labs' Dragon Green Beads (DG) were acquired on Beckman Coulter's Astrios EQ, MoFlo XDP with Propel Labs NanoView attachment, Gallios and Cytoflex flow cytometers. All instruments were peaked to maximize resolution and separation of populations (192, 520 and 780 nm). To further investigate Violet SSC as an alternative to the conventional mechanisms of EV detection, PCS control beads of 100, 200, 300 and 500 nm were evaluated. *Results*: The Cytoflex showed similarities in both resolution and dynamic range for all particles. Particles in the size range of 100–200 nm are not easily separated from one another on a Scatter Plot. Measuring differences in fluorescent intensity is the only means of separation for detection below 200 nm. *Summary/conclusion*: The interest in the identification and detection of submicron particles has increased in recent years. Flow Cytometry has become an important tool in EV research with instrumentation being developed to identify particles at the submicron level; such as cytometers optimized to improve light scattering collection and image cytometers. In this comparison study, it has been shown that the Cytoflex is compatible to the results obtained from the AstriosEQ, NanoView and Gallios. The ability to resolve and distinguish the populations as effectively as its counterparts, has proven the Cytoflex Violet SSC to be a viable alternative to the FSC PMT to detect EVs.

P-IV-22

Isolation and characterization of exosomes using flow cytometry and analytical ultracentrifugation

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Introduction: Historically, differential centrifugation has been the primary method for isolating extracellular vesicles (EVs), while additional technologies, such as filtration and polymer precipitation, have more recently entered the field. Recent advances in flow-cytometric detection technologies now allow for nanoparticles to also be detected by flow cytometry at the higher end of the exosome size range using side scatter (20–130 nm). Using flow cytometry-based sorting, exosomes can not only be characterized by their scatter profiles but also isolated at a rate of 25–50 K events per second (EPS). The goal of this research is to compare the exosome isolations from blood plasma, and precipitation; focusing on the optimization of exosome isolation by flow sorting. Subsequent analysis of the exosomes by analytical ultracentrifugation (AUC) provides additional information, such as mass distribution and the presence of debris. *Methods*: HeLa cells were cultivated using exosome-depleted foetal bovine serum (FBS) prepared by ultracentrifugation. HeLa and blood-plasma exosomes were initially separated by slow centrifugation and were then purified using flow sorting (Astrios EQ[1], Beckman Coulter), ultracentrifugation or Total Exosome Isolation Reagent (Life Technologies). Afterward, the purities of the exosome isolations were confirmed using CD63 Dynabeads (Life Technologies), analyzed by flow cytometry. The AUC optical interference module was standardized using polystyrene beads between 20 and 100 nm (MagnaBead) in order to confirm its ability to separate large particles, as well as to provide a reference for exosomes. Finally, the size and structure of the exosomes were confirmed using the AUC to measure the mass of the isolated exosomes. *Results*: All 3 methods can be used for purposes of isolation of exosomes with distinctive limitations and constraints for each method. The ultra-centrifugation is a long process requiring several rotors and steps. The flow cytometric method had issues with sub-threshold populations. The polymer precipitation has reagent to volume costs. The exosomes were able to be distinguished from optical noise by side scatter, and the purities of the exosome populations were confirmed using CD63 Dynabeads. The exosome isolations were further analyzed by AUC for exosome versus debris concentration for the 3 extraction methods. The AUC optical interference module was confirmed to be able to detect and distinguish the 20–100 nm polystyrene particles. *Summary/conclusion*: Flow cytometric sorting on a high-speed multiscatter enabled sorter allows for exosome sorting. The AUC was a complimentary method to use the sorted exosomes samples to determine its mass, mass distribution and presence of debris.

P-IV-23

Improvements to the characterization of extracellular vesicles by Nanoparticle Tracking Analysis: size and concentration

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Introduction: Extracellular vesicles (EVs, 30–100 nm) are released from endosomes in a wide variety of cells. It is increasingly accepted that exosomes are implicated and appear in a multitude of pathological conditions and show much promise as diagnostics for many different diseases such as cancer, heart disease, diabetes, Alzheimer's, pre-eclampsia, etc. However, developments in this area are constrained by limitations in the technology available for their measurement. Nanoparticle Tracking Analysis (NTA) (Malvern Instrument Ltd, Amesbury) offers the potential to both enumerate and speciates (through fluorescent markers) these microparticles in a rapid manner. *Methods*: In this method, the particles in suspension scatter laser light which is collected by a CCD (or sCMOS) camera via a microscope-type configuration. Particles (sized between 10 and 2000 nm) are tracked individually and their diffusion coefficient, and therefore size, calculated directly from their speed. This characterization gives a direct measurement of the concentration and size distribution of the particles in the field of view. *Results*: Here we present the improvement to concentration measurement through using a calibration algorithm (patent pending) to compensate for user settings and thus make the measurement independent of the user, halving the variability. We also demonstrate through the application of controlled flow and improved statistics that the precision can be further improved by a factor of 2. Implementing these changes significantly reduces the variability in concentration measurement. Sizing capability is also improved by compensating for the stochastic motion of Brownian motion allowing resolution of better than 1:1.25. *Summary/conclusion*: NTA is demonstrated to be a robust technology capable of measuring EVs down to a size of 30 nm to a sufficiently high resolution and delivering a system and user-independent concentration measurement.

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P-IV-24

Use of ApogeeMix beads to assess the performance of flow cytometers for small particle analysis

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Introduction: The complex relationship between particle size and the amount of light scattered at different collection angles makes it difficult to infer particle size from a flow cytometer's light scatter data. A population may be described as scattering an amount of light equal

to a reference particle (e.g. a latex or silica bead of known size) but same sized particles of different refractive index can give very different signal strengths (see data below). When comparing data between flow cytometers, the difficulties are compounded by differences in light scatter collection angles. Ideally, it would be possible to produce stable reference particles of known size and of a refractive index and structure similar to the bacteria or microvesicles of interest but such particles are not commercially available. Due to the refractive index difference, latex beads do not offer a precise means to assess a flow cytometer's light scatter performance for the study of biological particles. Instead, silica beads can be used as a better reference particle because silica's refractive index is closer to the refractive index of biological vesicles. *Methods*: The ApogeeMix product (Apogee Flow Systems Ltd, Cat #1493) is a convenient mixture of non-fluorescent silica beads and fluorescent latex beads with sizes from 110 to 1300 nm, which can be used to prepare flow cytometers for the analysis of small biological particles. It can be used to determine a region of interest on the flow cytometer's datagrams within which to count microvesicles. An Apogee A50-Micro + flow cytometer has been used to provide data on a range of sizes of silica and latex beads. *Results*: The flow cytometer's sensitivity and resolution are of interest to scientists wishing to study sub-micron biological particles which may be close to the instrument's noise limit. Data from the high sensitivity A50-Micro + flow cytometer demonstrates the ability of flow cytometry to count and resolve sub-micron particles. *Summary/ conclusion*: The ApogeeMix beads offer a fast and convenient method to assess your flow cytometer's optical performance.

Disclosure of Interest: O. Kenyon Conflict with: Director of Apogee Flow Systems Ltd.

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P-IV-25

Urine exosome mRNA in diabetic kidney disease

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Diabetic kidney disease (DKD) takes many years to develop. The earliest sign of kidney disease is indicated by the presence of small amounts of albumin in the urine, called microalbuminuria. Not all individuals with microalbuminuria, however, progress to end stage renal disease. Diagnostic biomarkers with improved sensitivity are necessary. We initiated this study in order to evaluate urine exosome mRNA as potential biomarkers in screening Type II diabetes patients for kidney disease. Exosomes and microvesicles (EMVs) from 10 mL urine (n = 2 control, n = 3 DKD) were captured and collected by a filter device called ExoComplete Isolation Tube. The EMV mRNAs were released by a lysis buffer and hybridized to a T7 promoter-linked oligo(dT) coated plate. The RNA was captured, amplified by in vitro transcription in solid phase, and then used as starting material for next generation RNA sequencing library preparation. Using CyberT software for data analysis, the most highly significant differentially expressed mRNA were those encoding for cytosolic ribosomes and mitochondrial components involved in translational elongation and oxidative phosphorylation, respectively. Validation by qPCR using 22 control and 18 DKD patient urine samples confirmed 6 potential mRNA biomarkers. Several of these biomarkers have been implicated to play a role in mediating oxidative stress. Further studies are necessary to validate their roles in the progression of end stage renal disease.

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Poster session V - EV therapeutics
Chairs: *Jason Aliotta and Su Chul Jang*

P-V-1

Powering the production of extracellular vesicles as drug delivery vectors using microfluidics

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Please see Oral with poster C

P-V-2

Extracellular vesicles from glioblastoma cells as potential drug-delivery vehicles to isotypic tumours

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Introduction: Glioblastoma Multiforme (GBM) is a highly aggressive tumour of the central nervous system, with only 5% surviving the 5th year post diagnosis. Extracellular vesicles (EVs) have been investigated over the past years for use in drug delivery systems with promising results. Since the composition of EVs resembles that of their cell of origin, a tendency for cancer EVs to be preferentially internalized by cancer cells exists. Thus, the source of GBM EVs might be the ideal choice as a drug-delivery vehicle in GBM treatment. This study seeks to investigate whether EVs isolated from primary GBM cell lines qualify as drug-delivery vehicles in GBM treatment with respect to their effects on cell proliferation. *Methods*: EVs were isolated from supernatants of 60–80% confluent cell cultures by sequential centrifugation followed by ultracentrifugation. EV quantification and size distribution were assessed with NTA, and EV morphology was characterized with TEM. To evaluate the proliferative potential of a primary GBM cell line (C16) EVs on C16 cells, electroporated and non-electroporated EVs were added to the culture medium and analyzed by flow cytometry. Protein analysis of GBM cells and EVs was done with mass spectrometry. Lastly, EVs were stained and uptake was visualized by confocal microscopy. *Results*: EV size distribution and quantities were comparable to those reported for other cancer cells. The morphology of EVs appeared round in shape and confirmed the general size distribution obtained from NTA. Electroporated and non-electroporated EVs showed no effects on cell proliferation compared to controls. In addition, GBM EVs were successfully internalized by GBM cells. Protein analysis revealed several proteins *only* present in the EV fraction that interact with surface molecules present in the cell fraction. *Summary/conclusion*: GBM EVs have a potential to be used as drug-delivery vehicles in the treatment of GBM with respect to their lack of stimulatory growth effects on the autologous cell line. GBM EVs were expected to stimulate proliferation of GBM cells, but no proliferative effect from electroporated or non-electroporated EVs was seen. To further establish GBM EVs as drug-delivery vehicles, internalization of GBM EVs in GBM cells was confirmed. The EV protein composition identified proteins only present in the GBM EV fraction which interact with proteins present on the surface of GBM cells. This might explain the interactions of GBM EVs with GBM cells with regards to cellular uptake. In order to fully conclude that GBM EVs qualify to be used clinically as drug-delivery vehicles in treating GBM, further functional analysis needs to be addressed, and an efficient way of loading EVs with relevant therapeutics needs to be optimized.

P-V-3

Characterization of exosome-encapsulated paclitaxel for the treatment of neoplasms

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Introduction: Exosomes are naturally occurring membrane-derived extracellular vesicles ~100 nm in size; they are produced by many cell types and are involved in intracellular communication by delivering cargo (e.g. proteins, nucleic acids) to recipient cells. Exosomes have recently generated interest as possible drug delivery vehicles due to their ability to be loaded with various cargo [e.g. siRNA, curcumin] and because of the absence of toxic excipients (e.g. Cremophor EL in the commercial formulation of paclitaxel, Taxol). Our lab has previously shown that macrophages loaded with a therapeutic are able to hone to sites of inflammation such as the tumour microenvironment. Exosomes released by macrophages may possess a similar ability to hone to sites of inflammation and thus represent a promising drug delivery platform. Paclitaxel (PTX), a water-insoluble small molecule chemotherapeutic commonly used for the treatment of various cancers, was incorporated into exosomes to increase its solubility and enhance its therapeutic efficacy against pulmonary metastases. The objective of this study was to explore the feasibility of an exosome based drug delivery platform for water insoluble chemotherapeutics, for example paclitaxel (PTX), for the treatment of solid tumours and tumour metastases. *Methods*: Herein, we compare different methods of loading exosomes derived from RAW 264.7 macrophages with PTX and characterize their size by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), uptake, release, stability and in vitro efficacy. Because the exosomal membrane is composed of a lipid bilayer, we believed that mild sonication would allow for paclitaxel to insert into the hydrophobic inner layer of the exosomal membrane without major disruption of exosomal membrane components. *Results*: We found that mild sonication conditions allows for the greatest drug loading of paclitaxel into exosomes (30.95 ± 1.38% loading capacity by μg protein). ExoPTX was taken up by 3LL-M27 Lewis Lung Carcinoma cells in significantly greater numbers than either liposomes or polystyrene nanoparticles in vitro and delivered PTX more efficiently than Taxol to MDCK WT and MDCK MDR1 (Pgp+) cells. ExoPTX was shown to exhibit a burst release profile followed by sustained release and good long-term stability. ExoPTX demonstrated significantly greater cytotoxicity against 3LL-M27 cells (13.57 ± 1.33 ng/ml) and MDCK WT (23.33 ± 3.77 ng/ml), as compared to Taxol (23.16 ± 1.88 and 69.54 ± 11.50 ng/ml, respectively). Furthermore, incorporation of PTX into exosomes appeared to somewhat mitigate resistance to PTX in Pgp+ cells (exoPTX IC50 for Pgp+ MDCK MDR1 cells was found to be 187.5 ± 38.65 ng/ml, for Taxol 1708.67 ± 299.93 ng/ml); the exact mechanism behind this phenomenon remains to be elucidated. *Summary/conclusion*: Our results demonstrate that this platform may provide a novel platform for the delivery of water insoluble chemotherapeutics to Pgp+ drug resistant cancer cells.

P-V-4

Hybrid exosomes by fusion with functional liposomes for drug delivery system

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Introduction: Exosomes are of great interest to drug delivery systems of biological molecules. In general, engineered exosomes have been prepared by genetic modification of parent cells with functional fusion membrane proteins. On the other hand, functional liposomes have been a focus of constant attention as drug carrier for therapeutically active agents. Hybrids of exosomes with liposomes are interesting to prepare functional exosomes. In this report, we propose a new method for modification of exosomes by exosome-liposome fusion. *Methods*: Exosomes were isolated form culture supernatants using ultracentrifugation protocol from CMS7wt cells and CMS7-HE cells which overexpress human HER2 receptor. The exosomes were characterized by FACS analysis, western blot analysis and nanoparticle tracking analysis (NTA). Interaction of exosomes with various liposomes (fusion between exosome and liposome) after freezing-thawing treatments was investigated by lipid mixing assay (FRET assay) and particle size measurement by NTA. Fluorescent labelled DOPC (non-ionic), DOPS (anionic), DOTAP (cationic) and DOPC: EPC (7:3) (cationic) liposomes were prepared by extrusion method through a polycarbonate filter with 100 nm pores. *Results*: The average sizes of exosomes were 141 ± 47 nm in the CMS7Wt and 127 ± 45 nm in the CMS7-HE. Phospho-HER2 was detected only CMS7-HE exosome. HER2 is activated through auto-phosphorylation at tyrosine residues, thus, the detection of phospho-HER2 provides an indication of HER2 activity. After freezing-thawing treatments of the mixture of exosomes and liposomes, effective lipid mixings and increases in sizes were observed. The results suggested that exosomes and liposome fused by freezing-thawing treatments. The cationic liposomes such as DOTAP and DOPC: EPC (7:3) liposomes showed high fusion efficiency. Phospho-HER2 was observed in the hybrid of exosome and liposome. *Summary/conclusion*: We prepared engineering exosome from CMS7-HE cell overexpressing human HER2 receptor and the HER2-containing exosome fused with various liposomes by freezing-thawing treatments. The hybrids of exosomes with functional liposomes offer a new option of wide utilization of exosomes in DDS.

P-V-5

Chemobiological and mechanobiological effects on endothelial cell-derived extracellular vesicle biogenesis

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Introduction: Extracellular Vesicles (EVs) are 30–100 nm lipid vesicles released by many cell types into the extracellular space or into biological fluids. EVs hold great potential as novel therapeutic delivery vehicles, however no scalable biomanufacturing processes currently exist to enable their widespread use. The first step in establishing such processes is to understand how EV production by cells is influenced by common parameters known to affect cell function. Here, we examined how mechanical substrate stiffness and cell media content effect EV biogenesis and cargo. *Methods*: Human umbilical vein endothelial cells (HUVEC) were used as a model EV-producing cell type. To determine effects of mechanical substrate stiffness, HUVEC were plated on 80-μm-thick polyacrylamide gels of varying physiological stiffnesses attached to glass coverslips and cultured in EV-depleted EGM-2 medium. After 24 hours, EVs were isolated by differential ultracentrifugation and were quantified and sized via a Nanosight LM10. Separately, HUVECs were cultured on typical polystyrene plates in the presence or absence of 50 uM ethanol, known to stimulate gene regulation changes in HUVEC. EVs were again charactertized, and RNA was isolated and assessed via agarose gel electrophoresis. miRNA content was analyzed using RT- qPCR, and a PCR-based microarray was performed to evaluate any EV-associated miRNA alteration after exposure to ethanol. *Results*: EV production by HUVECs increased with decreasing substrate stiffness. After ethanol exposure, HUVECs produced ~9% less EVs than the unstimulated controls; however, the RNA content of these EVs was almost 2.5 fold higher than in control cells. mIRnome analysis indicated a decrease in miRNAs associated with anti-angiogenic effects in ethanol-exposed HUVECs, consistent with the observation that ethanol increases angiogenesis in HUVECs. *Summary/conclusion*: We found that substrate stiffness and contents of the cell medium can influence EV production and cargo. Further exploration of these parameters may yield optimal conditions for EV production at large scale with selected desired cargo.

P-V-6

Optimization of exosome loading with the cytotoxic small molecule drug doxorubicin by electroporation

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Introduction: Exosomes loaded with small molecule drugs or siRNA are promising therapeutic agents as they are well tolerated by the immune system and can be directed to specific cells or tissues using peptide targeting motifs on their surface. In vitro loading by a number of methods such as soaking and electroporation has been previously described, however a strategy to optimize protocols for small molecule drugs has not been published so far. *Methods*: Herein, we describe a systematic approach to improve the loading of the hydrophobic small molecule drug doxorubicin into isolated exosomes using electroporation. The parameters analyzed were electroporation buffer composition, doxorubicin and exosome concentration, electroporation conditions, recovery times and strategies for the removal of unincorporated doxorubicin. The readouts used were particle number, doxorubicin fluorescence and cell viability. *Results*: We were able to show that high salt buffers containing sugars such as sucrose or trehalose support efficient incorporation of doxorubicin into exosomes while preventing doxorubicin precipitation occurring in low salt buffers. Longer recovery times promoted doxorubicin precipitation and hampered efficient removal of the non-incorporated doxorubicin from electroporated samples. Of the doxorubicin depletion procedures tested, only fast removal by spin desalting columns was able to retain the cytotoxic effects of the loaded exosomes. Additionally, we were able to show that the cytotoxic effect of drug-loaded exosomes correlate with exosome numbers and drug concentration although we report saturation effects in the latter. *Summary/conclusion*: Here we present a strategy to successfully incorporate a small molecule into exosomes by means of electroporation which may have a broader application.

P-V-7

Delivery of therapeutic agents by exosome-like nanoparticles made of grapefruit exosome-derived lipids

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Introduction: Exosomes have been found to contain more than 100 different types of miRNAs alone and can be taken up by different types of cells. Therefore, the same exosomes miRNA could have different biological effects on the different types of exosomes recipient cells. Addressing these issues will require tools to target and deliver specific miRNA for investigational purposes. Although the use of nanotechnology for the delivery of a wide range of medical treatments including miRNAs has potential to reduce adverse effects associated with drug therapy, tissue-specific delivery remains challenging. Recently, exosomes released from mammalian cells have been utilized for encapsulating drugs and siRNAs to treat brain-related diseases in mouse models. Although this approach is promising, production of large quantities of mammalian cell exosomes and evaluation of their potential biohazards has been challenging. *Methods*: Exosome-like nanoparticles were isolated in large quantities from the tissue of grapefruit using a standard sucrose gradient centrifugation method. A grapefruit-derived nano-vector (GNV) is assembled from grapefruit exosome-like derived lipids. *Results*: Exosome-like nanoparticles can

be isolated in large quantities from the tissue of grapefruit. We have demonstrated that exosome-like nanoparticles from grapefruit are composed of small RNAs, proteins and lipids. An grapefruit-derived nano-vector (GNV) assembled from grapefruit exosome-like derived lipids is capable of encapsulating siRNAs, and miRNAs, DNA expression vectors and proteins to different types of cells. We demonstrate the in vivo targeting specificity of grapefruit-derived nanovectors by co-delivering therapeutic agents with folic acid, which in turn leads to significantly increasing targeting efficiency to cells expressing folate receptors. The therapeutic potential of grapefruit-derived nanovectors was further demonstrated by enhancing the chemotherapeutic inhibition of tumour growth in 2 tumour animal models. Grapefruit-derived nanovectors are less toxic than nanoparticles made of synthetic lipids and, suggesting that they may be a useful tool for miRNAs and drug delivery. *Summary/conclusion*: Developing edible plant-derived exosomes like nanovectors, that is, the capability to deliver miRNAs in a tissue-specific manner, will move the exosomal miRNA biology field forward by providing opportunities to identify clear roles for specific exosomal miRNAs, not only in exosomes but in other vesicles. Based on the lipidomic profile of GNV, we propose that GNVs would be a versatile therapeutic carrier agent being capable of entrapping and delivering hydrophilic, hydrophobic and amphipatic agents.

P-V-8

Scalable production of exosomes and their potential use as a therapeutic for tendinopathy

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Introduction: Adipose-derived mesenchymal stem cells (MSCs) are characterized by their self-renewing capacity and their ability to differentiate into chondrocytes, adipocytes and osteocytes. This makes them attractive starting materials for tendon tissue engineering and regenerative medicine applications. While the recognized regenerative properties of stem cells show promise in bioengineering tendon constructs for repairs, there is limited evidence that direct injection of stem cells for tendon healing has beneficial effects. It has been suggested that much of the observed benefit of these stem cell injections arises from stem cell-secreted factors carried in discreet microvesicles called exosomes. These small vesicles contain bioactive components related to wound healing and present a potential new allogeneic therapy for tendinopathies. Here, we present our findings on the physical and functional characteristics of MSC exosomes relevant to wound healing and tissue regeneration. *Methods*: Data are presented on the proteomics of these exosomes, their ability to mediate cell migration and incorporate into the recipient cell membrane, and their ability to down regulate STAT3 phosphorylation as determined by western blotting. Scalable production of exosomes was accomplished using a hollow-fibre bioreactor. *Results*: A global analysis of the proteomic data identified α2-macroglobulin, which has been shown to inhibit MMP activities and improve tendon healing in animal models of rotator cuff and ACL injury, Lactoferrin (LTF), an iron binding protein with inflammation modulatory effects, can enhance fibroblast migration and proliferation, and an LTF-derived peptide has shown efficacy in reducing adhesions in a flexor tendon model system. Lipophilic dye transfer from labelled exosomes to cultured cell membranes indicated that the particles can deliver their payload to recipient cells. Treatment with exosomes significantly increases tenocyte proliferation to the level induced by FBS containing base medium, whereas the exosome depleted conditioned medium (Ex free) was ineffective. In addition, tenocytes treated with exosomes significantly increased collagen I production. Total bioreactor yield was approximately 10-fold greater than T-225 flask controls based on exosome number in addition to being at a 10-fold higher concentration. *Summary/conclusion*: Having successfully developed scalable exosome production, isolation procedures and in vitro assays to functionally characterize these particles which are secreted by the cultured MSCs, our goal is to leverage the regenerative and healing properties of adult stem cells by developing exosomes as a non-surgical and non-cellular treatment for tendon repair.

P-V-9

Establishment of an in vivo exosomal transfection as a new gene therapy

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Introduction: From genomic analyses, various diseases have been found to be caused by the mutation of specific genes. However, we do not have an effective treatment for these human genetic diseases because of the difficulty to apply the gene therapy to human patients. To overcome this, we now performed the establishment of an in vivo exosomal transfection strategy as a new gene therapy. *Methods*: We detected specific miRNA expression on the exosomal fraction by using real-time PCR when we transfected miRNA expression vector to HEK293T cells. Furthermore, we were able to significantly raise the production of exogenous miRNAs in exosomal fraction when the cell culture condition was modified and optimized. *Results*: When exosomes produced by large-scale preparation were checked by electron microscope, we did observe normal exosomes although some impurities on this exosomal fraction were evident. *Summary/conclusion*: Previously, our research group found that the inhibition of myostatin in muscle increases skeletal muscle mass and ameliorates dystrophic pathology in a model for Duchenne muscular dystrophy, decreases adipose tissue mass and prevents hepatic steatosis. We generated large-scale exosome which contains myostatin-shRNA and are ready to analyze their efficiency. We hope that this exosomal genetic silencing system will be a new therapy for muscle wasting diseases and even obesity.

P-V-10

Non-invasive imaging of radiolabelled exosome-mimetic nanovesicles using 99mTc-HMPAO

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Introduction: Exosomes known as nano-sized extracellular vesicles have attracted huge amount of interest due to its potential usefulness in drug delivery. Amid remarkable interest of exosome towards biomedical application, it is crucial to understand in vivo distribution and behaviour of exosome. Here, we developed a new method for radiolabelling of macrophage-derived exosome-mimetic nanovesicle (ENV) with ^{99m}Tc-HMPAO under physiologic condition and monitored in vivo distribution of ^{99m}Tc-HMPAO-ENVs using SPECT/CT in living mice. *Methods*: ENVs were produced from the mouse RAW264.7 macrophage cell line using a serial extrusion through filters with different pore sizes (10, 5 and 1 μm). The ENVs were labelled with ^{99m}Tc-HMPAO for 1 hour incubation, followed by removal of free ^{99m}Tc-HMPAO. SPECT/CT images were serially acquired at 30 minutes, 3 and 5 hours after intravenous injection of ^{99m}Tc-HMPAO-ENVs to BALB/c mouse. In vivo distribution study was also performed after sacrificing ^{99m}Tc-HMPAO-ENVs injected mice. *Results*: When ENVs were incubated with ^{99m}Tc-HMPAO at room temperature followed by purification, the radiochemical purify of ^{99m}Tc-HMPAO-ENVs was more than 90%. The expression of exosome-specific protein (CD63) in ^{99m}Tc-HMPAO-ENVs was not changed after labelling procedure. ^{99m}Tc-HMPAO-ENVs showed high serum stability (90%) which was similar with stability in phosphate buffered saline until 5 hours. SPECT/CT images of ^{99m}Tc-HMPAO-ENV injected mice exhibited high uptake in liver and no uptake in brain, whereas ^{99m}Tc-HMPAO only injected mice showed high brain uptake until

5 hours. *Summary/conclusion*: We firstly reported the radiolabelling method and non-invasive radionuclide imaging of ENVs. In vivo distribution of ^{99m}Tc-HMPAO-ENVs was investigated in living animals. The radiolabelled ENV imaging promises to provide useful information to understand in vivo behaviour of exosome for upcoming biomedical application.

P-V-11

Knockdown of human c-Myc by therapeutically exosome-mimetic nanovesicles in lymphoma

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Introduction: Myc (c-Myc) belongs to the class of transcription factors that regulates cellular growth, cell proliferation and differentiation. This proto-oncogene is activated by chromosomal translocation, and it is frequently deregulated in human cancers, such as lymphoma. The aim of this study was therefore to determine the knockdown efficiency of human Myc in the mouse lymphoma cell line λ820 driven by a human Myc transgene by therapeutic exosome-mimetic nanovesicles containing siRNA against human Myc. *Methods*: The NIH3T3 mouse cell line was lentivirally transduced with shRNA specifically designed to recognize human Myc and therefore over-expressing siRNA against human Myc. Exosome-mimetic nanovesicles from NIH3T3 were prepared by serial extrusion with different filters to get the desired sized of the vesicles containing the siRNA of interest. Vesicles were assessed for their size by Nanoparticle tracking analysis and were characterized by electron microscopy. Knockdown of Myc at the transcript level as well as at the protein level in λ820 cells were assessed by qPCR and western blot respectively. *Results*: Characterization of vesicles by Nanoparticle tracking analysis showed particle size around 170–200 nm. Electron microscopy revealed that the particles had intact vesicular structures that retain the properties of plasma membrane similar to exosomes. Treatment with exosome-mimetic nanovesicles to λ820 cells showed reduction of Myc at both the transcript and protein level indicating that siRNA against the Myc has been captured in the vesicles during the extrusion procedure. This treatment also reduced the number of viable cells in culture. *Summary/conclusion*: Taken together, cell-derived therapeutic nanovesicles that mimics exosomes could serve as vehicles carrying RNAi to efficiently knockdown several oncogenes to treat malignant tumours. Further assessment of effects of this cMyc targeting therapy in recipient cells will be pursued.

P-V-12

Mesenchymal stem cell EVs modulate cell adhesion homeostasis through its proteome

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Introduction: Mesenchymal stem cells (MSCs) are intimately associated with stem cell niches where they help regulate the local microenvironment to promote the engraftment of stem cells and calibrate their proliferation to the needs of the body. In recent years, MSC is increasingly shown to exert its biological activity through secreted EVs. Here we postulate that MSC's role in the maintenance of stem niches is partially mediated by EVs, and MSC EVs help regulate cell adhesion homeostasis in the niche through proteomic complementation. *Methods*: Purified MSC EVs were analyzed by mass spectrometry. The proteome were interrogated for proteins that are important for cell adhesion. The presence of some of these proteins was validated by immunoblotting, ELISA and enzymatic assays. The biological activity of some EV proteins was evaluated in cell assay systems or in animal models. *Results*: Proteomic analysis revealed that MSC EVs have >70 extracellular matrix (ECM) proteins, >20 cell adhesion molecules (CAMs) and ~20 ECM enzymes. In particular, we detected an abundance of collagen 7, a key protein that is mutated in epidermolysis bullosa, a disorder of epithelial adhesion. MSC EVs increased cellular adhesion in vitro and alleviate disease severity in collagen 7 deficient mice. *Summary/conclusion*: MSC EVs can modulate cell adhesion homeostasis through proteomic complementation.

P-V-13

Evaluation of therapeutic effect of neural stem cells and exosome mimetic nanovesicles derived from neural stem cell in 6-OHDA-induced mouse model of Parkinson's disease

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Introduction: Transplantation of neural stem cells (NSCs) has shown efficacy in animal models of Parkinson's disease (PD). Exosomes have been emerged as active component of the paracrine effect in stem cell therapy, yet have not been evaluated in PD model. The purpose of the study was to evaluate therapeutic effect of NSC and exosome-mimetic nanovesicles (NVs) derived from NSC in 6-OHDA induced mouse model of PD. *Methods*: The mouse model of PD was induced by unilateral injection of 6-OHDA in right striatum. Apomorphine induced rotation tests were conducted before, and 2, 4 weeks after the treatment. Human NSC line, HB1.F3, is transduced with an enhanced firefly luciferase retroviral vector (F3-effLuc cells). F3-effLuc-derived exosome-mimetic NVs (F3-NV) were obtained by serial extrusion and density gradient ultracentrifugation and labelled with Cy7. F3-effLuc cells or F3-NV were injected into the right striatum of mouse model of PD. Human foreskin fibroblasts transduced with effLuc (HFF-effLuc cells) and PBS were used as controls. In vivo bioluminescence imaging for cells and fluorescence imaging for F3-NVs were done. Mice were sacrificed 1 hour after injection of apomorphine at 4 weeks after transplantation, and immunostain for tyrosine hydroxylase (TH), dopamine transporter (DAT) and cFOS were done at the level of caudateputamen. *Results*: After transplantation of F3-effLuc or HFF-effLuc cells in PD mice, bioluminescence signals were visualized. Quantified bioluminescence intensity of the transplanted F3-effLuc and HFF-effLuc cells gradually decreased until it was undetectable by 10 days. Injected Cy7 labelled F3-NVs were visualized by in vivo fluorescence imaging at 3 hours after injection, but not visualized at 24 hours after injection. Side biased motor impairment was attenuated by F3-effLuc cell transplantation at 4 weeks after the treatment but not by HFF-effLuc, or PBS. F3-NVs-treated mice showed a tendency of improvement of side biased behaviour at 2 weeks after injection, but no significant improvement at 4 weeks after the treatment. There was no significant difference in, TH and DAT stain of PD models regardless of types of treatment (F3-effLuc, F3-NVs, HFF-effLuc and PBS). Striatal cFOS expression was lower in F3-effLuc-treated striatum than F3-NVs, HFF-effLuc or PBS-treated one. *Summary/conclusion*: Transplanted F3-effLuc cell and F3-NVs were successfully visualized by in vivo optical imaging. Side biased motor impairment and striatal supersensitivity of PD mouse was improved by F3-effLuc transplantation. F3-NVs showed tendency of behavioural improvement initially however the change was not sustained until 4 weeks after treatment.

P-V-14

Novel tools for labeling and manipulation of extracellular vesicle cargo to engineer intercellular delivery shuttles

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Introduction: Extracellular vesicles (EVs) are nano-sized vesicles that are shed by a variety of cell types in the body. Several lines of evidence suggest that exosomes function as messengers for intercellular communication by the transfer of functional proteins and RNA species. Their potential to serve as biomarkers for several pathologic conditions as well as vehicles for delivery of therapeutic agents has only begun to be explored. Development of tools that allow manipulation of the exosomal cargo and tracking exosome cargo delivery are key to optimizing EVs as therapeutic shuttles. *Methods*: Double-stranded DNA as well as RNA species ranging from as little as 22 and up to 5,000 nucleotides were successfully transfected into EVs using Exo-Fect technology (SBI). Functionality of the transfected nucleic acids in exosomes and their subsequent delivery in the recipient cells were evaluated by fluorescence microscopy. EV proteins were labeled using Exo-Glow (SBI) which is based on CFSE chemistry while trafficking of exosomal RNA was achieved by using Exo-Red (SBI), a fluorescent cationic dye that preferentially binds to single stranded RNA. EVs from various cell types including cancer cells, stem cells and neurological cells were harvested using ExoQuick-TC (SBI) and used for cargo labeling and transfection in these studies. *Results*: We show that EVs can be effectively transfected with siRNAs, mRNAs as well as plasmid DNA using a simple 2-step protocol developed for Exo-Fect. Importantly, we show that the transfected nucleic acids are translated into protein when delivered to recipient cells suggesting that they retain their functionality within the vesicles. EVs labeled with ExoGlow or ExoRed exhibited robust fluorescent signals at their emission wavelengths and were visible in cells after being internalized. Various cells types including cancer cells (MCF-7, MDA-MB-231), neurological cells (Schwann cells) as well as stem cells (Bone marrow and Adipocyte-derived MSCs) shed EVs in culture media and are available for biomarker discovery or cargo manipulation and tracking. *Summary/conclusion*: Taken together, the tools presented offer a comprehensive system for cargo labeling and manipulation to enhance applications in using EVs for therapeutic delivery. Furthermore, investigation of protein and/or RNA content in available, premade EVs harvested from various cell types will allow for the easy tracking of EVs through cargo labeling as well as accelerate the discovery of novel biomarkers.

Sponsored poster

P-V-15

Methods for engineering exosome cargo and programming target cell delivery

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Introduction: Extracellular vesicles (EVs) are naturally occurring nano-sized shuttles used by cells to communicate at both short range (i.e. across neural synapses) and at long range (i.e. between different tissue and organ types) within the body. There is great potential for EVs to be utilized in clinical settings to deliver specific RNAs, proteins, small molecule drugs and other biologics. To this end, we have developed novel technologies to package specific RNAs and proteins into engineered EVs. We have also built a system to efficiently display cell type specific surface ligands on EVs to program their delivery destinations. *Methods*: To achieve EV loading of miRNAs and anti-miRNAs into exosomes, a short nucleic acid motif was added to the 3′ end of a transfection ready mature miRNA oligo or the 3′ end of a hairpin miRNA sequence expressed from a lentivector. EV loading was validated using qRT-PCR, and target cell delivery was verified by 3′ UTR-linked luciferase reporter assays and Western blot analysis of cognate targets. To achieve EV loading of proteins, a sequence encoding a membrane inner leaflet targeting peptide was fused to the protein's open reading frame encoded in a lentivector, resulting in N-terminal fusion of the targeting peptide with the protein of interest. EV loading was validated using Western blots and enzymatic assays, and target cell delivery was confirmed using fluorescence microscopy, Western blots and enzymatic as well as functional assays. To achieve presentation of ligands on the EV membrane surface, the C1C2 domain of the MFG-E8 gene was fused to the ligand's open reading frame encoded in a lentivector, resulting in C-terminal fusion of the C1C2 domain to the ligand of interest. Cellular specific targeting was confirmed *in vitro* by analyses of the efficiency of labeled exosomal cargo delivery to various human cell lines and *in vivo* by mouse tail vein injection of luciferase containing exosomes displaying various surface ligands followed by whole body imaging. *Results*: We have confirmed EV loading and bioactivity within target cells of several miRNAs and anti-miRNAs. Additionally, we have shown EV loading of various reporter proteins (GFP, RFP, Luciferase and HRP) as well as confirmed target cell delivery. Further, we have shown that loading of the puromycin resistance gene in EVs and subsequent target cell delivery confers puromycin resistance to target cells, indicating that delivered proteins are functional within the target cell environment. We also show efficient EV coating of surface ligands, which specify target cell delivery by analysis of exosomal cargo delivery *in vitro* as well as *in vivo*. *Summary/conclusion*: Taken together, this complete system to engineer EV cargo and program target cell delivery enables the creation of designer EVs, which will deliver designated RNA and protein cargo to specific cell types, allowing steps forward in realizing exosomes as therapeutic shuttles.

Sponsored poster

Poster session VI - EVs in cancer I
Chairs: *Jason Webber and Kenneth W. Witwer*

P-VI-1

Immunosuppressive exosomes present in human ovarian tumour microenvironments rapidly and reversibly arrest T-cell activation

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Please see Symposium Session 2C

P-VI-4

Inhibition of uptake of tumour-derived EVs by monocytic cells: ''eat me'' versus ''don't eat me'' signals

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Introduction: Extracellular vesicles (EVs) are released by most tumours types, including glioblastoma (GBM) and play a key role in the creation of an immunosuppressive microenvironment via uptake by monocytic cells. GBM-derived EVs are considered to enable recruitment and modulation of monocytic cells to support tumour proliferation, invasion, vascularization and immune-evasion. Therefore, identifying EV ''eat me'' and ''don't eat me'' signals on monocytes and blocking selective EV internalization pathways may provide a new immunotherapeutic strategy against GBM. *Methods*: Using a druggable siRNA screen we explored potential ''eat me'' and ''don't eat me'' signals for GBM-derived EVs. We developed a flow cytometric based GBM-derived EVs uptake assay that allowed the monitoring of the uptake of PKH67-labelled GBM-derived EVs by $CD14^+$ human monocyte-derived macrophages in vitro. *Results*: These cells efficiently take up primary stem-like GBM-derived EVs in a dose-dependent manner. Notably, antagonist of a chemokine receptor inhibited EVs uptake by macrophages, whereas several ''don't eat me'' signals are subject of study. *Summary/conclusion*: These findings indicate that specific receptors can function as ''eat me'' or ''don't eat me'' signals for GBM-derived EVs on monocytic immune cells. Small molecule and antibodies directed against these receptors are currently being evaluated for their propensity to evoke immune attacks against GBM in a EVs-dependent manner in vitro and in vivo.

P-VI-5

Cell damage results in altered exosome profile of nasopharyngeal carcinoma cells

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Introduction: Head and neck cancers are the sixth most common cancer worldwide. One of the most remarkable malignancy of the head and neck is the cancer of the nasopharynx, with a tendency for spreading metastasis in the early stage. Beside the conventional pathways of metastasis formation, the information content of exosomes produced by the cancer cells may play a key role in the metastatic disease. The purpose of this study was to investigate how cytostatic therapy changes the characteristics of the tumour-derived exosomes. *Methods*: In our experimental model we compared the quantity and content of exosomes produced by a nasopharyngeal carcinoma cell line (5–8F) under conventional (chemoterapy) and alternative ($AgTiO_2$ catalyzed reactive oxygen species generation) cytostatic treatment. MicroRNA content of the nasopharyngeal cell-derived exosomes was analyzed with SOLiD 5500xl technology. The sequences were annotated in CLC Genomics Workbench version 5.5.1. Exosome quantitation was performed with Nanosight NS500 device. *Results*: The cytostatic activity of $AgTiO_2$ in a photo-catalytic process was commensurable with a classic chemotherapeutic agent (doxorubicin). We have proven that the tumour cell devastation altered both the number and the content of the exosomes. We have found significant changes of the expression rate of mir-205, mir-451a, mir-125a, mir-30d, mir-30c-1, mir-30c-2, mir-425 and mir-17. *Summary/conclusion*: The significantly increased quantity of the exosomes may potentiate the information transfer from tumour cells to the surrounding stroma cells, perhaps this could promote metastasis formation during cytostatic treatment. Since the microRNA profiles showed significant differences after cytostatic versus photocatalytic treatment, our results point to the role of cell damage in of exosome production.

P-VI-6

Melanoma-derived EVs as drivers of immunosuppression

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Introduction: Evolution of metastatic melanoma from a primary tumour of the skin to widespread dissemination is crucially dependent on early regional lymph node metastases. Characterization of tumour-draining, sentinel lymph nodes (SLNs) in patients reveals an immunosuppressed state amenable to tumour growth and progression. Importantly, this immune profile was detected in the SLN irrespective of metastatic status. The observation that regional immunosuppression is independent of nodal involvement suggests the lymphatic microenvironment is altered prior to clinical evidence of metastasis and, therefore, an alternative mechanism independent of tumour cells is responsible for initiating this process. In the current study, we evaluated melanoma-derived EVs for their potential to polarize immunity towards an immunosuppressive, tumour-promoting state. *Methods*: EVs were isolated from human melanoma cell lines (A375, SKMEL28, C32TG), cultured under normoxic or hypoxic conditions and characterized by protein and RNA composition, size distribution and concentration. Proteomic and RNA profiling was analyzed by Metacore pathway analysis and comparative analysis using the PlateletWeb proteomic database. For functional analysis, melanoma-derived EVs were co-cultured with human monocyte-derived dendritic cells and dendritic cell maturation and activation was assessed by phenotyping surface marker expression and mixed lymphocyte reaction proliferation assays. *Results*: Hypoxic stress increased EV production and size distribution in all melanoma cell lines assessed. The proteomic and genomic analysis of vesicular cargo identified immune signatures both unique to each and shared among

all melanoma cell lines including ICAM1 and Galectin 3 binding protein. Of note, striking homology exists between proteomes of melanoma-derived EVs and platelets, suggesting platelets serve as a valuable model to study EV functions. Melanoma-derived EVs demonstrate a functional capacity to dramatically suppress dendritic cell maturation and activation in vitro. *Summary/conclusion*: The enrichment of melanoma-derived EVs for immune mediators and ability of the vesicles to suppress dendritic cell function in vitro supports our proposed model in which melanoma-derived EVs traffic to regional lymph nodes in advance of nodal involvement to foster an immunosuppressive environment promoting metastasis. We are currently translating these findings into SLNs of patients to identify evidence of melanoma-derived EVs in metastasis.

P-VI-7

The adenosine pathway in ovarian carcinoma: tumour cells and tumour-derived exosomes express CD39 and CD73 ectonucleotidases, produce adenosine and mediate immune suppression

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Introduction: Ectonuleotidases CD39/CD73 have been reported to play an important role in functional suppression of various immune cells via adenosine that is generated locally in the tumour microenvironment. In patients with ovarian cancer (OvCa) exosomes released by tumour cells (TEX) are abundant in plasma or ascites and may be involved in tumour progression. Since TEX usually carry proteins that are expressed in tumour cells from which TEX originate, we hypothesized that CD39+ and CD73+ TEX could deliver these enzymes to distant immune cells and suppress their functions or elevate suppressor activity of regulatory T cells (Treg). *Methods*: We investigated the expression and clinical significance of CD39, CD73, adenosine deaminase (ADA) and CD26 in OvCa tissues by immunohistochemistry (IHC) and in TEX isolated from patients' plasma by Western blots. ATP hydrolysis by TEX was measured using a luciferase detection assay. The phenotype of NK cells and Treg separated from PMBC of normal donors was evaluated by flow cytometry after co-culture with TEX. *Results*: By IHC in tissue sections, 70% of tumour cells were CD39+, 77% were CD73+ and 100% were CD26+ADA+. Expression levels of the ectonucleotidases varied from strong to moderate, and patients with a more advanced disease stage had tumours showing strongest CD73 expression ($p < 0.05$). Exosomes isolated from plasma of OvCa patients, in contrast to TEX from healthy donors, carried CD39, CD73, ADA and TGF-β1 as well as the tumour markers MAGE3/6 and Ep-CAM. They hydrolyzed more exogeneous ATP and produced more adenosine ($p < 0.05$) than did TEX from OvCa cell line supernatants and from healthy donors ($p < 0.05$). After co-incubation with TEX, normal NK cells downregulated expression of NKG2D, NKp44 and NKp46 ($p < 0.05$) and Tregs up-regulated expression of perforin, FasL, CCR7 ($p < 0.05$) and showed increased suppression of responder cells ($p < 0.01$). *Summary/conclusion*: Exosomes isolated from the plasma of OvCa patients were found to carry enzymatically-active ectonucleotidases and to produce extracellular adenosine. These exosomes were capable of down-regulating NK cell functions and up-regulating Treg activity in vitro. These TEX-mediated mechanisms could contribute to tumour-induced immune suppression characteristic of OvCa, resulting in tumour immune escape and OvCa progression.

P-VI-8

Exosomes confer pro-survival signals to alter the phenotype of prostate cells in their surrounding environment

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Introduction: Since their discovery in 1969 by Anderson, EV research has grown exponentially. Cancer cell-derived exosomes (CEV) in particular have been one of the main areas of interest for EV scientists. These effects occur via blocking or inducing specific pathways and are possible as a result of their extensive range of bioactive molecules. It is very well known that cancer cells produce many of their own growth factors to sustain independent proliferative growth signalling. MAPK and PI3K/Akt pathways are recognized as the main cytoplasmic signalling pathway that play a central role in growth signalling. Due to their large array of proteins as well as their role in disease progression, numerous studies have already looked into the effects of CEV on different signalling pathways within the neighbouring cells in their microenvironment. *Methods*: Exosomes were purified from the conditioned media of AR+/− ve PCa cell lines. Further analysis using nanosight, western blot and transmission electron microscopy validated the size, purity and integrity of isolated exosomes. Different functional assay including apoptosis, *real Time cell analysis,* 3D migration and cell motility were performed to investigate the role of PCa-derived exosomes in PCa progression. Finally LNCaP xenografted nude mice were treated with 2 different dosage of PCa-derived exosomes, and the level of PSA and tumour growth were assessed. *Results*: We investigate the role of AR +/− ve, PCa CEV on PCa tumour growth and progression. We have also reported the effects of exosomes derived from PCa cells on the PSA level and tumour growth of mice bearing human PCa tumour xenografts when they have been systemically introduced via IV injection. While the primary emphasis of this research was to understand the effects of different PCa-derived exosomes, with distinct AR phenotypes, on cell-cell communication as they confer changes in cellular properties of neighbouring cells in a tumour population, further studies are required to achieve a deeper and more precise understanding of the role of exosomes at the molecular level as it pertains to cancer progression and metastasis. *Summary/conclusion*: Our experimental evidence indicates that exosomes with different AR phenotype attribute positively in most of mechanisms that contribute to prostate cancer progression.

P-VI-9

Identification of exosomal markers for taxane-resistance and progression of prostate cancer

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Introduction: In order to allow a specific diagnosis of cancer by examining blood exosomes, it is a prerequisite to identify exosomal markers associated with disease states. We have recently reported that exosomes derived from prostate can be isolated from blood using magnetic beads conjugated with anti-PSMA (prostate-specific membrane antigen) antibody. In the present study, we aimed to identify exosomal protein markers related to taxane-resistance and progression of prostate cancer. *Methods*: Exosomes were isolated by differential centrifugation of culture media from docetaxel-resistant prostate cancer PC-3 cells (PC-3R) and their parental PC-3 cells. Exosomes were subjected to Western blot analysis and iTRAQ-based quantitative proteomic analysis. Silencing of protein expression was conducted by siRNA transfection. Exosomes were isolated from the serum of patients by differential centrifugation and subjected to Western blot analysis. This study was approved by the Bioethics Committees and written informed consent was obtained from all patients. *Results*: P-glycoprotein (P-gp) encoded by multidrug resistance protein 1 (MDR1) gene plays an important role in drug-resistance.

The expression of P-gp was higher in exosomes as well as cell extracts from PC-3R cells than in those from PC-3 cells. Unlike other taxanes such as docetaxel and paclitaxel, a novel taxane, cabazitaxel, has poor affinity for P-gp and is thereby effective in docetaxel-resistant, castration-resistant prostate cancer (CRPC). Indeed, cabazitaxel effectively killed PC-3R cells and MDR1 knockdown improved the sensitivity of PC-3R cells to docetaxel but not to cabazitaxel. The P-gp level in blood exosomes was relatively higher in patients with clinically docetaxel-resistant patients than in therapy-naïve patients. We also performed proteomic analysis of exosomes isolated from cells and found that expression of Integrin, beta 4 (ITGB4) was increased in PC-3R cells compared with PC-3 cells. Silencing of ITGB4 expression inhibited migration and invasion, but did not affect proliferation and taxane-resistance in PC-3R cells, suggesting its importance in progression and metastasis. *Summary/conclusion*: We identified P-gp and ITGB4 as exosomal markers for docetaxel-resistance and progression of prostate cancer, respectively, and also demonstrated that P-gp expression in blood exosomes was elevated in docetaxel-resistant prostate cancer patients than in therapy-naïve patients. Increased ITGB4 expression has been shown to be associated with progression and poor prognosis in various cancers including prostate and pancreatic cancer. When combined with a surface marker specific for a certain type of cells (such as PSMA for prostate epithelial cells), detection of exosomal P-gp and ITGB4 in blood would allow us to select treatment (such as docetaxel or cabazitaxel) and predict prognosis as well as to diagnose drug-resistance and the stage of progression.

P-VI-11

Altered compartmentalization of KIT enhances death receptor 5 and KIT-enriched exosome release by imatinib-treated gastrointestinal stromal tumours

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Please see Oral with poster B

P-VI-12

The effect of extracellular vesicles (EVs) from cancer-associated fibroblasts (CAFs) on oral/mobile tongue cancer cell (HSC3) invasion in vitro

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Introduction: Oral cancer is among the most common malignancies worldwide. Although treatment has improved, a major challenge lies in its early-diagnosis. Cancer microenvironment and cancer-associated fibroblasts (CAFs) are shown to regulate cancer growth and invasion by producing for example growth factors and cytokines. CAFs may load these molecules into extracellular vesicles (EVs) and mediate their effects this way. The current project studies if EVs of CAF origin affect invasion of tongue cancer cell line, HSC3, differently compared to EVs of normal fibroblasts (NFs). *Methods*: Four CAF (CECGA, CECORO, MCA, MCD) and four NF cell lines (MNFB, MNF3, MNF4, TGN1) were used. To produce EVs, cells were plated at $0.5 \times 10^6/175\ cm^2$. After 24 hours serum-free medium was exchanged on cells and conditioned media were collected 48 hours later. EVs were isolated with ultracentrifugations of 10,000 $\times$ g; 90 minutes and 100,000 $\times$ g; 90 minutes at 4°C. Composition and number of EVs was assessed using nanoparticle tracking instrument (NanoSight NS300). The effect of EVs on HSC3 cell invasion was studied in transwell invasion assay. HSC-3s in serum-free medium were plated with 5.76×10^6 EVs (determined using NanoSight) into 24-well transwells, pre-coated with gelatinous protein mixture and allowed to invade for 48 hours. At the end of invasion assay cells were fixed and stained, and the number of invading cells was studied using a microplate reader. We are yet to characterize EVs using transmission electron microscopy (TEM) and to finish mass spectrometry analyses for all cell lines (LC-MS/MS). *Results*: The transwell invasion assay showed that EVs of CAF origin induced invasion of HSC3s significantly compared to EVs of NFs. Preliminary results show differences between proteomes of CAF and NF EVs. *Summary/conclusion*: EVs of CAF origin induce invasion of HSC3s compared to EVs of NF. The final results will enlighten the interaction between CAFs and HSC3s, and the significance of CAF produced EVs in HSC3 invasion.

P-VI-13

Marrow stromal cell exosomes frustrate temozolomide treatment of glioblastoma

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Introduction: Bone marrow stromal cells (MSCs) produce exosomes which can be found in circulating blood. Temozolomide (TMZ) is an alkylating agent used for the treatment of glioblastoma multiform. As TMZ is distributed systemically, and therapy can result myelosuppression, we tested the response of TMZ upon MSC exsomes, and the profile of non-coding RNAs within the exosomes. Furthermore, we tested if MSC exosomes altered the effect of TMZ upon glioblastoma cells. To this end, we treated MSCs with TMZ in vitro and performed miRNA PCR arrays upon exosomes produced by the MSCs (tMSC-exo). We also treated glioblastoma cell lines with tMSC-exo and measured the effect upon glioblastoma cell viability, growth and response to TMZ. *Methods*: Exosomes were harvested from primary human MSCs using ExoQuick-TC precipitation solution. Real-time PCR was employed to detect miRNA or vault RNAs in MSC exosomes or U87 human glioblastoma cells. BrdU, LDH and MTT assays were performed to test tumour cell proliferation, TMZ cytotoxicity and cell viability, respectively. The Izon qNano system was used to analyze and quantify harvested exosomes. MSCs were treated with a dose range of TMZ of 5–100 μM. *Results*: tMSC-exo elicit a protective effect upon U87 glioblastoma cells treated with TMZ. TMZ treatment significantly alters the miRNA profile of MSC exosomes and increases exosome vault RNA. The protective effect of tMSC-exo is likely mediated by inducing senescence in tumour cells. *Summary/conclusion*: Our findings indicate that MSCs exosomes may diminish the efficacy of TMZ treatment of glioblastoma.

P-VI-14

Proteomics of prostate cancer stroma reveal the onset of similar myofibroblasts in response to exosomes or soluble TGF-beta as a stimulating factor

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Introduction: In prostate cancer, the stroma surrounding the tumour becomes perturbed, resulting in the chronic presence of myofibroblastic cells supporting tumour growth and ultimately disease progression. Cancer cell TGF-beta1 is a documented factor responsible for driving stromal differentiation to myofibroblasts. We recently demonstrated cancer exosomes can also activate this phenotype-from primary normal prostatic stromal cells. It is currently unclear which stimulus (exosomes or TGF-beta) gives a myofibroblast phenotype that best reflects the perturbed, cancer-educated stromal

myofibroblast in vivo. We hypothesized that exosomes and not soluble TGF-beta are key to generating disease-relevant myofibroblasts, and we used a proteomic profiling approach to address this question. *Methods*: Pairs of prostate biopsies were taken at the cancer site (representing disease) or from the other half of the prostate with no clinical disease (representing normal stroma). Primary stromal cell cultures were generated from these (6 patients). Normal stroma was stimulated with soluble TGF-beta or prostate cancer (Du145 cell) exosomes. Following 3 day differentiation whole cell lysates were prepared, and tryptic digests performed with a 4-plex iTRAQ labelling protocol (AB Siex). Samples were analyzed by LC/MALDI and the protein profiles were compared. Differentially expressed proteins were identified, and some confirmed by western blotting. *Results*: TGF-beta or exosomes successfully triggered myofibroblastic differentiation of normal stroma in 6/6 donors. Comparing the proteome of normal versus naturally occurring diseased cells revealed differential expression of over 40 proteins, including elevated CALD1, TAGLN, CALM1 and reduced BASP1, ALDH1A1 and CAV1 ($FC \geq \pm 1.5$, $p < 0.001$). Changes triggered by TGF-beta or exosomes showed some overlap in phenotype with downregulated BASP1 and ALDH1A1. Robust FN elevation was however unique to TGF-beta stimulation and not a property of exosome-induced or natural myofibroblasts. *Summary/conclusion*: We document changes in several proteins related to cytoskeleton and extracellular matrix, which may show utility for defining cancerous-stroma in future studies. Exosomes or TGF-beta induce a similar profile however that is not readily distinguishable.

P-VI-15

Extensive analysis of four ovarian cancer cell lines and their production of extracellular vesicles during normoxic and hypoxic conditions

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Introduction: Extracellular vesicles (EVs) are one of several tools cells use to communicate with each other. It is therefore expected that cell culture supernatants contain EVs with specific phenotypes reflecting the cells producing them. The hypoxia-induced release of EVs from cancer cells has been hypothesized to cause the malignant transformation of normal recipient cells, which results in malignant cell proliferation and migration. Taken together this suggests that EVs play a part in cancer in general and in the spread of cancer. Understanding the EVs signalling in cancer cell communication and the phenotype of cancer cells and cell-derived EVs may help to overcome the therapeutic challenges in cancer treatment. *Methods*: Different concentrations of the ovarian cancer cell lines COV 504, SKOV 3, OAW 42 and Pt 4 were subjected to normoxic or hypoxic conditions. The cells and the cell culture supernatants were harvested after 12 and 24 hours. The cellular phenotype was analyzed by flow cytometry using 5 cellular markers (CAIX, CAXII, CD9, CD81 and CD151). The EVs were phenotyped (for 31 protein markers) and the amount estimated (a-CD9, -CD63 and -CD81) using the EV Array (Jørgensen et al., 2013, JEV). *Results*: Data showed that independent of cell line, confluence and cellular concentration, cells subjected to hypoxic conditions for 24 hours produce more EVs than cells subjected to normoxic conditions, even though, there were more living cells after 24 hours incubation in normoxia versus hypoxia. Furthermore, the cellular phenotype is only slightly affected by time, cellular concentration and hypoxia, whereas the EV phenotype seems to be considerably more affected by these conditions. *Summary/conclusion*: These data indicate that cells subjected to normoxia focus mostly on proliferation whereas the cells subjected to hypoxia focus on EV release. In addition, data suggests that, EVs are used by the cells for fast and effective communication with their surroundings.

Reference

1. Jørgensen M, Bæk R, Pedersen S, Søndergaard EKL, Kristensen SR, Varming K. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. Journal of Extracellular Vesicles. June 2013.

P-VI-16

Platelets and extracellular vesicles in host–glioblastoma communication

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Introduction: Extracellular vesicles (EVs) are released from many cell types, including platelets. In recent years, it has become increasingly clear that both EVs and platelets play a role in cancer progression and metastasis. In addition, platelets are known to release high numbers of EVs upon activation (platelet EVs are also called platelet microparticles or PMPs). Activation of platelets and subsequent release of their EVs may contribute to tumour growth. While this has been suggested for some tumour types, many aspects are still unknown, especially in the case of glioblastoma (GBM). In this project we attempt to study the interactions that take place between platelets, GBM and EVs. *Methods*: We created mT/mG reporter cell lines of both human GBM8 and mouse GBM cells. These reporter cells carry a floxed allele for membrane-targeted tdTomato (mT) that can be excised by expression of Cre-recombinase (Cre). Excision results in alternate expression of eGFP, effectively switching red fluorescent cells to green. EVs of Cre-expressing cells are expected to contain Cre mRNA that may drive Cre expression in target cells. We used the PF4-Cre transgenic mouse model that expresses Cre in megakaryocytes and platelets. Nude mice were intracranially (i.c.) injected with human GBM8-mT/mG cells. Platelets were isolated from the blood of PF4-Cre mice and injected intravenously (i.v.) via the tail vein into the GBM8-mT/mG recipient mice. As a negative control, we injected isolated platelets from wildtype mice. For syngeneic mouse GBM models, we also performed PF4-Cre bone marrow transplantation in FVB:129/Ola (F1) recipient mice followed several weeks later by i.c. injection of mouse GBM-mT/mG tumour cells. *Results*: Fluorescence imaging of brains of nude mice bearing human GBM8-mT/mG tumours showed switching of tumour cells from tdTomato (red) to GFP (green) in the mice injected with PF4-Cre platelets, but not in control mice. Studies in syngeneic GBM mouse models are on-going and will be reported at the ISEV 2015 meeting. *Summary/conclusion*: This red-to-green switch in recipient GBM reporter cells suggests that platelets home to the tumour site, where they are activated and release their EV payload. These mT/mG reporter models will allow functional studies to determine the effect of platelets and their EVs on GBM in physiologically relevant in vivo tumour models.

P-VI-17

Cetuximab treatment alters effects of tumour cell-derived extracellular vesicles on recipient cells

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Introduction: Due to overexpression or mutations, epidermal growth factor receptor (EGFR) is overactive in several cancer types. Hyperactivity of EGFR leads to excessive growth of tumours and is associated with increased angiogenesis and metastasis. EGFR is a validated target for cancer therapy with several inhibitors in the clinic. Cetuximab is a monoclonal antibody that binds to EGFR and thereby competes with its natural ligand epidermal growth factor (EGF). Extracellular vesicles (EVs) released from tumour cells bear oncogenic

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proteins such as EGFR and promote angiogenesis in endothelial cells. Here, we assessed the effects of cetuximab treatment on content and function of tumour cell-derived EVs. *Methods*: Viability of A431 cells after cetuximab treatment was assessed using MTS assay. EVs were isolated using differential (ultra)centrifugation. Nanoparticle tracking analysis (NTA) and microBCA protein assay were used to determine EV number and protein content. EV markers and EGFR were detected by Western Blotting. Effects of EVs on endothelial cells were assessed in angiogenesis assays and using Western Blotting for downstream signalling pathways. *Results*: Cetuximab inhibited viability of A431 cells in a dose-dependent manner. EVs released from cetuximab-treated A431 cells differed from EVs released by non-treated cells in content, but not in number. Levels of Akt, pAkt, MAPK and pMAPK were lower in EVs derived from cetuximab-treated cells. When endothelial cells were treated with EVs originating from cetuximab-treated A431 cells, cellular activation was reduced. *Summary/conclusion*: Our results indicate that besides its direct effect on tumour cells, cetuximab may have indirect effects on endothelial cells by altering the content and function of tumour cell-derived EVs.

P-VI-18

The role of extracellular vesicles in oral cancer progression

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Introduction: Oral cancer includes cancers of the throat, tongue and mouth. The latest Cancer Research UK figures place the annual number of new cases and deaths at 6,767 and 2,119 respectively; worryingly oral cancer mortality rates are slowly on the rise increasing by 10% in the last decade. Efforts to improve survival rates are hampered by a limited understanding of the molecular complexity of the disease. Recently, interest has grown in the contribution of extracellular vesicles (EVs) to cancer pathogenesis. EV's are produced by most cell types but are produced in higher quantities by cancer cells. Since the discovery of mRNA and miRNA in EVs they have been considered as a major signalling system capable of exerting powerful behaviour changing effects on local or distant cells. Developing tumours exist as a complex milieu comprising multiple cell types each capable of producing a range of EVs with pleiotropic functions. The aim of this work is to explore the role of EV's and in particular their miRNA cargo, in oral cancer progression. *Methods*: EV's were extracted from the culture media of oral cancer cell lines using ultracentrifugation and then characterized using TEM and western blotting. EV's were labelled with fluorescent markers and transferred to cells of a different line to visualize transfer of RNA. *Results*: We have successfully isolated EVs from a panel of cell lines representative of the stages of oral cancer development and confirmed their presence by western blot and transmission electron microscopy. Using fluorescently labelled EV's the horizontal transfer of RNA between oral cancer cells and stromal cells that would be present in the tumour microenvironment has been visualized. *Summary/conclusion*: Using a combination of techniques the beginnings of an EV mediated signalling network in the oral cancer microenvironment has been revealed. Future work will identify the RNA and protein cargo of the isolated EVs in order to identify their roles in oral cancer progression.

P-VI-19

On the role of breast cancer exosomes in platelet activation and aggregation

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Introduction: Thrombosis is a major cause of mortality in cancer patients. There is increasing evidence supporting the participation of tumour-derived extracellular vesicles in cancer-associated thrombosis. Tumour-derived vesicles may carry tissue factor (TF), the clotting initiator protein, as well as to modulate platelet function. In this study we evaluated the ability of tumour-derived exosomes in promoting platelet activation, aggregation and plasma coagulation. *Methods*: We employed 2 human mammary carcinoma cell lines: MCF-7 (non-metastatic) and MDA-MB231 (highly metastatic). Exosomes were isolated from conditioned media using Exoquick^{TM}, and particle size was further confirmed on a LM10 nanoparticle analyzer. Platelet/exosome interaction was evaluated by confocal microscopy and flow citometry analyses. Platelet aggregation was measured on a Chronolog aggregometer. *Results*: The mean size of tumour-derived extracellular vesicles ranged from 146 to 151 nm for both cell lines. Incubation of fluorescently labelled exosomes with platelets promoted P-selectin exposure, a known platelet activation marker. Such effect was more pronounced upon incubation with MDA-MB231-derived exosomes. On the other hand, exosomes did not induce platelet aggregation. Exosomes from MDA-MB231 cells showed a higher TF level and procoagulant activity as compared to MCF-7-derived exosomes. Accordingly, MDA-MB231-derived exosomes accelerated platelet aggregation on platelet rich plasma in a TF-dependent fashion. *Summary/conclusion*: Our results suggest that mammary carcinoma-derived exosomes interact with platelets, mediate their activation, and also favour plasma coagulation and platelet aggregation through TF-dependent thrombin generation. This work was supported by CAPES, CNPq and FAPERJ.

P-VI-20

Development of a biomarker-based microvesicle assay for prostate cancer prognosis

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Introduction: The use of PSA as the gold standard for diagnosing prostate cancer (PCa) has been challenged recently, and the search for new PCa biomarkers has increased. Although the PSA test has acceptable sensitivity, it lacks the necessary specificity to discriminate benign prostatic diseases, resulting in overdiagnosis and overtreatment. New, more specific biomarkers for PCa are needed to prevent unnecessary surgical biopsies. The analysis of extracellular microvesicles in biofluids is an exciting new area of investigation. *Methods*: A diagnostic test must differentiate microvesicles originating from PCa cells versus non-cancerous origins and also define a biomarker which is enriched in the PCa microvesicle population. Three prostate-specific membrane antigen (PSMA) antibodies, each of highly specific for PCa cells, were tested; 3/E7, 3/A12 and J591. As a potential biomarker, the CD151 antibody 1A5, which recognizes a subset of the tetraspanin family, integrin-free CD151 was tested. All antibodies were directly conjugated to fluorophores and tested for functionality by assessing surface expression in LnCap cells. Plasma and urine were collected from non-PCA, treated PCa and drug-naïve metastatic PCa patients. Initial studies examined plasma preparation and storage as a source of variation. Next, assay conditions were optimized. Finally plasma was sequentially centrifuged to provide platelet rich, plate poor and exosomal fractions and then assayed to determine the levels of PSMA+, PSCA+ and CD151^{free}+ microparticles using the Apogee A50 micro-flow cytometer. *Results*: Plasma processing had significant effects on PSMA+ populations. Fresh plasma had more PSMA+ microvesicles than any other treatment group. Assaying fresh patient plasma is not practical, and plasma fully processed within 2 hours and frozen to -80°C was considered standard. Plasma was sequentially centrifuged to provide platelet rich, platelet poor, platelet depleted and exosomal fractions. No differences between PSMA levels were detected between the platelet-rich plasma of non-PCA and drug-naïve metastatic patients but PSMA+ and 1A5+ microvesicles were significantly enriched in platelet poor plasma

microvesicles. Further centrifugation did not improve this enrichment. The 3/E7 and J591 PSMA antibodies had similar enrichments of PSMA and 1A5 in the drug-naïve metastatic plasma samples. PSCA and 3/12 did not yield any significant differences between groups. *Summary/conclusion*: Using PSMA antibodies to identify prostate-derived microvesicles, we have shown that variations in plasma processing can significantly change the PSMA+ microvesicle population. To this end, we have standardized a 2 hours blood processing protocol for all prospective sampling and have determined that the platelet poor plasma fraction is optimal to see differences in PSMA+/1A5 microvesicles between non-PCA and metastatic PCa patients. In summary, we have developed an assay that can distinguish metastatic PCa from non-PCa using a simple flow cytometry assay.

P-VI-21

Differential profiling of smoker versus non-smoker broncho alveolar lavage extracellular vesicles: towards tobacco smoke exposition biomarkers identification?

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Introduction: Evaluation and understanding of tobacco health effects are of major interest worldwide and answer to important societal concerns. Identification of new biomarkers of exposure to tobacco smoke potentially implicated in lung carcinogenesis would allow a better observation of tobacco exposed population, thanks to screening establishment at reversible stages of pathological processes. In this study, Bronchial Alveolar Lavages (BALs) were carried out on 10 smokers and 10 non-smokers, and Extracellular Vesicles (EVs) were isolated from the supernatants, for identification of characteristic RNAs or specific membrane markers. Our purpose was to evaluate EVs as potential biomarkers of tobacco exposure. *Methods*: *BALs obtention:* The experimental design was approved by the ethical committee (Comité de Protection des Personnes Nord-Ouest, France; ECH11/03 DC-2011-1393). BALs were performed by bronchoscopic procedure. *EVs isolation*: BALs were submitted to differential centrifugations, 0.2 μm filtration and ultracentrifugations. *RNA extraction and RT-qPCR*: Small and large RNAs were obtained separately from BAL EVs thanks to columns extraction kit. RNAs quantity and quality were measured by a Bioanalyzer. RT-qPCR was performed with Taqman procedure. *Flow cytometry:* BAL EVs were fixed to magnetic beads thanks to exosome-specific antibodies and -specific markers were quantified. *Results*: Expression of miR-26b, miR-223 and miR-30a, miRNAs known as induced by environmental pollutants or expressed in lung cancers, was shown to be higher in EVs isolated from smokers versus non-smokers. In parallel, specific surface markers like EGFR, identified as potential lung cancer biomarker on blood exosomes surface, or MMP-14, a membrane protein found on EVs derived from tobacco smoke exposed macrophages were measured. *Summary/conclusion*: This preliminary study revealed that EVs could be used as biomarkers of exposure to tobacco smoke. Other parameters will be assessed to confirm these results. The most relevant biomarkers could be then used to exposure profiling in blood samples.

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Poster session VII - Late breaker, EVs in disease
Chairs: *Norman Haughey and Pamela Wearsch*

P-VII-1

Expression of microRNAs in plasma exosomes collected in the first trimester of pregnancy complicated by preeclampsia, intrauterine growth restriction or gestational diabetes

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Introduction: Common pregnancy complications such as preeclampsia, intrauterine growth restriction and gestational diabetes are related to placental dysfunction. Recent case-control studies have reported that placenta from pregnancies complicated by preeclampsia and/or intrauterine growth restriction differentially expressed certain microRNAs compared to those from uncomplicated pregnancies. Most of these studies have been conducted on full term placenta samples following birth, which is not appropriate for early biomarker discovery. A recent study reported that placental-specific miRNAs were released into maternal circulation through exosomes, tissue-specific nanovesicles of 30–10 nm diameter secreted by all types of cells including trophoblastic cells. Our objective is to examine the microRNA profile of exosomes in pregnancies complicated by preeclampsia or intrauterine growth restriction or gestational diabetes using blood samples collected in early pregnancy. *Methods*: We performed a case-control study nested in a prospective cohort of pregnant women enrolled at the first trimester of pregnancy, in Centre Hospitalier Universitaire, Sherbrooke, Canada. Five cases of preeclampsia, five cases of intrauterine growth restriction and twenty-three cases of gestational diabetes were selected and each case was matched for parity and gestational age at sampling with 2 controls (uncomplicated pregnancy). Based on literature review, we selected 18 microRNAs (placenta-specific or not) reported with placenta altered expression in preeclampsia and/or intrauterine growth restriction pregnancy or gestational diabetes. Plasma exosomes (including placental exosomes) and microRNAs were purified using ExoRNeasy method. Presence of placental exosomes was verified with immuno-electron microscopy and microRNAs quantification was done using small-RNA chip. The analysis of relative expression of the 18 microRNAs was done by quantitative real-time polymerase chain reaction with home-made assays. MicroRNA *C. elegans* miR-39 was used as ''spike-in'' to allow normalisation of the results. *Results*: We have shown the presence of placental exosomes in blood sample collected between 6 and 15 weeks of pregnancy. For preeclampsia pregnancy we have overexpression of 8 microRNAs (fold change $>1,35$), for intrauterine growth restriction we have shown underexpression of 4 microRNAs (fold change $<-1,23$) and for gestational diabetes we have overexpression of 7 microRNAs. *Summary/Conclusion*: Exosomes as well as miRNAs represent a new avenue in the area of early diagnostic of pregnancy complications. The miRNA profile of placental exosomes may be used as an early biomarker of placental dysfunction. To validate our results we currently conduct a larger prospective clinical study of placental exosomes using blood and urine repeated sampling during first and second trimesters of gestation.

P-VII-2

Organ targeting and enhancement of therapeutic effect of exosome by surface modification with cationized polysaccharide

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Introduction: Exosome is distributed to many part of a body through the bloodstream once it is intravenously injected. Surface modification of exosome with a technique of bioengineering would add the some ability of tissue targeting and enhancement of effect of exosome. Pullulan is a polysaccharide polymer consisting of maltotriose units. It has an ability to be taken-up by hepatocytes through asialoglycoprotein receptor (AGPR) and its application as an efficient transporter of plasmid DNA to hepatocytes has been reported. This research is undertaken to evaluate an application of cationized pullulan as a biomaterial for surface modification of exosome. *Methods*: Cationized pullulan (CP) was made from pullulan with molecular weight 47,300 and spermine. Exosome was collected from the supernatant of mesenchymal stem cell from bone marrow of C57B6 mice with ultracentrifugation. CP was introduced on the surface of exosome by simple mixing. The change of diameter and zeta potential of exosome with CP was analyzed. Presence of CP on the surface of exosome was confirmed by the aggregation induced by *Ricinus communis* Agglutinin that could cause aggregation of pullulan. HepG2 was used for the evaluation of taken-up of fluorescence-labelled exosome with CP *in vitro*. Accumulation of exosome with CP was also observed under the presence of asialofetuin, a competitive inhibitor of AGPR, to confirm the involvement of the receptor. Animal experiments were conducted under a protocol approved by our institutional review board. Exosome with or without CP was injected intravenously into liver injury C57B6 male mice prepared by the intravenous injection of concanavalin-A. Liver and serum samples were collected 24 hours. later to evaluate the histological findings, the alanine aminotransferase (ALT) and cytokine mRNAs expression levels. Phenotypical change of non-parenchymal liver cells was evaluated by flow cytometry. Liver localization of fluorescence-labelled exosome after the injection was also observed. *Results*: Gradual increase of diameter and positive change of zeta potential was observed along with the increase of the concentration of CP added. Increase of number of fluorescent particles with HepG2 was observed in a group of exosome with CP while the number was decreased by asialofetuin treatment. The ALT level was suppressed and histological finding of liver inflammation was improved in the group of exosome with CP in vivo. More fluorescent particles were observed in the same group than a group of exosome without CP. The proportion of regulatory T cell was also increased. *Summary/Conclusion*: Surface modification of exosome with a polysaccharide polymer and the evaluation of modified exosome were done and the enhancement of liver accumulation and the effect of modified exosome was observed. This study would shed light on the novel usage and future potentials of biomaterials for the modification of exosome.

P-VII-3

Lung cancer–derived extracellular vesicles triggered oncogenic signals and increased vascular permeability in an autocrine/paracrine fashion

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Introduction: Extracellular vesicles (EVs) have been shown to play important roles in many diseases including tumour. However, the role of EVs in lung cancer is still largely unknown. In this study, we tried to find out the biological functions of EVs in lung cancer. *Methods*: EVs were isolated from culture supernatants, serum, and malignant pleural effusion (MPE) using ultra-centrifugation and ultra-filtration and then evaluated by TEM, cryo-EM, Nanosight, and western blotting. The biological functions of EVs were analyzed in both *in vitro* cell line model and *in vivo* animal model. *Results*: EVs could be isolated from culture supernatants, serum, and MPE samples using these two methods with different capacity revealed by EM and Nanosight. Specific EV markers including Alix, CD63, and, Tsg101 were detected in the isolated EVs. The EVs carried various RNA species

that small RNAs seemed to be enriched. Furthermore, the EVs could be taken up by lung cancer cells and trigger oncogenic signals such as Stat3 and Akt in an autocrine/paracrine fashion. Previously, we have shown that IL-6/Stat3/tissue factor (TF)/VEGF pathway plays an important role in lung cancer angiogenesis and metastasis as well as the formation of MPE. Here, we showed that EVs from lung cancer samples carried high level of VEGF and TF and triggered vascular permeability changes in mice. *Summary/Conclusion*: Using these methods, we isolated EVs not only from culture supernatants but also various lung cancer associated clinical samples. Furthermore, the EVs triggered oncogenic signals and increased vascular permeability in an autocrine/paracrine fashion. These results may help the understanding of the biological functions of EVs in lung cancer and also the discovery of novel biomarkers and potential drug targets.

P-VII-4

Dendritic cell-derived exosomes/EVs as maintenance immunotherapy after first line chemotherapy in NSCLC

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Introduction: Dendritic cell–derived exosomes (Dex) are small extracellular vesicles (EV) secreted by viable dendritic cells (DC). In the two phase I trials that we conducted using the first generation of Dex (IFN-γ-free) in end-stage cancer (1,2), we reported that Dex exerted NK cell effector functions in patients (3). A second generation of Dex (IFN-γ-Dex) was manufactured from matured DC with the aim of boosting T cell immune responses (4). *Methods*: We carried out a phase II clinical trial testing the clinical benefit of IFN-γ-Dex loaded with MHC class I and class II–restricted cancer antigens as maintenance immunotherapy in HLA-A2^{+} patients bearing inoperable non-small cell lung cancer (NSCLC) responding to or stabilized after induction chemotherapy. Informed consent was obtained and the trial was registered (CSET 2008/1437 IDRCB 2008-A1171-54; NCT01159288). The primary endpoint was to observe at least 50% of patients with progression-free survival at 4 months after chemotherapy cessation. *Results*: Feasibility of IFN-γ-Dex manufacturing was 89% with 75% of products exhibiting a ''mature'' phenotype. Twenty-two patients received IFN-g-Dex and only one patient exhibited grade 3 toxicity. The median time to progression was 2.2 months and median overall survival was 15 months. Seven patients (32%) experienced stabilization of >4 months. The primary endpoint was not reached. One patient with disseminated metastases became operable after 2 years of IFN-γ-Dex maintenance therapy. Although no significant induction of Dex antigen-specific T cells was observed, an increase in NKp30-dependent NK cell functions was evidenced after IFN-γ-Dex treatment in the NSCLC patients presenting with defective NKp30 expression. *Summary/Conclusion*: Corroborating the findings of the two phase I studies, this phase II trial confirmed the capacity of Dex to boost the NK cell arm of antitumor immunity in patients with advanced NSCLC.

References

1. Escudier B, Dorval T, Chaput N, André F, Caby MP, Novault S, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. J Transl Med. 2005 Mar 2;3(1):10.

2. Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. J Transl Med. 2005 Feb 21;3(1):9.

3. Viaud S, Terme M, Flament C, Taieb J, André F, Novault S, et al. Dendritic cell-derived exosomes promote natural killer cell activation and proliferation: a role for NKG2D ligands and IL-15Ralpha. PLoS One. 2009;4(3):e4942.

4. Viaud S, Ploix S, Lapierre V, Théry C, Commere PH, Tramalloni D. Updated technology to produce highly immunogenic dendritic cell-derived exosomes of clinical grade: a critical role of interferon-γ. J Immunother. 2011 Jan;34(1):65–75.

P-VII-5

Severe injury alterations in plasma microparticle phenotypes are associated with transfusion requirements and mortality

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Introduction: Microparticles (MP) are small vesicles shed from damaged or activated cells. They express phosphatidylserine, adhesion receptors, and tissue factor, rendering them highly procoagulant. Circulating MPs are predominantly of platelet origin and support haemostasis and vascular function. Severe injury often presents with increased bleeding (requiring transfusion of blood products) and mortality. Injury provokes cellular activation and release of MPs. The roles of MPs in survival after severe injury are largely unknown. We hypothesized that altered MP phenotypes would be associated with transfusion requirements and poor outcomes. *Methods*: This is a single centre study that was approved by the Institutional Review Board. The study cohort consisted of 166 patients with major trauma who required a blood product transfusion and 26 normal blood donors. Plasma samples for MPs were collected at admission to emergency department ($n = 166$), and post resuscitation ($n = 44$) upon admission to the ICU. Samples were analyzed by flow cytometry for MP counts and cellular origin using antibodies for platelet (PMP; CD41), leukocyte (LMP; CD45), erythrocyte (RMP; CD235a), endothelial cell (EMP; CD146), tissue factor (TFMP; CD142), and Annexin V (AVMP). These data were compared over time (pre vs. post resuscitation), and analyzed with comprehensive demographic, injury, mortality, and other clinical data. *Results*: The median cohort age was 34 (IQR 23, 51), 72% were male, Injury Severity Score was 29 (IQR 19, 36), and 24 hours mortality was 13% (22/166). During the first 24 hours of admission patients received a median of 11 (IQR 5, 23) units of total blood products (median RBC 5U, plasma 6U, platelets 12U). MP levels and phenotypic distribution were different between patients and controls. Highly elevated admission EMPs were found both in survivors (409/μl) and non-survivors (393/μl) compared to controls (23/μl, $p < 0.001$), and remained elevated over time. Admission levels of PMP, AVMP, RMP, and TFMP were significantly lower in patients who died within 24 hours of admission compared to survivors (PMP 1828/μl vs. 2448/μl, p=0.003; AVMP 1753/μl vs. 2719/μl, $p = 0.001$; RMP 230/μl vs. 481/μl, $p = 0.005$; TFMP 180/μl vs. 251/μl, $p = 0.03$). Patients with lower MP levels at admission were transfused with the highest volumes of blood products within the first 24 hours. PMP levels increased over time in patients who survived and decreased in non-survivors, resulting in significantly lower levels at ICU admission in non-survivors compared to survivors (1245 PMP/μl vs. 2655 PMP/μl, $p = 0.005$). *Summary/Conclusion*: Severe injury results in endothelial activation and altered MP phenotypes. Significant differences in specific MP phenotypes were associated with blood product requirements and 24-hour mortality.

P-VII-6

Hypoxia-induced lipid accumulation in prostate cancer cells controls extracellular vesicles biogenesis promoting growth and invasiveness

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Introduction: Hypoxia in prostate cancer (PCA) is associated with an aggressive phenotype and poor prognosis; however, the precise underlying mechanism is still unclear. Here, we studied the role of cellular lipids in extracellular vesicles (EVs) biogenesis as well as growth and invasiveness of hypoxic PCA cells. *Methods*: Ultracentrifugation, NTA and GC-MS techniques were used to isolate and characterize EVs; Immunoblotting to assess protein levels; and shRNA to stable knock-down carnitine palmitoyltransferase 1A (CPT1A) expression. *Results*: Human PCA cells and secreted EVs were enriched in lipids under hypoxic (1% O_2) condition due to the activation of lipogenesis-related enzymes and signalling molecules. Importantly, omega-6 fatty acids linoleic acid (18C:2) and arachidonic acid (20C:4) were strongly represented in the EVs triglycerides ($\sim$5-fold, $p < 0.01$) underscoring their role as bioactive lipid carriers. These fatty acids were also increased in the triglycerides of hypoxic PCA cells, creating a reservoir of bioactive lipids that could stimulate growth following oxygen exposure. As expected, higher proliferation was observed in hypoxic PCA cells (LNCaP, C4-2B and DU145) following reoxygenation associated with rapid use of the lipids. Notably, inhibition of lipid -oxidation pathway by CPT1 inhibitor etomoxir or shRNA-mediated CPT1A knockdown compromised hypoxic PCA cells proliferation following reoxygenation. Also, COX2 inhibitor celecoxib caused strong reduction in the growth and invasiveness of hypoxic PCA cells following reoxygenation, and strongly inhibited the invasiveness induced by hypoxic PCA EVs, establishing the vital role of COX2 enzymatic products therein. Importantly, concentration and loading of EVs secreted by hypoxic PCA cells were strongly reduced under delipidized serum condition and by lipogenesis inhibitors (fatostatin and silibinin). *Summary/Conclusion*: Present study suggests an important role of cellular lipids in EVs biogenesis and in PCA aggressiveness under hypoxia.

P-VII-7

Plasma exosome profile in patients with cardiovascular diseases

Bing Wang[1], Hendrik Zimmet[1], Maria Jose Torres[2], Henry Krum[1], Gregory Rice[2] and Carlos Salomon[2]
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Introduction: Recent studies highlight the putative utility of exosomes in the diagnosis of disease onset and treatment monitoring. To date, there is a paucity of data defining changes in the release, role and diagnostic utility of exosomes in various stress and disease conditions, however, the role of these nanovesicles in subjects with cardiovascular disease remains to be fully elucidated. The aim of this study was to quantify the exosomes vesicles in plasma from patients with Chronic and Acute Heart Failure compared with healthy volunteers. *Methods*: Plasma was obtained from patients with acute (ADHF, $n = 12$), chronic decompensated heart failure (CDHF, $n = 10$) and healthy volunteers (control, $n = 10$). Exosomes were isolated by differential and buoyant density centrifugation using a sucrose continuous gradient and characterised by their size distribution and morphology using the nanoparticles tracking analysis (NTA; Nanosight™) and electron microscopy (EM), respectively. The total number of exosomes and placenta-derived exosomes were determined by quantifying the immunoreactive exosomal marker, CD63 using an ELISA kit (ExoELISA kit, System Biosciences). *Results*: Exosomes were identified as spherical vesicles and diameters ranging from 50 to 100 nm and were positive for CD63. The number of exosomes particles in ADHF subjects averaged $4.8 \pm 1.8 \times 10^9$ number of exosomes vesicles per ml plasma (NEV/ml) and was significantly higher ($p < 0.05$) than that observed in the plasma of subjects with CDHF ($0.87 \pm 0.88 \times 10^9$ NEV/ml) and control ($1.4 \pm 1.3 \times 10^9$ NEV/ml). No significant difference between control and CDHF was identified ($p = 0.39$). Exosomes in patients with ADHF was $\sim$5-fold higher compared to CDHF. *Summary/Conclusion*: This study demonstrated that patient who experience ADHF have higher amount of plasma exosomes compared with CDHF. While the role of exosomes in the onset and development of cardiovascular diseases has not been established, the quantification of these nanovesicles would provide an opportunity to develop and evaluate appropriate intervention strategies to limit acute adverse sequel.

P-VII-9

Regulation of astrocyte EAAT2 gene expression by EV derived from HIV-1-infected cells

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Introduction: Neuronal dysfunction and degeneration are the causative mechanisms for the HIV-associated neurocognitive disorders (HAND). In this study, we hypothesize that EVs (exosomes) released by HIV-1 infected macrophages down regulate expression of astrocyte excitatory amino acid transporter 2 (EAAT2), whose dysregulation is implicated in the pathogenesis of HAND by glutamate mediated excitotoxicity. *Methods*: Exosomes were isolated from conditioned media of U1 cells and U937 cells by ultracentrifugation and Iodixanol (optiprep) gradient centrifugation. The size and concentration of vesicles were analyzed with nanoparticle tracking analysis (NTA) and scanning electron microscopy. The EVs were characterized for markers of exosomes such as Tsg101 and Alix, and Acetylcholinesterase enzyme (AchE) activity. Human foetal brain astrocytes were prepared from brain tissues of gestational age week 16. The expression level exosomal miRNAs were examined by microarray and real time PCR. The expression level of target proteins was detected by western blot. *Results*: The expression levels of numerous miRNA were upregulated in U1 cell-derived exosomes in comparison to the parental cell-derived exosomes. We detected down regulation in the expression of EAAT2, which was predicted in silico using a miRNA-targeting mRNA prediction algorithm, as a target of miRNAs, miR-200b and miR-30a-5p. These two miRNAs were up-regulated in U1 cell-derived exosomes. Furthermore, these miRNAs were validated to target EAAT2 experimentally using luciferase assays. *Summary/Conclusion*: Collectively, these observations demonstrate that miRNA cargo in exosomes derived from HIV-1-infected cell dysregulate the expression of EAAT2 a predominantly astroglial glutamate transporter responsible for the majority of synaptic glutamate clearance in the mammalian brain.

P-VII-10

Exosome fractions of conditioned media from normal and pre-eclamptic placental villi are selectively anti-angiogenic and reversed by low molecular weight heparin

Kalpana Pillai[1], Dora Baczyk[1] and John Kingdom[1]
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Please see Oral with poster B

P-VII-11

Cancer-associated urinary extracellular vesicles as novel bladder cancer biomarker

Yusuke Yoshioka[1], Yuki Konishi[2], Hideo Sasaki[3], Nobuyoshi Kosaka[2], Hideki Ohta[4], Hiroyuki Okamoto[4], Hikaru Sonoda[4] and Takahiro Ochiya[2]
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Introduction: Small membranous extracellular vesicles (EVs) are naturally secreted by almost all cell types including cancer cells

and have been found in various body fluids. Recently, EVs attract much attention as potential biomarker because tumour cells have been shown to release EVs into circulation which mirror their cellular origin. Therefore, detection of tumour-associated EVs in body fluids from cancer patients could serve as a non-invasive liquid biopsy for diagnosis and monitoring of cancer. The main objective of this study is to investigate the potential use of urinary EVs from bladder cancer patients. *Methods*: We have developed a bead-based proximity assay named ExoScreen, which is based on AlphaLISA technique (Yoshioka et al., 2014 Nat Commun). In this assay, EVs are captured by two antibodies modified in distinct ways. One is a biotinylated antibody, and the other is an antibody conjugated with AlphaLISA acceptor beads. To characterize the membrane components of tumour-associated EVs, we performed proteomic analysis using urinary EVs of bladder cancer patients and those of healthy donors. Using ExoScreen system, we then explored the feasibility of the identified membrane proteins as biomarker for bladder cancer patients. *Results*: We first confirmed that ExoScreen using anti-CD9 and -CD63 antibodies enabled us to detect EVs present in 5 μl of healthy donor urine. Our proteomic analysis using clinical samples identified several transmembrane proteins. Of these, we selected 3 proteins as candidate biomarkers, and performed ExoScreen using antibodies against these urinary EV proteins. Urine from bladder cancer patients contained EVs that are double positive for one of these three proteins and a general EV marker, CD9 or CD63, more abundantly than that from healthy donors. *Summary/Conclusion*: ExoScreen propose a novel liquid biopsy technique to detect bladder cancer-specific urinary EVs.

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Scientific Program ISEV 2015 meeting
Friday April 24, 2015
Oral Presentations

Registration	08:00-09:00
Setting up posters (Poster sessions VIII, IX, X, XI, XII, XIII)	08:00-09:00
Ballroom D Meet the experts I: Diversity of EVs Chair: *Clotilde Théry* Speakers: *Jan Lötvall and Stephen J. Gould*	08:00-08:45
Ballroom E Meet the experts II: Stem cells and EVs as therapeutics Chair: *Steven M. Jay* Speakers: *Bernd Giebel and Sai Kiang Lim*	08:00-08:45
Ballroom F-H Meet the experts III: EVs in cardiovascular disease Chair: *Chantal Boulanger* Speakers: *Eduardo Marban and Dominique de Kleijn*	08:00-08:45
Sponsor Exhibition	10:00-18:00

Ballroom D-E
Plenary session - EVs and cancer
Chairs: *Fred Hochberg and Janusz Rak* 09:00-10:15

Xandra O. Breakefield, Ph.D.
Harvard Medical School, Massachusetts General Hospital
The evil little things about cancer: EVs as infiltrators and informants

O-PL-1

Tumour-derived large oncosomes contain kinase activity and can modulate transcription factor activation in the tumour microenvironment

Valentina René Minciacchi[1,2], Lorenzo Cavallini[3], Cristiana Spinelli[1,2], Mandana Zandian[1,2], Rosalyn M. Adam[4], Emanuele Cocucci[5,6], Michael R. Freeman[1,2,4,7] and Dolores Di Vizio[1,2,4,7]
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Introduction: Tumour-derived extracellular vesicles (EVs) can mediate intercellular communication within the tumour microenvironment through activation of signal transduction pathways as well as through direct transfer of their molecular cargo. We recently demonstrated that rapidly migratory ''amoeboid'' prostate cancer (PCa) cells shed large, bioactive EVs, termed large oncosomes (LO), whose abundance correlates with tumour aggressiveness. The goal of this study was to investigate the role of LO in the tumour-stroma cross-talk. *Methods*: We used high-speed centrifugation, iodixanol gradient centrifugation and filtration for EV isolation; flow cytometry and confocal microscopy for EV quantitation and imaging; western blotting (WB); immunoprecipitation and kinase assay; promoter-reporter assay for transcription factor (TF) activity; and tube formation as an angiogenesis assay. *Results*: We demonstrated that LO derived from PCa cells overexpressing MyrAkt1, which harbour significantly higher levels of a constitutively active form of Akt1 in comparison with exosomes, can be internalized by different cell types at different rates. Inhibition of LO internalization by selective block of endocytic pathways suggested that LO uptake occurs via phagocytosis. LO internalization in fibroblasts resulted in altered TF activity, including Myc and Ets factor TFs. Treatment of endothelial cells with conditioned media from fibroblasts previously exposed to LO significantly increased the rate of tube formation, an effect that was potentiated by direct exposure of HUVEC cells to LO. *Summary/conclusion*: Our results demonstrate that tumour-derived LO contain active kinases and can modulate TF activation in the tumour microenvironment. These novel findings suggest important functional roles for LO as mediators of stromal responses to the presence of aggressive cancers.

This study was supported by grants from the National Institutes of Health (NCI NIH R00 CA131472 (to D.D.V.); NIH UCLA SPORE in Prostate Cancer award P50 CA092131 (to D.D.V.); Avon Foundation Fund 02-2013-043 (D.D.V.).

O-PL-2

Extracellular vesicle-mediated transfer of functional RNA in the tumour microenvironment

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Introduction: Extracellular vesicles (EVs) have been shown to transfer various molecules including functional RNA between cells and this process has been suggested to be particularly relevant in tumour-host interactions. However, data on EV-mediated RNA transfer has been obtained primarily by in vitro experiments or involving ex vivo manipulations likely affecting its biology, leaving its physiological relevance unclear. *Methods*: We engineered glioma and carcinoma tumour cells to express Cre recombinase showing their release of EVs containing Cre mRNA in various EV subfractions including exosomes. Transplantation of these genetically modified tumour cells into mice with a Cre reporter background leads to frequent recombination events at the tumour site. *Results*: In both tumour models the majority of recombined cells are CD45+ leukocytes, predominantly Gr1+CD11b+myeloid-derived suppressor cells (MDSCs). In addition, multiple lineages of recombined cells can be observed in the glioma model. In the lung carcinoma model, recombined MDSCs display an enhanced immunosuppressive phenotype and an altered miRNA profile compared to their non-recombined counterparts. *Summary/conclusion*: Cre-lox-based tracing of tumour EV RNA transfer in vivo can therefore be used to identify individual target cells in the tumour microenvironment for further mechanistical or functional analysis.

Networking coffee 10:15-10:45

Poster viewing sessions VIII, IX, X, XI, XII, XIII
Posters not attended by authors 10:15-10:45

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Ballroom D

Symposium session 3A - Novel developments in EV characterization

Chairs: *Erez Eitan and Edwin van der Pol* 10:45-12:00

O-3A-1

A methodology for comparable measurements and characterization of extracellular vesicles in plasma as an international standardization collaboration

Shona Pedersen[1], Sigrid M. Lund[1], Reink Nieuwland[2], Murray Broom[3], Raymond Schiffelers[4], Marike Broekman[5], Andrew Hill[6], Meta Kuehn[7], Anthony de Maio[8], Sybren Maas[5], Edit Buzas[9], Xabier Osteikoetxea[9], Benjamin Scicluna[6], Mitch Shambrook[6] and Amy Phillips[3]
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Introduction: Recently, novel platforms for the detection of extracellular vesicles (EVs) have emerged but clinical evaluations of these new technologies are lacking. To promote effective research of EVs, a crucial pre-requisite is the ability to compare work across different groups using optimized and standardized methods of characterization, analysis and reporting. We report on the results of such a methodology being developed to allow consistent analysis of vesicles isolated from plasma and other complex body fluids. *Methods*: The isolation method is based on size exclusion chromatography (SEC) which is gentle and takes less than 20 minutes. The characterization technique used is Tunable Resistive Pulse Sensing (TRPS), as it is possible to standardize operation to provide repeatable measurements across a defined size range and analyze concentration over a defined population size-range. *Results*: The protocols have been tested using a liposome control and citrated plasma sample measured by 7 groups. Each group will perform 5 measurements to demonstrate the reproducibility across SEC isolation, TRPS measurement and users. Preliminary evaluation of the liposome model across users showed that the TRPS method was capable of 40% coefficient of variation (CV). An improved protocol that mitigates the variation of the nanopore and biological sample interactions has been developed and tested using a double blind study involving 8 participants where a CV of 30% was calculated. *Summary/conclusion*: Accurate, high-resolution characterization of EVs is critical to understanding their properties, function and potential use as predictive markers or therapeutic agents. Isolating EVs using SEC combined with TRPS as high-resolution particle-by-particle analysis technique can provide much needed detail to better understand the use of EVs. We have presented results of this standard measurement protocol that allows for comparable characterization, analysis and reporting of clinical samples.

O-3A-2

Determination of the concentrations of the main populations of extracellular vesicles in normal plasma

Alain R. Brisson, Nicolas Arraud, Céline Gounou, Romain Linares and Delphine Turpin
CNRS-UMR CBMN, University of Bordeaux, Pessac, France

Introduction: Extracellular vesicles (EVs) are proposed to play major roles in health and disease. Many papers have reported increased EV levels in various diseases, yet methods used until now for quantifying EVs suffer from well-known drawbacks. It is, therefore, critical to design simple methods for determining precisely EV concentrations. We have shown recently that a simple flow cytometry (FCM) method, based on fluorescence triggering, enabled to detect 50 × more Annexin-5-positive (Anx5+) EVs than the conventional FCM methods based on light scattering triggering (1). Here, we will present the extension of this approach to the phenotyping of the main EV populations in plasma, namely those derived from erythrocytes and platelets, as well as their sub-populations that bind or do not bind Anx5. *Methods*: Platelet free plasma (PFP) samples were either single labelled with Anx5-Cy5, anti-CD41-PE or anti-CD235a-PE mAb or double labelled with Anx5-Cy5 and either 1 of anti-CD41-PE or anti-CD235a-PE mAb. EV concentrations were determined by triggering detection either on a fluorescence signal or on the forward scatter intensity, using a Gallios flow cytometer. In addition, EVs were labelled with 10 nm gold particles conjugated with Anx5, anti-CD41 or CD235a, and were enumerated after sedimentation on EM grids, as described in (2). *Results*: Using the fluorescence triggering approach, the following EV concentrations were obtained: 30,000 ± 6,000 (n = 5) Anx5+ EVs, 22,000 ± 6,000 CD41+ EVs and 13,000 ± 5,000 CD235a+ EVs, expressed per μL pure PFP. These concentrations are significantly higher than those determined by conventional light scattering triggering, namely 40 × for Anx5+ EVs, 75 × for CD41+ EVs and 15 × for CD235a+ EVs. About 30% of Anx5+ EVs were found to be of platelet origin and only 3% of erythrocyte origin. These studies were performed at saturating concentration for each of the 3 markers. In addition, we verified that there was no contribution of coincidence effect, by showing that EV concentrations decreased linearly for a series of two-fold dilutions of PFP, while their mean fluorescence intensity remained constant. EV concentrations determined by EM after on-grid sedimentation were close to those determined by fluorescence triggering in FCM. The fluorescence detection limit was found to correspond to about 1,000 Anx5-Cy5 and anti-CD41-PE or anti-CD235a-PE molecules. *Summary/conclusion*: The method of EV detection and phenotyping by fluorescence triggering in FCM is reliable, sensitive and simple. The catalogue of EVs in normal plasma constitutes a baseline level for future studies of EVs in diseases. This study constitutes an important first step towards the development of a standardized approach for quantifying EVs.

References

1. Arraud N, Gounou C, Linares R, Brisson AR. A simple flow cytometry method improves the detection of phosphatidylserine-exposing extracellular vesicles. J. Thromb. Haemost. 29 Nov 2014.
2. Arraud N, Linares R, Tan S, Gounou C, Pasquet JM, Mornet S, et al. Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. J. Thromb. Haemost. 2014;12(5):614–27.

O-3A-3

Nano-plasmonic exosome (nPLEX) platform

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Introduction: Exosomes, membrane-bound phospholipid nanovesicles actively secreted by mammalian cells, have emerged as novel biomarkers for clinical diagnostics as they carry molecular constituents reflective of originating cells. Despite the clinical potential of

exosomes, detecting and molecularly profiling exosomes has been a challenging task that often requires time-consuming sample purification and labelling processes. Here, we describe a label-free, high-throughput approach for real-time quantitative analyses of exosomes directly from clinical samples. *Methods*: We specifically developed a nano-plasmonic exosome (nPLEX) sensor, which comprised multiple arrays of periodic nanoholes (200 nm in diameter and 450 nm in periodicity) patterned in a thin gold film (200 nm) on a conventinal glass microscope slide. With a multichannel microfluidic flow cell constructed on top of the sensor arrays, each nanohole array was functionalized with a different type of antibody. Target-specific binding of exosomes on the sensor array induced a spectral shift in the optical resonance wavelength. Such a shift was proportional to the number of bound exosomes and correlated sensitively with overall cancer antigen abundance, thereby, enabling quantitative molecular profiling. *Results*: Compared to conventional protein analysis methods (e.g. Western blot and enyzme-linked immunosorbent assay/ELISA), this platform offers improved sensitivity at least by 100 times and assay speed ($<$60 minutes) and enables portable operation when integrated with miniaturized optics. We profiled the expression levels of molecular markers in exosomes isolated from cancer cell culture, whose profiling results showed an excellent match with those of parental cells. This indicates that cancer-derived exosomes reflect the molecular signature of their primary tumour. We also used the nPLEX assay to detect ovarian cancer exosomes in ascites samples. Our study showed that cancer-derived exosomes can be identified by the elevated expression of specific protein markers. In addition, changes in exosomal protein profiles further showed potential as indicators of treatment response. *Summary/conclusion*: With its capacity for sensitive, label-free molecular detection, the nPLEX platform would be a promising new tool for comprehensive exosomal molecular analyses for clinical trial testing. These efforts could ultimately enhance patient care opportunities through clinical trial testing and improve disease monitoring and rational therapy selection to derive greater clinical benefits.

O-3A-4

Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters

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Introduction: Accurate spatiotemporal assessment of extracellular vesicle (EV) delivery and cargo RNA translation requires specific and robust live-cell imaging technologies. For fluorescent proteins conjugated to proteins enriched in EVs, labelling may be restricted to subpopulations of EVs, limiting their wider use to observe multiple EV types. Meanwhile, fluorescent dyes for EV lipid labelling including PKH may not reflect true half-life of EVs and can be retained in association with other lipid entities for long periods, thus misguiding spatiotemporal assessment of EV dynamics, especially over extended periods. *Methods*: Here we engineered optical reporters to label multiple EV populations to visualize tumour EVs both in culture and in vivo. Enhanced green fluorescence protein (EGFP) and tandem dimer Tomato (tdTomato) were fused at their NH_2-termini with a palmitoylation signal (PalmGFP, PalmtdTomato) for inner EV membrane labelling. To monitor EV-RNA cargo, transcripts encoding PalmtdTomato were tagged with MS2 RNA binding sequences and detected by co-expression of bacteriophage MS2 coat protein fused with EGFP. By multiplexing the fluorescent and a bioluminescent EV membrane reporters (GlucB), we further examined the dynamics of EV uptake and translation of EV-delivered cargo mRNAs in cancer cells. *Results*: PalmGFP/PalmtdTomato enabled visualization and tracking of tumour EV release, uptake and exchange between cell populations in culture by confocal microscopy, as well as in vivo via multiphoton intravital microscopy. The reporters also allowed microscopic semi-quantification of EVs and flow cytometry analysis of EV uptake by the recipient cells. We further visualized EV-mRNA cargo and revealed the rapid dynamics of tumour EV uptake and translation of EV-delivered mRNAs that occurred within 1-hour post-horizontal transfer between cells. *Summary/conclusion*: These studies confirm that EV-mediated communication is dynamic and multidirectional between cells with delivery of functional mRNA.

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O-3A-5

Assessment of small RNA sorting into different extracellular fractions revealed by high-throughput sequencing of breast cell lines

Juan P. Tosar[1,2], Fabiana Gámbaro[1], Julia Sanguinetti[1], Braulio Bonilla[1], Kenneth W. Witwer[3] and Alfonso Cayota[1,2]
[1]Functional Genomics Unit, Institut Pasteur de Montevideo, Montevideo, Uruguay; [2]Department of Medicine, Universidad de la República, Montevideo, Uruguay; [3]Molecular and Comparative Pathobiology, Johns Hopkins University, Baltimore, MD, USA

Introduction: Intercellular communication can be mediated by extracellular small regulatory RNAs (sRNAs). Circulating sRNAs are intensively studied as potential minimally invasive disease biomarkers. To date, most attention is centred on exosomes (small extracellular vesicles and EVs) and microRNAs as the vectors and the secreted species, respectively. However, an increased understanding of the plethora of sRNAs secreted by different cell types in different extracellular fractions is needed. It is still not clear to what extent specific sRNAs are selected for secretion or whether sRNA secretion is mostly passive. *Methods*: Using high-throughput sequencing, we compared the intracellular sRNA content of breast epithelial cell lines (MCF-7 and MCF-10A) with extracellular fractions enriched in microvesicles (large EVs), exosomes and ribonucleoprotein complexes via standard and widely used differential centrifugation methods. Lung and cervical lines were also evaluated. Selected results were confirmed by droplet digital PCR. *Results*: Our results are consistent with a non-selective secretion model for most microRNAs. Nevertheless, a small number of microRNAs were statistically significantly enriched in the vesicle or protein complex enriched fractions, consistent with preferential secretion. In contrast with most microRNAs, 5′ tRNA halves and 5′ RNA Y4-derived fragments of 31–33 nucleotides in length were greatly and significantly enriched in the extracellular space. This was the case even in non-mammary cell lines. Specific tRNA halves were predominantly detected as part of ribonucleoprotein complexes of approximately 45 kDa. Vault RNAs were detected mostly in the EV fractions. *Summary/conclusion*: Overall, we show that, while many microRNAs do not appear to be selectively released from the examined cell lines different sRNA families have characteristic secretion patterns. These findings should prompt further investigations of the role of these sRNAs in the extracellular space.

Ballroom E
Symposium session 3B - EVs in cardiovascular disease
Chairs: *Peter Quesenberry and Costanza Emanueli* 10:45-12:00

O-3B-1

Exosomes from human cardiac progenitor cells preserve cardiac function long term after myocardial infarction

Lucio Barile[1], Elisabetta Cervio[1], Vincenzo Lionetti[2], Marco Matteucci[2], Tiziano Torre[3], Tiziano Moccetti[3] and Giuseppe Vassalli[1]
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Introduction: Recent evidence suggests cardiac progenitor cells (CPC) may improve cardiac function after injury. The underlying mechanisms are indirect but their mediators remain unidentified. Exosomes (Exo) act as paracrine signalling mediators. Here we report that Exo secreted by human CPC are crucial cardioprotective agents that improve left ventricular ejection fraction (LVEF%) in long-term animal model of infarct. *Methods*: Medium from CPC or normal human dermal fibroblasts (NHDF) was conditioned for 5–7 days and subjected to differential centrifugation for Exo isolation. Exo from CPC (Exo-CPC) were tested in-vitro for their functional activity such as anti-apoptotic and pro-angiogenic effects and compared with Exo from NHDF (Exo-F). The content of micro-RNA (miRNA) has been analysed by real-time PCR in Exo-CPC versus Exo-F. Exo-CPC derived from 6 patients were pooled and intramyocardially injected in-vivo in animal model of permanent left anterior descending (LAD) coronary ligation. One and four weeks after injection LVEF was evaluated by echocardiography and hearts were processed for histological analysis. *Results*: Exo-CPC inhibited apoptosis in cardiomyocytes enhance tube formation in human endothelial cells in-vitro compared to Exo-F. Exo-CPC were enriched in miR-210, miR-132, miR-146a and miR-181a compared to Exo-F. In gain-of-function studies, miR-210 and miR-146a inhibited apoptosis in cardiomyocytes by downregulating their targets ephrinA3/PTP1b and Nox4 respectively. miR-132 downregulated its target RasGAP-p120 and enhanced angiogenesis. Moreover, Exo-CPC, but not Exo-F, downregulated anti-apoptotic factors in cardiomyocytes. Infarcted hearts injected with Exo-CPC significantly preserved the LVEF after 1 week (84.00 $\pm$ 1.6%) and the effect was preserved after 4 weeks (80.57 $\pm$ 2.3%) compared with animals injected with Exo-F (60.71 $\pm$ 7.4%; 48.00 $\pm$ 4.6% 1 and 4 weeks respectively). Moreover Exo-CPC injected hearts showed significantly reduced scar size (6.7 $\pm$ 2.0% Exo-CPC vs. 19.25 $\pm$ 3.4% Exo-F). *Summary/conclusion*: Exo are the active component of the paracrine secretion of human CPC. They are enriched in miRNA with cardioprotective and proangiogenic activities. Exo-CPC preserves heart function in a long-term animal model of permanent LAD ligation. As a cell-free approach, Exo could circumvent many of the limitations of cell transplantation.

O-3B-2

Selective internalization of human stem cell-derived exosomes mediates angiogenesis and ischemic tissue repair

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Introduction: Locally transplanted human CD34$^+$ cells have been shown to improve exercise tolerance in patients with myocardial ischemia and promote angiogenesis in animal models. Earlier, we have demonstrated a novel mechanism that CD34$^+$ cells secrete pro-angiogenic exosomes (CD34Exo), which constitute a critical component of the paracrine activity of the cells. *Methods*: We used a murine model of myocardial ischemia to determine the therapeutic efficacy of CD34Exo. Langendorff digestion was used to prepare single cell suspensions; confocal microscopy and flow cytometry was used to study uptake of exosomes; RNA was analyzed by Taqman assays. *Results*: Cell-free CD34Exo mimicked the therapeutic activity of CD34$^+$ cells by significantly improving myocardial ischemia (ejection fraction 42 $\pm$ 4 vs. 22 $\pm$ 6%; capillary density 113 $\pm$ 7 vs. 66 $\pm$ 6/HPF; fibrosis 27 $\pm$ 2 vs. 48 $\pm$ 7%; $p < .005$, $n = 7–12$) compared with PBS control. Trafficking studies revealed that CD34Exo and CD34Exosomal miRNAs such as proangiogenic miR126 was selectively internalized to endothelial cells and cardiomyocytes, but not to fibroblasts in the CD34Exo-injected ischemic hearts. CD34Exo injection induced the expression of miR126 and several pro-angiogenic genes in mouse ischemic myocardium, but did not affect the endogenous synthesis of miR126, suggesting a direct transfer of miR126. CD34Exo lacking in miR126 had decreased angiogenic and therapeutic activity both in vitro and in vivo, indicating that exosomes-mediated transfer of miR126 was important for the therapeutic function of CD34Exo. Our recent studies suggest that surface proteins of CD34Exo are involved in the internalization into endothelial cells and induction of angiogenic activity. *Summary/conclusion*: Our results reveal a novel molecular mechanism of CD34Exo that involves selective uptake of stem cell-derived exosomes in the ischemic heart. Exosomes-shuttled miRNAs may signify amplification of stem cell function and may explain therapeutic benefits associated with human CD34$^+$ cell therapy.

O-3B-3

Characterisation of the human pericardial fluid microRNAs and exosome content

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Introduction: The pericardial fluid (PF) has been proposed as a biologically active compartment able to induce myocardium responses. Nonetheless, this novel concept has not been sufficiently tested. Recently, it has been shown that cells release functionally active microRNAs (miRs) often contained into exosomes (nanovesicles) or other kinds of extracellular vesicles (EVs). In vitro, EVs shuttle miRs other molecules from cells to cell, thus helping in cell-to-cell communications. The functional relevance of EVs has not been yet proved in vivo, especially in human studies. In this study, we have aimed at (a) providing the first characterization of the miRs and exosomes present in the human PF; (b) investigating the biological function elicited by PF-exosomes on cultured cardiac cells. *Methods*: A series of samples were collected (under ethical approval) as leftover from aortic valve (AV) surgery: PF, plasma, thoracic aorta and right atrium appendage (RAA). The PF miR content was determined by an Exiqon miR array. The top expressed miRs were validated by RT-PCR and also measured in plasma, thoracic aorta and RAA. Exosomes in PF and plasma were quantified using a nanoparticle tracking analysis system (Nanosight) and the presence of exosomes in both biological fluid was confirmed by TEM. Next, exosome preparations were enriched from the PF and plasma (Exoquick method) and validated by TEM before being employed in cell biology experiments. Following confirmation of florescence-conjugated exosome incorporation by human endothelial cells (ECs) and human iPSC-derived cardiac myocytes, cells were treated with either

exosomes, exosome-free PF or vehicle and submitted to cell death/survival and cell migration assay (limited to ECs). *Results*: Analyses of miR array data and single RT-PCR revealed that a cohort of miRs, known to be highly expressed by vascular cells and cardiomyocytes are enriched in the PF in comparison to plasma samples. Exosomes are abundant in the PF of cardiac surgery patients, even if less concentrated than in plasma. In pilot experiments, PF exosomes appear to regulate cell death in both cardiac myocytes and ECs and they also regulated EC migration. More experiments are ongoing. *Summary/conclusion*: This study provides the first characterization of miRs and exosomes in the human PF, showing that PF is an important source of miRs produced by cardiac cells. Finally, for the first time, we provided evidence of a functional role of PF-derived exosomes harvested from cardiovascular patients.

O-3B-4

The exosome subpopulation of extracellular vesicles is central to the pathogenesis and reversal of monocrotaline-induced pulmonary hypertension in mice

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Introduction: We have demonstrated that extracellular vesicles (EVs) isolated from mice with monocrotaline (MCT)-induced pulmonary hypertension (PH) induce right ventricular (RV) hypertrophy and pulmonary vascular remodelling when infused into healthy mice (Aliotta et al., Cardiovasc. Res. 2013). These changes can be completely reversed in MCT-injured mice after infusion of EVs isolated from murine bone marrow-derived mesenchymal stem cells (mMSCs) (Aliotta et al., 2014 ATS abstract). As the EVs used in these studies represent a mixture of micro vesicles (MVs) and exosomes (EXs), we wished to determine which EV subpopulation influenced pulmonary hypertensive changes. *Methods*: Platelet-free plasma isolated from MCT-injured and vehicle-injected mice was ultracentrifuged at 10,000*g* for 1 hour. The pelleted material (MVs) was saved. The remaining supernatant was then ultracentrifuged at 100,000 for 1 hour and the pelleted material (EXs) was saved. Alternatively, platelet-free plasma was ultracentrifuged at 100,000*g* (without the 10,000*g* step) and the pelleted material was saved (whole population: MVs + EXs). The same EV subpopulations were isolated from mMSC cell-free conditioned media. Injury study: cohorts of healthy mice were infused via tail vein with an equal amount of plasma-derived MVs, EXs and MVs + EXs from MCT-injured and vehicle injected mice. Twenty eight days after injections, recipient mouse right ventricular (RV) hypertrophy was assessed by RV-to-body weight (RV/BW) ratio (mg/g) and pulmonary vascular remodelling by blood vessel wall thickness-to-diameter (WT/D) ratio. Reversal study: cohorts of mice with established MCT-induced PH were infused via tail vein with an equal amount of mMSC MVs, EXs and MVs + EXs. Twenty-eight days after injections, mice were analyzed as previously described. *Results*: Injury study: RV/BW and WT/D ratios were higher in mice infused with MCT EXs and MVs + EXs versus control mice (0.139 ± 0.015, 0.135 ± 0.011 vs. 0.071 ± 0.014 mg/g; 0.121 ± 0.011, 0.123 ± 0.012 vs. $0.062 + 0.009\%$, $p < 0.05$, $n = 5$/cohort). RV/BW and WD/T ratios were similar in mice infused with MCT MVs and control mice. Reversal study: RV/BW and WT/D ratios were higher in MCT-injured mice infused with mMSC MVs versus control mice (0.141 ± 0.02 vs. 0.073 ± 0.012 mg/g; 0.124 ± 0.018 vs. $0.059 + 0.011\%$, $p < 0.05$, $n = 5$/cohort). RV/BW and WD/T ratios were similar in MCT-injured mice infused with mMSC EXs, mMSC MVs + EXs and control mice. *Summary/conclusion*: The EX subpopulation but not the MV subpopulation of EVs induces RV remodelling and pulmonary vascular remodelling in healthy mice when isolated from MCT-injured mice and reverses RV remodelling and pulmonary vascular remodelling in MCT-injured mice when isolated from mMSC. These studies serve as the basis for characterizing EVs involved in these important biological observations.

O-3B-5

Systemic delivery of microRNA-146a mimics in lipid microparticles substitutes for ApoE in suppressing inflammation and atherosclerosis in hyperlipidemic mice

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Introduction: Apolipoprotein E (apoE) exerts anti-inflammatory properties that protect against atherosclerosis and other inflammatory diseases beyond reducing blood lipid levels. We tested the hypothesis that apoE enhances levels of microRNA-146a to control NF-κB signalling in leukocytes. We also tested whether the systemic delivery of miR-146a mimics emulsified in lipid microparticles could substitute for apoE in suppressing leukocyte activation and thereby the progression of atherosclerosis in hyperlipidemic *Apoe*$^{-/-}$ mice. *Methods*: Male wildtype and *Apoe*$^{-/-}$ mice served as a source of bone marrow monocytes and peritoneal macrophages. Chow-fed 16 week-old male *Apoe*$^{-/-}$ mice were infused via tail vein twice a week for 6 weeks with 1 nmol miR-146a mimics or scrambled control miRNA emulsified in cationic phospholipids. At sacrifice, levels of miR-146a and its mRNA targets IRAK-1 and TRAF-6 along with the inflammatory cytokines IL-6 and TNF-alpha were detected in isolated monocytes and macrophages, and the extent of atherosclerosis was assessed in the aortic root and abdominal aorta. *Results*: An absence of *Apoe* expression in macrophages and monocytes resulted in reduced levels of miR-146a and enhanced NF-κB signalling and inflammatory responses upon stimulation with LPS. Accordingly, the cellular enrichment of miR-146a through the systemic delivery of miR-146a mimics into *Apoe*$^{-/-}$ mice substantially attenuated systemic inflammation and atherosclerosis in the absence of plasma lipid reduction. *Summary/conclusion*: Our findings demonstrate that apoE suppresses NF-κB-mediated inflammation and atherosclerosis by enhancing miR-146a levels in monocytes and macrophages. Our findings also demonstrate that the systemic delivery of miR-146a can reproduce the beneficial effects of apoE to control inflammation and atherosclerosis. On-going studies are exploring the impact of apoE on the cellular release of miR-146a into exosomes, and HDL that can be communicated to leukocytes and vascular cells to suppress inflammation and atherosclerosis.

Ballroom F-H
Symposium session 3C - EVs and viral infection
Chairs: *V. Craig Bond and Jennifer Jones* 10:45-12:00

O-3C-1

Extracellular vesicles in HIV-1 isolates and their contribution to infection

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Introduction: It is known that HIV preparations contain not only infectious viruses but predominantly non-infectious ''defective'' particles. These particles seem not to be inert ballast but rather to be implicated in viral infection. Here, using a recently developed flow technique (''flow virometry'') that permits analysis of the antigenic composition of small ($<$300 nm) cell-derived particles, we studied the composition of HIV-1 preparations and the role of EVs in HIV infection. *Methods*: Membrane vesicles and viral particles were captured with 15 nm magnetic nanoparticles (MNPs) coupled to ''capture'' antibodies against membrane antigens of interest. MNPs were separated on magnetic columns from non-bound antibodies and particles that do not carry a capture antigen and analyzed by a flow cytometer triggered on fluorescence. The role of EVs in HIV infection was studied in a TZM-bl cells and in human tonsillar explants. *Results*: Using capture MNPs decorated with antibodies against HIV gp120 in various conformations, or against surface antigens of non-HIV particles (CD45+) as well as against various cellular tetraspanins that are incorporated in EVs, we analyzed HIV-1 preparations and identified HIV virions with functional gp120, HIV virions that carry gp120 in a non-functional conformation, and, as well, non-HIV CD45+ EVs. Some of these EVs nevertheless carry HIV protein gp120 representing a hybrid between an EV and a virion. Also, there were gp120+ particles that did not carry CD45 but carried cellular proteins that are typical for EVs: CD81 and/or/CD63. By depleting or enriching HIV preparations with EVs, we found that EVs modulate HIV infection. *Summary/conclusion*: What we call ''HIV suspensions'' are mixtures of different particles: Some of them are true viruses carrying gp120 in different conformations, some are not viruses but rather EVs carrying gp120 and some are intermediates between viruses and EVs. We found that EVs play an important role in HIV infection.

O-3C-2

An analysis of extracellular vesicles and small RNA in cervicovaginal lavage of HIV-infected and uninfected individuals

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Introduction: We recently conducted a pilot study of extracellular vesicles (EV) and miRNA in cervicovaginal lavage (CVL) from sex workers with frequent exposure to HIV. The results of this study indicated that HIV positive individuals had fewer liberated EV in CVL and differential expression of miRNAs including miR-223. We have now expanded this study from 5 to 15 individuals per category and have added an additional exposure group, hypothesizing that EV and their cargo may be an indicator of mucosal conditions associated with HIV infection and/or reduced susceptibility to HIV. *Methods*: Forty-five archived CVL samples from the Pumwani sex worker cohort were obtained, representing 15 individuals each in the categories HIV+, HIV highly exposed seronegative (HESN or resistant), and HIV negative. Based on our pilot study, we selected 48 miRNAs with high probability of expression in CVL and CVL EVs and prepared a custom low-density array. RNA isolated from differential centrifugation-enriched EV pellets was profiled in duplicate for each of 45 CVL samples. Nanoparticle tracking analysis was performed to assess number of particles liberated. Analyses were performed by group and also accounted for donor characteristics such as age, antiretroviral drug status, infections and sexual and hygiene practices. *Results*: Thirty two of the 48 miRNAs were detected in most CVL EV samples. The most abundant miRNAs in CVL EVs included miRs-186, -205 and -223 which were detected in all replicates of all samples. While fewer particles were found in CVL of HIV-infected than in resistant or negative individuals, there was substantial variability of counts and the differences did not reach statistical significance. Differentially expressed miRNAs included several that have characterized roles in inflammation and/or that had been found in our previous studies of HIV-infected individuals, for example, miR-223, miR-29 family members, and miR-328. *Summary/conclusion*: Our results support the idea that EV in cervicovaginal mucus contain small RNAs and that certain RNAs associate with HIV infection status. However, initial enthusiasm that EV counts in CVL could be an indicator of infection status has been dampened by the high variability observed in mucosal samples.

O-3C-3

Role of exosomes from HIV-1 infected cells in neurodegeneration

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Introduction: Neuronal dysfunction and degeneration are the causative mechanisms for the HIV-associated neurocognitive disorders (HAND) in the era of combined antiretroviral therapy (cART). In this study, we assessed the effects of exosomes derived from PMA activated promonocytic cells U1 that are latently infected with HIV-1, U1 cells treated with HIV-1 protease inhibitor, Ritonavir and parental cells U937 on human fetal neurons. *Methods*: Exosomes were derived from conditioned media of U1 cells and U1 cells cultured in the presence of HIV-1 protease inhibitor ritonavir and U937 cells by ultracentrifugation and Exoquick techniques. Human fetal brain neurons were prepared from brain tissues of gestational age week 16. *Results*: Neuronal cultures treated with U1 cell and U1/ritonavir-derived exosomes were found to be severely compromised in their ability to maintain existing neuronal network as well as their ability to form neurites in a scratch-wound assay. In addition, neuronal cultures treated with U1 exosomes demonstrated increase in reactive oxygen species (ROS) generation and low levels of superoxide dismutase (SOD) activity indicating heightened oxidative stress. Analysis of the phosphorylation status of protein kinases in neurons treated with U1 exosomes using phosphoproteome array demonstrated dysregulation of regulators of signal transduction, cell proliferation and survival. Specifically, we observed dysregulation of cAMP response element binding protein (CREB)-target gene expression and proteins involved in actin assembly. *Summary/conclusion*: Collectively, these observations demonstrate that exosomes derived from HIV-1 infected cell and HIV-1 cells treated with HIV-1 protease inhibitor Ritonavir can cause neuronal dysfunction and degeneration.

O-3C-4

Activated monocyte-derived exosomes have a decrease in miR-223 and stimulate ICAM-1 in endothelial cells: implications of chronic immune activation in cardiovascular disease

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Introduction: Widespread use of antiretroviral therapy (ART) by HIV-infected subjects has improved their health and extended their lives. However, with increased longevity cardiovascular disease has become a significant comorbidity. HIV-infected individuals continue to have chronic immune activation in spite of effective therapy lowering viral load to undetectable. We recently reported that individuals with HIV infection have a type 1 interferon (IFN alpha) phenotype with elevated circulating lipopolysaccharide (LPS). We modelled this activation in vitro to determine the effect of activated monocyte-derived exosomes on intracellular adhesion molecule (ICAM-1) expression in human endothelial cells. Stimulation of ICAM-1 would facilitate monocyte migration through the endothelium and promote the development of atherosclerosis by plaque formation. *Methods*: TaqMan low-density human miRNA arrays were performed on human monocyte-derived exosomes from controls (N = 3) and HIV-infected subjects (N = 5). Monocyte exosomes were harvested using a polymer-based exosome precipitation method, labelled with DiI-C16 and incubated with human umbilical vein endothelial cells (HUVECs) to confirm entry. To determine the effect on HUVECs, monocytes were treated with IFN, LPS or a combination for 3 hours before media exchange and further incubated for 24 hours. Exosome-treated HUVECs were lysed to isolate total RNA and probed by RT-PCR for miR- 223 and expression levels of ICAM-1 were determined. *Results*: miR arrays on monocyte exosomes from HIV-infected and control subjects showed miR-223 to be the most abundant miR. However, miR-223 was decreased in HIV-infected subjects compared to controls. Monocytes treated with a combination of IFN/LPS released exosomes with a marked decrease in miR-223 compared to IFN or LPS alone or untreated monocytes. Normal HUVECs did not express miR-223. However, when exosomes were internalized, miR-223 was expressed. Monocytes treated with LPS or IFN/LPS released exosomes with low miR-223 expression that when incubated with HUVECs stimulated ICAM-1 expression. *Summary/conclusion*: Normal human monocyte-derived exosomes have abundant miR-223 that is suppressed when treated with IFN/LPS, an activation pattern we find in HIV-infected subjects. When untreated monocyte exosomes were incubated with endothelial cells, they did not stimulate ICAM-1. However, exosomes from IFN/LPS-stimulated monocytes activated ICAM-1 expression, suggesting that miR-223 is protective and a decrease in this miR may enhance monocyte migration into the endothelium and contribute to the development of cardiovascular disease.

O-3C-5

Phosphatidylserine vesicles enable efficient en bloc transmission of multiple enteroviruses

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Introduction: A central paradigm within virology is that each viral particle largely behaves as an independent infectious unit. *Methods*: Here we investigate the assembly, exit and subsequent infection processes of enteroviral particles using a combination of imaging techniques including confocal microscopy, super-resolution light microscopy, correlative light electron microscopy along with single molecule RNA fluorescence in situ hybridization (FISH), proteomic and biochemical approaches. *Results*: We demonstrate that clusters of enteroviral particles are packaged within phosphatidylserine (PS) lipid-enriched vesicles that are non-lytically released from cells and provide greater infection efficiency than free single viral particles. We show that vesicular PS lipids are co-factors to the relevant enterovirus receptors in mediating subsequent infectivity and transmission, in particular to primary human macrophages. We demonstrate that clustered packaging of viral particles within vesicles enables multiple viral RNA genomes to be collectively transferred into single cells. *Summary/conclusion*: This study reveals a novel mode of viral transmission, where enteroviral genomes are transmitted from cell-to-cell en bloc in membrane-bound PS vesicles instead of single independent genomes. This has implications for facilitating genetic cooperativity among viral quasispecies as well as enhancing viral replication.

Networking lunch 12:00-13:00

Poster viewing sessions VIII, IX, X, XI, XII, XIII
Posters not attended by authors 12:30-13:00

Poster walk by chairperson, Session VIII, IX, X, XI, XII, XIII
Posters not attended by authors 13:00-14:00

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Ballroom D
Symposium session 4A - EVs in tumour progression
Chairs: *Takahiro Ochiya and Dolores Di Vizio* 14:00-15:30

O-4A-1

Extracellular vesicle induced phenotype switching in malignant and non-malignant colon cells

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Introduction: Colorectal (CRC) cancer is the second most prevalent cancer in women and the third most prevalent cancer among men, globally. Recent declines in mortality can be attributed to improved screening processes and treatment plans including surgical resection of tumour tissue, adjuvant chemotherapy and radiation. However, even with medical advances, the average 5-year survival rate of patients is a moderate 65%. There is a need for a more expansive body of targeted therapies to improve clinical outcome. An emerging body of research focuses heavily on understanding the tumour microenvironment, specifically soluble factors secreted and taken up by cells. Extracellular vesicles (EVs) are secreted from many cells carrying cargoes including proteins and nucleic acids. Studies have shown that EVs play a role in a variety of biological processes including immunity, bone formation and recently they have been implicated in promotion of a metastatic phenotype. *Methods*: EVs were isolated from HCT116 colon cancer cells, 1,459 non-malignant colon epithelial cells and tumour and normal colon tissue from a patient sample. Co-cultures were performed with 1,459 cells and malignant vesicles, as well as HCT116 cells and non-malignant vesicles. Malignant phenotype was measured using soft agar colony formation assay. Co-cultures were also analyzed for protein levels using mass spectrometry. The importance of 14-3-3 zeta/delta in transfer of malignant phenotype was explored using siRNA. Additionally, luciferase reporter assay was used to measure transcriptional activity of NF-kB. *Results*: This study demonstrates the ability of EVs derived from malignant colon cancer cell lines and malignant patient tissue to induce the malignant phenotype in non-malignant colon cells. Similarly, EVs derived from non-malignant colon cell lines and normal patient tissue reversed the malignant phenotype of HCT116 cells. Cells expressing an EV-induced malignant phenotype showed increased transcriptional activity of NF-κB, a protein implicated in both anti-apoptotic and inflammatory processes in the development and progression of CRC. We also used proteomic analysis to identify an up regulation of 14-3-3 zeta/delta resulting from an EV-mediated switch to the malignant phenotype. Further study demonstrated that knock down of 14-3-3 zeta/delta reduced anchorage-independent growth of HCT116 cells and 1,459 cells co-cultured with HCT-derived EVs. *Summary/conclusion*: Evidence of EV-mediated induction of malignant phenotype, and reversal of malignant phenotype, provides rational basis for further study of the role of EVs in tumorigenisis. Identification of 14-3-3 zeta/delta as up-regulated in malignancy suggests its potential as a putative drug target for the treatment of colorectal cancer.

O-4A-2

Androgen-stimulated extracellular vesicles regulate cellular proliferation in androgen-deprived prostate cancer cells

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Introduction: Androgen hormones are required for cellular proliferation and maintenance in prostate cancer. Treatment for advanced prostate cancer has focused on inhibiting the action of androgens in activating a nuclear receptor, an androgen receptor (AR), which upon binding with androgens can mediate the DNA transcription process. However, androgens can also induce non-genomic effects by facilitating the activation of plasma membrane receptors that lead to the activation of kinase signalling cascades, bypassing the genomic AR activity. *Methods*: We investigated the effect of androgens in regulating extracellular vesicle (EV) secretion in AR+ prostate cancer cells. EVs were isolated using differential ultracentrifugation method and characterised using EM and qNANO. We performed mass spectrometry LC MS/MS followed by quantitative analysis using Scaffold4, bioinformatic analysis using R and analysed with Ingenuity Pathway Analysis to investigate activated pathways. Changes of EV markers under various treatments were investigated using qRT PCR and confocal microscopy. CD9 were analysed using western blot and TR-FIA assay. Functional analysis was performed using a life imaging Incucyte assay. Plasma samples from patients under therapy were collected longitudinally and analysed using CD9 TR-FIA assay. *Results*: We found that treatment with androgen, androgen deprivation or using androgen agonists altered the secretion of 150 nm vesicles in LNCaP cell lines. Western blot followed by quantitative analysis showed that the increased secretion of these vesicles corresponded to an increased amount of CD9 in isolated EVs. This effect was not due to AR activation, as androgens did not regulate CD9 expression. In contrary, androgen manipulation altered mRNA expression of another EV marker, TSG101. The CD9-enriched EVs isolated from androgen-treated LNCaPs were able to increase the proliferative rate of androgen-deprived cells, implying a role of CD9 in mediating cellular proliferation via EVs. Comparison of mass spec data and microarray gene expression profiling showed that the effect of androgen upon AR transcription activity only minimally influenced the EV content, suggesting an alternative pathway(s) is responsible for this process and requires further investigation. *Summary/conclusion*: While we have just begun to unravel the role of EV in prostate cancer progression, our data confirms that androgens can regulate the secretion of subpopulations of EVs, the CD9-enriched EVs, leading to modulation of paracrine signalling in LNCaP cells. Isolation and characterisation of CD9+ vesicles may have prognostic value in advanced prostate cancer patients.

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O-4A-3

Exosome-mediated transfer of alphaV integrins promotes cell-cell communication in prostate cancer

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Introduction: We have investigated whether integrins are transferred among different subsets of prostate cancer (PrCa) cells through exosomes and have the ability to induce functional aberrations in recipient cells. Recent studies have characterized integrin expression in exosomes but have not investigated whether integrins are actually transferred to recipient cells and whether they modulate the phenotype of these cells. We have focused our study on the alphaVbeta3 and alphaVbeta6 integrins since they are highly up-regulated in cancer and metastasis. *Methods*: Exosomes from cell cultures were purified by differential ultracentrifugation. The quality of our preparations was tested by electron microscopy, continuous sucrose gradient and biochemical characterization using antibodies to CD63, CD81 and Flotillin-1. Exosome internalization was determined by confocal microscopy of red PKH26-labeled exosomes followed by Z-stack image analysis. Immunoblotting, FACS and RT-PCR analysis were used to investigate whether these integrins are transferred as proteins to recipient cells and are localized to the cell surface. Cell migration assays on integrin specific substrates were also performed. Exosomes from sera were purified using the ExoQuick kit (SBI). *Results*: We show here that the alphaV integrins are present in exosomes of several PrCa cells and are transferred as proteins from donor to recipient cells. The size range (40–100 nm), the characteristic cup shape of these vesicles, the density range of 1.15–1.17 g/ml as well as CD63, CD81 and Flotillin-1 enrichment were also confirmed. Furthermore, FACS analysis demonstrated that they are localized to the cell surface indicating they are functional. The active state of alphaVbeta6 in recipient cells was confirmed in cell migration assays on an alphaVbeta6 specific ligand, latency-associated peptide-TGFBeta. To evaluate the physiological relevance of our findings, we purified exosomes from sera of PrCa patients as well as TRAMP mice which had developed PrCa. The results show that the alphaVbeta3 integrin is expressed in these exosomes. *Summary/conclusion*: Overall, this study shows that alphaV integrins are transferred among different subsets of PrCa cells through exosomes and promote cell migration through interaction with specific ligands, suggesting that this novel pathway may lead to increased PrCa metastasis in distinct distant sites. In addition, the results show that the alphaVbeta3 integrin is expressed in exosomes purified from serum of PrCa patients and is a potential biomarker for PrCa.

AS and CF., Co-first Authors

AS, Biochemistry – Molecular Pharmacology Graduate Program

RDR, Genetics – Cancer Biology Graduate Program

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O-4A-4

Exosomes as microenvironmental cue for engaging mesenchymal stem cells in osteosarcoma progression

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Introduction: Osteosarcoma (OS) is a very aggressive malignant bone tumour mainly affecting children and adolescents. No major breakthrough in the treatment of OS has been reported in the last 3 decades, and survival in the presence of metastases has stagnated at a dismal 30%. Thus, alternative therapeutical approaches urgently need to be explored. Because OS onset occurs during the adolescent growth spurt at sites of rapid bone growth, microenvironmental signals provided by stromal cells could have a defining role in OS development and progression. We hypothesized that tumour cell-secreted exosomes play a pro-tumorigenic role in OS progression by controlling mesenchymal stem cell (MSC) physiology. To test this hypothesis, we explored the properties of OS-exosomes "educated" MSCs in a xenograft model of OS. *Methods*: To study the effect of OS exosomes on MSC behaviour, we injected OS exosome-educated-MSCs in a bioluminescent orthotopic OS xenograft model and monitored tumour growth and metastasis formation by bioluminescence imaging. The effects of OS exosomes on MSC cell cycle and cytokine expression were assessed in vitro by FACS, qPCR and ELISA. To investigate the role of exosome-induced IL-6 production we used a specific IL-6 blocking agent in vivo that is assayed for its anti-inflammatory effects in clinical trials. *Results*: We found that OS-secreted exosomes promote tumour progression by reprogramming MSCs into a pro-tumorigenic and pro-metastatic phenotype. In vitro, OS exosomes stimulate cell cycle and induce the expression of IL-6, a cytokine associated with tumour-inflammation. The use of an anti-IL-6 antibody in OS-bearing mice completely abrogated the tumour- and metastasis-promoting effects of exosome-educated-MSCs. *Summary/conclusion*: Our study suggests that interfering with exosome-mediated tumour-MSCs communication may represent a new therapeutic option to fight OS, and underscore the importance for studying the mechanism behind exosome-mediated IL-6 activation in human tumours.

O-4A-5

Exosomal TSPAN8 and CD151 drive tumour progression in host and non-metastatic tumour cells

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Introduction: Tspan and CD151 are metastasis-promoting tetraspanins and a knockdown (kd) of Tspan8 or CD151 and most pronounced of both tetraspanins affects the metastatic potential of the rat pancreatic adenocarcinoma line ASML. We elaborated the impact of ASML^{wt}, -CD151^{kd} and/or Tspan8^{kd} exosomes on metastatic progression as exosomes play a major role in tumour progression and tetraspanins are suggested to be engaged in exosome targeting. *Methods*: In vitro and in vivo control of exosome activity of a metastasizing tumour line depending on Tspan8 and CD151 expression. *Results*: ASML-CD151/Tspan8^{kd} cells poorly metastasize but regain metastatic capacity when rats are pretreated with ASML^{wt}, but not with ASML-CD151^{kd} and/or Tspan8^{kd} exosomes. Both exosomal CD151 and Tspan8 contribute to host matrix remodelling due to exosomal tetraspanin-integrin and tetraspanin-protease associations. ASML^{wt} exosomes also support stroma activation with upregulation of cytokines, cytokine receptors and proteases and promote inflammatory cytokine expression in leukocytes. Finally, CD151-/Tspan8-competent exosomes support EMT gene expression in poorly-metastatic ASML-CD151/Tspan8^{kd} cells. These effects are not seen or are weakened using ASML-CD151^{kd} or –Tspan8^{kd} exosomes. Thus, multiple activities of exosomal Tspan8 and CD151 cooperate in promoting metastasis. (a) binding and uptake of ASML-CD151/Tspan8^{kd} exosomes is severely impaired due to defects on tetraspanin-integrin complexes; (b) by the reduced recruitment of MMP2, MMP9 and TACE into the Tspan8 or CD151 exosomal tetraspanin web, matrix degradation becomes inefficient, which together with the reduced integrin expression hampers mobility and strikingly affects invasiveness; (c) the strong impact of exosome-uptake on host cells is at least in part due to exosomal miRNA that affects target cell-specific mostly cell cycle regulators, chemokines and chemokines receptors; (d) induction of EMT though not exclusively relying on Tspan8 and CD151 may become initiated be the impact of Tspan8 on the recruitment of E-cadherin targeting miRNA into exosomes. *Summary/conclusion*: We interpret these findings that the contribution of Tspan8 and CD151 to tumour progression mostly relies on exosomal activities of these 2 tetraspanins.

O-4A-6

Melanoma cell-derived exosomes alter the microenviroment of malignant tumours via re-education of mesenchymal stem cells by miRNAs

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Introduction: The mechanism of its rapid metastasis formation, high genetic variability and effective immune escape of malignant melanoma are not explained yet. Exosomes are among the potential mediators of communication between melanoma cells and their environment. To explore the interaction of melanoma cell-derived exosomes (mcde) and their microenvironment, we investigated their effect on syngeneic mesenchymal stem cells (MSC). *Methods*: Murine primary MSCs from adipose tissue were pretreated with B16F1 melanoma cell-derived exosomes. Exosomes were stained by lipidophilic dyes, and their uptake into recipient cells was visualized. The rate of apoptosis and expression of multipotent stromal cell markers were analyzed by flow cytofluorometry. To explore the potential in vivo tumour-promoting effect of exosomes, tumour bearing mice were injected with mcde-conditioned MSCs i.v.; the control mice received untreated MSC. Because we hypothesized that exosomal information might be carried by their miRNA content, we sequenced the whole miRNA spectra by SOLiD 5500xl technology, and then the sequences were annotated in CLC Genomics Workbench version 5.5.1. *Results*: After 1 hour incubation the labelled exosomes were internalized into MSCs. The internalization was the highest after 4 hours. We have found marked differences in the expression CD44, α4 integrin, CD29, CD106, CD73 and Sca-1 after induction. The ratio of late apoptotic or necrotic/early apoptotic cells decreased after mcde treatment (0.43/1 vs. 0.98/1). In animal experiments, the survival rate at day 42th was 38% after mcde-conditioned MSC treatment versus 85% non-conditioned MSCs. We have found highest expression levels of mir205, mir31, mir21a, mir15b, respectively. *Summary/conclusion*: Our data suggest that melanoma cell-derived exosomes could re-educate mesenchymal stem cells, giving rise to a cell population that supports metastasis formation. The potential inducer of MSC re-education might be the miRNA content of exosomes.

Ballroom E
Symposium session 4B - EVs and the nervous system
Chairs: *Shilpa Buch and Paula Saá* 14:00-15:30

O-4B-1

The choroid plexus epithelium plays a crucial role in both systemic and brain inflammation via the production of extracellular vesicles

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Introduction: The blood-cerebrospinal fluid barrier (BCSFB) forms a unique interface between blood and brain. It consists of a single cell layer, called choroid plexus epithelium (CPE), situated at the interface of blood and cerebrospinal fluid (CSF). The CPE forms a barrier to protect the brain from fluctuations in peripheral blood thereby assuring brain homeostasis, produces CSF and is responsible for the active removal of toxic molecules from the brain. In recent years, the BCSFB has gained increasing attention, especially its role in inflammatory and age-related diseases. *Methods*: We made use of different sepsis mouse models, namely endotoxemia, that is, intraperitoneal (ip) injection of lipopolysaccharide (LPS), caecal ligation and puncture and ip injection of the cytokine TNF. As mouse models for Alzheimer's disease, we used the transgenic APP/PS1 mice and intracerebroventricular (icv) injection of amyloid β oligomers. We performed miRNA and mRNA profiling, using the NanoString and Illumina technologies, respectively. Both TEM and 3D SEM were used to visualize the CP tissue. Primary CPE cells and mixed cortical cultures were used to study the effects in vitro. *Results*: We observed that both sepsis and Alzheimer's disease are associated with an increase in amount of miRNA containing extracellular vesicles (EVs) in the CSF. Interestingly, we identified the CPE as main source of these EVs. Indeed, different inflammatory triggers were able to activate the exosome machinery in the CPE cells, reflected by an increase in exosome containing multivesicular bodies (MVBs), which could be blocked by an exosome inhibitor. Additionally, the CPE-derived EVs were able to cross the ependymal layer that lines the ventricles, were taken up by astrocytes and microglia, and induced eventually miRNA target repression and inflammatory gene up-regulation. Analysis of the EV content revealed the presence of several pro-inflammatory miRNAs such as miR146 and miR155 and proteins involved in vesicular transport and inflammation. *Summary/conclusion*: We found that the CPE releases EVs into the CSF in response to inflammatory stimuli, thereby affecting brain homeostasis.

O-4B-2

Neuronal origin plasma exosomes provide novel biomarkers for neuronal intracellular dysfunction in Alzheimer's disease

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Introduction: Using a methodology for enriching blood exosomes for neuronal origin, we introduced a series of novel biomarkers for Alzheimer's disease (AD), including pathogenic proteins (p-tau, Aβ(1–42)) and insulin signalling factors. Here, we turn our attention to lysosomal function and cellular stress responses, both previously implicated in AD pathogenesis. *Methods*: Blood exosomes were obtained from patients with AD (AD = 24) and controls (AC = 24), and from 20 others when cognitively normal and 1–10 years later when diagnosed with AD. Exosomes were enriched for neuronal origin by immunoabsorption with anti-L1CAM antibody. Exosomal cathepsin D (CD), type-1 lysosome-associated membrane protein (LAMP1), ubiquitinylated proteins (UBP), heat-shock protein-70 (HSP70), low-density lipoprotein receptor-related protein-6 (LRP6), heat-shock transcription factor-1 (HSF-1) and repressor element 1-silencing transcription factor (REST) were quantified by immunoabsorption assays. We examined their performance in diagnostic classification of AD using Stepwise Discriminant Classification (cross-validated using leave-1-out) and Receiver Operating Characteristic (ROC) analyses. *Results*: AD patients had significantly higher mean exosomal CD, LAMP1, and UBP and lower exosomal HSP70, HSF-1, LRP6, and REST versus controls (e.g. for CD, 18 vs. 8 ng/ml; for REST, 67 vs. 667 pg/ml; all $p < 0.001$; Figure displays CD and REST values for AD and AC). Stepwise Discriminant Classification models combining these markers achieved 100% diagnostic accuracy. In the longitudinal cohort, preclinical and clinical levels of all proteins were indistinguishable and different versus controls (all $p < 0.001$). *Summary/conclusion*: Exosomal levels of lysosomal proteins and transcription factors that mediate neuronal defences against stresses distinguish patients with AD from controls and predict AD diagnosis. Besides their potential implications for early disease detection and therapeutic monitoring, these results confirm in living AD patients early neuronal lysosomal dysfunction and abnormal responses to cellular stresses.

O-4B-3

Extracellular vesicles from amyotrophic lateral sclerosis tissue have misfolded SOD1 cargo and are implicated in propagation of protein misfolding

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Introduction: Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease wherein motor neurons progressively degenerate over time, and pathology spreads spatiotemporally through the neuroaxis from one contiguous area to the next. Mutations in the Cu/Zn superoxide dismutase (SOD1) gene are linked to inherited cases of ALS, and misfolded SOD1 is found in neuronal tissues from sporadic ALS patients. We have previously shown that wild-type human SOD1 in cultured cells can be induced to misfold by physical contact with mutant misfolded SOD1, and that cell-to-cell transmission of misfolding can occur via the uptake of exosomes collected from misfolded SOD1-expressing cells. However, the mechanism by which misfolded SOD1 is propagated in vivo remains ambiguous. We hypothesized that vesicles found in the extracellular spaces of ALS neuronal tissues would bear misfolded SOD1 and participate in propagation of SOD1. *Methods*: We isolated EVs from frozen neuronal tissues of ALS mouse models and human ALS patients by serial centrifugation and purification by density gradient. Vesicles were examined by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), immunoblotting, and a high-throughput fluorescence quantitation immunoassay, cellomics. *Results*: We found that vesicle populations pelleted at 10,000 × *g* and 100,000 × *g* and localized to the ~30% sucrose density fraction displayed characteristic circular cup-shaped morphology by TEM. Both EV populations were positive for neuronal EV-specific markers prion protein, flotillin1, and SOD1. Markers of intracellular contamination were largely absent in the isolations, confirming cellular integrity during isolation. Vesicles under TEM displayed a highly variable size distribution, which was

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confirmed using NTA technology. Immunoprecipitation using conformation-specific antibodies that detect misfolded SOD1 showed the enrichment of misfolded SOD1 on human and mouse ALS tissue-derived EVs compared to control tissues. We applied the secreted EV-containing fraction from mutant-SOD1 expressing cells onto wild-type cells in culture and observed an induction of SOD1 misfolding, a phenomenon that was abolished by heat-denaturation of the EV-containing fraction. Finally, our preliminary results suggest that the application of ALS mouse model tissue-derived EVs onto fresh HEK cell cultures also causes misfolding of wild-type SOD1 in those cells. *Summary/conclusion*: In summary, we have successfully isolated EV populations from ALS patient and murine neuronal tissues, and shown these vesicles to specifically carry misfolded SOD1. Our results suggest that EVs bearing misfolded SOD1 are competent to induce misfolding of wild-type SOD1, implicating EV dissemination in the propagation of SOD1 misfolding seen in ALS.

O-4B-4

Characterization of blood-derived exosomes from glioblastoma (GBM) patients following oral loading with Gliolan® (5-ALA)

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Introduction: Gliolan® (5-aminolevulinic acid hydrochloride) is currently approved in Europe for the intraoperative visualization of malignant tissue during glioma surgery (WHO grade III and IV). The agent is currently being studied as an adjunct to fluorescent-guided surgery to maximize tumour resection. The present study was undertaken to define if tumours loaded with Gliolan® (5-ALA) could shed circulating microparticles containing PPIX derived fluorophore as a novel tool to endogenously label, track and quantify tumour derived microparticles. *Methods*: Serum samples from GBM patients ($n = 31$) undergoing surgery were collected prior to, and at different time points up to 48 hours following oral dosing with Gliolan® (20 mg/kg). Microparticles were isolated by gel filtration and characterized using Nanoparticle Tracking Analysis (NTA) and BCA for microparticle size/number and protein content. Endogenous fluorescence from the microparticles was assessed using NTA in the fluorescence detection mode (lex = 405 nm, lem > 430 nm). Western blot analysis for canonical protein and CPOX converting enzyme forming PPIX were utilized. *Results*: Microparticles (mode diameter of 50–100 nm) expressing Tenascin-C, CD63 and CD9 are present at a concentration of $\sim 10^{11}$ particles/ml of serum (protein content = 283.5 + 47 μg/ml of sera). Multiple microparticle phenotypes based upon size ($\sim$20 nm to $\sim$200 nm) were observed under fluorescence mode implying capture of cytosolic fluorophore during biogenesis of at least 2 major populations of shed microparticles. Microparticles from GBM patients administered Gliolan® contain an fluorescent species unique to PPIX that is observed in a small ($\sim$0.1%) fraction of the total number of microparticles after dosing suggesting that cellular cytosol may circulate with shed microparticles. *Summary/conclusion*: The evidence demonstrates that a small molecule drug following oral dosage can be uptaken by tumour cells, enzymatically modified and detected in the macroscopic tumour guiding resection and shed back into circulating microparticles within hours of dosing. This direct measure of tumour function affords potential diagnostic opportunities for the early detection of tumour recurrence using a "liquid biopsy" procedure.

O-4B-5

Plasma extracellular vesicles (EVs) as biomarkers in a new murine model of variant Creutzfeldt-Jakob disease (vCJD)

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Introduction: vCJD belongs to the group of human transmissible spongiform encephalopathies (TSEs). Transfusion of non-leukoreduced red blood cells from asymptomatic donors that subsequently developed vCJD resulted in disease transmission to 4 recipients. Recently, disease-associated prion protein, PrPres, was detected in whole blood of vCJD cases. These findings and the uncertainty about the number of vCJD-infected people question the safety of blood-derived products and stress the need of reliable tests for early detection of infected individuals. We reported the colocalization of PrPres, with plasma extracellular vesicle (EV) preparations of pre-clinical and clinical wild-type (wt) mice infected with mouse (mo)-adapted-vCJD and Fukuoka. In this study, we used another model, transgenic Tga20 mice overexpressing mo-PrP, to reinforce our previous findings that blood-derived EVs may serve as vehicles for the transfusion transmission of the TSEs. *Methods*: Groups of Tga20 mice were intracerebrally inoculated with 1% mo-vCJD, or normal brain homogenate as control. They were euthanized periodically during the preclinical and clinical phases. Blood, brains and spleens were removed for biochemical, histological and PMCA studies. EVs were isolated from plasma with ExoQuick. *Results*: We established a new mo-vCJD model, with Tga20 mice manifesting clinical signs after shorter incubation time than wt mice (104 vs. 140 days). We confirmed PrPres in plasma EV preparations by PMCA and determined the shortest post-infection period for detection. The temporal pattern of PrPres in EVs has been compared with PrPres occurrence in spleen (potential source of prions in blood) and brain. *Summary/conclusion*: Our data confirm the potential of our strategy as a novel diagnostic approach for vCJD detection in blood, contribute to elucidate the prionemia origin, and establish a model to assess the effectiveness of prophylactic or therapeutic interventions.

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O-4B-6

HIV Tat-induced miR-9 released from astrocyte EVs contributes to microglial migration: Implications for HAND

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Introduction: Increased microglial activation and migration are contributing hallmark features of HIV-associated neurological disorders (HAND). HIV-1 Tat protein is toxic for various CNS cells often resulting in cellular apoptosis, activation and/or migration. Molecular mechanism(s) by which Tat mediates its effects is the focus of this study. MiRNA-mediated regulation of disease pathogenesis represents an evolving area of research that has ramifications for identification of potential therapeutic targets for various neurodegenerative disorders. The highly conserved brain enriched miR-9 plays critical roles in neurogenesis as well as axonal extension. Its role in microglial migration, however, remains poorly understood. The goal of the current study was to examine how HIV Tat mediates induction and release of miR-9, which in turn, regulates the cross-talk between astrocytes and microglia, thereby contributing to disease pathogenesis. *Methods*: Using real-time PCR we demonstrated increased expression of miRNA-9 in the frontal cortices of HIV/SIV-infected humans/macaques. Transmission electron microscopy and western blot analyses was used to characterize EVs. Functional end point of cell migration was assessed by examining migration of cells in Boyden chambers. To validate the specificity of miR-9, target protector transfection using lipofectamine was performed. *Results*: Our findings demonstrated that HIV Tat exposure resulted in increased induction/release of miR-9 in the EVs isolated from astrocytes. MiR-9-enriched EVs, were in turn, taken up by the microglia, resulting in increased migration of these cells. Treatment of microglia with Dotap liposomal formulations containing miR-9, resulted in increased microglial migration and reciprocally, formulations containing anti-miR-9 failed to mediate migration. Mir-9 mediated migration of microglia involved downregulated expression of the key target protein, monocyte

chemotactic protein-induced protein 1 and downstream signalling via the β-catenin pathway. In vivo validation of these findings further confirmed the role of miR-9. *Summary/conclusion*: HIV-Tat can induce the expression and release of miR-9 in astrocytes, which are subsequently taken up by microglial cells via the EVs, leading ultimately to migration of these latter cells. These studies are likely to reveal new mechanism(s) and regulatory strategies in the paracrine-mediated regulation of miRNAs with relevance to microglia dysfunction. EV-loaded anti-miRs could be developed as a potential therapeutic strategy for HAND.

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Ballroom F-H
Symposium session 4C - EV proteomics and lipidomics
Chairs: *Hidetoshi Tahara and An Hendrix* 14:00-15:30

O-4C-1

In-depth proteomics analysis of human breast milk-derived extracellular vesicles to reveal their origin and targets in the infant's developing immune system

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Introduction: Besides providing nutrition, breast milk delivers important signals that stimulate the infant's developing immune system. It has been postulated that extracellular vesicles (EV) in milk support the instruction and/or development of neonatal immunity. However, little is known about the composition of milk-derived EV, partly due to the difficulty to purify EV from other components in milk. *Methods*: In this study, an extensive LC-MS/MS proteomic analysis was performed, whereby EV were isolated from breast milk of 7 individual donors using our recently established optimized density gradient-based isolation protocol [1]. High-density, non-floating complexes were included to compare the contents of EV to other macromolecular structures in milk. A comprehensive protein network was composed tracing the possible cellular origins of milk-derived EV and the potential targets in the gut. *Results*: An average of 579 proteins was identified in EV, compared to 205 proteins in the non-floating fraction. Interestingly, EV associated proteins like ANXA5 and Flotillin were exclusively identified in EV, while CD9, CD63 and CD81 were also present in non-floating protein complexes. Additionally, MHC-II was identified in the EV fraction only, suggesting that antigenic epitopes may be delivered via EV released from antigen-presenting cells. Besides MHC-I, the mammary epithelial cell marker beta-1,4-galactosyltransferase (lactose subunit) was identified in the EV fraction only, demonstrating EV of epithelial origin. Furthermore, several adhesion molecules (ICAM-1, CEACAM-1) were associated to EV which could allow EV binding to gut epithelial cells and gut resident immune cells. *Summary/conclusion*: In-depth proteomic analysis and compilation of an extensive network of EV proteins involved in immunity demonstrates that milk-derived EV originate from multiple cellular sources and have the ability to target various cell types in the gut.

O-4C-2

Verification of predictive biomarker candidates of colorectal cancer metastasis in serum extracellular vesicles by selected reaction monitoring-based targeted proteomics

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Introduction: Recently, the release of extracellular vesicles such as exosomes into biological fluids calls attention to their promise as circulating biomarkers in the surveillance of disease state such as cancer progression. Since proteins in the extracellular vesicles are very stable, they are the ideal resource for proteomics based biomarker discovery. In addition, high abundance serum/plasma proteins can be removed during isolation process of the extracellular vesicles, which can eliminate a huge potential source of interference in mass spectrometric analysis. *Methods*: A quantitative proteomic analysis for biomarker discovery was performed by iTRAQ labelling using membrane protein of colorectal cancer tissue. Serum extracellular vesicles were prepared by ultracentrifugation. The biomarker candidates identified in colorectal cancer tissue were verified in serum extracellular vesicles obtained from health controls (n = 20), colorectal cancer patients with and without metastasis (n = 18 each) by SRM/MRM targeted proteomic approach using stable isotope-labelled reference peptides. *Results*: We have previously identified and verified biomarker candidates of colorectal cancer in membrane fractions of colorectal cancer tissues using iTRAQ and SRM/MRM methods. Among about 5,500 proteins identified, 105 membrane and extracellular proteins were shown to be differentially expressed between adenomas, cancer without metastasis and with metastasis. These biomarker candidates were verified by SRM/MRM using stable synthetic isotope-labelled peptides as an internal control (Kume et al., Mol Cell Proteomics 13: 1471–84, 2014). Then, we investigated if we can detect and quantify the biomarker candidates in the extracellular vesicles containing fraction of serum by SRM/MRM. Among 100 biomarker candidates, we could quantitate more than 20 proteins in the extracellular vesicles containing fraction of serum. Moreover, we were able to verify 3 predictive biomarker candidates for colorectal cancer metastasis by SRM/MRM. Intriguingly, genes corresponding to the 3 biomarker candidates in extracellular vesicles have been reported to reside in 19q13 region that is amplified in advanced colorectal cancer. Further validation of the predictive biomarker candidates are currently under investigation. *Summary/conclusion*: These results suggest that targeted proteomic technology is a powerful tool to identify and verify novel promising biomarkers for diagnosis in the extracellular vesicles of biological fluids.

O-4C-3

Selection of extracellular vesicle subsets markers by proteomics

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Introduction: Extracellular vesicles (EV) are a heterogeneous population of nano-sized vesicles with important regulatory roles. EV attributes depend on the type and status of the cell secreting them. We hypothesize that the targets and functions of EVs are determined by their constituent protein, lipid, and RNA components, and that EV subsets produced by different cells (and under different conditions) have functional differences that are due to and identifiable by their distinctive component repertoires. Although certain proteins (such as CD9, TSG101, and Alix) are recognized as common components among EVs, we hypothesize that subset-specific components contribute to subset-specific functions. In order to determine whether EV subsets are relevant in performing regulatory effects, we must be able to isolate EV subsets from the bulk population of EVs and

perform functional studies. Whether we isolate EV subsets by preparative nanoFACS sorting, or by coupling to beads, we need subset-specific surface markers. In this study we present the use of high sensitivity proteomics to identify surface markers for further analysis and sorting of EV subsets. *Methods*: We isolated exosomes by ultracentrifugation from irradiated (25 Gy) and non-irradiated 4T1 mammary carcinoma cell line, after 48 hours culture in exosome-depleted medium. Exosome isolation was performed by serial centrifugation and ultracentrifugation. 10 μg of protein were used for proteomic analyses (Thermo Orbitrap Fusion Tribrid Mass Spectrometer). *Results*: Based on redundant peptide fragments, we identified a total of 549 proteins. 160 proteins were uniquely expressed on irradiated samples. Among the proteins uniquely identified in the irradiated EV preparation, 41 were plasma membrane associated, and 3 of these (Mcam, Plexin A1, and Stom) were very highly represented in the analysis. Of the proteins expressed in both irradiated and unirradiated EV samples, 204 proteins were overrepresented at least 2-fold in the irradiated EVs, 82 were plasma membrane proteins. In total, 37 proteins were overrepresented by more than 10-fold, and 6 were plasma membrane proteins with high peptide numbers. These were Rap2b, Rab8a, Stxbp3a, Slc9a3r1, CD109, Clic4. Mcam, PlexinA1 and Stom have been selected for further testing as radiation-specific EV markers. *Summary/conclusion*: EVs have tremendous potential as biomarkers and regulators of disease. However, to leverage this potential, we need ways to identify relevant EV (sub)populations. One of our goals is to understand the impact of radiation on the immune system, and, to this end, we sought to identify markers for radiation-associated EVs that could be used to investigate the functions of radiation-induced EVs. We identified candidate radiation-specific markers with protein identification mass spectrometry and are proceeding to test the utility of these markers for subset discrimination.

O-4C-4

Evaluation of the intravesicular versus extravesicular protein cargo in extracellular vesicles

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Introduction: The protein cargo of extracellular vesicles (EVs) is important to determine their biogenesis and function, as well as their putative biomarker roles in disease. Isolation of these vesicles often leads to co-isolation of likely non-vesicular proteins. The aim of this study is therefore to dissect the exosomal protein cargo to determine which proteins that are truly intravesicular and which are co-isolated together with the EVs, or present on the EV surface. *Methods*: Human mast cell (HMC-1) EVs were isolated by ultracentrifugation and were further separated on density gradient, resulting in 2 fractions, 1 with lower density (LD-EV) and 1 with higher density (HD-EV) respectively. Enzymatically digestions with proteinase K (PK) was used to remove proteins not protected by a lipid membrane. PK and not treated (NT) samples were analyzed by LC-MS/MS. Western blot and electron microscopy was used in validation experiments. *Results*: Electron microscopy revealed intact vesicles after PK treatment and Western blot confirmed protection of ''exosome-markers.'' Together this suggests that the PK treatment used was not harmful to the vesicle integrity. Mass spectrometry revealed 1,826, 1,628, 1,480 and 1,001 proteins for the LD-EV NT, LD-EV PK, HD-EV NT and HD-EV PK samples respectively. Thus, several proteins were found to be protected from PK digestion, suggesting that these are intravesicular. In addition a number of proteins were found to be digested by PK treatment which is indicating that they are present outside the EV lipid membrane. *Summary/conclusion*: These data indicate that proteins not protected by a lipid bilayer are co-isolated with EVs even after a density separation. Presence of proteins uniquely found in the PK treated samples indicate a masking of certain proteins by the more abundant shared and co-isolated proteins. These data can be useful to identify-specific intravesicular proteins.

O-4C-5

Proteogenomic analysis of exosomes and ectosomes reveals that exosomes are more oncogenic

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Introduction: Exosomes (40–100 nm) are produced through the endocytic pathway via the multivesicular bodies while ectosomes (100–1,000 nm in diameter) are released through budding of the plasma membrane. Despite the differences in the mechanism of biogenesis, there are no well-defined molecular markers that can distinguish between the 2 types of extracellular vesicles (EVs). In recent years, many studies have focused on the role of exosomes in various disease states; however, ectosomes remains poorly characterized. With the goal of finding a better demarcation for these 2 types of vesicles in terms of their physical, biochemical and functional properties, a comprehensive proteogenomic analysis was done with exosomes and ectosomes isolated from 3 different human cell types. *Methods*: The EVs were isolated by ultracentrifugation and OptiPrep density gradient centrifugation. In addition, 10,000 *g* centrifugation (10K pellet) was performed to isolate larger EVs. Fraction similar to the density of exosomes (1.10 g/ml), higher density fraction (1.14–1.20 g/ml) and 10K pellet were analysed by transmission electron and atomic force microscopic techniques. Quantitative proteomics analysis was performed on the isolated exosomes, ectosomes (1.14–1.20 g/ml), 10K pellet and whole cell lysate of 3 different cell types (neuroblastoma cells SH-SY5Y and SK-N-BE2; colorectal cancer cells LIM1215). Exome sequencing analysis was performed on SH-SY5Y cells and a proteogenomic analysis was performed to identify mutant proteins secreted via exosomes and ectosomes. To characterize the functional attributes of exosomes and ectosomes, MTS-based cell proliferation and wound healing-based migration assay was performed. *Results*: Microscopic analysis confirmed the enrichment of smaller EVs in exosome fractions and larger EVs in ectosome fractions. Western blotting confirmed that many of the so-called exosomal markers are also present in ectosomes. However, the markers are exosome enriched. Quantitative proteomics analysis highlighted that exosomes are enriched or uniquely contain the ESCRT machinery proteins, annexins, flotillins, tetraspanins and integrins. For the first time, the analysis also highlighted the presence of an ectosome protein signature that can be used to discriminate between the EV types. Proteogenomic analysis revealed the secretion of multiple mutant proteins that are implicated in neuroblastoma via exosome and ectosomes. A follow up bioinformatics analysis based on proteogeomics data, COSMIC database and EST expression data revealed that exosomes contain more oncogenic potential than ectosomes. Treatment of recipient cells with exosomes induced significant cell proliferation and migration compared to ectosomes. *Summary/conclusion*: This study will help us not only decode the biogenesis and cargo-sorting mechanisms during different types of vesicle formation but also elucidate the pathophysiological roles of these EVs, especially the less studied ectosomes.

O-4C-6

The ether lipid precursor hexadecylglycerol stimulates the release and changes the composition of exosomes derived from PC-3 cells

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Introduction: The molecular machinery involved in the formation and release of exosomes is still not well understood. Interestingly, lipids are emerging as important constituents of this machinery.

Ether lipids are important structural components of membranes and have several biological functions, for example, in signalling and membrane fusion and trafficking. In this study we have analyzed the changes produced in the cellular and exosomal lipidome after addition of an ether lipid precursor, and we have investigated whether the release of exosomes is affected in these conditions. *Methods*: Prostate cancer PC-3 cells were treated with or without the ether precursor hexadecylglycerol (HG) for 24 hours in complete medium, and then for 17–19 hours with similar substance concentrations in serum-free medium. Exosomes were isolated by sequential centrifugation (1,000 g, 10,000 g, 100,000 g) and subjected to several analyses: Western blot, electron microscopy, silver staining, nanoparticle tracking analysis. The lipidome of cells and exosomes were analyzed by mass spectrometry. *Results*: The ether lipid precursor HG was used to increase the cellular levels of ether lipids in PC-3 cells. Lipidomic analyses confirmed that the cellular levels of ether lipids were increased in HG-treated cells compared to control cells. Furthermore, increased levels of ether lipids were also found in exosomes released by these cells. Interestingly, cells containing high levels of ether lipids released more exosomes per cell than control cells, and these exosomes were similar in size to control exosomes. Moreover, silver staining and Western blot analyses showed that the protein composition of exosomes released in the presence of HG was changed. Finally, we used quantitative electron microscopy in an attempt to get more information about the stage of exosome release that was affected by HG. A lower number of multivesicular bodies (MVBs) per cell profile were observed in HG-treated cells. This could indicate that more MVBs have fused with the plasma membrane in HG-treated cells and more intraluminal vesicles have been released. Considering that ether lipids have previously been implicated in membrane fusion, it is possible that the addition of HG increases the fusion of MVBs with the plasma membrane. *Summary/conclusion*: These results show that an increase in cellular ether lipids is associated with changes in the release and composition of exosomes. It is at the moment unclear how HG alters exosome release and at which step the compound affects exosome release. However, our result may indicate that the addition of HG affects the fusion of MVBs with the plasma membrane.

Networking coffee 15:30-16:00

Poster sessions VIII, IX, X, XI, XII, XIII
Posters not attended by authors 15:30-16:00

Ballroom D
Symposium session 5A - EVs as cancer biomarkers I
Chairs: *Suresh Mathivanan and Göran Ronquist* 16:00-17:45

O-5A-1

Identification of vesicular RNA and protein that may contribute to the radiation-induced bystander effect

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Introduction: Tumours are often treated with ionizing radiation, which induces DNA damage and leads to apoptosis of cancer cells. However, DNA damage can also be observed in neighbouring cells that were not directly treated with radiation. This curious phenomenon, known as the bystander effect (BE), can lead to long term genomic instability in bystander cells. We have recently shown that the radiation-induced BE is caused by extracellular vesicles (EVs) released by irradiated cells. We also demonstrated that treatment of EVs with RNase abrogated the ability of EVs to induce BE, suggesting an RNA molecule is involved. In order to identify potential RNA and protein species involved in the bystander effect we performed transcriptomic and proteomic screens. *Methods*: Human MCF-7 breast carcinoma cells were irradiated with 0 Gy or 2 Gy of X rays and the EVs were harvested from conditioned media. Total RNA and protein was extracted from EVs and differential incorporation was assessed using RNA-seq and mass spectrometry, respectively. *Results*: Our preliminary data highlight the differences in the proteome and transcriptome of EVs released by cells under normal and stressed conditions. The proteomic screen reveals a number of proteins that are up-regulated EVs released following irradiation. The transcriptomic study shows a number of coding and non-coding RNAs to be enriched in radiation-induced EVs. *Summary/conclusion*: The results of these unbiased approaches have identified a number of candidate molecules that may be carried by EVs and mediate the radiation-induced bystander effect. This work offers insight into the responses of tumour cells to treatment and has implications for cancer radiotherapy.

O-5A-2

Development and validation of non-invasive blood-based vesicle-miRNA assays for response monitoring to support personalized treatment in lymphoma patients

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Introduction: Upcoming treatment strategies for malignant lymphoma patients are aimed towards personalized therapy, increasing survival and reducing chemotherapy-induced side effects. In patients that respond poorly or still progress after initial cycles of chemotherapy, clinicians may decide for treatment intensification (escalation) or a switch to alternative ''targeted'' therapies. However, for patients with an excellent response, de-escalation should be considered. Ideally, clinicians are fully aware of the tumour status and clinical response allowing them to modulate treatment in a timely and adequate fashion. For lymphoma this is most pertinent in classical Hodgkin lymphoma (cHL) and diffuse large B-cell lymphoma (DLBCL). *Methods*: We developed a single-step size-exclusion chromatography method that enables detection of miRNAs enriched in plasma vesicles using self-prepared size-exclusion chromatography columns. We extracted RNA from the vesicle fractions and measured 10 miRNAs that were identified as candidate biomarkers by RNAseq. *Results*: We discovered that lymphoma cells actively secrete small vesicles (exosomes) (8) carrying a defined pattern of 22 nt microRNA (miRNAs) transcripts. Using a customized size-exclusion chromatography approach, we isolated uncontaminated vesicles directly from plasma and performedRNAseq and/or RT-PCR. We measured elevated levels of defined miRNAs in plasma vesicles fromcHL patients compared to healthy donors. In a first clinical evaluation of cHL samples before and during treatment, we observed significant decreases in several circulating miRNAs that corresponded with FDG-PET images. Importantly, miRNA levels remain ''low'' in remission patients during follow-up after the end of treatment, precluding a direct chemotherapy-related effect. Increase of miRNA levels were seen at relapse. We concluded that vesicle-bound tumour-miRNAs may be suitable for non-invasive treatment monitoring in lymphoma patients. *Summary/conclusion*: We demonstarted a strong correlation between defined circulating vesicle-miRNAs with the presence (or absence) of vital tumour in cHL patients determined by interim FDG-PET and via serum TARC levels, a chemokine produced exclusively Hodgkin tumour cells.

O-5A-3

Circulating hTERT (human telomerase) mRNA: mechanism of action and potential use for early diagnosis of malignancy

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Introduction: In contrast to current impressive advances in biological knowledge and achievements in the therapy of cancer, the field of early diagnosis lags behind and presents an unmet need. We hereby present the human telomerase as a potential tool for early diagnosis of cancer. Telomerase activation is a prerequisite for the perpetuation of the malignant clone during cancer progression as it elongates telomeres in each cell division. We established a method for the detection and quantification of hTERT mRNA products in exosomes derived from cancer cell lines growth media and human sera for future use as a diagnostic tool for the early detection of cancer. *Methods*: Exosomes were isolated by exosome isolation kit. qPCR was performed to assess the hTERT mRNA levels in exosomes and in cells. Verification of exosome isolation, hTERT transcription factors and telomerase levels were estimated by Western blotting. Telomerase activity was assessed by qPCR-based TRAP assay. *Results*: In 4 cancer cell lines, exosomal hTERT mRNA expression was detected and correlated with telomerase activity and intracellular hTERT mRNA expression, compared to a non-telomerase expressing cells. We studied the crosstalk between T cell leukemia derived exosomes and primary human fibroblast cells and revealed that the secreted exosomes transfer the hTERT mRNA from the ''donor'' cancer cell into the ''recipient'' non-telomerase expressing fibroblasts. These transcripts were successfully translated into a mature and fully active telomerase 24 hours post exposure. To establish a method for early diagnosis of malignancies, we screened sera from 130 patients with various malignancies and compared their exosomal hTERT mRNA levels to that of healthy volunteers. The results have shown that the expression of hTERT is variable among malignancies and between different patients with the same cancer type. To exclude some variables, such as drugs, that may influence exosome secretion on our chosen experimental cell lines, we examined the effect of the

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chronically administered drugs: aspirin, simvastatin and captopril. While simvastatin significantly decreased hTERT mRNA in exosomes derived from 1 cell line, neither aspirin nor captopril effected the secretion of exosomes, indicated by a similar hTERT expression in the relevant treated cells. Furthermore, no change in the intracellular hTERT expression and telomerase activity after drug exposure was evident. *Summary/conclusion*: In light of these results we confirmed that exosomes derived from tumour cells can affect the surrounding microenvironment by exploiting the recipient cell mechanism and promoting the activation of telomerase in those cells. Understanding these mechanisms may have a strong impact on deciphering metastases formation. Exosomal hTERT may serve as a valid marker for the detection of malignancy, also in patients treated with commonly used medications. Hopefully, these results will be translated into the development of a new diagnostic tool for the early diagnosis of cancer.

O-5A-4

Glioblastoma-specific miRNA signature in the extracellular vesicle and soluble fractions of clinical cerebrospinal fluid specimens

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Introduction: Glioblastoma is the most common form of primary brain neoplasm and remains one of the deadliest of human cancers. While glioblastoma tumour burden has been shown to predict therapeutic response, the opacity of the skull creates technical challenges in the quantitative assessment of tumour burden. Moreover, the inherent eloquence of the brain translates into potentially devastating consequences for repeated biopsies. In this context, minimally invasive biomarkers of glioblastoma tumour are urgently needed as a foundation for meaningful therapeutic development. There have been significant advances toward the development of Extracellular Vesicles (EVs) as a platform for fulfilling this critical need. The underlying premise of this biomarker platform is observation that EVs isolated from glioblastoma cell lines contain tumour-specific mRNA and miRNAs. Here we provide a comprehensive profile of miRNAs derived from the cerebrospinal fluid (CSF) of 108 glioblastoma patients and 50 patients diagnosed with non-neoplastic conditions. *Methods*: The CSF was fractionated into a soluble fraction and an EV fraction that were profiled separately. miRNA profiling was performed using the TaqMan OpenArray platform, where 754 miRNA TaqMan assays were immobilized onto a solid state cartridge. *Results*: Distinct miRNA signatures were derived from the soluble and EV fractions of CSF that discriminated glioblastoma patients from patients with non-neoplastic diseases. Interestingly, these profiles are distinct from those miRNA that are most over-expressed in the glioblastoma tumour specimen. *Summary/conclusion*: This work represents the largest effort of CSF miRNA profiling for glioblastoma patients to date and lays the foundation for CSF EV miRNA as a platform for glioblastoma biomarker development.

O-5A-5

Detection of exosomal EGFRvIII mutation and EGFR amplification in CSF of glioblastoma patients – report of the Exosomal Glioma Biomarker Consortium

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Introduction: A non-invasive way of diagnosis glioblastoma and glioblastoma genetic subtypes may provide the opportunity to diagnose patients at less cost and at lower risk than open or stereotactic brain biopsy. It may also provide the opportunity to follow patients in a minimally invasive manner over time for changes in tumour genetic content. We sought to test this paradigm of minimally invasive diagnosis based upon analysis of exosomal EGFRvIII mutation present in preoperative cerebrospinal fluid (CSF). *Methods*: A consortium provided GBM tissue and CSF under stringent ascertainment. CSF, before operation, followed by tumour tissue was obtained from 70 patients who then had confirmation of glioblastoma histology. Patients were typed for wild-type EGFR (EGFRwt) expression as well as EGFRvIII status in both tumour and CSF exosome RNA relative to the housekeeping genes GAPDH and 18S rRNA. Concordance of EGFRvIII detection in CSF exosome RNA and tissue was determined. *Results*: Of the 70 patients, 25% were positive for EGFRvIII in tissue. The sensitivity of CSF detection of EGFrvIII was 50% and specificity of 98%. CSF of cisternal origin had a higher sensitivity of 67%. Interestingly, a new EGFR deletion (now named EGFRv6) was discovered in this project and could be detected in the CSF exosomes. EGFrvIII expression in tumour tissue was correlated with amplification of EGFRwt in tumour tissue (p-value < 0.001). Expression of EGFrvIII in CSF was also correlated with amplification of EGFRwt in CSF (p-value $= 0.006$). 6 of the patients had RNA levels that were too low to detect EGFRwt and were deemed ''sample not sufficient'' for analysis. Five additional patients with EGFrvIII+ tumours had EGFrvIII in CSF upon tumour recurrence. *Summary/Conclusion*: Exosomes from preoperative CSF contain tumour RNA of diagnostic value with a sensitivity over 50% and specificity 98%. These finding support the utility of CSF EGFRvIII detection to provide tumour-specific diagnosis. This mutation is strongly associated with the classical subtype of GBM that can be targeted by chemotherapy and immunization therapies.

O-5A-6

Characterization of functional cargo of large oncosomes

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Introduction: Tumour cells can release a typically large extracellular vesicles (EVs) (1–10 μm), called large oncosomes (LO), which may play an important role in cell-cell communication. Studies from our group and others have shown that EVs are bioactive extracellular organelles. Here we profiled LO protein and DNA cargo. *Methods*: EVs were purified by differential centrifugation and density gradient

centrifugation, imaged by confocal microscopy, and profiled by mass spectrometry, next generation sequencing (NGS) and qRT-PCR. *Results*: Quantitative mass spectrometry (SILAC) demonstrated that, in comparison to exosomes, LO from prostate cancer cells were enriched in proteins involved in cell cycle regulation, vesicle-mediated transport, cell motility and invasion. CK18 emerged as an LO-enriched protein and was elevated in EV preparations from the blood of patients with prostate cancer in comparison to healthy control subjects. LO were also enriched in proteins involved in glutamine, glucose and amino acid metabolism and altered glutamine metabolism in recipient cells. LO cargo was enriched in chromatin proteins, including H2B, H2A, and NPM1 (FDR < 0.05). Imaging demonstrated that H2B was exported into LO and co-localized with DNA, suggesting that chromatin complexes are transferred by LO into recipient cells. LO DNA demonstrated a single strand (ssDNA) to double strand (dsDNA) ratio of 5:1. Whole genome sequencing of glioma and prostate cancer cells, and derived LO, demonstrated that DNA in LO encompassed all 23 chromosomes. LO DNA contained genetic aberrations of the cell of origin, including point mutations and copy number variations. MYC gain and PTEN loss, important features of prostate cancer, as well as EGFR gain, typically found in glioma, were detectable in LO. Finally, LO-transferred drug resistance to sensitive cells. *Summary/Conclusion*: Our data suggest that LO-derived DNA and protein can be used as a circulating source of tutor-derived information. They further support the hypothesis that LO transfer from tumour cells might mediate functional alterations in trans. Whether this process is mediated by DNA or protein requires further investigation.

O-5A-7

Identification of markers of endometrial cancer in exosome-like vesicles of uterine aspirates using a super SILAC quantitative proteomics approach

Irene Campoy[1], Evgeny Kanshin[2], Lucia Lanau[1], Tamara Sequeiros[1], Elena Martínez[1], Laura Devis[1], Peter Kubiniok[2], Montserrat Cubo-Albert[3], Silvia Cabrera[3], Jordi Temprana[4], Xavier Matias-Guiu[5], Jaume Reventos[1,6,7], Antonio Gil-Moreno[3†], Pierre Thibault[2†] and Eva Colás[1,5†]

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Introduction: Endometrial cancer (EC) accounts for 8,500 deaths per year in the US alone. There is an urgent need to develop non-invasive tests that can provide early detection of ECs. This study focuses on the identification of markers in exosome-like vesicles (ELVs) isolated from uterine aspirates by combining subcellular fractionation and quantitative proteomics. Uterine aspirates have advantages over other body fluids, as it is the closest body fluid to the neoplastic endometrium cells, and those cells are likely secreting key proteins during disease progression. *Methods*: This study obtained approval from the institutional review board. Written informed consent was obtained from all patients. Protein extracts from purified ELVs were obtained following ultracentrifugation of uterine fluid from age-matched groups of control, endometrioid and serous EC patients (10 patients/group). The quality of isolated ELVs was monitored by Nanoparticle Tracking Analysis, and immunoblots, and only ELVs of diameter 100 ± 50 nm showing exosomal markers were selected for subsequent experiments. To profile protein abundance across different groups, we develop a super SILAC approach where ELV proteins from 3 different EC cell lines grown in heavy Lys and Arg amino acids were combined with ELV protein extracts of each patient. Proteins were separated by SDS-PAGE, and gel-isolated bands were digested with trypsin and analyzed by Mass Spectrometry. *Results*: Our proteomics analyses identified key exosome markers including CD81, CD63, CD9 and TSG101, thus confirming the quality of subcellular fractionation. Quantitative proteomics in ELVs also enabled the identification of protein biomarkers previously identified at tissue level and novel biomarkers for endometrioid and serous EC diagnosis. *Summary/conclusion*: Our study describes, for the first time, the proteome of uterine aspirate ELV and enabled the identification of several putative biomarkers to improve EC diagnosis.

[†]These authors have contributed equally to the work.

Ballroom E
Symposium session 5B - EVs and the immune system
Chairs: *Marca Wauben and Prasun Datta* 16:00-17:30

O-5B-1

Immunogenic and tolerogenic dendritic cell subsets release phenotypically different extracellular vesicle populations with distinct immune-modulatory properties

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Introduction: Antigen presenting dendritic cells (DC) are key regulators of the adaptive immune system. Different subsets of DC can either act as potent stimulators of T cell-mediated immune responses or function to maintain immunological tolerance. Previous data indicate that DC-derived extracellular vesicles (EV) can contribute to DC-T cell communication and regulation of immune responses. Furthermore, it is known that the molecular composition of EV can be very heterogeneous and depends on the differentiation and activation state of the producer cell. We therefore aimed to comprehensively characterize the molecular contents of EV from immunogenic and tolerogenic DC in order to understand how these EV may differentially affect adaptive immune responses. *Methods*: We investigated differences in the quantity, quality, and function of EV released by lipopolysaccharide (LPS)-stimulated immunogenic murine bone marrow-derived DC and 1α, 25-dihydroxyvitamin D3 (VitD3)-treated tolerogenic DC. To this end, we developed a velocity gradient centrifugation-based method to segregate the pool of released EV into subpopulations, which were characterized by high-resolution flow cytometry and western blotting. *Results*: The EV subpopulations segregated on velocity gradients differed in light scatter profiles as well as CD9 surface protein levels. Although major histocompatibility (MHC) class II levels on the cell surface of VitD3-treated tolerogenic DC remained unchanged, we observed substantially reduced surface MHC class II levels on EV released by these cells. Furthermore, functional characterization of the various EV populations indicated that EV from VitD3-treated and LPS-stimulated DC have different modulatory effect on cytokine production during antigen-driven DC-CD4$^+$ T cell interactions. *Summary/conclusion*: This study sheds more light on how phenotypically different EV populations may contribute to the immune-modulatory function of immunogenic and tolerogenic DC subsets.

O-5B-2

Epithelial-to-mesenchymal transition by mast cell extracellular vesicles: Mechanism of epithelial damage

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Introduction: Disruption of lung epithelium is a major attribute of several lung diseases. Mast cells have been reported to be involved in many lung diseases, and are known to release significant quantities of different extracellular vesicles (EVs). The aim of this study is to determine how the epithelial to mesenchymal transition is induced by mast cell derived EVs. *Methods*: EVs were isolated from human a mast cell line (HMC-1) with differential centrifugations. Cell migration related to EV exposure was evaluated using a reversed Boyden chamber assay. The expression of mRNA involved in epithelial to mesenchymal transition was evaluated with qRT-PCR. Protein level validation of matrix metalloproteinase activity and TGFb-1 was performed using zymography and ELISA respectively. *Results*: Mast cells derived EVs induce epithelial to mesenchymal (EMT) like phenotype in A549 cells associated with increased MMP transcripts and MMP release. HMC-1 EV-exposed epithelial cells showed significantly elevated levels of the classical EMT markers TWIST1 and N-cadherin. A dose dependent increase in cell migration was observed. This feature was also associated with release of TGFb-1 from epithelial cells after EVs exposure. *Summary/conclusion*: Taken together our results suggest that EVs from mast cells exhibit potent features to instruct epithelial cancer cells to shift to a mesenchymal phenotype. Experiments are underway to dissect which molecules mediate this cellular effect.

O-5B-3

Nanovesicles released by apoptotic endothelial cells and characterized by an active 20S proteasome trigger autoimmunity and enhance vascular rejection

Mélanie Dieudé[1], Christina Bell[2], Julie Turgeon[1], Deborah Beillevaire[1], Luc Pomerleau[1], Katia Hamelin[1], Shijie Qi[1], Matthieu Rousseau[3], Diane Gingras[4], Eric Boilard[3] and Marie-Josée Hébert[1]
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Introduction: The causal and associative role of autoantibodies to LG3/perlecan with acute vascular rejection was recently described in renal transplant patients. One of the most intriguing observations is the appearance of anti-LG3 autoantibodies before transplantation in absence of an autoimmune disease context. Since we know that LG3 fragment is produced by apoptotic endothelial cells (apoEC) and that uremic patients show enhanced endothelial apoptosis, we aimed at evaluating whether membrane vesicles (MV) released by apoEC contribute to autoimmunity to LG3 and risk of rejection. *Methods*: MV released by apoptotic endothelial cells, apoptotic bodies (apobodies) and apoptotic nanovesicles (apoNano), were purified by sequential centrifugation and analysed by small particle flow cytometry, electron microscopy and comparative proteomics. C57Bl/6 mice transplanted with an aortic graft from a MHC-mismatched BALB/c donor or non-transplanted C57Bl/6 mice were injected intravenously with apoptotic MV for up to 3 weeks. *Results*: Injection of apobodies (size 500 to 7,000 nm), characterized by the presence of histones, failed to induce an immunogenic response. Injection of apoNano (30–100 nm) characterized by the presence of the autoantigen perlecan/LG3, induced a strong anti-LG3 IgG response, both in non-transplanted ($n = 11$, $p < 0.001$) and transplanted ($n = 12$, $p < 0.05$) mice. Aorta recipients injected with apoNano significantly increased neointima formation ($n = 6$, $p < 0.01$), compared to recipients injected with apobodies or vehicle. Proteomic analyses identified all sub-components of the 20S proteasome specifically in apoNano. All 3 proteolytic activities of the 20S proteasome were significantly increased in apoNano (trypsin-like ($p < 0.05$), chymotrypsin-like ($p < 0.01$) and caspase-like ($p < 0.01$)) compared to apobodies or cell extracts. Proteasome inhibition in endothelial cells with bortezomib, did not reduce the secretion of apoNano but significantly impeded their proteolytic activity ($p < 0.001$). Injection of proteasome-inhibited apoNano led to reduced circulating levels of anti-LG3 IgG ($n = 11$, $p < 0.05$). *Summary/conclusion*: These results identify endothelial cell-derived apoptotic nanovesicles characterized by active 20S proteasome as novel inducers of autoimmunity and accelerators of vascular remodelling.

This work is supported by the Canadian Institute of Health Research (CIHR) and the Canadian National Transplantation Research Program (CNTRP)

O-5B-4

The role of MSC-derived EVs in immunomodulation: the role of purinergic signalling

Erja Kerkelä[1], Anita Laitinen[1], Sami Valkonen[2], Kaija Alfthan[3], Matti Höyhtyä[4], Petri Lehenkari[5], Pia Siljander[2] and Saara Laitinen[1]
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Introduction: Recent observations suggest that 1 important function of mesenchymal stem/stromal cells (MSCs) is to protect against excessive inflammatory responses. MSCs have a promising therapeutic potential in many immunological disorders and in tissue repair/engineering. The functional mechanism of these cells is not fully understood. Currently the main therapeutic route is considered to be mediated via paracrine interactions with immune cells. We investigated the production of extracellular vesicles (EVs) from 2 different sources: human umbilical cord blood derived MSCs (hUCBMSC) and bone marrow derived MSCs (hBMMSC) and further the role of purinergic signalling in immune cell interactions. *Methods*: hMSCs were cultured in serum free media and EVs were collected from conditioned media by ultracentrifugation. Production of the vesicles was characterized by NTA and EM. The content of the exonuclease important in purinergic signalling (CD39 and CD73) was analyzed using indirect immune fluorescence (IIF) and flow cytometry (FACSAria and Apogee). The measurement of ectonuclease activity of the MSCs and MSC-EVs was performed using co-culture assays and the production of different nucleotides analyzed by high performance liquid chromatography (HPLC). The immunological effects on proliferation of different T-cell subtypes were analyzed by co-culture assays and flow cytometry. *Results*: Both MSCs and the EVs derived from them were able to generate adenosine from adenosine 5′-monophosphate (AMP). The extracellular vesicles secreted from both hBMMSCs and hUCBMSCs (MSC-EVs) highly expressed active CD73, but low amount of CD39. We show that the adenosine production by MSCs was specific to CD73 since it was inhibited by a specific CD73 inhibitor. We also show that the interplay between MSCs and T cells is important for the adenosine mediated immunosupression. The CD39 expression is higher in T-cells. They produce more efficiently adenosine 5′-monophosphate (AMP) from adenosine 5′-triphosphate (ATP) compared to MSCs. On the other hand AMP is hydrolyzed to adenosine less efficiently by T-cells alone, but this is enhanced in presence of MSCs or MSC-derived EVs. *Summary/conclusion*: We demonstrate that the purinergic signalling especially the adenosine production is important functional pathway that MSCs use to regulate immune cells. Part of this regulation is mediated via MSC-EVs. This route may be important route in situations of tissue damage when there are high amounts of ATP liberated from tissue. The interplay between T-cells and MSCs is needed and we show for the first time how purinergic signalling is working in concert between these cells.

O-5B-5

Platelet microparticles reprogram macrophage gene expression and function

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Introduction: Platelets contain an abundant and diverse array of messenger RNA (mRNA)-regulatory microRNAs that can be released in microparticles (MPs) upon activation. The relative abundance of microRNAs in platelet MPs, the crosstalk between platelets and macrophages as well as the capacity of macrophages to mediate clearance of platelet MPs prompted us to investigate if platelet MPs can regulate gene expression and influence the phenotype of macrophages through the delivery of functional microRNAs. *Methods*: MPs isolated from thrombin-activated platelets were counted and characterized by flow cytometry prior to co-culture with human primary macrophages. Internalization of MPs was analyzed by confocal microscopy, whereas the mRNA and microRNA profiling of macrophages was performed by micro-array and quantitative PCR (qPCR). *Results*: We demonstrate that platelet MPs can be internalized by primary human macrophages and deliver functional miR-126. The increase in macrophage miR-126 levels was not prevented by actinomycin D, suggesting that it was not due to de novo gene transcription. Platelet MP-derived miR-126 downregulated expression of 4 predicted gene targets at the mRNA level. These effects were abrogated by expression of a miR-126 sponge, implying that the mRNA downregulatory effects of platelet MPs are mediated by miR-126. Platelet MPs induced a significant upregulation of 34 microRNAs and a concomitant downregulation of 277 RNAs, including mRNAs encoding for cytokines/chemokines CCL4, CSF1 and TNF. These changes in macrophage gene expression were associated with a significant reduction in macrophage CCL4, CSF1 and TNF mRNA levels and cytokine/chemokine release and accompanied by a marked increase in the phagocytic capacity of macrophages. *Summary/conclusion*: These findings suggest that platelet MPs can modify the microRNA and mRNA transcriptomes of macrophages, and reprogram macrophages towards a phagocytic phenotype. Platelet MPs may thus act as a natural vehicle for microRNAs, contribute to intercellular signalling and condition the circulatory system under specific health conditions associated with platelet activation.

O-5B-6

Vesicle-associated microRNAs released by different human CD4+ T-cell subsets can mediate intercellular communication

Paola de Candia[1], Anna Torri[1], Donatella Carpi[2], Maria Cristina Crosti[2], Monica Moro[2], Mirjam Hoxha[3], Valentina Bollati[3], Maria Cristina Gagliani[4], Carlo Tacchetti[4] and Sergio Abrignani[1]
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Introduction: $CD4^+$ T helper (Th) lymphocytes can differentiate into various cell subsets, with defined biological functions in orchestrating adaptive immune responses. The aim of the present work has been to analyse nature and microRNA content of extracellular vesicles (EV) they release upon activation. *Methods*: To this goal, human $CD4^+$ Th1, Th2, Th17 (T conventional) and T regulatory cells were purified from peripheral blood of healthy donors and activated *in vitro*. EVs released after activation were purified by microfiltration, differential precipitation and differential centrifugation in parallel; characterized by nanoparticle tracking analysis, fluorescence activated cell sorting and transmission electron microscopy to evaluate size distribution and concentration, membrane integrity and RNA content, and obtain visual validation. We also observed the actual passage of RNA material from cell to cell through SYTO RNAselect fluorescent staining of vesicle-releasing cells and then profiled EVs derived from the different human primary lymphocyte subsets for microRNA content by RT-qPCR. *Results*: We showed that T regulatory vesicles are characterized by a very distinct pattern of microRNAs compared to T conventional, as assessed by Pearson correlation analysis and Spearman's rank-based hierarchical clustering. Descriptive Principal Component Analysis enforced these findings allowing the identification of a T regulatory-derived vesicle-associated microRNA signature. Moreover, GO biological processes linked to lymphocyte activation and immune response were significantly over represented in the list of literature-curated validated targets of T regulatory (but not T conventional) vesicle-enriched microRNAs and our preliminary data support the hypothesis that these microRNAs down-modulate messenger RNAs in receiving cells. *Summary/Conclusion*: In conclusion, our results suggest that the inhibitory activity of human T regulatory cells can, at least in part, utilize the regulatory function of vesicle-associated microRNAs.

Ballroom F-H
Symposium session 5C - EV biogenesis II
Chairs: *Guillaume van Niel and Alissa Weaver* 16:00-17:30

O-5C-1

Deciphering the biogenesis of extracellular miRNAs-Argonaute complexes using the RUSH-RISC system

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Introduction: miRNAs associate with Argonaute (AGO) proteins as part of RNA-induced silencing complexes (miRISCs) that repress expression of mRNAs displaying miRNA sequence-complementarity. Over the last years, several studies demonstrated the presence of microRNAs (ex-miRNAs) and/or AGO proteins in several mammalian biofluids, either associated with extracellular micro- vesicles/particles or as free protein complexes. Although there is a raising interest in these extracellular miRNAs as biomarker or as potential cell-to-cell mediator, their origins and biogenesis pathways remains mostly unknown. Interestingly, our laboratory and others previously uncovered the tight association of RNA silencing machinery with cell endo-membranes such as Endoplasmic Reticulum (ER), Golgi or MultiVesicular Bodies (MVB). This association is notably important for regulating miRISCs loading, turnover and activity but might also be key to drive RNA silencing complexes toward secretion pathways. Here we present the RUSH-RISC model, an innovative system allowing us to manipulate the endomembrane association of AGO in living cells to investigate its intra- and extra-cellular dynamic. *Methods*: RUSH-RISC is based on the RUSH system that relies on the co-expression of a membrane-specific hook (e.g. golgi, endoplasmic reticulum ER, multivesicular bodies, or MVB) fused to streptavidin, along with a protein bait (here Argonaute 2 – AGO2) tagged with streptavidin-binding protein (SBP) and a fluorescent protein (FP). The streptavidin-SBP complex is stable in cells, allowing trapping of the bait in a specific compartment (here specific endomembranes). Upon biotin addition, a potent streptavidin binder, the tagged-Argonaute is released, allowing us to monitor its cellular trafficking using microscopy but also to purify associated complexes using affinity resins followed by mass-spectrometry (IP-MS). *Results*: We successfully established RUSH-RISC stable cell lines in which AGO2 accumulate respectively on the cytoplasmic face of ER-, Golgi- or MVB-compartments. Using this system, we not only identified differences in the trafficking dynamic of these ''subcellular-specific'' pools of AGO2, but also characterized proteins specifically associated with these fractions using IP-MS. Moreover, we demonstrate that specific tethering of AGO2 onto the cytoplasmic face of MVB (e.g. key organelle for exosomes formation) dramatically enhances the production of extracellular EV-associated pools of AGO2. *Summary/conclusion*: In conclusion, the RUSH-RISC allows, for the first time, to investigate dynamic and composition of endomembrane-bound Argonaute fraction. This system will also not only be key in deciphering the mechanisms associated with ex-miRNAs/Argonaute complexes biogenesis, topology and composition, but also has great potential to engineer extracellular vesicles dramatically enriched in bioactive AGO2-bound miRNA/siRNA that could be used for therapeutic purposes.

O-5C-2

Sortilin mediates the release and transfer of exosomes in concert with two tyrosine kinase receptors

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Introduction: Cancer cells can influence their cellular microenvironment through intercellular communication. Growing evidence indicates that microenvironment control is supported by the release of extracellular microvesicles, called exosomes. Exosomes have the capacity to transfer their cellular content to neighbouring cells and modify their microenvironment to promote tumour-induced immune suppression, angiogenesis, and pre-metastatic niche formation. Still, the function of proteins found in the exosomal cargo and their mechanisms in membrane transport and the signalling events is not clearly defined. The multifaceted receptor, neurotensin (NT) receptor-3, also called sortilin plays a multitude of roles in the cell as a receptor or a co-receptor, in protein transport to the plasma membrane and to lysosomes, and in the regulated secretion. Numerous studies indicate that sortilin expression is elevated in several human cancers. In this study, we examined closely the secretion mechanism utilized for the extracellular domain of sortilin from human lung cancer cells (A549) and the effect on the microenvironment. *Methods*: We use a number of experimental approaches in our studies including lentivirus-mediated RNAi, metabolic labelling, immunoprecipitation, immunofluorescence (indirect and confocal), Western blot, exosome purification, EM, FACs analysis, co-culture using AMNIS, microarray, invasion assays, ELISA and the chicken embryo chorioallantoic membrane (CAM) model. *Results*: Here, we show for the first time that sortilin uses a ''canonical pathway'' and can be found in exosomes. We demonstrate that sortilin is a key component of exosomes mediating communication between A549 and endothelial cells. Sortilin is already known to play a prime function in cancer cells; however we report herein that it plays new role in both assembly of a tyrosine kinase complex and its exosome release. This novel complex (TES complex) expressed by exosomes results in the linkage of 2 tyrosine kinase receptors, TrkB and EGFR with sortilin. Using in vitro and ex vivo models, we demonstrate that this complex containing sortilin exhibits a control on endothelial cells and angiogenesis activation through exosome transfer. *Summary/conclusion*: Taken together our data suggest a paracrine function for sortilin and its partners in exosome transfer and the control of the microenvironment. This novel complex containing sortilin could play the role of as a molecular switch in cancer progression by promoting angiogenesis.

O-5C-3

Exosome secretion by vascular smooth muscle cells is regulated by the cytoskeleton

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Introduction: Vascular smooth muscle cells (VSMCs) normally regulate vascular tone however pathological stresses such as inflammation or calcium and phosphate imbalance induce VSMC trans differentiation to a ''repair'' phenotype characterized by proliferation and secretion of exosomes. Exosomes form the nidus for vascular calcification which is an independent risk factor for cardiovascular morbidity and mortality. VSMC phenotypic conversion is associated with cytoskeletal re-arrangements so in the present study we tested how components of the cytoskeleton impact on exosome secretion. *Methods*: Exosomes were quantified by a CD63-bead capture assay.

VSMCs were imaged by Scanning electron microscopy (Gemini 1525 FEGSEM). VSMCs transfected with DNA vectors encoding fluorescently-labelled organelle markers and actin tracer by electroporation were imaged by time-lapse acquisition of optically-sectioned z-volumes captured using spinning disk confocal microscopy (Nikon). *Results*: PDGF treatment induced loss of VSMC contractile marker expression, increased proliferation and stimulated exosome secretion and calcification. Using scanning electron microscopy we found organized outward budding vesicular structures at the cell surface following cytoskeletal filaments. Using immunofluorescent staining we also observed colocalisation between CD63-positive multivesicular bodies (MVB) and F-actin cables. Disassembling of the actin cytoskeleton triggered exosome secretion by VSMCs whilst inhibition of ROCK kinase using Y27632 prevented MVB tethering to F-actin cables and inhibited exosome secretion. Finally by using time-lapse microscopy we observed fast trafficking of CD63-positive organelles along F-actin cables associated with extensive organelle fusion and fission. *Summary/conclusion*: Our findings suggest that phenotypic conversion increases exosome production by affecting actin filaments. MVB destined for secretion are tethered to F-actin in a Rho-kinase dependent manner and can be transported to exosytosis sites where local disassembly of F-actin filaments triggers exosome secretion.

O-5C-4

Rab27a controls HIV-1 assembly by regulating plasma membrane levels of phosphatidylinositol 4,5-bisphosphate

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Introduction: During the late stages of the HIV-1 replication cycle, the viral polyprotein $Pr55^{Gag}$ is recruited to the plasma membrane (PM), where it binds phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] and directs HIV-1 assembly. The cellular mechanisms underlying $PI(4,5)P_2$ synthesis at the site of HIV assembly are not known. We hypothesized that exosome secretion and HIV-1 assembly are functionally coupled, and therefore, we undertook the analysis of the role played by Rab27a in HIV-1 assembly. *Methods*: Rab27a expression was silenced in Jurkat cells as well as in primary CD4+ T lymphocytes and macrophages. Cells were subsequently infected with HIV-1, and viral production was analyzed by ELISA. Alterations in exosome secretion were analyzed by immunoblot. Modifications in endosome trafficking and Gag distribution were analyzed by confocal microscopy. *Results*: We show that Rab27a controls the trafficking of multivesicular endosomes (MVEs) carrying phosphatidylinositol 4-kinase type 2 alpha (PI4KIIa) towards the PM of CD4+ T cells, promoting exosome secretion, high levels of PM phosphatidylinositol 4-phosphate and the localized production of $PI(4,5)P_2$. This phosphoinositide subsequently promotes Gag recruitment and HIV-1 assembly. Rab27a also controls $PI(4,5)P_2$ levels at the virus-containing compartments of macrophages. *Summary/conclusion*: We conclude that by directing the trafficking of PI4KIIa-positive endosomes towards the PM, Rab27a controls exosome secretion and PI $(4,5)P_2$ production at the site of HIV-1 assembly.

O-5C-5

Role of cytoskeletal organization in the biogenesis of matrix vesicles during odontoblast-supported mineralization

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Introduction: Matrix vesicles (MVs), a specific population of extracellular vesicles, are initial sites of mineralization of bone, dentin and cartilage. Mineralization-competent MVs are enriched in tissue-non–specific alkaline phosphatase (TNAP), PHOSPHO1 phosphatase and PO_4^{-3} and Ca^{2+} transporters, which support mineralization. Earlier, we demonstrated that 17AII11 preodontoblast cell line deficient for the Trps1 transcription factor showed impaired mineralization and produced fewer MVs than the parental cell line. However, the mechanisms of MVs biogenesis are not clear. Here, we investigated the role of cytoskeletal organization and activation of Rho/Rac1/Cdc42 GTP-ases in the biogenesis of mineralization-competent MVs. *Methods*: Preodontoblast-derived 17IIA11 cell line was used as a model of MV-initiated mineralization. MVs induced by osteogenic medium (10 mM sodium phosphate and 50 mg/ml ascorbic acid) were analyzed by Nanosight NS300 and Western blot. Remodelling of cytoskeleton components was inhibited by cytochalasin D, nocodazole, dynasore and forchlorfenuron. *Results*: Ten-fold increase of the number of MVs/cell in extracellular matrix was observed 12 hours after induction of osteogenic differentiation in comparison with untreated cells. MVs release was accompanied by activation of Rac1 and Cdc42, but not RhoA. The Rac1 and Cdc42 activation was diminished in Trps1-deficient cells, in which MVs biogenesis and mineralization is impaired. MVs release was increased by non-cytotoxic levels of inhibitors of cytoskeletal organization, with the strongest effect of nocodazole. Comparative Western analyses of MVs and cells under osteogenic and non-osteogenic conditions revealed differential distribution of TNAP and PHOSPHO1 isoforms between MVs and cells. *Summary/conclusion*: Our results suggest that cytoskeletal changes and activation of Rac1 and Cdc42 play a role in MVs biogenesis and that osteogenic properties of 17AII11 cell line rely in MVs released by these cells.

Poster viewing (not attended by authors) 17:15-18.00

ISEV networking event - Dinner Cruise 19:30-22:30
Buses to the dinner location leave from the Marriott Conference Center entrance at 18:00

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Poster Presentations

Poster session VIII - EVs in cardiovascular disease
Chairs: *Dylan Burger and Dimitrios Kapogiannis*

P-VIII-1

Microvesicles from activated endothelial cells are decreased by weight loss and correlate with dyslipidaemia and cardiovascular risk in severe obesity

Maja H. Christensen[1], Morten H. Nielsen[1], Stine B. Thomsen[2], Henrik Vestergaard[2] and Aase Handberg[1]
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Introduction: Obesity, elevated triglycerides (TG) and low HDL-cholesterol are all factors related to the metabolic syndrome (MetSy), a state that includes increased cardiovascular risk. Endothelial CD36 is involved in TG clearance from TG-rich lipoproteins and up-regulated by oxidative stress, both potentially activating endothelial cells. Microvesicles (MVs) may be markers and mediators of endothelial dysfunction having impact on the cardiovascular state during obesity. The aim of this study was to evaluate the effect of weight loss on levels of total MV number, activated endothelial MVs (EMVs) and CD36 positive EMVs, and their correlation to HDL, TG and MetSy. *Methods*: Twenty obese individuals (BMI of 43.0 ± 5.4 kg/m^2) were included. Informed consent was obtained, and the study was approved by the local ethical committee. Fasting blood samples were collected at baseline and 3 months after weight loss. Plasma MV profile was analyzed in accordance to a published flow cytometric method by this group that includes the whole MV size range of 100−1,000 nm. MVs were stained with lactadherin-FITC, CD62e-PE and CD36-APC antibodies. Statistical analysis included the Wilcoxon signed rank test and Spearman's Correlation test. *Results*: Gastric bypass reduced BMI with 20% ($p < 0.001$). Total MV number decreased by 39% ($p = 0.025$), total EMV number by 47% ($p = 0.002$) and CD36 positive EMVs by 73% ($p = 0.004$). At baseline, total MV count correlated with TG (rho = 0.5, $p = 0.015$) and TG/HDL ratio (rho = 0.6, $p = 0.009$). Total EMVs correlated with HDL (rho = −0.6, $p = 0.005$), TG (rho = 0.5, $p = 0.02$) and TG/HDL ratio (rho = 0.8, $p < 0.001$). When data from baseline and after weight loss were pooled, total MV number was increased by 1.7 ($p < 0.012$), total EMV by 1.74 ($p < 0.067$) and CD36+EMV by 3.45 fold ($p < 0.01$) in participants with MetSy. *Summary/conclusion*: Significant weight loss in obese individuals leads to a decrease in total MV number. The decrease in EMVs and CD36+ EMVs may reflect reduced endothelial dysfunction and lower cardiovascular risk.

P-VIII-2

Transplanted bone marrow-derived endothelial progenitor cells isolated from mice with monocrotaline-induced pulmonary hypertension cause pulmonary hypertension in healthy mice

Jason Aliotta, Mandy Pereira, Mark Dooner, Michael Del Tatto, Elaine Papa, Yan Cheng, Laura Goldberg, Sicheng Wen, James Klinger and Peter Quesenberry
Rhode Island Hospital, Alpert Medical School of Brown University, Providence, RI, USA

Introduction: We have demonstrated that whole, undifferentiated bone marrow (WBM) cells isolated from mice with monocrotaline (MCT)-induced pulmonary hypertension (PH) cause pulmonary hypertensive changes when transplanted into lethally-irradiated mice (Aliotta et al., 2014, ATS abstract). Endothelial progenitor cells (EPCs) have been implicated in the pathogenesis of human PH as more have been noted to be present in the circulation and remodelled pulmonary vasculature of these patients compared with healthy humans. We wished to determine if bone marrow-derived EPCs isolated from mice with MCT PH were the cells responsible for inducing PH in healthy mice. *Methods*: Sca-1+/ckit+/flk1+ cells (EPCs) and sca−1−/ckit−/flk1− cells (non-EPCs) were isolated from the bone marrow of MCT-injured and vehicle-injected mice. Cohorts of lethally-irradiated mice were transplanted with EPCs (2,000 EPCs + 200,000 helper WBM cells/recipient) or non-EPCs (200,000 non-EPCs + 200,000 helper WBM cells/recipient) from the bone marrow of MCT-injured and vehicle-injected mice. Twenty-eight days after transplantation, recipient mouse right ventricular (RV) hypertrophy was assessed by RV-to-body weight (RV/BW) ratio (mg/g) and pulmonary vascular remodelling by blood vessel wall thickness-to-diameter (WT/D) ratio. *Results*: RV/BW ratios of mice transplanted with EPCs from MCT-injured mice (0.136 ± 0.032 mg/g) were similar to those of MCT-injured mice (0.144 ± 0.031 mg/g, p = NS, n = 5/cohort) but elevated compared to mice transplanted with EPCs and non-EPCs from vehicle-injected mice (0.076 ± 0.011 and 0.081 ± 0.024 mg/g, $p < 0.05$, n = 5/cohort). RV/BW ratios of mice transplanted with non-EPCs from MCT-injured mice (0.108 ± 0.028 mg/g, n = 5/cohort) were lower than those of MCT-injured mice but elevated compared to mice transplanted with cells from vehicle-injected mice. WT/D ratio measurements were not completed by the time of this abstract submission. *Summary/conclusion*: These findings suggest that EPCs are among the pathogenic bone marrow cells in MCT-injured mice that are responsible for inducing RV hypertrophy, a key feature of PH, upon transplantation into healthy mice.

P-VIII-3

Modulation of exosome release from endothelial cells causes alterations in cell proliferation and miRNA-126 expression

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Introduction: Exosomes are usually defined as extracellular particles released from cell membranes via exocytosis with size between 30 and 100 nm in diameter. For exosome release, there are 2 major mechanisms: Endosomal Sorting Complex Required for Transport (ESCORT) mechanism and ESCORT-independent mechanism. Ceramide is one of the most important factors in ESCORT-independent mechanism. Neutral sphingomyelinase (nSMase) is an enzyme that breaks sphingomyelin into ceramide which can upregulates exocytosis in plasma membrane. In this study, we used an nSMase inhibitor (GW4869) and an analogue of ceramide (C6-ceramide) to modulate the release of exosomes in endothelial cells (ECs), and to test the effects of these modulations on EC proliferation and miRNA126 expression. *Methods*: Endothelial cells were treated with

GW4869 in 5, 10, 15 μM and C6-ceramide in 10, 15, 20 μM for 24 hours. Exosomes were isolated form cell culture medium by ultracentrifuge at 120,000 g for 2 hours after filtration with 100 nm filter. Exosome size and concentration were analyzed by Nanoparticle Tracking Analysis (NTA) technique. Cell proliferation was detected by MTT. The levels of miRNA126 expression in ECs and isolated exosomes were detected by RT-PCR. *Results*: GW4869 significantly decreased exosome release (by 33%) and C6-ceramide significantly increased exosome release (by 42%) in ECs. C6-ceramide significantly decreased the cell proliferation rate in a dose-dependent manner but GW4869 had no effects on it. GW4869 decreased miRNA126 expression (by 9%) in ECs and their released exosomes (by 57%). C6-ceramide showed the opposite effects in ECs, increased miRNA126 expression (by 18%) in cells but also decreased the miRNA126 expression (by 56%) in exosomes. *Summary/conclusion*: Inhibition of EC exosome release results in reduction of miRNA126 expression in both ECs and their released exosomes without affects the cell proliferation, whereas increase in exosome release showed opposite effects on ECs but not EC derived exosomes and also decreased the cell proliferation rate. Our data demonstrate that exosome release could affect EC function which may imply in vascular physiology and pathology.

P-VIII-4

Increased miR-107 in pulmonary vascular endothelial cells cultured with extracellular vesicles known to induce pulmonary hypertension in mice

Jason Aliotta, Mandy Pereira, Mark Dooner, Michael Del Tatto, Elaine Papa, Yan Cheng, Laura Goldberg, Sicheng Wen, James Klinger nd Peter Quesenberry
Rhode Island Hospital, Alpert Medical School of Brown University, Providence, RI, USA

Introduction: We have demonstrated that lung, plasma and bone marrow cell-derived extracellular vesicles (LEVs, PEVs, BEVs) from mice with monocrotaline (MCT)-induced pulmonary hypertension (PH) induce features of PH when infused into healthy mice. In addition, murine pulmonary vascular endothelial cells (mPVECs) cultured with LEVs, PEVs and BEVs from MCT-injured mice have lower rates of apoptosis and have increased expression of anti-apoptotic genes compared with mPVECs cultured with EVs from healthy mice. We wished to determine if changes in miR expression of mPVECs exposed to EVs from MCT-injured mice might be connected to this apoptosis-resistant phenotype. *Methods*: mPVECs (Lonza) were cultured with EVs from MCT-injured and vehicle (PBS)-injected mice for 7 days. miRNA microarray analysis was performed on cultured mPVEC. miRNA species of interest were defined as miRNAs uniquely up- or down-regulated in mPVECs after culture with EVs from MCT-injured mice (up- or down-regulated >2-fold in mPVECs cultured with MCT-EVs vs. mPVECs but not up- or down-regulated in mPVECs cultured with vehicle mouse EVs vs. control mPVECs). *Results*: We identified 15, 35 and 26 distinct miR species that were up-regulated, and 19, 28 and 24 distinct miR species that were down-regulated in mPVECs cultured with MCT-LEVs, PEVs and BEVs but not in mPVECs cultured with those EVs from vehicle mice. miRs-107 and -449a were commonly up-regulated in mPVECs exposed to all 3 MCT-EV populations (125-, 213-, 124-fold increase in mPVECs cultured with MCT-LEVs, PEVs and BEVs vs. control mPVECs). *Summary/conclusion*: miR-107 is commonly up-regulated in mPVECs after culture with 3 EV populations previously demonstrated to induce features of PH in healthy animals and an anti-apoptotic phenotype in cultured mPVECs. It has been reported that miR-107 is among the hypoxia-induced miRNAs and its over expression leads to the decrease in pro-apoptotic signalling in a variety of cancer cell lines. Although these studies did not determine if EV-based miR-107 is delivered to mPVECs in culture or if miR-107 is expressed de novo in response to exposure to these EVs, they suggest that mPVEC expression of miR-107 may contribute to pathogenesis of MCT EV-induced PH.

P-VIII-5

Use of outgrowth endothelial cell vesicles to modulate retinal angiogenesis

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Introduction: When the blood supply to the retina is impaired this can result in uncontrolled proliferation of new fragile blood vessels, which leak blood and fluids. This causes the loss of vision experienced in several eye diseases such as diabetic retinopathy, retinal vein occlusion and retinopathy of prematurity. Current therapeutic strategies aimed at blocking the proliferation include inhibiting VEGF, however there are mounting concerns over the effects of chronic VEGF inhibition. Our strategy is to tackle the cause of the initial impaired blood flow and therefore prevent the growth of the leaky vessels. A subtype of endothelial progenitor cells (EPCs), termed outgrowth endothelial cells (OECs) provide an attractive alternative therapy to address the underlying pathology and promote revascularization of the ischaemic retina. We have previously demonstrated the selective extracellular export of specific regulatory miRNAs into extracellular vesicles (EVs) which may contribute to this response. We aim to harness the vesicles released from OECs to promote vascular regeneration. *Methods*: OECs were isolated from umbilical cords using established protocols. EVs were isolated from conditioned OEC culture medium by ultracentrifugation and characterized by electron microscopy and flow cytometry. OEC and OEC EV miRNA content was investigated by deep sequencing. EVs were labelled with CellTracker™ CM-DiI and delivered by intravitreal injection to a mouse model of oxygen-induced retinopathy (OIR model) at P13 and P20. Various markers of vascular cells were used to identify recipient cells. *Results*: The population of EVs ranged in size from 50 to 2,000 nm and were positive for CD9 and CD63. Specific miRNAs were enriched in OEC EVs. The EVs are taken up by retinal microvascular endothelial cells (HRMECs) in culture. Intravitreal injection of OEC EVs in the OIR model showed a trend towards decreased retinal avascular area. Following intravitreal injection, labelled EVs can be detected in the retina for >7 days and predominantly target perivascular cells. Co-localization studies suggest that these may be macrophage/microglia. *Summary/conclusion*: EVs derived from OECs can be successfully delivered to the retina and become localized around vessels. They therefore have the potential to modulate revascularization and reduce pathologic angiogenesis. We postulate future modification of EV miRNA content may enhance this activity.

P-VIII-7

Endothelial-derived extracellular vesicles in inflammation stimulate monocyte migration in vitro and is mediated by Endoglin/CD105

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Introduction: Endothelial cell-derived extracellular vesicles (EVs) are released under basal conditions and in response to inflammatory stimuli. Since endothelial cells are activated in a range of inflammatory pathologies from atherogenesis to metastatic cancer, it is plausible that EVs may act as a signal for monocyte mobilization and recruitment. The aims of this study were to assess the effect of endothelial-derived EV on monocyte chemotaxis/migration in vitro. This study also looked at the effect of Endoglin/CD105, a RGD-containing transmembrane glycoprotein highly expressed on endothelial EV, reported to play a role in leucocyte migration. *Methods*: EVs

were generated from human umbilical endothelial cells stimulated with TNF-α, IL-1β, IL-4 or IL-6 for 24 hours. EVs were isolated using differential ultracentrifugation, re-suspended in chemotaxis buffer (RPMI, HEPES and 0.1% BSA) and characterized according to established markers. Chemotaxis studies were performed using a transwell assay (Corning Fluroblok™) with calcein AM fluorescently stained THP-1 monocytes. To look at effects of CD105 on THP-1 migration, EVs were pre-incubated with a blocking antibody to CD105 (R&D systems) for 1 hour prior to chemotaxis assays. Fluorescence in the lower chamber was measured over 2 hours, and the number of cells migrated quantified by comparison to a standard curve. *Results*: EV generation was significantly increased with TNF-α (15.5×10^8/ml) and IL-1β (14.3×10^8/ml), compared to basal (7.1×10^8/ml). Monocyte migration was significantly increased (2.5-fold, $p < 0.01$) towards EV generated after TNF-α and IL-1β treatment, compared to basal. When TNF-α EV were incubated with the monocytes prior to chemotaxis assays, migration was significantly increased compared to that seen in response to EV alone (3-fold, $p < 0.01$). THP-1 migration was also increased by 3.5-fold ($p < 0.01$) to recombinant CD105 (10 ng/ml) as well as to TNF-α-derived EV, pre-incubation of EV with a CD105 blocking Ab abolished this response. *Summary/conclusion*: This study demonstrates that under conditions of inflammation, the generation of endothelial EV is increased. Exposure to EV increased monocyte movement/migration which is likely due to an increase in motility, rather than exerting a chemoattractant effect. The increase in monocyte movement may in part be mediated by CD105.

P-VIII-8

Monocyte-derived microvesicles are elevated in morbidly obese individuals and associated with oxidized LDL

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Introduction: The main cause of increased mortality among obese individuals is cardiovascular disease. Early atherosclerosis involves foam cell formation in the vessel wall induced by CD36-induced accumulation of oxidized LDL (oxLDL) in macrophages. Obesity is a state of insulin resistance and increased oxidative stress. Insulin resistance increases monocyte CD36 expression, and we propose that the interaction between oxLDL and circulating monocyte CD36 induce release of monocyte-derived microvesicles (MMVs). In this study, we aimed to investigate the association between oxLDL and MMVs and CD36+MMVs, respectively, and to evaluate the effect of weight loss on MMV and CD36+MMV number. *Methods*: Twenty morbidly obese individuals were studied before and 3 months after weight loss due to gastric bypass. The study was approved by the local ethical committee, and informed consent was obtained before inclusion. MVs stained with lactadherin, anti-CD14 and anti-CD36 were analyzed by flow cytometry. A mix of calibrated fluorescent beads was used to define the MV region (100–1,000 nm). Circulating oxLDL concentration was determined by ELISA. Statistical analysis included the Wilcoxon signed-rank test and Spearman's Correlation test. *Results*: Gastric bypass reduced BMI from 42.7 ± 5.4 to 34.3 ± 5.2 kg/m^2 ($p < 0.0001$). Total number of MMVs and CD36+MMVs were reduced by 77% ($p = 0.073$) and 80% ($p = 0.002$), respectively. At baseline oxLDL correlated with MMVs (Rho = 0.49, $p = 0.028$) as well as with CD36+MMVs (Rho = 0.49, $p = 0.029$), whereas total MV number was unrelated to oxLDL. When data from baseline and after weight loss were pooled, CD36+MMV number correlated with oxLDL (Rho = 0.36, $p = 0.023$), hsCRP (Rho = 0.38, $p = 0.015$) and insulin resistance estimated as HOMA-IR (Rho = 0.37, $p = 0.018$). *Summary/conclusion*: MMVs and CD36+MMVs are increased in obesity possibly induced by monocyte CD36 interaction with oxLDL and may be an early marker of the on-going pathophysiological process of atherosclerosis.

P-VIII-9

Pro-inflammatory microvesicle release by insulin-resistant human macrophages in vitro

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Introduction: The prevalences of pre-diabetes and Type 2 diabetes (T2D) have increased dramatically recently and both are underpinned by insulin resistance (IR). These conditions are both important risk factors for the development of atherosclerosis, which is a chronic inflammatory disease of the large arteries, involving activation of the vascular endothelium, vascular inflammation and oxidative stress. Trans-endothelial migration of monocyte subsets and their differentiation into macrophages is an important component of the inflammatory phenotype. Both endothelial inflammation and oxidative stress are stimulated by the hyperglycaemia associated with both T2D and pre-diabetes. The aim of this study was to determine if IR induces microvesicle (MV) release from human macrophages and if these can directly cause endothelial dysfunction in vitro. *Methods*: Conditioned medium was collected from Phorbol myristate acetate-differentiated human THP-1 macrophages that had been treated with a saturated fatty acid [palmitic acid (PA); 250–750 μM] to induce IR. Numbers of <1 μm MVs released into the supernatant were counted by flow cytometry using enumeration beads and 1.1 μm sizing beads, after labelling of particles with annexin V. Human umbilical vein endothelial cells (HUVEC) were incubated with concentrations of glucose (between 6 and 50 mM) for 24 hours, followed by collection of medium to measure MV numbers. After culture in increasing[mec1] glucose concentrations, HUVEC were loaded with dihydrorhodamine-1,2,3 and treated with MVs isolated from PA-treated THP-1 supernatants. Production of reactive oxygen species was measured by fluorescence changes for up to 24 hours. *Results*: Treatment of THP-1 with 500 or 750 μM PA stimulated a moderate increase in annexinV$^+$ MV release ($p = 0.052$ one way ANOVA). There was no alteration of MV release after culture of HUVEC in high glucose medium. ROS production by HUVECs cultured for up to 48 hours in high glucose concentrations (12.5, 25, 50 mM) was increased compared to normal levels (6 mM). However, addition of MVs isolated from THP-1s treated with 500 or 750 μM PA led to a significant further increase in ROS production in HUVECs cultured in high glucose concentrations versus those in 6 mM glucose ($p = 0.0062$ one way ANOVA). *Summary/conclusion*: These data suggest that high glucose concentrations may prime EC for oxidative stress upon further pro-inflammatory insult. Insulin resistant macrophages may provide this insult by release of MV, as well as by cytokines and other pro-inflammatory molecules. This may provide a novel mechanism whereby hyperglycaemia and elevated plasma lipids jointly contribute to the development of atherosclerosis in pre-diabetic and T2D patients.

P-VIII-11

Mitral valve interstitial cell growth is improved by canine Wharton's Jelly mesenchymal stem cell exosomes

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Introduction: Mitral valve prolapse is the most common adult valvular heart disease in industrialized countries. Histologic changes include disarray of collagen and elastin fibres, which are known as myxomatous changes. In dogs with myxomatous mitral valvular disease (MMVD), the histological changes and functional consequences are virtually identical to those in humans. Canine valve interstitial cells (VICs) from diseased heart valves have a greater prevalence of cells with a myofibroblastic phenotype, growth retardation and lower

viability in vitro. The changes seen in VICs in diseased valves suggest a progressive fibrotic process. Given that MSC and its exosomes can exert anti-fibrotic effects, we investigated the effects of exosomes derived from pooled Wharton's Jelly MSC lines (WJ-MSC, n = 7) on the growth potential of canine VICs. *Methods*: Histopathology was used to confirm the disease state of the valves. VICs were isolated by enzymatic digestion from normal (n = 2), mildly affected (n = 3) and severely affected valves (n = 2) and then cultured in DMEM-F12 + 15% FBS. Immunohistochemistry and RT-PCR were used to evaluate the expression of aSMA, vimentin, elastin, fibronectin, tenascin, collagen and CTGF. Conditioned media (CM) was collected from WJ-MSC cultured in aMEM + 10% FBS for 24 hours. Exosomes were isolated with centrifugation at 300 × g for 10 minutes and 2,000 × g for 10 minutes, followed by either Vivaspin 6 100 kD ultrafiltration or by ultracentrifugation at 100,000 × g for 90 minutes for further concentration. NanoSight (NTA) was used to characterize particle size distribution, and the purity of exosome pellets was analyzed using size exclusion HPLC and flow cytometry. Internalization of WJ-MSC stained with either PKH or SYTO RNASelect by VICs was evaluated. *Results*: Culturing with WJ-MSC CM resulted in an increased number of viable VICs with both fibroblastic and myofibroblastic phenotypes compared to non-CM. When CM was depleted of exosomes, cell growth was markedly reduced, and the opposite effect was seen when the exosome fraction was added back to non-CM. PKH and RNA staining confirmed EV uptake into VIC cytoplasm and nucleus, although RNA containing exosomes were internalized by < 50% of VICs. *Summary/conclusion*: Exosomes from WJ-MSC CM improve both cell growth and viability for fibroblastic and myofibroblatic VICs and have potential therapeutic benefits for the treatment of MMVD.

P-VIII-12

Exosomes in cardiac preconditioning with isoflurane and hypoxia

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Introduction: Cardiac preconditioning with brief cycles of hypoxia or volatile anaesthetics like isoflurane leads to a significant protection from subsequent ischemia/reperfusion injury. Lately, the crosstalk between different cardiac cell types (cardiomyocytes, fibroblasts, endothelial cells) has come into focus of current research. One possibility is the intercellular exchange of cardioprotective mediators like heat shock proteins (HSP). In this context, exosomes have emerged as possible communication mediators. Recent publications indicate that exosomes might be involved in hypoxic preconditioning, but mechanistic data are still missing. In addition, it remains to be investigated, whether other preconditioning stimuli, like volatile anaesthetics also trigger the release of vesicles and if these exosomes differ in their protein composition and play a role in preconditioning. *Methods*: Primary cardiac cells (fibroblasts and cardiomyocytes) from neonatal rats were exposed to different preconditioning stimuli like isoflurane (1.5 Vol%, 4 hours) hypoxia (< 1% O_2, 1 hour) or argon (50%, 1 hour). Supernatants were collected at different time points after preconditioning, and exosomes were isolated via differential centrifugation with a final ultracentrifugation step. Isolated exosomes were quantified and characterized via different methods: (a) protein quantification using micro BCA assay, (b) western blot analysis with detection of the exosomal marker proteins CD63, Flotilin-1 and HSC70, (c) nanoparticle tracking (NTA), (d) electron microscopy. *Results*: Preconditioning with isoflurane and hypoxia triggers the release of extracellular vesicles from cardiomyocytes and fibroblasts. Interestingly, the composition of these vesicles differs depending on the applied stimulus. Preconditioning of fibroblasts with isoflurane leads to the release of CD63, HSC70 and Flotilin-1 positive vesicles. In contrast, treatment with hypoxia triggers CD63 and Flotilin-1 positive but HSC70 negative vesicles whereas vesicles after argon treatment are only Flotilin-1 positive. This implies that different preconditioning stimuli indeed trigger the release of vesicles with varying protein compositions. *Summary/conclusion*: We were able to show, that primary cardiac cells actively secrete exosomes after different preconditioning stimuli. The protein composition of these vesicles differs, depending on the stimulus used and might indicate differential roles during cardiac preconditioning.

P-VIII-13

Systemic delivery of microRNA-146a mimics in lipid microparticles substitutes for ApoE in suppressing inflammation and atherosclerosis in hyperlipidemic mice

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Please see Symposium Session 3B

P-VIII-14

The predictive role of circulating microparticles in patients with chronic heart failure

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Introduction: The study aim was to evaluate whether circulating microparticles with apoptotic or none-apoptotic phenotypes are useful for risk assessment of 3-year cumulative fatal and non-fatal cardiovascular events in CHF patients. *Methods*: It was studied prospectively the incidence of fatal and non-fatal cardiovascular events, as well as the frequency of occurrence of death from any cause in a cohort of 388 patients with CHF during 3 years of observation. Circulating levels of NT-pro brain natriuretic peptide (NT-pro-BNP), high-sensitivity C-reactive protein (hs-CRP), endothelial apoptotic microparticles (EMPs) were measured at baseline. *Results*: Median follow-up was of 2.32 years (IQR = 1.8–3.1). During follow-up, 110 cardiovascular events (including 43 fatal cases) were determined. Additionally, 74 subjects were hospitalized repetitively due to worsening CHF and also 16 subjects were readmitted in the hospital due to other cardiovascular reasons. In the univariate logistic regression analysis, the main factors independently related with cumulative end-points were creatinine, fasting glucose, HbA1c, total cholesterol, uric acid various types of EPMs, NT-pro-BNP, hs-CRP, NYHA class, decreased left ventricular ejection fraction (LVEF) less 45% and type 2 diabetes mellitus. In multivariate model NYHA class, decreased LVEF (less 45%), NT-pro-BNP, hs-CRP, CD144 + / CD31 + /annexin V + EMPs and CD31 + /annexin V + EMPs remained statistically significant for cumulative end-point. Adding of CD144 + / CD31 + /annexin V + EMCs and CD31 + /annexin V + EMCs to the standard ABC model may improve the relative IDI for cumulative end-point by 11.4 and 10.5% respectively. *Summary/conclusion*: Apoptotic phenotype of circulating microparticles may relate 3-year combined clinical outcomes in CHF patients.

P-VIII-15

Muscle-released exosomes act as paracrine signal during the development of lipid-induced insulin-resistance

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Introduction: Skeletal muscle (SkM), the largest organ in the human body, is responsible for whole-body metabolism. Although communication between SkM and other tissues appears of importance in the development of diabetes, the possibility that SkM-derived exosomes act as a mode of communication has hitherto never been described. In this study, we have tested the hypothesis that SkM-released exosomes might transmit specific signals during insulin-resistance (IR) induced by saturated fatty acids. *Methods*: C57/black6 mice were fed either with a standard diet (SD mice) or with SD enriched with 20% palm oil (palmitic acid 44.5%, oleic acid 38.5%) (HP mice). In vitro, C2C12 cells were incubated with 0.5 mM palmitate (C16:0), the most abundant saturated fatty acid of palm oil (EXO-Palm) or with BSA as control (EXO-BSA). In addition, C57/black6 mice were given single i.v. injection of labelled exosomes from control- or palmitate-exposed muscle cells to determine their biodistribution in vivo. *Results*: (a) Feeding mice with palm oil modified the exosome secretion rate from SkM. This result was confirmed in vitro as increasing the amount of palmitate in the medium of C2C12 induced an increase in exosome release. (b) HP mice had reduced expressions of markers of muscle differentiation indicating that palm oil had a deep impact on muscle homeostasis in addition to IR. All these alterations were reproduced in vitro when muscle cells were treated with EXO-Palm compared with EXO-BSA suggesting that muscle released exosomes could transfer the deleterious action of palmitate between muscle cells. In agreement, EXO-palm was enriched in palmitate compared with EXO-BSA indicating that muscle-released exosomes likely transfer lipids in recipient cells. (c) Fluorescent labelled muscle-exosomes injected into mice were incorporated into 8 different tissues including pancreas and liver. This result suggested that SkM might transfer specific signals through the exosomal route to key metabolic tissues. *Summary/conclusion*: We provide a proof-of-concept that SkM cells might communicate to other insulin-sensitive tissues through exosome secretion. Alterations in this signalling system during high fat diet may eventually contribute to the development of IR and type 2 diabetes. We also demonstrated that muscle-released exosomes act as "endocrine-like" signals which can locally affect muscle homeostasis during lipid-induced IR.

P-VIII-16

Extracellular vesicles secreted from adipocytes exposed to hypoxia and their effects on macrophage chemotaxis and phenotype

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Introduction: Localized areas of hypoxia are associated with expanding adipose tissue, particularly in obesity. This hypoxia is a strong stimulus for angiogenesis to supply the expanding tissue bed with new blood vessels. Expanding adipose tissue can also become inflamed resulting in further recruitment of inflammatory cells such as macrophages therefore driving obesity-related metabolic disorders. Adipocytes have been shown to release extracellular vesicles (EVs) in vitro that can promote angiogenesis in vivo. The aim of this work was to determine whether adipocyte EV production was altered following exposure to a hypoxic episode, and whether these hypoxic EVs could stimulate macrophages to a pro-inflammatory state. *Methods*: 3T3-L1 adipocytes were incubated in either 1 or 95% oxygen for 24 hours in serum-free media which was then collected for EV isolation. EV size and concentration were measured using nanoparticle tracking analysis, and changes in fatty acid composition were analyzed using gas chromatography with flame ionization detection. Changes in EV protein content were measured using a sticky plate ELISA. *Results*: Hypoxia (1% oxygen) was shown to increase EV production by 3T3-L1 adipocytes compared to 95% oxygen controls (1%; 391 ± 90 EVs/viable cell, 95%; 158 ± 23 EVs/viable cell, $p > 0.05$). Hypoxic adipocyte-derived EVs were then analyzed for changes in their lipid composition and their protein content compared to 95% oxygen controls. Data will be presented showing the functionality of hypoxic adipocyte-derived EVs tested using THP1 macrophages in a Dunn chamber, and their ability to stimulate a phenotypic shift towards an M1 phenotype using qPCR. *Summary/conclusion*: EV production is increased in adipocytes in response to hypoxia. These EVs may aid a phenotypic shift in tissue-resident macrophages towards a more pro-inflammatory phenotype and also promote further migration of macrophages into adipose tissue. Together, this may help to enhance the inflammation of adipose tissue and exacerbate obesity-related metabolic disorders.

P-VIII-17

Age- and sex-specific differences in blood microvesicles from healthy humans

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Introduction: Incidence and prevalence of cardiovascular disease (CVD) varies by sex and age. Cellular activation associated with progression of CVD could induce shedding of cell membrane-derived bioactive microvesicles (MV) into the blood. Concentrations of cell-specific MV in blood have the potential to be a diagnostic/prognostic marker of pathology, but ranges of MV must first be established in healthy humans. This study identified cellular origin of blood-borne MV in blood of healthy women and men by decades of life. *Methods*: Venous blood was collected into protease inhibitor anticoagulant (hirudin plus soybean trypsin inhibitor) from age- and sex-matched apparently healthy blood donors ($n = 153$; 65 men and 88 women; 20–70 years of age) participating in the Mayo Clinic Individualized Medicine Biobank. Blood MV (> 0.2 micron) were isolated by differential centrifugation and analyzed by digital flow cytometry. Cellular origin of MV was verified by 2 different marker antibodies (e.g. $r^2 = 0.96$; CD41 vs. CD42a for platelets) and analyzed as MV/μl plasma. *Results*: Platelet-derived MV were the most abundant type of MV in blood from men and women in all age groups. Total numbers of platelet- and endothelium-derived MV were significantly ($p < 0.05$) greater in women than men. In women there was a positive relationship between age and erythrocyte-derived MV ($p = 0.009$); while in men adipocyte-derived MV increased with age ($p = 0.007$). *Summary/conclusion*: This study provides ranges for various cell-derived blood-borne MV > 0.2 microns in apparently healthy men and women from 20 to 70 years of age. Future studies need to validate these reference ranges in other healthy individuals for comparison to patient populations or asymptomatic individuals at risk for cardiovascular, thrombotic or other diseases.

P-VIII-18

Future potential of human cell-derived extracellular vesicles in cardiovascular research

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Introduction: Healthcare systems are facing increasing costs for the diagnosis and treatment of patients with cardiovascular disease.

This demands for more extensive development of personalized diagnostics and therapy. Current advances in biosensors, bio-imaging technology and functional biomarker discovery hold potential for a new generation of personalized diagnostics. A wide range of functional biomarkers such as lipid, protein, mRNA and miRNA are principally carried through biological fluids by secreted extracellular vesicles (EVs) for intercellular communication and autocrine signalling. Moreover, it has become clear that the cargo of EVs is tightly linked to the stage of a disease. Hence, the molecular profiling of EVs to identify a cell-specific signature has emerged as a new tool in personalized diagnostics. Our aim is to identify disease-associated biomarkers and to develop label free methodology for biomarker profiling and quantification of EVs in relation to the risk for cardiovascular disease. *Methods*: Different analysis tools such as Transmission electron microscopy (TEM) and dynamic light scattering were used to study the size distribution and the morphology of isolated EVs from different cell lines and from primary cultures of endothelial cells derived from patients with different risks for cardiovascular disease. Biomarker profiling and quantifying of isolated EVs were carried out using different label dependent and label free methodologies. *Results*: Vesicles having an approximate size range between 25 and 100 nm were successfully isolated using different isolation processes. We observed that stress applied to cells is resulting in changes in the number and biomarker profile of secreted EVs. The number of isolated EVs from conditioned cells with TNF-α were 1.2−1.4 fold higher as compared to untreated cells. The observed changes in the biomarker profile of EVs were verified using electron immune-gold labelling, FACs, ELISA and SPR techniques. In addition, we discovered that CD63 also has potential to discriminate between EVs derived from normal or from stressed endothelial cells. *Summary/conclusion*: Our results support the idea that labelled and unlabelled biomarker based approaches for EV profiling can be used to study stress related responses in cells of patients with different risk profiles.

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Poster session IX - Protein analysis of EVs
Chairs: *Eric Boilard and Tommaso Leonardi*

P-IX-1

Simplified protocol for Tamm-Horsfall protein depletion from urinary extra dimensional electrophoresis-based proteome analysis

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Introduction: The main problem encountered during purification of urinary extracellular vesicles (EVs), including exosomes, is that Tamm-Horsfall protein (THP), which is the most abundant protein in urine of healthy subjects secreted from the thick ascending limb of Henle's loop, co-precipitates in the EV fraction because of its aggregation property. The aim of this study was to design a simple and easy protocol for THP depletion using polyvinylidenedifluoride (PVDF) membrane filters for urinary EVs for proteomic analysis based on 2 dimensional electrophoresis (2DE). *Methods*: Urinary EVs were purified from a pooled urine sample of healthy subjects by 2-step ultracentrifugation. Isolated EVs were analyzed by 2DE. PVDF membrane filtration was performed (a) before centrifugation at 17,000 $\times$ g, (b) before ultracentrifugation at 200,000 $\times$ g or (c) after ultracentrifugation at 200,000 $\times$ g. The resulting 2DE protein profiles were compared with that of the unfiltered sample. Protein spots were subsequently trypsin-digested and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Results*: The most appropriate timing for PVDF membrane filtration was step (c), which resulted in the detection of 206 $\pm$ 5 proteins. Compared to the unfiltered EV sample (183 $\pm$ 6), the protein pattern after (c) showed clear background and no loss of the detected spots, resulting in increased number of visible protein spots than with the unfiltered sample. In steps (a and b), the numbers of urinary EV protein spots were 40 $\pm$ 7 and 48 $\pm$ 6, respectively, which were significantly lower than those in both the unfiltered sample and filtered sample after step (c). Among the 89 proteins identified in the present proteome analysis, 35 proteins have not been published or listed in the major database of urinary EVs, indication that these 35 proteins were newly identified in this study. *Summary/conclusion*: Highly aggregated THP molecules were trapped in the PVDF membrane upon filtration, leading to better resolution of urinary EV proteins in 2DE analysis. We determined the appropriate timing for PVDF membrane filtration for THP removal, and this easy-to-use approach facilitated improved identification of urinary EVs proteins.

P-IX-2

An enzyme-linked immunosorbent assay to quantify water and salt transporters in urinary extracellular vesicles

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Introduction: Urinary extracellular vesicles (uEVs) are nanometre sized particles that contain many disease-related proteins from renal epithelial cells, including water channels and sodium transporters. Protein abundance in uEVs may reflect the physiological or pathophysiological state of the kidney. uEVs are usually isolated using ultracentrifugation, making it less suitable for high throughput clinical application. For this reason, we developed an enzyme linked immune sorbent assay (ELISA) that requires less starting material, allows more simultaneous measurements and could ultimately be applicable for clinical use. As proof of principle, the ELISA was designed to quantify the water channel aquaporin-2 (AQP-2) and the sodium chloride co-transporter (NCC) in uEVs. *Methods*: The sandwich ELISA that we constructed consists of a primary antibody that captures uEVs, a secondary antibody directed against the protein of interest (AQP-2 or NCC) and a tertiary antibody for luminescent detection (horse radish peroxidase). The capture antibody is directed against the tetraspanin CD9, which is abundantly expressed on the plasma membrane of uEVs. Because our secondary antibodies are directed against intracellular domains of AQP-2 and NCC, the use of detergents to permeabilize uEVs is required, for example using sodium dodecyl sulphate (SDS) or triton X-100. *Results*: Our uEV ELISA setup allowed sensitive detection of AQP-2 and NCC in a concentration-dependent matter. Intra-assay coefficient of variation for AQP-2 and NCC was estimated to be 8%. As expected, permeabilization of uEVs was required to obtain a signal. Compared to other detergents, SDS yielded the highest signal to noise ratio. Subsequently, thirsting and water loading experiments in healthy volunteers were performed. This allowed verification whether physiological changes in the water balance regulating hormone vasopressin were reflected in AQP-2 and NCC abundance in uEVs. Indeed, normalized for urinary creatinine, fasting increased the abundance of AQP-2 and NCC in uEVs, while water loading decreased the abundance of both proteins in uEVs. The abundance of CD9, however, remained constant in both experiments. *Summary/conclusion*: We successfully developed an ELISA to capture and quantify uEV content and validated this technique for analysis of AQP-2 and NCC. The abundances of AQP2 and NCC in uEVs changed with thirsting and water loading, recapitulating their known regulation by vasopressin. The uEV-marker CD9, however, paralleled urinary creatinine, suggesting that the number of excreted uEVs remained constant. Our uEV ELISA set-up may be used as platform for other uEV proteins of interest.

P-IX-3

Proteomic profiling of exosomes from cancer cell lines to identify biomarker candidates for colon cancer

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Introduction: Despite current biomarkers such as CEA and CA 19-9 are used in hospital, there is an increasing demand for biomarkers for early detection of colon cancer because of their low sensitivities and specificities. Exosomes are informative vesicles harbouring unique information about the physiological or pathophysiological state of cells and provide early biomarker candidates. In this study, we focus on identification of specific biomarkers for diagnosis of colon cancer through differential proteomic approaches to exosomes from colon cancer and normal cell lines. *Methods*: We isolated exosomes from colon cancer cell lines (HT-29 and HCT-116) and colon normal cell line (CRL-1541) by a differential ultracentrifugation method. These exosomes are characterized by western blot and FACS analysis using exosomal markers, and nanoparticle tracking analysis (NTA). Exosomal proteins were trypsinized and subsequently analyzed using liquid chromatography coupled to mass spectrometry (LC-MS/MS). *Results*: We have obtained the exosomes from cultured media of colon cancer cell lines and identified a profile of differentially expressed proteins from 3 cell lines. Most of the proteins identified in colon cancer cells exosomes compared to normal cell exosomes were part of biological process involved in signal transduction and protein metabolic process. Some candidates are selected among 316 differentially expressed proteins and confirmed by western blot analysis. *Summary/conclusion*: Some exosomal proteins identified from cell lines will be confirmed in a large-scale of clinical samples to increase the potential of early diagnostic biomarker for colon cancer.

P-IX-4

Inadequate isolation methods mask the genuine exosomal proteome

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Introduction: Despite the enormous interest in the role of exosomes in cancer, one important challenge remains the lack of standard methods to obtain highly pure exosome populations for transcriptome and proteome studies. We recently compared the performance of 4 exosome isolation protocols and determined the transcriptome of the resultant samples. We found OptiPrep density gradient (ODG) producing the purest exosomal isolates, while commercially available kits such as ExoQuick (EQ) co-isolate contaminants and introduce bias when identifying the exosome-specific RNA content (Van Deun et al. ''The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling.'' JEV. 18 Sept 2014). In this follow-up study, we examine proteomics data obtained from both OptiPrep and EQ samples to determine to what extent a suboptimal isolation procedure affects the identified proteome. *Methods*: Liquid chromatography – tandem mass spectrometry was performed on lysates of exosomes isolated from breast cancer cell-conditioned medium via the ODG or EQ method. To validate the data, the Vesiclepedia database was used whereby we selected for human proteins identified in vesicles designated as ''exosomes.'' Data were further analyzed using the DAVID or UniProt database for Gene Ontology enrichment analysis. *Results*: Comparison of ODG-exo and EQ-exo against the Vesiclepedia database revealed that 90% of ODG-exo and only 72.5% of EQ-exo were already identified previously. When selecting for proteins in exosomes isolated through density gradient, this percentage dropped for EQ-exo but stayed high for ODG-exo. DAVID GO Cellular Compartment analysis annotated ODG-exo proteins most significantly to compartment categories of vesicular nature, while for EQ-exo this was not the case. Performing GO Molecular Function analysis, the most significant categories identified for ODG-exo were GTPase activity and GTP binding, while for EQ-exo these categories were (poly-A) RNA binding and structural constituent of ribosome. This is in agreement with previous observations where we found an enrichment of the RNA-binding protein Argonaute-2 in EQ-exo samples compare to ODG-exo. Although Ago2 itself was not identified in EQ-exo, members from the same family were. *Summary/conclusion*: We have shown that the ODG sample is much more enriched in proteins related to exosomes than the EQ sample. The presence of RNA-binding proteins in EQ-exo provides an explanation for the previously identified discrepancy in mRNA profile. Our combined results indicate that both transcriptome and proteome of exosome samples are highly dependent on the performance of the isolation technique. This has considerable implications for functional and biomarker studies performed with exosomes isolated using an inadequate method.

P-IX-5

Characterization of the protein-to-lipid ratio of extracellular vesicles by infrared spectroscopy

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Introduction: Extracellular vesicles (EVs) attract great interest due to their potential use as non-invasive biomarkers. However, comparison of results in the field and evaluation of clinical studies are hampered by the lack of standardization and characterization of vesicle preparations. The total protein and lipid content of a preparation is a key property that characterizes the subtype and quality of the EV sample. *Methods*: In this study, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was used for the first time to characterize the protein-to-lipid ratio of different EV preparations. EVs isolated from Jurkat cell line and from erythrocyte concentrates were studied, and the calibration of the method was performed by using synthetic liposome – protein mixtures, lipoproteins and erythrocyte ghost membrane samples. To describe the amount of proteins in each sample, the Amide I bond at 1,690 to 1,600 cm^{-1} was used, while the C = 0 band (at 1,765 to 1,710 cm^{-1}) and the CH_2 bands (at 3,000 to 2,800 cm^{-1}) characteristic to lipid moieties was used for the phospholipids. *Results*: The characteristic lipid and protein bands can be clearly identified on the IR spectra of the studied EV preparations. Using the above mentioned reference samples, we could relate the measured band intensities to concentration values. In accordance with previous investigations applying classical biochemical assays, we found characteristic protein-to-lipid ratio values for the subtypes of EVs. Moreover, we found that the IR spectra of EV preparations also carry information about the presence of non-vesicular materials, like lipoproteins and protein complexes. *Summary/conclusion*: ATR-FTIR was found to be an effective method for the rapid and reliable characterization of the protein-to-lipid ratio of a vesicle preparation. Moreover, the difficulties associated to the interfering compounds in biochemical assays can be overcome by using this method.

P-IX-6

Meta-EVO: a meta-analysis with extracellular vesicular ontology to identify biomarkers

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Introduction: Extracellular vesicles (EVs) have major biological effects and tremendous potential as biomarkers. There is a prevailing understanding that different cells, under different conditions, release distinctive types EVs that have specific effects that are due to specific protein, lipid or RNA repertoires of those different types of EVs. However, we do not yet know what the relevant protein, lipid and RNA repertoires are for most of the types of EVs that have been identified. Dexosomes, Texosomes, Prostasomes and countless other types of EVs have been reported, but we lack relevant subset-specific annotation for EVs, and most mixed samples of such EVs, could not be precisely sorted to separate one EV type (or subset) from another. In order to facilitate the development of the necessary bioinformatics infrastructure that would enable EV researchers to identify relevant subset-specific EV components, we propose a draft EV Ontology (EVO) framework that identifies biological parameters that are important to the classification of EV subsets. Furthermore, we analyzed ExoCarta data to determine what information relating to (candidate) subset-specific markers can be drawn from public databases at this time. *Methods*: We defined the following EVO attributes, below, as characteristics that we would want to include in analyses of EV meta-data, in order to identify EV subset-specific biomarkers. EVO terms 1, 2 and 6 are annotated in ExoCarta.

1. Cell/tissue source of the EV preparation?
2. How were the EVs prepared & analyzed?
3. What was the state of the source cells/tissue? (ischemic, irradiated, etc.)
4. What were the target tissues/cells where the EVs had an effect?
5. What effects did the EVs have?
6. Route(s) of EV travel (serum, urine, cerebrospinal fluid, etc.)?
7. Any genetic variations known in the source?

We extracted the December 2014 ExoCarta database and analyzed the data, with basic methods, such as Venn Diagrams and exploratory analyses, including t-SNE multidimensional data visualization, to identify structure in the underlying dataset. *Results*: Our analyses identified a limited number of candidate subset-specific proteins, and we found clusters of proteins associated with specific cell types

(adenocarcinoma of the prostate and pancreatic cancer) and with pleural effusions. However, multidimensional analysis demonstrated that some of the "weights" of selected subset markers are attributable, in part, to expected similarities within data sets from individual studies. *Summary/conclusion*: Although ExoCarta is the most comprehensive data repository that exists for tissue-, disease- or treatment-related EV biomarkers, additional annotation and input from the EV community is needed to capture relevant information for future researchers. We put forward this proposed EV Ontological framework as a starting point, and our meta-analysis of the ExoCarta database as one example of the potential uses of this valuable resource.

P-IX-7

Systematic comparative lipidomic and proteomic analysis of exosomes: a path toward reverse engineering and artificial exosome development

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Please see Oral with Poster A

P-IX-8

Proteomic profiling of detergent resistant membranes (lipid rafts) of prostasomes and their revesiculation

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Introduction: Prostasomes are exosomes derived from prostate epithelial cells. The membrane lipid composition has a high contribution of sphingomyelin at the expense of phosphatidylcholine and has a high cholesterol/phospholipid ratio. Lipid rafts are liquid-ordered domains that are more tightly packed than the surrounding bilayer. Lipid rafts are proposed to be submicroscopic assemblies that float freely within the liquid membrane bilayer and some proteins preferentially partition into the ordered raft domains. We asked the question whether lipid rafts do exist in prostasomes and which proteins might be associated with them. *Methods*: Prostasomes were subjected to ultracentrifugation in a sucrose density gradient containing PBS plus 1% Triton X-100 with capacity for banding at 1.10 g/mL, that is the classical density of lipid rafts. Prepared prostasomal lipid rafts were analyzed by mass spectrometry and electron microscopy. Lipid raft associated proteins were identified and several of them were involved in intraluminal vesicle formation for example tetraspanins, ESCRTs and Ras-related proteins. *Results*: This is the first comprehensive LC-MS/MS profiling of proteins in lipid rafts derived from exosomes. Prostasomal lipid rafts and control prostasomes displayed similar spherical shapes but the former were more electron lucent than controls. *Summary/conclusion*: Prostasomal lipid rafts also presented a bilayered membrane, and we hypothesized that they underwent revesiculation. Accordingly, prostasomes contain lipid rafts that may be functional vesicular entities.

P-IX-9

Exosomes produced by myeloid-derived suppressor cells contain ubiquitinated proteins

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Introduction: Myeloid-derived suppressor cells (MDSC) are present in most cancer patients where they promote tumour growth by inhibiting anti-tumour immunity. We have recently shown that exosomes from MDSC polarize macrophages towards a tumour promoting phenotype and mediate their chemotaxis of MDSC (1). The current study uses proteomic techniques to test the presence of ubiquitinated proteins in exosomes produced by MDSC (2). Although ubiquitination has been shown to signal both the internalization of surface proteins and the sorting of endosomal proteins into luminal vesicles, the basis for differentiation between lysosomal degradation and exocytosis is not yet understood in exosome formation. Ubiquitin forms an isopeptide bond through its C-terminus to the ε-amino group of a lysine on a substrate protein. Tryptic digestion cleaves ubiquitin at Arg74, which leaves a diglycine remnant on the modified lysine of the substrate protein. *Methods*: MDSC were harvested from BALB/c mice carrying 4T1 mammary carcinoma tumours secreting interleukin-1β and plated overnight in serum-free medium. MDSC-derived exosomes were purified from culture supernatants. Ubiquitinated proteins from an exosome lysate were enriched using agarose beads coupled to anti-ubiquitin antibodies (cell signalling). Following tryptic digestion, enrichment was repeated using antibodies specific for gylcyl-glycyl-lysine. LC-MS/MS analyses were performed on a Shimadzu Prominent nanoHPLC (Shimadzu BioSciences) in-line with an LTQ-orbitrap XL (Thermo Fisher Scientific). Peptide identifications were made by the meta-search engine PepArML with the UniProt Mouse database. The mass corresponding to diglycine-modified lysine was used as a variable modification. *Results*: A total of 65 tryptic peptides containing modified lysines correspond to 50 ubiquitinated proteins. Each protein reported as ubiquitinated has been identified with at least one peptide containing a diglycine remnant. From this total, only 10 proteins have previously been identified in MDSC-derived exosomes. Additionally, a polyubiquitin fragment was characterized with multiple branch sites. Several histones were observed to be conjugated at multiple unique and non-overlapping sites, and the proinflammatory high mobility group protein B1 (HMG B1) was found to be ubiquitinated. Among the cohort of 50 proteins, 34 have not been previously reported to be ubiquitinated. *Summary/conclusion*: Mass spectrometry-based proteomics has been used successfully to identify 50 ubiquitinated proteins from MDSC-derived exosomes. Based on protein assay results, approximately 10% of the MDSC-derived exosome lysate is composed of ubiquitinated proteins. Among the list of identified proteins, 5 are associated with endosome and exosome formation. These proteins of interest include sorting nexin 13, keratin type I cytoskeletal 14 and 42, leucine zipper EF hand-containing transmembrane protein 1 (LETM1) and endoplasmin.

References

1. Burke M, Choksawangkarn W, Edwards N, Ostrand-Rosenberg S, Fenselau C. Exosomes from myeloid derived suppressor cells carry biologically active proteins. J Proteosome Res. 2014;13(2): 836–43.
2. Burke MC, Oei MS, Edwards NJ, Ostrand-Rosenberg S, Fenselau C. Ubiquinated Proteins in Exosomes Secreted by Myeloid-derived Suppressor Cells. J Proteosome Res. 2014;13(12):5965–72.

P-IX-10

Comparative biophysical and lipidomic analysis of mammalian and nematode exosomes

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Introduction: Exosomes and micro-vesicles represent a rapidly expanding field with an explosion over the last few years in the number of publications showing their role in intercellular transfer of proteins and genetic information. Studies in a diverse range of organisms suggest that vesicles secretion is ubiquitous across Bacteria, Eukaryotes and Archaea. A number of reports on exosomes secreted by various parasitic organisms also suggest their role in host-pathogen interactions. We recently identified vesicles secreted by a gastrointestinal nematode, *Heligmosomoides polygyrus*, which infects mice. These vesicles contain small RNAs and proteins that are homologs to mammalian exosomes proteins. Furthermore, these vesicles are taken up into mouse epithelial cells, presumably through a conserved uptake pathway. Since very little is known about nematode exosomes, here we examine the lipid content and biophysical properties of these vesicles in comparison to mammalian exosomes from epithelial and macrophage cells. *Methods*: Exosomes isolation by ultracentrifugation and TEM imaging, western blot analysis of exosomes markers, silver staining of exosomes proteins, LC-MS analysis of exosomes lipids content and dynamic light scattering analysis of exosomes size and charge. *Results*: *H. polygyrus* exosomes show similar size, morphology and protein content to mammalian-derived exosomes. Nonetheless, biophysical characterization highlights subtle differences in the surface electrostatic charge of the vesicles (Z-potential). Nematode and mammalian exosomes present very similar sphingolipids contents with Sphingomyelin as the most abundant species. However, the global phospholipids analysis reveals some differences in the enrichment of unique phospholipids species. All together these results suggest that *H. polygyrus* secreted exosomes have similar but distinct lipid composition compared to mammalian exosomes. They also display some subtle differences in their size and charge. *Summary/conclusion*: We speculate that the unique lipid profile of *H. polygyrus* exosomes might mediate their stability and functional properties within the host environment. This work suggests that, in general, the lipid content of mammalian exosomes from different cell types is similar to one another but very distinct from nematode-derived vesicles.

P-IX-11

Proteomic analysis of Phytomonas serpens-derived microvesicles

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Introduction: *Phytomonas serpens* is along with other members of the genus *Phytomonas* an economically important trypanosomatid parasite that causes crop diseases in coconut, oil palms, cassava and coffee tree, being highly abundant in tomato fruits. The transmission to plants occurs through the saliva of phytophagous hemipteran. *Methods*: *P. serpens strain 15T* isolated from tomato fruit (*Lycopersicum esculentum*) was cultured in GYPMI medium (glucose, yeast extract, peptone and meat infusion) at 28°C. Three days after peal, a billion parasites were washed with PBS and cultured in RPMI medium for 2 hours at 28°C. Supernatant free from parasites were filtered in a 0.45 μm membrane, and extracellular microvesicles isolation was performed by ultracentrifugation at 120,000 × g during 2 hours. Sample characterization was performed using NanoSight LM10. Protein levels were measured by Qubit fluorometric quantification. Three microvesicle sample preparations, independently obtained from *P. serpens* were separated by SDS-PAGE. The gel lanes were excised, sliced and digested with trypsin. Five micrograms of protein were analyzed in triplicate by LC-MS/MS in a Thermo Scientific Easy-nLC 1000 system coupled to a LTQ Orbitrap XL ETD. Peaklist picking, protein identification, were done using the MaxQuant version 1.5.0.25 and Pattern Lab platform. *Results*: Microvesicles medium size was 150.7 ± 6.6 nm, mode 134.3 ± 7.3 nm, with a concentration of 1.00e + 012 particles/sample. Proteomics analysis identified 1,411 proteins and 5,495 peptides. In the absence of *Phytomonas* genome data, we compared the peptides with those from other trypanosomatids (NCBI/Taxon ID 5654) with available genome data. The results showed and enrichment for peptides corresponding to heat shock proteins. *Summary/conclusion*: This is the first characterization of *P. serpens* microvesicles. Proteomic analysis indicates that there is an enrichment in proteins involved in stress response.

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P-IX-12

Proteome analysis of extracellular vesicles from patients with systemic sclerosis

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Introduction: Systemic sclerosis (SSc) is a complex systemic autoimmune disease of unknown aetiology characterized by extensive fibrosis of the skin and of inner organs. In SSc, vascular damage may lead to local inflammation and activation of endothelial cells resulting in changed EV levels which may be associated with the disease phenotype. The current study was initiated to characterize EVs from SSc patients for comparison with EVs from healthy controls (HCs) in the search for new disease biomarkers and improved understanding of disease mechanisms. *Methods*: We profiled proteins from EVs isolated from a total of 61 samples including 37 patients diagnosed with SSc (n = 37) and 24 HC (n = 24). EVs were isolated from platelet-poor plasma by 4 times wash at 18,890 × g, 30 minutes. Washed EVs were precipitated, resolubilized in urea and digested with endo-Lys C followed by continued digestion using trypsin. Digested samples were analyzed by nano-LC-tandem mass spectrometry on an Orbitrap XL. Peptide data were processed for label-free protein quantification and protein identification and were correlated with diagnosis and measures of disease severity. *Results*: In total, 1,029 unique proteins were identified and quantified. The obtained protein list was subjected to Gene Ontology analysis using DAVID and showed a significant overrepresentation of proteins associated with membrane-bound vesicles, platelet alpha-granules, mitochondria and extracellular matrix. The Mann–Whitney U-test was applied to identify proteins showing statistically significant different levels between SSc patients and HCs followed by a Benjamini-Hochberg correction of p-values to correct for multiple hypothesis testing. This resulted in a list of 222 proteins showing significant differences between HCs and SSc patients ($p < 0.05$). Proteins increased in abundance in EVs from SSc patients included TGF-beta, extracellular matrix proteins (fibulin-1, fibronectin), complement proteins and proteins related to coagulation (von Willebrand factor, fibrinogen, plasminogen) in agreement with disease pathology. Proteins decreased in abundance included proteins of mitochondrial origin (maleate dehydrogenase, isocitrate dehydrogenase, several ATP synthase subunits). *Summary/conclusion*: Circulating EVs isolated from SSc patients consistently show altered protein profiles relative to healthy controls in accordance with disease pathology and severity.

P-IX-13

Exosomal proteomic profiling of Niacin-treated Type 2 Diabetes (T2D) patients by isobaric tags for relative and absolute quantitation (iTRAQ) labelling mass spectrometry

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Introduction: Type 2 diabetes (T2D) is associated with an increased risk of atherosclerotic cardiovascular disease. Current available

treatment in managing atherosclerosis remains limited with niacin being the most potent but poor patient tolerance due to side effects. Exosomes are cell-derived vesicles thought to facilitate cellular communication and have been exploited as novel reservoir for disease biomarker discovery. Niacin effect on exosomal proteomic profiles has not been described. This study aimed to compare the proteome profiles of exosomes isolated from plasma of niacin-treated T2D patients. *Methods*: Seven male subjects with T2D and low high-density lipoprotein-cholesterol (HDL-C) were recruited. Fasting plasma was collected before and after 12-week of niacin therapy. Exosomes were isolated using 2 ligands, Annexin-V (AV) and Cholera Toxin B (CTB). Pooled exosomes were lysed for proteomics profiling using iTRAQ mass spectrometry. Only identified proteins with fold-change ≥ 1.2 compared to baseline and p-value < 0.05 were included in pathway analysis using IPA. *Results*: Niacin treatment raised HDL-C from 0.8 mmol/l (95% CI: 0.7–0.9) to 0.9 mmol/l (95% CI: 0.8–1.1). In AV-isolated exosomes, 131 differential proteins were identified, of which 38% were found to be signal peptide-associated and 11% were involved in acute-phase response signalling. In CTB-isolated exosomes, among the 110 differential proteins identified, 25% were involved in intracellular transport. They were also found to be associated to integrin-linked kinase, 2-ketoglutarate dehydrogenase complex, acute-phase response signalling. *Summary/conclusion*: Proteomic compositions of 2 exosome isolation methods may represent 2 distinct signalling pathways. Enriched pathways identified are related to known and novel mechanistic actions of niacin. Future studies may reveal the pleiotropic effects of niacin.

P-IX-14

Hepatocyte-secreted extracellular vesicles in endogenous and xenobiotics metabolism

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Introduction: Hepatic function is essential for homeostasis of the organism and outcome of different endogenous and external stresses greatly depends on the integrated response ejected by this organ. Liver injury ranging from mild infection to life-threatening liver failure is a serious worldwide health issue, and a major goal in liver pathology is the identification of molecular markers for its early detection, that is before clinical manifestations are produced. *Methods*: By applying metabolomics, proteomics and transcriptomics technologies as well as specific biochemical tools, our group is studying the physiological role of extracellular vesicles in the hepatic function in normal and pathological conditions to identify novel low-invasive markers for liver injury. *Results*: Our group demonstrated that hepatocytes are able to secrete exosome-like vesicles enriched in metabolic enzymes. We are currently achieving a thorough qualitative and quantitative analysis by transcriptomics, proteomics and metabolomics of hepatocyte-derived extracellular vesicles challenged to different model toxins as well as the effect that these vesicles have on blood homeostasis. We have detected a significant number of RNAs, proteins and enzymatic activities in these vesicles that are altered by the liver toxins. *Summary/conclusion*: Our work provides a repertoire of low invasive candidate markers for liver damage. In addition, we have detected a number of metabolites and enzymatic activities that are enriched in hepatocytes-released vesicles that support a physiological role of these vesicles in several cellular pathways.

P-IX-15

Extensive analysis of plasma exosomes in healthy donors – does gender, age or smoking influence the contents of plasma exosomes?

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Introduction: Plasma-derived exosomes are raising increased attention as they potentially serve as a novel, inexpensive and minimally invasive biomarker reservoir to screen for various diseases and thereby might increase the chance of early detection of chronic diseases or cancers especially. In order to use exosomes as biomarkers, it is important to know the biological variance of healthy individuals. The purpose of the study was to examine the exosomal contents and their distribution of various proteins in plasma of a healthy cohort of 160 persons with focus on gender, age and smoking status. *Methods*: The novel multiplexed platform of the extracellular vesicle array (EV Array, 1) was used for profiling the exosomes. The assay is based on the antibody capture of exosomes and subsequent detection of the captured exosomes by biotin-labelled anti-tetraspanin antibodies (CD9, CD63 and CD81). Antibodies against 37 different exosome biomarkers were used to capture the exosomes. The panel of antibodies contained the well-known exosome markers (CD9, CD63, CD81 and HLA-ABC), and 33 other membrane or membrane-associated markers related to, for example, cancer and inflammation. *Results*: Using the EV Array, it was possible to detect and profile exosomes for 37 analytes simultaneously using only 10 μl of plasma. In the cohort of 160 healthy donors, the amount of exosomes varied greatly. The distribution of the 37 exosomal markers varied greatly among the donors, and all data were analyzed with respect to the gender, age and smoking status of the donors. Smokers had a significantly higher amount of CD9 and CD151 on their exosomes compared to the non-smoking cohort. However, none of the analyzed antigens tends to be influenced by the gender of the donor. Concerning age, it was observed that the amount of AREG significantly lowers by age. *Summary/conclusion*: Generally, a large variation (in both amount and expressed antigens) was seen in the healthy cohort, which should be taken into consideration when looking for disease biomarkers.

Reference

1. Jørgensen M, Bæk R, Pedersen S, Søndergaard EKL, Kristensen SR, and Varming K. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. J. Extracell. Vesicles. June 2013.

P-IX-16

Redefining the breast cancer exosome proteome using unbiased quantitative proteomics and multivariate cluster analysis

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Introduction: Exosomes are microvesicles of endocytic origin constitutively released by multiple cell types into the extracellular environment. With evidence that exosomes can be detected in the blood of patients with various onco-malignancies, the development of a platform that uses exosomes as a diagnostic tool has been proposed. However, it has been difficult to truly define the exosome proteome due to the challenge of contaminant proteins identified via mass spectrometry from various exosome enrichment strategies. *Methods*: We combined differential and density ultracentrifugation exosome enrichment together with Tandem-Mass-Tag based proteomic analysis. Three conditioned media derived fractions corresponding to a 10,000 g cellular debris pellet, a 100,000 g crude exosome pellet and an Optiprep exosome enriched pellet, were isobaric labelled and analyzed via LC-MS/MS. Reporter ion intensities of identified peptides, from the 3 fractions in our analysis, enabled calculation of enrcihment ratios as a result of our exosome isolation strategy. To unbiasedly determine which proteins localized within exosomes, a Support Vector Machine (SVM) cluster analysis of our enrichment ratios was performed using known exosome markers (ALIX, TSG101 and Syndecan) and non-exosomes markers (PARP1, ATP synthase subunit δ, ATP citrate synthase). *Results*: Employing SVM cluster analysis allowed us to classify 455 proteins as confident exosomal cargo proteins out of 4,124 quantified proteins in our proteomic analysis. Annotation revealed breast cancer derived exosomes were enriched in plasma membrane proteins with known

associations in breast cancer oncogenesis. *Summary/conclusion*: Our analysis shows our ability to define the exosome proteome by discriminating between contaminant proteins and high-confidence exosome cargo proteins. This study represents a robust proteomic pipeline and conceptual framework for the future development of using exosomes as potential multiprotein marker phenotyping tool in breast cancer diagnosis.

P-IX-17

Proteome-wide profiling of circulating exosomes for identification of scirrhous gastric cancer biomarkers

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Please see Oral with poster B

P-IX-18

Identification and characterization of invasive-associated EV proteins by using proteomics analysis

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Introduction: Exosomes are extracellular vesicles (EVs) released from cancer cells and various cells into the extracellular space. They are small particle membrane proteins with a diameter 50–100 nm and contain microRNA, and protein that could be transferred to target cells. Their EVs were speculated to facilitate local invasion and metastatic spread. To elucidate biological function and role of EV on cancer cell, we performed a shotgun proteomic analysis of EVs released from pancreatic cancer cell line. *Methods*: Firstly, we investigated the invasive potential of 5 pancreatic cancer cells in matrigel invasion. Secondly, EVs were isolated from culture medium of their pancreatic cancer cell lines by differential ultracentrifugation and profile of their EVs-proteins was analyzed by a quantitative proteomics approach using SWATH MS analysis. *Results*: The matrigel invasion assay indicated that their cell lines have a differential invasive potential. In the SWATH MS experiments, a total of 645 proteins were identified with high confidence by a quantitative proteomics approach in 5 pancreatic cancer cell lines. A total of 91 proteins were found high correlation and reversed correlation between invasion rate and fold change of proteins and 13 proteins of them were annotated as membrane proteins by Gene Ontology analysis. Moreover, the High invasive pancreatic cancer cell line derived EVs enhanced invasive potential of Low invasive pancreatic cancer cell line and knockdown of EV-protein X by siRNA lead to reduction of invasive potential. *Summary/conclusion*: Our results suggested that EV-protein X was a key protein involved in the invasion activity of cancer-derived EVs. These results provide not only understanding the molecular biology of invasion mechanism but will novel diagnostic biomarkers or therapeutic targets of pancreatic cancer.

P-IX-19

Proteomic study of prostate cancer extracellular vesicles for identification of potential diagnostic markers

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Introduction: Prostate cancer is the most common non-cutaneous malignancy and the second deadliest form of cancer in American men. Currently, there is a need for more reliable prostate cancer diagnostic markers. Our study uses a proteomic approach to identify potential diagnostic marker candidates using extracellular vesicles (EVs) derived from 2 prostate cancer cell lines and 1 prostate-derived non-cancerous cell line. *Methods*: Prostate cancer cell lines LNCaP and 22RV1, and prostate-derived non-cancerous cell line RWPE1, were seeded in 150 cm dishes and allowed to shed EVs into serum-free media for 48 hours. The cell supernatant was then collected and centrifuged at 300, 3,000 and 10,000 $\times$ g to eliminate cell debris and apoptotic bodies. The samples were then ultracentrifuged at 100,000 $\times$ g for 2 hours (EV2) followed by an ultracentrifugation at 100,000 $\times$ g for 16 hours of the resulting supernatant (EV16). The presence of EVs was confirmed for both fractions from each cell line using transmission electron microscopy. The enriched EV fractions were lysed, and proteins were subjected to reduction and alkylation prior to trypsin digestion. LC-MS/MS analysis of the resulting peptides was done using an Ultimate 3000 RSLC nano system coupled to a Thermo Q Exactive Orbitrap mass spectrometer. Bioinformatic analysis was conducted using Proteome Discoverer with Sequest HT algorhythm and Scaffold software programs. Proteins were identified with a 5% false discovery rate and a minimum of 2 unique peptides per protein. *Results*: A total of 339, 362, 403, 532, 386, 408 proteins were identified for 22RV1 EV2, 22RV1 EV16, LNCaP EV2, LNCaP EV16, RWPE1 EV2 and RWPE1 EV16 samples, respectively. Our results show a difference in the proteome of EVs derived from prostate cancer cell-lines LNCaP and 22RV1 in comparison to prostate-derived non-cancerous cell line RWPE, with a total of 186 and 209 cancer-cell-unique proteins in EV2 and EV16, respectively. Among the proteins identified uniquely in prostate cancer cell lines are PTK2, angiopoietin1, both of which are important for cell proliferation, migration and angiogenesis, and β-catenin, which has been shown to increase androgen receptor activity important for cancer cell survival and proliferation. *Summary/conclusion*: This study shows that there is a difference in EV proteomes from prostate cancer and healthy cell lines. Further testing of biofluids from healthy and prostate cancer positive patients is planned in order to validate our identified proteins as promising diagnostic marker candidates.

P-IX-20

A multiplex bead platform for the protein characterization of cell culture and plasma-derived exosomes

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Introduction: Exosomes are released by a variety of cell types either constitutively or stimulation-induced. Depending on the originating cell, exosomes are loaded with a specific set of proteins, lipids and nucleic acids. To investigate the origin, composition and function of exosomes in biological fluids (e.g. plasma), specific markers are needed. We established a multiplex bead-based array consisting of capture and detection antibodies to analyze the composition of exosomal surface proteins in a given sample by flow cytometry. *Methods*: To produce exosomes from defined origins, blood cell populations were isolated, cultivated in exosome-free medium and stimulated if required. Exosomes were isolated from conditioned cell media or healthy plasma by serial centrifugation, filtration and ultracentrifugation. Isolated exosomes were incubated with a mix of 39 distinct fluorescently labelled capture antibody beads. The bead populations were discriminated by flow cytometry, and specifically bound exosomes were detected by a second set of fluorescently labelled antibodies recognizing the exosome markers CD9, CD63 and CD81. To compare the surface protein profiles from different exosome sources, signals were normalized to the mean signal intensity of anti-CD9, anti-CD63 and anti-CD81 coupled beads. *Results*: The multiplex bead platform allows the analysis of exosome surface markers and their relative abundance. Capture antibodies were chosen with focus on blood cell markers. Well characterized cell markers were detected on the originating cells as well as on the secreted exosomes, for example CD42a on platelet exosomes and

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CD3 on T cell exosomes. We could show that the common exosome markers CD9, CD63 and CD81 were not equally distributed on the investigated populations. CD9 was underrepresented on NK exosomes, the signal intensity for CD63 was relatively low on plasma exosomes and platelet exosomes bound to a smaller extent on anti-CD81 beads. *Summary/conclusion*: Exosomes harbour specific sets of proteins on their surface depending on the originating cell type and its status. The composition of exosomes in biological fluids can be deduced from this specific protein set under different physiological and pathological conditions, for example to monitor disease progression or to evaluate immune responses after therapy.

Poster session X - EVs in immune and inflammatory disorders
Chairs: *Saara Laitinen and Paola de Candia*

P-X-1

Role of miRNA-132 and extracellular vesicles in liver fibrosis

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Please see Oral with poster C

P-X-2

Circulating extracellular vesicles and microRNAs in hereditary angioedema

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Introduction: Hereditary angioedema (HAE) with C1INH deficiency has tremendously diverse clinical presentation. Despite the recent advances in treatment, no reliable biomarkers are available for identifying patients with higher risk/potential for frequent attacks. We assessed feasibility of using extracellular vesicles (EVs) and microRNAs (miRNAs) as potential biomarkers for HAE attack frequency. *Methods*: Peripheral blood was collected from 3 separate groups (8 each): HAE patients with more than 12 attacks in the past 12 months; HAE patients with less than 6 attacks in the last 12 months; and healthy controls. Circulating miRNA was isolated from plasma. Profiling of 754 human miRNAs was done with the QuantStudio OpenArray platform. The top 10 differentially expressed miRNAs were selected for validation by individual qPCR assays. EVs were sized and counted by nanoparticle tracking analysis. *Results*: Members of the paralogous miR-17-92 clusters are estrogen-sensitive and were identified to be differentially expressed between cases and controls. The largest differences were observed between controls and the HAE with frequent attack group. miR-384 was detected by array almost exclusively in healthy controls but not in HAE patients with frequent attacks. Individuals with higher attack frequency had lower EV counts than individuals with lower attack frequency ($p = 0.04$). *Summary/conclusion*: Circulating EVs and miRNAs can potentially serve as biomarkers for predicting HAE attack frequency. The presence of certain miRNA(s) may affect the clinical presentation of HAE patients. Larger studies, as well as studies including longitudinal sampling, are warranted.

P-X-3

High-resolution flow cytometric analysis of synovial fluid-derived extracellular vesicle populations during joint inflammation

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Please see Oral with poster C

P-X-4

Circulating miR-21 in extracellular vesicles could serve as a biomarker of β cell death in Type 1 Diabetes Mellitus

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Introduction: Recent emphasis has been placed on the role of intrinsic β cell stress pathways that may activate or accelerate autoimmune-mediated β cell death during the progression of Type 1 diabetes (T1D). Altered β cell and serum microRNA (miRNA) profiles have been shown in T1D, suggesting a role for these small, non-coding RNAs in β cell pathology. Specifically, β cell miR-21 expression is increased in models of T1D, but whether miR-21 promotes β cell death or survival in developing T1D remains controversial. β cells release extracellular vesicles (EVs) containing multiple miRNAs. Therefore, interrogation of EV cargo could yield insights regarding activation of β cell intrinsic stress pathways and provide clinically useful biomarkers of inflammatory stress within the islet during T1D development. *Methods*: To test this, INS-1 832/13 β cells were treated with a cytokine mix to mimic the early T1D milieu, combined with or without transfection of a miR-21 mimic. Exoquick TC was used to isolate EVs from treated cell media. C57BL6/J mice were treated with multiple low doses of streptozotocin to induce diabetes, and Exoquick was used to isolate EVs from terminal plasma 1 week after treatment. *Results*: Cytokine treatment increased β cell miR-21 levels and, consistent with previous data, miR-21 overexpression decreased the proapoptotic protein Programmed Cell Death 4. However, transfection of a miR-21 mimic with or without cytokines decreased cell count and viability (via AO/PI staining) and increased cleaved caspase 3 levels. Interestingly, cytokine treatment increased miR-21 levels in treated cell media ~5-fold. This effect was doubled in EVs isolated from media. However, in mice with chemically induced T1D, no increase in EV miR-21 was seen compared to controls. *Summary/conclusion*: Our findings show that in contrast to a prosurvival role in other systems, β cell miR-21 increased apoptosis. Our results also suggest that cytokine-induced miR-21 production in β cells could increase circulating miR-21 levels during T1D development. Lack of increase in circulating EV miR-21 in diabetic mice may have resulted from our choice of T1D model, or may reflect a need for further selection of β cell-specific EVs. Future studies will verify elevations of exosomal miR-21 in serum from mice and humans with developing T1D.

P-X-6

Urinary exosome-associated miR-146a as a potential lupus nephritis biomarker

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Introduction: Changes in urinary miRNAs have been reported in several renal diseases, having a strong potential to be biomarkers of renal injury. Here we investigated if miRNAs in urine are concentrated in exosomes and whether a glomerular disease as lupus nephritis (LN) modifies the distribution pattern. *Methods*: We used urine of patients with systemic lupus erythematosus (SLE) (6 active, 10 inactive and 12 absence of LN) and healthy controls ($n = 12$), quantifying miRNAs by RT-qPCR in cell-free urine (CFU), exosome-depleted supernatant (Sn) and exosome pellet (Exo). Selected miRNA were: ubiquitously detected in urine (miR-302d and miR-335*), a urine kidney damage biomarker (miR-200c) and altered in glomeruli of LN (miR-146a). Vesicles were characterized by electron microscopy, western blot and NanoSight. *Results*: In active LN, all miRNAs were significantly higher in urinary Exo compared to Sn fraction, especially miR-146a (57-fold change, $p < 0.01$). Furthermore, when

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we compared miRNAs of each urinary fraction among pathological groups with controls, we observed that miRNAs of exosome pellet in active LN were significantly increased compared to that in controls, being the miRNA-146a the most augmented (103-fold change, $p < 0.001$). Inactive LN only showed a significant increase for miR-146a ($p < 0.05$). Finally, urinary exosomal miRNA-146a had the highest diagnostic role of active LN compared to SLE in the absence of LN (AUC 0.960, $p < 0.01$) and between active and inactive LN (AUC 0.867, $p < 0.05$). In addition, logistic regression was performed, and a significant odds ratio for exosomal miR-146a was obtained (OR 24.00, $p < 0.05$). *Summary/conclusion*: This study confirms that urinary miRNAs are enriched in exosome-containing pellet. In the presence of active LN, the quantity of miRNAs was increased, especially in isolated exosomes. The exosomal miRNA 146a showed a high diagnostic accuracy of active LN. These results remark the attractiveness of exosome-associated miRNAs in urine as renal disease markers.

P-X-7

Dendritic cell-derived exosomes carry the major cat allergen fel d 1 and induce allergic immune response

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Introduction: Exosomes are nano-sized membrane vesicles released from a wide variety of cells, and they can be found in several body fluids. Depending on their cellular origin, they have been demonstrated to have immune stimulatory-, inhibitory- or tolerance-inducing effects. However, it is still unclear what role exosomes play during human inflammatory diseases. We here explored whether dendritic cell (DC)-derived exosomes can carry aeroallergens, such as the major cat allergen Fel d 1, and if they thereby contribute to the pathogenesis of allergic disease. *Methods*: Monocyte derived DCs from cat allergic and healthy blood donors were co-cultured with rFel d 1 (Exos-rFel d 1) or cultured alone. Exosomes from cultures were isolated by ultracentrifugation and characterized by iEM, flow cytometry and NanoSight. ELISA and TEM were used for detection of rFel d 1 on exosomes. ELISpot co-cultures of peripheral blood mononuclear cells and exosomes were used to verify whether Exos-rFel d 1 are able to induce IL-4 responses in cat allergic donors. The study was approved by the local ethics committee at Karolinska Institutet and informed consent was obtained from the participants. *Results*: Isolated exosomes had a size with a diameter ranging from 135 to 150 nm and exhibited a typical phenotype of DC-derived exosomes, showing the presence of HLA-DR, CD63 and CD81 on the surface. Results showed that Exos-rFel d 1 carry rFel d 1, which was both verified by ELISA and TEM. ELISpot results showed that Exos-rFel d 1 are able to induce IL-4 responses in the cat allergic donors. *Summary/conclusion*: Our results demonstrate a novel pathway for distribution of aeroallergens via exosomes derived from DC. These exosomes are able to present allergens and thereby induce T-cell T(H)2-like cytokine production in allergic donors. Thus, these exosomes may be important immunestimulatory factors in allergic immune responses and important targets or engineered tools in immunotherapy.

P-X-8

Distribution of phospholipase A2 isoforms in nanovesicles from bronchoalveolar lavage fluid

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Introduction: Nanovesicles, including exosomes, play a significant role in intercellular communication. They are of endosomal origin, present in almost all biological fluids. They carry proteins including active enzymes, mRNAs, miRNAs and bioactive lipids. Bronchoalveolar Lavage Fluid (BALF) is a useful diagnostic tool for studying lung pathology, but the presence and the role of nanovesicles in this fluid is still unclear. The aim of our work is to investigate the phospholipases A2 (PLA2) distribution in nanovesicles fractions isolated from BALF and their association with different lung pathologies. These enzymes hydrolyse the *sn*-2 acyl chain of phospholipids giving rise to bioactive molecules, such as arachidonic acid and platelet-activating factor. They are associated with inflammatory conditions or even to signal transduction processes. *Methods*: In this regard, we isolated BALF nanovesicles with differential ultracentrifugations from mechanically-ventilated patients with acute lung inflammation (ARDS) and controls, without cardiopulmonary disease. Purification of nanovesicles was performed using continuous sucrose gradients. The fractions were collected and characterized morphologically by TEM and DLS, as well as by blotting CD63 and Grp78 as positive and negative markers, respectively. sPLA2-IIA, cPLA2 and p-cPLA2 were analysed in the different vesicular fractions by Western Blotting. *Results*: We found that exosomes are present in BALF from both ARDS and our control patients without obvious infection, indicating constitutive or induced exosomal production. sPLA2-IIA was mainly detected in BALF exosomes from patients with acute lung inflammation. This possibly suggests a novel way of sPLA2 transportation. cPLA2 was not detected in any sample, whereas we identified traces of its active form, p-cPLA2. *Summary/conclusion*: The localization of different PLA2 isoforms in exosomes could indicate a direct delivery of the enzymes to target cells in order to alter the microenvironment conditions in the lung under acute inflammation.

P-X-9

Oral administration of bovine milk-derived extracellular vesicles ameliorates arthritis in two mouse models

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Introduction: Development of rheumatoid arthritis is associated with environmental factors, and several studies show a connection with diet. In this study, we investigated the effect of oral intake of bovine milk-derived extracellular vesicles (BMEVs) on collagen-induced arthritis (CIA) and the spontaneous polyarthritis model in IL1Ra$^{-/-}$ mice. *Methods*: Isolated BMEVs from semi-skimmed milk were characterized by nanoparticle tracking analysis, electron microscopy, anti-CD63 staining and PCR. Cellular uptake of PKH-67 labelled BMEVs was analyzed by flow cytometry and microscopy. Active TGFb was measured using a (CAGA)12-fLuc reporter assay. Naïve T cells were cultured for 5 days with an inflammatory cocktail in the presence of BMEVs, and Th17 or Treg differentiation was assessed by RORgT or FoxP3 mRNA expression, respectively. IL-1Ra$^{-/-}$ mice received a daily dose of BMEVs by oral gavage starting at week 5 of age. CIA mice received BMEVs via drinking water a week before immunization. Arthritis was scored macroscopically and histologically. Serum IgG levels were measured by ELISA and T-cell specific gene expression in LPS stimulated splenocytes by Luminex and RT-qPCR. *Results*: BMEVs expressed CD63 and contained milk-specific mRNA, immunoregulatory miRNAs and bioactive TGFb. Incubation of naïve T cells with BMEVs induced Th17 that could be blocked by anti-TGFb1,2,3 antibodies. Also induction of Treg differentiation was observed. BMEVs treatment of mice showed a delayed onset of arthritis in both the IL-1Ra$^{-/-}$ and CIA model and diminished

cartilage pathology and bone marrow inflammation. BMEVs also reduced the circulation levels of MCP1 and IL-6 levels and their production by splenic cells. In CIA, serum anti-collagen type II IgGa level and splenic Th1 and Th17 numbers were reduced. *Summary/conclusion*: BMEVs are immunoregulatory possibly by their TGFb content, and oral delivery ameliorates disease in two autoimmune arthritis models.

P-X-10

LL37-associated extracellular vesicles regulate Behçet's disease severity

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Introduction: The relationship between LL37 associated plasma microparticle (MP) level and severity of Behcet's disease (BD) is unresolved. This study aimed to identify the inflammatory action of LL37-MPs to different clinical states of BD. *Methods*: Plasma MPs of 72 BD and 22 healthy subjects were analyzed by FACS to determine the cell types beget MP to plasma. Intracellular localization, binding and internalization kinetics of MP by PBMCs were assessed either by confocal microscopy or by FACS. Differential cytokine secretion from PBMCs or mouse spleen cells in response to MPs or LL-37 associated MPs were studied either by intracellular cytokine staining or by ELISA. *Results*: Data revealed 65% of all MPs derived from platelets in active BD patients. The plasma MP concentration in active BD patients was 4.8-folds higher than healthy individual MP concentration. In culture, 40% of PBMCs became MP+ by 8 hours post-incubation. Active BD patient's plasma contained 5-fold more LL37 than healthy plasma, whereas MP associated fraction constituted 65% of all LL37. Treatment of healthy PBMCs or mouse splenocytes with BD MPs induced differential IL-6, IL12 and IFNγ production. Cytokine inductive capacity of BD patients was dependent on disease severity. When healthy MPs were mixed with LL-37, their activity reproduced active BD patients' MPs. LL37 mediated MP internalization and induced >2X more IL1β from monocytes and >20X IFNα from pDC. *Summary/conclusion*: This study established that BD severity is correlated with elevated circulating LL37 associated MPs. LL37 mediates enhanced MP internalization by immune cells thereby led to an exacerbated immune milieu and this contributed to sustained BD activity.

P-X-11

Neutrophil microvesicles resolve acute gout by inhibiting C5a priming of the inflammasome

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Introduction: Gout is a highly inflammatory but self-limiting joint disease induced by the precipitation of monosodium urate crystals (MSU). While it is well established that inflammasome activation by MSU mediates acute inflammation, little is known about the mechanism controlling its spontaneous resolution. *Methods*: The peritoneum of C57BL/6, MerTK$^{-/-}$ and C5aR$^{-/-}$ mice was stimulated with MSU. The peritoneal compartment was assessed for the number of infiltrating neutrophils (PMN), neutrophil microvesicles (PMN-Ecto), concentration of cytokines (IL-1β, TGFβ) and complement factors (C5a). To determine whether PMN-Ecto have an anti-inflammatory effect, the peritoneum was pre-treated with PMN-Ecto prior to MSU stimulation. Alternatively, liposomes containing either phosphatidylserine (PS) or control phosphatidylcholine (PC) were used to implicate PS expression in the regulatory mechanism of PMN-Ecto. Furthermore, PMN-Ecto were isolated from synovial exudates of patients undergoing an acute gouty attack. Their ability to inhibit inflammasome priming and IL-1β release was tested in vitro on human macrophages. *Results*: Intraperitoneal injection of MSU induced a rapid rise in complement factor C5a, which subsequently primed the inflammasome of resident macrophages for IL-1β release. Neutrophils (PMN) infiltrated the peritoneum and released phosphatidylserine (PS)-positive microvesicles (PMN-Ecto) in response to C5a early on in the course of inflammation. Treatment of the peritoneum with these PMN-Ecto resulted in the suppression of C5a priming of the inflammasome and consequently 3-fold inhibition of IL-1β release and PMN influx. Ectosome-mediated suppression required the presence of the PS-receptor MerTK and could be reproduced using PS-expressing liposomes. In addition, ectosomes triggered the release of TGFβ independent of MerTK. TGFβ, however, was not sufficient to control MSU-driven inflammation in vivo. Finally, PMN-Ecto were found to be present in significant amounts in joint aspirates of patients with gouty arthritis and had anti-inflammatory properties in vitro. *Summary/conclusion*: These results show that C5a simultaneously initiates and limits gouty inflammation by inducing the release of PMN-Ecto. Ectosome-mediated control of inflammasome-driven inflammation is a compelling concept of autoregulation initiated early on during PMN activation in gout.

P-X-12

Microparticle subtypes in decompensated liver cirrhosis using imaging flow cytometry

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Introduction: The origin of circulating microparticles in cirrhosis and portal hypertension remains unclear. We set out to measure platelet-, endothelial- and leukocyte-derived MPs in patients with decompensated cirrhosis with a more sensitive detection technique utilizing imaging flow-cytometry. We also evaluated the effect of freezing and thawing on resulting sample data. *Methods*: Plasma was obtained from 9 adult hospitalized patients with decompensated cirrhosis and 8 healthy controls (HC). Enumeration and phenotyping of MP were accomplished with imaging flow cytometry (Imagestream XMKII) and surface markers: annexin V (AV), platelets (CD41), monocytes (CD14, CD45) and endothelial cells (CD105, CD31). Levels of MPs were compared between groups directly after phlebotomy. An aliquot was frozen at -80°C and MPs were measured again 4 weeks later after thawing. *Results*: Biochemical profiles differed significantly between patients and controls, including peripheral platelet levels ($p < 0.0001$). PMP constituted the largest proportion of AV+ and AV− phenotypes in both groups. Levels of AV+ MP were not significantly different between patients and controls (3.9×10^6/ml patients and 3.5×10^6/ml, $p = 0.84$). Phenotypes of AV+ MP revealed no significant differences between groups: PMP (2.6×10^6/ml patients and 2.5×10^6/ml controls, $p = 0.94$), EMP (4.0×10^3/ml patients and 1.9×10^3/ml controls, $p = 0.44$) and MMP (1.6×10^4/ml patients and 1.1×10^4/ml in controls, $p = 0.26$). Levels of MMP were significantly higher in the entire cohort when measured after freezing and storing plasma for 1 month (mean difference 1.0×10^4/ml, $p = 0.0054$). *Summary/conclusion*: Levels of AV+ and AV− MPs, especially PMP, appear similar between decompensated cirrhosis patients and HC. Freezing and storing plasma might alter numbers of subtypes of MPs. Further study is warranted to explore other MP phenotypes and function, which may relate to mechanisms underlying the pathophysiology of portal hypertension and cirrhosis.

P-X-15

Characterization of murine cytotoxic T-cell-derived exosomes in the prevention of tumour growth

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Introduction: Activated $CD8^+$ T cells recognize tumour-associated antigen peptides on the context of major histocompatibility complex class I molecules and are capable to regress tumour progression via their-producing cytokines and cytotoxic agents in favourable condition. Thus, although direct interaction of $CD8^+$ T cells with tumour cells has been well received, the characterization of $CD8^+$ T-cell-released exosomes on the tumour formation and growth is not fully understood. *Methods*: Splenocytes of TCR gene transgenic DUC18 mice whose $CD8^+$ T cells recognize the mutated ERK2 protein-derived 9-mer (QYIHSANVL) tumour antigen peptide/H-$2K^d$ molecule were used as $CD8^+$ cytotoxic T-lymphocyte (CTL) source. After 7-day cultivation of DUC18 splenocytes with 9-mer peptide, the obtained culture medium was subjected to the microfiltration (0.45 and 0.22 μm) and the super centrifugation (100,000 × g) for exosome purification. At 10–14 days after inoculation of $mERK2^+$ CMS5a, or $mERK2^-$ tumour cells (CMS7 or B16) s.c. in respective BALB/c or B6 mice, DUC18 $CD8^+$ CTL-released exosomes were injected in each tumour. *Results*: DUC18 $CD8^+$ CTL-released exosomes were confirmed to be present in the culture medium at approximately 1 mg/ml concentration, and characterized as 100–150 nm diameter, and $CD8^{+/-}$, $CD9^+$, $CD63^-$, $CD90^{++}$ and $TCRVbeta8.3^+$ surface phenotypes. DUC18 $CD8^+$ CTL-released exosomes attenuated the growth of not only specifically relevant CMS5a but also unrelated CMS7 or B16. Furthermore, non-specific $CD8^+$ T cells obtained from CD3-mediated activation of BALB/c splenocytes also released exosomes with similar property to DUC18 CTL exosomes, indicating that $CD8^+$ T-cell-released exosomes function as a tumour inhibitor non-specifically. Interestingly, $CD4^+$ T cells obtained from CD3-activated BALB/c splenocytes did not produce inhibitory exosomes for tumour growth. Tumour growth inhibition by the activated $CD8^+$ T-cell-released exosomes seemed to be mediated by the depletion of mesenchymal stroma cells rather than the direct attenuation of tumour cells. *Summary/conclusion*: In this study, we demonstrated for the first time the inhibitory roles of $CD8^+$ T-cell-released exosomes in the progression of tumours. This information on the inhibitory roles has a possibility for the development of therapeutic strategy against cancer.

P-X-17

Exosomes from human semen impair antigen-presenting cell function and decrease antigen-specific T-cell responses

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Introduction: Exposure to semen is the primary route of transmission for many sexually transmitted infections, including HIV. Accumulating evidence suggests that components in semen directly impair leukocytes, which could compromise the protective efficacy of vaccine-induced immune responses in the mucosa. Seminal plasma contains large numbers of extracellular microvesicles or exosomes (SE). Thus, we explored the effects of SE on blood and mucosal immune responses. *Methods*: SE isolated from semen donated by healthy men were pooled from multiple donors. Dendritic cells (DC) were derived from peripheral blood mononuclear cell (PBMC) monocytes. DC and PBMC cultures were exposed to SE and assayed for SE uptake, immune function and cytokine production by confocal microscopy, flow cytometry and quantitative PCR. *Results*: Extracellular vesicles from semen are present at an average concentration of 2.2×10^{13} particles per ejaculate (n = 18 donors). These SE were taken up extensively by peripheral and vaginal DCs, to a lesser degree by B cells, and scarcely at all by T cells after 2 hours. In PBMC cultures, SE impaired memory T-cell function, reducing the production of TNFα and/or IFNγ in response to CMV, EBV or influenza-derived peptides by 73% for CD4+ and 55% for CD8+ T cells, in a dose-responsive manner (n = 4 donors). SE also impaired vaginal T-cell responses to a superantigen. CD8+ T-cell responses were also inhibited (by 51%, n = 3 donors) when just DC, as opposed to bulk PBMC, were exposed to SE. This effect occurred even when only a fraction of DCs (as low as 20%) were exposed to SE. *Summary/conclusion*: Exposure to SE inhibits adaptive immune responses, apparently by interfering with antigen presentation, as exposing only DCs to SE recapitulates the effect of exposing mixed cultures. Understanding how semen alters programmed immune responses is important to develop the next generation of vaccine and preventative treatments against sexually transmitted diseases.

P-X-18

Murine mesenchymal stem cell-derived exosomes have immunomodulatory effects on T-cells, especially suppressing Th17, but not Th1 differentiation

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Introduction: Mouse mesenchymal stem cells (MSCs) have been reported to alleviate the disease severity in the neurodegenerative disease models such as experimental autoimmune encephalitis (EAE). Although increasing evidences suggest that these cells can contribute to modulate immune responses, the underlying mechanisms are not fully understood. *Methods*: Exosomes which derived from mMSC were isolated by ultracentrifuge (100,000 g). Then these nanovesicles were used at in vitro and in vivo model. 1. Exosomes were treated at Th17&Th1 differentiation condition using naive CD4+ T cells. 2. Exosomes were injected into mouse tail intravenous, after induced EAE (experimental autoimmune encephalomyelitis). *Results*: In the present study, the exosomes were purified from the culture supernatant of MSCs using ultracentrifugation and tested for their immunosuppressive effects on T cells. Exosomes exhibited potent suppressive effects on T-cell proliferation through the cell cycle arrest. Specifically, treatment with MSC-derived exosome significantly reduced the level of RORγt expression at Th17 condition. However, the expression level of T-bet showed no difference between the exosome-treated and control group under Th1 condition. To prove the effect of the exosomes in vivo, EAE was induced in four cohorts of mice and each group was given with exosome, exosome-enriched, exosome-lowered culture supernatant or saline in the disease initiation phase. The development of EAE was suppressed by exosome or exosome-enriched culture supernatant. Importantly, the level of IL-17 was 2 times lower in exosome-treated group than that of control mice, but IFN-γ level was not different between 2 groups in ELISPOT analyses. Furthermore, proteasome 20s were detected in MSC-exosomes using immune-blot, and microarray data showed that even T cell's proteasomes were increased after treatment of exosomes. Interestingly, Th17 suppressive effects of exosomes were reversed in the presence of proteasome inhibitor (MG132). These mean that it could be one of candidates about suppression of RORγt. *Summary/conclusion*: In conclusion, exosomes secreted from MSCs can suppress T-cell activation and specifically abrogate Th17 T-cell differentiation in vitro and in vivo.

P-X-19

Platelet microparticles reprogram macrophage gene expression and function

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Please see Symposium Session 5B

P-X-20

Binding of exosomal miR-138 to TLR7 promotes microglial activation and contributes to morphine-associated neuroinflammation

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Introduction: Opiate abuse and HIV-1 have been described as 2 linked global health crises, and despite the advent of anti-retroviral therapy, abuse of opiates is on a rise and has been shown to result in increased neuroinflammation in HIV-associated neurological disorders (HAND). This finding was corroborated in a morphine-dependent model of rhesus macaques (RMs) infected with CCR5-utilizing SIVR71/17E. Mechanism(s) by which opiates such as morphine contribute to increased neuroinflammatory responses remains less understood. In recent years, microRNAs (miR), known regulators of translational regulation of genes, have emerged as key paracrine signalling mediators, regulating disease pathogenesis and cellular crosstalk specifically, via the transfer of miRs via extracellular vesicles (EV). In this regard, we have demonstrated that EV-mediated shuttling of miR-29 from the HIV protein Tat & morphine-exposed astrocytes regulated neuronal dysfunction. *Methods*: Using realtime PCR, expression of miRNA-138 was examined in the basal ganglia of SIV-infected RMs with or without morphine dependence. Transmission electron microscopy and western blot analyses were used to characterize EVs. RNA-immunoprecipitation assay was used to verify miRNA-TLR7 interaction in microglial cells. Microglial activation was assessed by realtime PCR and ELISA for cytokines such as IL-6 & TNF-a both in wild type and TLR7$^{-/-}$ mice. *Results*: MiR-138 was upregulated in the basal ganglia of the morphine-dependent, SIV-infected RMs compared with SIV-infected RMs. Exposure of astrocytes in culture to morphine resulted in increased induction and release of miR-138 in the EVs isolated from the culture media of these cells. Released EVs, in turn, were taken up with microglia, resulting in their activation, via the TLR7-dependent pathway. Mutation of the GUUGUGU motif in miR-138 that is homologous to a TLR7 binding domain ablated this activation. Corroboration of these cell culture findings was further demonstrated in vivo. In wild-type mice morphine administration resulted in activation of microglia, and in TLR7$^{-/-}$ mice morphine failed to mediate this effect. *Summary/conclusion*: Exposure of astrocytes to morphine leads to increased expression and release of miR-138, which when taken up by microglial cells results in their activation via the TLR-7 dependent pathways. These findings have ramifications for the development of EV-loaded anti-miRNAs as therapeutics for neuroinflammation associated with opiate abuse.

P-X-21

The innate immune response to Plasmodium falciparum-infected erythrocytes and parasite-derived exosomes

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Introduction: The malaria parasite *Plasmodium falciparum* infects erythrocytes in the human host during the asexual stage of its life-cycle. Recent findings demonstrate that during this stage, parasites can use extracellular vesicles as a mode of cell–cell communication. We have shown that these parasite-derived vesicles are distinct from uninfected erythrocyte vesicles, and contain exosome- and parasite-specific proteins. *Methods*: We investigated the innate immune response to parasite exosomes using human primary cells, multi-parameter flow cytometry and multiplex cytokine ELISA. In addition, the immune response to parasite exosomes was compared to the response to infected erythrocytes. *Results*: We found that when peripheral blood mononuclear cells were stimulated with parasite exosomes, CD14+ monocytes were activated, whereas no responses from either NK cells or $\gamma\delta$ T cells were detected. When analyzing total cytokine release by isolated monocytes, the response to exosomes was comparatively lower than the response to infected erythrocytes. *Summary/conclusion*: Our findings suggest that although parasite exosomes activate host monocytes, the low immunogenicity of exosomes compared to infected erythrocytes may allow cell–cell communication via exosomes to proceed without major immune interference. Further investigations into the potential immunomodulatory effects exerted by these parasite exosomes on the host are underway.

P-X-22

Circulating microparticles associate to severe radiation proctitis consecutive to abdomino-pelvic radiotherapy

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Introduction: Microparticles (MPs) are membrane fragments with biological activities shed from damaged or activated cells. MPs have been studied as biomarkers in several inflammatory diseases and as central players in intercellular communication. In this study, we investigated the potential use of MPs as biomarkers in patients suffering from radiation proctitis consecutive to complications of abdomino-pelvic radiotherapy. In addition, we evaluated the relationship between circulating MPs and endothelium-dependent responses. *Methods*: We included 217 patients who were over-exposed to ionizing radiation during their treatment against prostate cancer. According to their rectal bleeding grade, patients were divided into 4 severity grades, 0, 1, 2 and 3, which included 77, 81, 39 and 21 patients, respectively. Platelet-free plasma was obtained from blood samples after centrifugation at 1,500 g for 15 minutes to remove cell debris. After centrifugation at 13,500 g for 5 minutes, MPs from the supernatant were quantified, and their phenotype was analysed by flow cytometry. For functional assays, MPs were isolated by 3 consecutive centrifugations at 20,000 g for 120 minutes, and tested individually or in pools according to patients' grade. *Results*: Flow cytometry analysis of platelet-free plasma indicated that circulating levels of annexin V^{+} MPs displayed a 3-fold increase in grade 3 patients (SOMALENT scale) as compared to patients with grade 0, 1 and 2. Moreover, platelet-derived CD41^{+} MPs constituted the major sub-population compared to leukocyte, monocyte, endothelial and red blood cells in all groups. Using a clotting assay, we measured the procoagulant activity of MPs, and we found that thrombin generation velocity tended to decrease in grade 3 patients compared to other groups. Finally, MPs from grade 3 patients did not affect endothelium activation when compared to other grades. *Summary/conclusion*: Our data demonstrate that high level of circulating MPs is correlated to the grade 3 patients with radiation proctitis. These results suggest that detection of circulating MPs may be valuable for the prognostic of radiotherapy complications. Eventually, this study could contribute to propose a new anti-MPs therapeutic approach for the treatment of radiation-induced pelvic disease.

Poster session XI - EVs in CNS and kidney diseases
Chairs: *Norman Haughey and Uta Erdbruegger*

P-XI-1

Molecular characterization of neurally enriched exosomes from human plasma

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Introduction: Neural cell adhesion molecules L1CAM and NCAM are highly expressed in brain tissues. We have developed a methodology for enriching blood exosomes for expression of these surface markers and have successfully implemented it for neurodegenerative disease biomarker discovery. Here, we further characterize subsets of plasma exosomes isolated from the total population by sequential immunoabsorption: first with anti-L1CAM antibody ($L1CAM^+$) and then with anti-CD81 antibody ($CD81^+/L1CAM^-$). *Methods*: Total exosomes were extracted from half of a millilitre of human plasma from n = 16 healthy control subjects with age range 20–70 years using ExoQuick exosome isolation kit (System Biosciences, Mountain View, CA, USA), followed by enrichment of exosomes from neural sources using immunoabsorption with anti-L1CAM and anti-NCAM, in 12 subjects. Exosomes in suspensions were counted with a NS500 nanoparticle tracking system (Nanosight, Amesbury, United Kingdom). For identification and comparison of known surface markers in different populations of exosomes ($L1CAM^+$, $L1CAM^-/CD81^+$), we used Exo-Check exosome Antibody Array (System Biosciences, Mountain View, CA, USA) and western blot. Finally, we performed real-time PCR analysis for relatively neuronal-specific HuD mRNA. *Results*: Exosomes expressing L1CAM or NCAM similarly comprised 5% of total exosomes. When visualized with electron microscopy, there were no differences in size or morphology between $L1CAM^+$, $L1CAM^-/CD81^+$ and total exosomes. No exosome population expressed GM130 (mitochondria) or EEA1 (early endosome) suggesting lack of cellular contamination. $L1CAM^-CD81^+$ exosomes expressed significantly higher levels of ICAM1 (endothelial), and EpCAM (epithelial) and $L1CAM^+$ showed much higher levels of NCAM, higher TSG101 and ALIX (endosomal sorting complex). $L1CAM^+$ exosomes contained significantly higher levels of HuD mRNA. *Summary/conclusion*: We provide strong circumstantial evidence that we are able to isolate a subpopulation of exosomes enriched for neural origin, on the basis of co-expressing functionally and structurally unrelated L1CAM, NCAM and HuD mRNA. The distinctive set of proteins and RNAs in neural-enriched blood exosomes create unique opportunities for studying normal and diseased brain functions.

P-XI-2

Methods for quantifying extracellular vesicles (EVs) in clinical cerebrospinal fluids (CSF)

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Introduction: Extracellular vesicles (EVs) have emerged as a promising biomarker platform for glioblastoma patients. However, the optimal method for quantitative assessment of EVs in clinical bio-fluids remains a point of contention. Multiple high-resolution platforms for quantitative EV analysis have emerged, including methods grounded in diffraction measurement of Brownian motion (Nanosight), Tunable Resistive Pulse Sensing (qNano), vesicle flow cytomery (VFC) and EV particle count based on Transmission Electron Microscopy (TEM). Here, we compared quantitative EV particle assessment using cerebrospinal fluids derived from glioblastoma patients using these methods. *Methods*: EVs were isolated from CSF by differential centrifugation and resuspended in PBS. Nanoparticle Tracking Analysis (NTA) was performed on a Nanosight LM-10HS equipped with a 405 nm laser. TRPS analysis was performed using an Izon qNano. VFC was performed using a fluorogenic lipid probe (di-8), fluorescently labelled antibodies and a custom high sensitivity flow cytometer. *Results*: For particles with median sizes of 100 nm, Nanosight and qNano yielded comparable particle count and distribution. VFC particle counts were consistently 2- to 3-fold lower than Nanosight and qNano, suggesting that non-lipid aggregates contributed to particle count while using these platforms. TEM yielded meaningful data in terms of EV morphology; however, particle counts were consistently under-estimated by an order of magnitude relative to Nanosight and qNano. The number of larger particles (diameter of 200 nm or greater) detected by Nanosight were underestimated by an order of magnitude relative to qNano. *Summary/conclusion*: These results unveil the strength and pitfalls of each quantitative method for assessing EVs derived from clinical cerebrospinal fluids and suggest that thoughtful synthesis of multiplatform quantitation will be required to guide meaningful clinical investigations.

P-XI-3

MicroRNAs in human cerebrospinal fluid as biomarkers for Alzheimers disease

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Introduction: Alzheimer's disease (AD) is the most common form of dementia. There are currently no clinical biomarkers to confirm the onset of AD, but such a tool would allow earlier initiation of treatments that can slow disease progression. Here, we examined the utility of microRNAs (miRNAs) in cerebrospinal fluid (CSF) to serve as biomarkers for AD. *Methods*: We examined miRNA expression in CSF of living donors (47 healthy, 47 AD) obtained from the Oregon Alzheimer's Disease Center using TaqMan Human MicroRNA Arrays. Total RNA was isolated from CSF using the mirVana PARIS kit and concentrated using Zymo Clean & Concentrator-5 columns. RNAs were reverse transcribed, pre-amplified and run on Taqman miRNA arrays. Ct values were calculated using automated baseline and threshold calculations in QuantStudio Software. *Results*: We analyzed the data using a 2-step approach. First, we considered presence/absence of miRNAs utilizing a Fisher's Exact test ($p < 0.05$) of the 215 miRNAs detected in 20–80% of the CSF samples. We found 18 miRNA biomarker candidates: 17 with decreased presence in AD and 1 with increased presence in AD, relative to control. Second, we ran inferential tests based on the log-rank statistic and Kaplan Meier curves, which utilize Ct and ΔCt relative to reference: U6 small RNA. Of 643 miRNAs evaluated, 74 had log-rank $p < 0.05$ and 28 had $p < 0.01$. The top 20 most significantly differentially expressed miRNAs ($p < 0.0061$) had false discovery rate of ~20%. We also evaluated linear combinations of subsets of the 20 top miRNAs via best subsets logistic regression, and computed the area under the receiver operating characteristic (ROC) curve to ascertain classification performance. Top-performing linear combinations of 3,

4 and 5 miRNA have areas under the ROC of 0.80–0.87. *Summary/conclusion*: These studies highlight the potential of miRNAs in human CSF as clinical biomarkers for AD, and suggest that the use of multiple miRNAs improves the sensitivity and specificity of performance. Future analyses will expand the pilot cohort, refine the statistical approach and validate identified candidate miRNA biomarkers in an independent set of banked CSF samples. Funded by NIH NCATS UH2 TR000903 (JAS & JFQ).

P-XI-4

Lysosome status modulates exosome function in intercellular signalling and intracellular protein disposal

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Please see Oral with poster B

P-XI-5

Age-related alterations in the number of exosomes derived from APP transgenic mouse CSF

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Introduction: Increased levels of amyloid-β peptide (Aβ) in human brain are linked to the pathogenesis of Alzheimer's disease (AD). We have previously reported that intracerebral administration of neuronal exosomes into brains of AD model mice resulted in reductions in Aβ levels, suggesting that the exogenously treated exosomes have potential ability to sequestering Aβ. However, it remains unclear whether the exosomes, which originate from brain resident cells, also contribute to Aβ metabolism. In this study, we evaluated tissue Aβ levels and exosome densities in the cerebrospinal fluid (CSF) during aging of AD model mice. *Methods*: Transgenic mice expressing the human APP (amyloid precursor protein) bearing the Swedish and Indiana (KM670/671NL, V717F) mutations (J20 strain) were used as AD model mice. The exosomes were isolated from CSF of the mice at ages 2–23 months by sequential ultracentrifugation. The particle size and densities of the exosomes were analyzed using qNano System (Izon Science, Ltd). Aβ levels were determined with an ELISA kit. *Results*: The CSF-derived exosomes were confirmed by electron microscopy to mainly consist of small membrane vesicles 50–200 nm in diameter. Aβ was detectable in the CSF-derived exosome fractions, but the levels of exosome-associated Aβ were markedly lower in the aged brains compared to the young subjects. Particle analysis revealed that the densities of exosomes in the CSF at 6–7 months old decreased compared to 2–3 months old, these low levels were maintained through 12–13 months of age and thereafter drastically declined in 23-month-old mice. In contrast, tissue levels of Aβ continued increasing during aging. *Summary/conclusion*: Our data demonstrated that exosomes in the mouse brains declines during aging, inversely correlated with Aβ levels. This raised the possibility that endogenous exosomes may play a role in modulating Aβ metabolism.

P-XI-6

Stress-induced changes in exosomal histone secretion

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Please see Oral with poster C

P-XI-7

Organotypic brain slices for studying the role of microglial-derived microvesicles in neonatal stroke

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Introduction: Neonatal stroke is as frequent as stroke in adults and causes life-long debilitating effects. Neuroinflammation plays a major role in stroke, with microglial cells serving as injury modifiers. Using age-appropriate model of neonatal stroke, a transient middle cerebral artery occlusion (tMCAO) in postnatal day 7 (P7) rats, we previously showed that microglial cells protect rather than harm (Faustino et al., *J. Neurosci* 2011), but the role of microglia-derived microvesicle particles (MMVP) as means of cell–cell communication after neonatal stroke has not been explored. *Methods*: P9 Cx3cr1-eGFP/Ccr2-RFP mice were subjected to tMCAO, and organotypic brain slices were dissected 4 hours after reperfusion from injured and contralateral cortex and maintained for up to 5 days. GFP+-MMVP were isolated by differential centrifugation from slice supernatants and characterized by flow cytometry (ImageStreamX) using markers specific for microvesicles, such as phosphatidylserine (PS). RFP allowed detection of Ccr2-RFP+ monocytes. *Results*: Injury (Nissl and presence of cleaved caspase-3) and lack of RFP+monocytes were confirmed in slices from individual mice. The number of MMVP collected from contralateral organotypic slices 3–5 days after plating was sufficient for MMVP measurements (n = 6). Our preliminary data show that the number of MMVP and the numbers of CD45+/RFP-, CD45+/GFP+ and CD45+/PS+ MMVP in injured regions increased significantly compared to those in contralateral regions. Structural characteristics of MMVP were affected by injury. *Summary/conclusion*: Our preliminary results indicate that stroke increases the number and characteristics of MMVP in organotypic slice cultures obtained from injured regions, suggesting that slice cultures can be used to study cellular interactions and signals after stroke to better understand the role of microglial activation in neonatal stroke pathology.
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P-XI-8

Blood-spinal cord barrier disruption after contusive spinal cord injury rapidly recovers following intravenous infusion of bone marrow mesenchymal stem cells (MSCs) or MSC-derived exosomes

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Introduction: Traumatic spinal cord injury (SCI) damages the blood spinal cord barrier (BSCB) and allows immune cells and toxic molecules to infiltrate into the cord, contributing to the neuronal loss, axon severing and demyelination that can lead to paralysis. Intravenous infusions of mesenchymal stem cells (MSCs) can reduce the severity of experimental spinal cord injury (SCI), but mechanisms are not fully understood. *Methods*: Young adult rats were subjected to a moderate contusive spinal cord injury (SCI) at the T9 level, i.v. infused

with 1×10^6 rat MSCs or media 1 week post-SCI, and assessed for functional recovery, BSCB permeability, or distribution of transplanted MSCs. Alternatively, a small number of rats were infused with exosomes isolated from MSC-conditioned media. Locomotor function was assessed using the Basso–Beattie–Bresnahan (B-B-B) rating scale. Spatial and temporal changes in BSCB integrity were assessed by i.v. infusions of Evans blue (EvB) with ex vivo optical imaging and spectrophotometric quantitation of EvB leakage into the parenchyma. Distribution of DiR-labelled GFP-expressing MSCs was assessed by in vivo and ex vivo imaging of organs and fluorescence microscopy of frozen sections. *Results*: SCI resulted in diffuse and persistent BSCB leakage. BSCB leakage was reduced in MSC transplanted rats, and locomotor function was improved beginning 1 week post-MSC infusion. However, i.v infused MSCs were not detected within the spinal cord at any time point, but appeared to traffic transiently to the lungs. Preliminary data showed that exosomes isolated from MSC conditioned media also reduced BSCB permeability and improved locomotor functioning 1 week post -infusion. *Summary/conclusion*: Infusions of either MSCs or MSC-derived exosomes 1 week after contusive SCI reduced BSCB permeability and improved functional recovery. Our preliminary data suggest that the therapeutic effect of MSCs on SCI may be mediated by MSC-derived exosomes.

P-XI-9

Astrocyte-derived exosomes regulate dendritic complexity and synaptic protein expression in neurons

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Please see Oral with Poster B

P-XI-10

Extracellular vesicles from the choroid plexus propagate pro-inflammatory message in CNS upon peripheral inflammatory stimulus

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Introduction: The main enigmatic question regarding the central nervous system (CNS) is how periphery communicates with CNS and vice versa, in normal as well as pathophysiological conditions. Here, we hypothesized that the choroid plexus epithelium (CPE), a unique single layer of epithelial cells situated at the interface of the blood and the cerebrospinal fluid (CSF), forming the blood-CSF barrier (BCSFB), might be equipped to do this. In recent years, the BCSFB has gained increasing attention, especially its role in inflammatory and neurodegenerative diseases. Our lab recently showed that systemic inflammatory conditions such as sepsis compromise BCSFB barrier functionality in vivo, allowing components of the blood to gain access into the CNS via the CSF. *Methods*: Several high throughput technologies, such as NanoString and advanced mass spectrometry (MS) were used, together with in vitro and in vivo qPCR, western blot and immunohistochemistry analyses. *Results*: Here, we found that systemic inflammation induced a fast decrease in miRNA expression levels in the CPE, and this was inversely correlated with increased miRNAs levels in the CSF, such as the pro-inflammatory miRNAs miR146, miR155, miR9 and miR1a. This was linked with an increase in the amount of extracellular vesicles (EVs) in the CSF. Using transmission electron microscopy (TEM), we also observed in the CPE cells a time-dependent increase in multivesicular bodies (MVBs) filled with EVs, called exosomes, upon inflammatory stimulation in vivo. In vitro studies revealed that these secreted EVs are taken up by brain parenchymal cells and are able to transfer a message from the blood to the CNS. In vitro and in vivo pharmacological inhibition of the exosome production reduced the inflammation-induced exosome release and resulted into accumulation of miRNAs in the CPE cells. Proteomic analysis of EVs isolated from the CSF revealed that they carry myriad number of protein molecules that act as key signalling molecules in the recipient cells. *Summary/conclusion*: In conclusion, we identified CPE-derived EVs as a new mechanism of blood-CNS communication during peripheral inflammation by transferring a pro-inflammatory message from the BCSFB to the recipient brain parenchymal cells.

P-XI-11

Specific population of urinary extracellular vesicles identifies structural pathology of the kidney as detected by computed tomography and biopsy among healthy humans

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Introduction: Diagnosis of chronic kidney disease relies almost exclusively on glomerular filtration rate (GFR) and albuminuria without invasive assessment of structural pathology, thus occult injury can be missed. This study assessed whether populations of urinary extracellular vehicles (EVs) reflect underlying renal structural features of adults with normal GFR and urinary albumin. *Methods*: Computer tomography (CT) angiogram and random urine samples were obtained from age-stratified (18–70 years) healthy kidney donors ($n = 69$ men and 69 women) prior to kidney donation. A kidney biopsy was obtained during transplantation. Macro-structural CT analyses included indices of nephron hypertrophy (increased cortical volume) and nephrosclerosis (decreased cortical volume and surface roughness). Micro-structural biopsy analyses included indices of nephron hypertrophy (glomerular volume, cortical volume per glomerulus and mean profile tubular area) and nephrosclerosis (% fibrosis, % glomerulosclerosis, arteriosclerosis and arterial hyalinosis). EVs (>0.2 micron) in cell-free urine were analyzed by digital flow cytometry using fluorophore conjugated cell-specific antibodies with or without annexin-V, and normalized to urine creatinine. *Results*: Nephron hypertrophy negatively correlated ($p < 0.05$) with urinary EVs positive for phosphatidylserine (PS) and monocyte chemoattractant protein-1 (MCP-1), and those derived from mesangial cells and parietal epithelium of Bowman's capsule. Nephrosclerosis positively correlated ($p < 0.05$) with urinary EVs positive for PS and MCP-1 and negatively ($p < 0.05$) with exosome marker and EVs derived from podocytes or parietal cells. *Summary/conclusion*: Specific markers positive populations of urinary EVs from cells in the nephron may identify underlying structural kidney pathology.

P-XI-12

Exosomal RNA isolated from kidney transplantation preservation fluid provides a biomarker source for organ quality

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Introduction: Kidney transplantation is the preferred option for chronic kidney disease patients, and the shortage of donor kidneys is an increasing problem. Molecular and histological analysis of 0-hour biopsies can assess kidney quality and have predictive value for short- and long-term outcome after transplantation but require an invasive procedure and may not reflect the quality of the entire organ. Alternative, non-invasive and more representative methods to assess graft quality are required. We hypothesized that donor kidney preservation fluid contains exosomes which harbour biomarkers associated with graft quality, allowing prediction of kidney function and risk for complications after transplantation. *Methods*: We developed a protocol to collect transplant preservation fluid. Preservation fluid from donor kidneys from different donor types (living, heart-beating or non-heartbeating) which are associated with different risks for adverse kidney function after transplantation was collected and analyzed for the presence of exosomes by ultracentrifugation or commercially available reagents followed by nanoparticle tracking, electron microscopy and sucrose density gradient analyses. Small RNA content was analyzed by next generation sequencing. *Results*: Exosomes of around 132 nm with a density of 1.11 g/ml could be isolated from the donor kidney preservation fluid. RNA was isolated and analyzed by next-generation sequencing, identifying $>$2,000 small RNAs, including miRNAs and tRNAs. The most abundant miRNAs included many miRNAs known to be highly expressed in endothelial cells, suggesting that isolated exosomes are derived from the renal vascular endothelium. Comparing small RNAs derived from exosomes from different donor types identified a panel of 83 small RNAs that distinguish between donor types. *Summary/conclusion*: Here, we present the proof of concept for a novel approach to assess donor kidney quality and potentially post-transplantation function. This exosome-based approach is non-invasive, easy to implement and can be easily translated to other donor organs.

P-XI-13

Significant expansion of plasma bandwidth for biomarker discovery with plasma extracellular vesicles

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Introduction: Plasma as the main conduit of secreted molecules, and clinically accessible, is the ideal source of biomarkers. However, low abundance disease biomarker discovery in plasma necessitates either enrichment of low abundance proteins or depletion of high abundance proteins. Cholera toxin B chain (CTB) and annexin V (AV) bind 2 distinct groups of extracellular vesicles (EVs) in the plasma via their respective receptors, namely GM1 ganglioside and phosphatidylserine. We compared the effectiveness of using whole plasma, plasma CTB- or plasma AV-binding EVs or their various combinations in identifying biomarker candidates. *Methods*: Plasma from 5 pre-eclamptic (PE) patients and 5 matched healthy control; 6 spontaneous pre-term labour (PTL) patients and 6 matched healthy controls and 6 stroke patients and 6 matched healthy controls were respectively pooled into 3 sets of patients' and matched controls' plasma pools. CTB- and AV-EVs were extracted using magnetic-bead technology. Proteins extracted from plasma and EVs were analyzed by an array of 656 antibodies of which 475 targeted human antigens. GFP was used as the background control. *Results*: In the PE group, 276 antigens were detected (118 in AV-EVs, 153 in CTB-EVs and 200 in plasma) in the patient group versus a total of 272 in control group (190 in AV-EVs, 168 in CTB-EVs and 170 in plasma). Of all the detected antigens, 40 were in the patient only and 36 in the control only, thus representing candidate biomarkers. In the PTL group, 143 antigens were detected (113 in AV-EVs, 116 in CTB-EVs and 120 in plasma) in the patient group versus a total of 205 in the control group (120 in AV-EVs, 171 in CTB-EVs and 158 in plasma). Of all the detected antigens, 12 were in the patient only and 74 in the control only, thus representing candidate biomarkers. In the stroke group, 375 antigens were detected (352 in AV-EVs, 330 in CTB-EVs and 238 in plasma) in the patient group versus a total of 279 in control group (190 in AV-EVs, 168 in CTB-EVs and 170 in plasma). Of all the detected antigens, 131 were in the patient only and 35 in the control only, thus representing candidate biomarkers. The candidate biomarkers for the 3 diseases that were exclusively in the plasma were 51% (39/76) for PE, 24% (21/86) for PTL and 1.8% (3/166) for stroke. Those that were exclusively in the EVs were 45% (35/76) for PE, 56% (48/86) for PTL and 66.3% (110/166) for stroke. This exclusivity in EVs enhanced plasma bandwidth for discovery. *Summary/conclusion*: Using plasma EVs in addition to whole plasma enhanced the bandwidth of plasma for biomarker discovery by 2- to 30-fold. The unique distribution of a biomarker in 3 different plasma fractions (whole plasma, plasma CTB-EV & plasma AV-EV) suggests that the presence of a candidate biomarker, in one but not any of the fractions, could be diagnostic in itself. The level and distribution of a biomarker in plasma EVs with respect to whole plasma, could enhance the rigour, robustness and specificity of the biomarker.

Poster session XII - EVs in cancer II
Chairs: *Michael Graner and Devasis Chatterjee*

P-XII-1

Leptin modulates exosome release and composition in epithelial ovarian cancer

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Introduction: Leptin has been identified as a "cytokine-like hormone" acting as growth factor in different cancers. Leptin exerts its effects through activation of OB-Rb isoform and its overexpression has been associated with worse prognosis in several cancers, including epithelial ovarian cancer. Recently, exosome release has been identified as one of the key events regulating cell–cell communication and the interaction between cancer cells and their hosting microenvironment, particularly during metastasis process. What factors contribute to modulate exosome release and composition in ovarian cancer is almost unknown. Here, we propose that leptin could modulate exosome release and miRNA content in ovarian cancer cells. *Methods*: Two ovarian cancer cell lines (HEY and SKOV3) maintained in free-exosome culturing media were treated with or without leptin (100 ng/ml for up to 48 hours). Upon leptin or MOCK exposure, exosomes floating in the medium were isolated using ExoQuick-TC according to manufacturer instructions. To confirm proper isolation, exosomes were characterized using TSG101, HSP70 and CD63 markers by immunoblot. To assess exosome release and detecting any change in number or morphology of exosomes, induced by MOCK or leptin exposure, vesicles were characterized and counted using images taken by electron microscopy to extraction aliquots. To investigate changes in exosome composition, we analyzed miRNA exosome levels of 5 representative miRNAs previously characterized in ovarian cancer (miR-21, miR-200a, -200b, -200c and let-7a) by Taqman-Real time PCR. RNU48 expression we used as internal control. *Results*: Isolated exosomes from both cell lines expressed TSG101, HSP70 and CD63 as measured by immunoblot. An increase in TSG101 expression was found HEY cells but not in SKOV3 cells upon leptin treatment. HSP70 levels increase in both cell lines. In contrast, CD63 levels did not change in both cell lines. By analyzing images captured by electron microscopy, we detected an increase in the number but not change in morphology of exosome vesicles released upon leptin treatment in both cell lines. We also found changes in miRNA expression in both cell lines. Specifically, we found an increase in miR-200a and miR200b expression in HEY cells and increase in miR-21, miR-200a and miR-200c in SKOV3 cells. *Summary/conclusion*: Here, we demonstrated that leptin indeed increases exosome release and modifies the expression pattern of exosome markers and miRNA composition in the cell lines tested. These results suggest a leptin role in modulating exosome cell–cell communication and a potential explanation for leptin participation in cancer progression.

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P-XII-2

Analysis of the specific microRNA motifs and basic properties of the activated human T-cell-released extracellular vesicles

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Introduction: $CD8^+$ T cells play a central role in the rejection of established tumours in an appropriate antigen-restricted manner. The activated $CD8^+$ T cells have been well-known to produce cytokines such as IFN-g and TNF-a in addition to cytotoxic nature; however, the characterization of $CD8^+$ T-cell-released extracellular vesicles (EVs), including exosomes, is not fully understood. Recently our group has revealed by murine study that EVs obtained from the activated $CD8^+$ T cells attenuate the growth of various tumours in association with microRNAs (miRNA) by intratumoral administration (unpublished data), indicating the importance of exosomal miRNAs on the deep understanding of $CD8^+$ T cells. In this study, we investigated the miRNA profile and basic characters of the activated human $CD8^+$ T-cell-derived EVs. *Methods*: PBMCs isolated from healthy donors (who obtained an informed consent) were expanded and activated with anti-human CD3 mAb (OKT-3), IL-2 and RetroNectin. The obtained culture supernatant was subjected to microfiltration (0.45 and 0.22 μm) and ultracentrifugation (120,000 × g, 70 minutes) for EV purification. Diameter and protein concentration of the PBMC-released EVs were measured by NTA and BCA assay, respectively. The expression of tetraspanins and T-cell markers was observed by flow cytometric analysis of the latex beads bound with EVs. The miRNA profile in EV cargos was examined by miRNA microarray. *Results*: $CD8^+$ T cells enriched in the culture process of PBMCs produced EVs of approximately 160 nm size. The $CD8^+$ T-cell-released EVs showed the expression of not only tertaspains (CD9, CD63, CD81) but also CD8 and HLA-I molecules. By the analysis of the EV miRNAs in comparison with the parent $CD8^+$ T-cell-containing miRNAs, large parts of EV dominant miRNAs showed the guanine rich motifs including GGGG, likely GGAG with a result from other group. In addition, the $CD8^+$ T-cell-released EVs contained miRNAs with antitumor property. Our results suggest that EVs are released from $CD8^+$ T cells under the precise regulation to exert cytotoxicity. *Summary/conclusion*: Our results regarding the guanine rich motifs as GGGG and cytotoxic nature of miRNAs in $CD8^+$ T-cell-released EVs exhibit a new possibility for the treatment of patients with cancer.

P-XII-3

Mechanisms and impacts of exosomal microRNAs on lung adenocarcinoma tumourigenesis

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Introduction: microRNAs (miRNA) are small non-coding RNAs that modulate the activity of protein coding genes. The miRNAs have been shown to have a number of functions in cancer cells including tumour suppression and oncogenesis. The miRNAs have recently been shown to be selectively packaged into exosomes which may be a tumour specific mechanism. Exosomes and their contents have been shown to affect many cancers, however, the mechanism behind this selection process and the function of the miRNAs are not well understood. We aim to determine novel mechanisms driving exosome packaging and to determine the function of miRNAs enriched in exosomes in lung adenocarcinoma. *Methods*: The miRNA profiles were generated from exosomal and cellular fractions of 5 lung adenocarcinoma cell lines, using qRT-PCR for a panel of 742 different miRNAs. Candidate miRNAs with at least a 4-fold change between the 2 fractions were selected for further analysis. The miRNAs up-regulated in the exosomal fractions were then analyzed with MEME suite, a motif-based sequence analysis tool, to determine if selected mature miRNA sequences shared a common motif. To assess the biological role of the miRNA candidates selectively packaged into exosomes, lentiviral miRNA inhibitors and miRNA mimics were used. *Results*: On average, we detected the expression of 264 miRNAs in the exosomes and 258 miRNAs in the cells. Of those miRNAs, an average of 15 were observed to be

selectively released in exosomes and 14 were selectively retained in cells. Comparing across all cell lines, we noted that miRNAs 223-3p, 142-3p, 451a, 144-3p and 150 were up-regulated in 5/5 exosome samples tested, and 145-5p and 605-5p were found to be up-regulated in 4/5 exosome samples relative to the cells they were derived from. The miR-502-5p was the only miRNA found to be selectively retained in 5/5 cell lines relative to the exosomes. The miRNAs up-regulated in exosomes regulate key oncogenes, including EGFR and c-Myc. Exosomal miRNAs were enriched for a known RNA binding motif UGUA. *Summary/conclusion*: We have identified a set of miRNAs that are commonly enriched in lung adenocarcinoma cell line exosomes. These miRNAs appear to regulate key oncogenes in lung adenocarcinoma. The discovery of novel exosomal miRNA function and mechanisms could directly impact patient care through the development of novel therapeutics.

P-XII-4

Extracellular vesicles as a potential mediator of microRNA-linked ovarian cancer drug resistance

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Introduction: Ovarian cancer is the deadliest gynaecological cancer. A major contributor to the poor survival rate is the development of chemoresistance to platinum-based therapies such as cisplatin and carboplatin. We aimed to test the role of miRNAs in the acquisition of drug resistance in ovarian cancer and whether vesicular transfer between cells could be a route by which resistance can develop. *Methods*: We used microarrays to measure miRNA levels in the ovarian cancer cell line A2780 and its cisplatin-resistant derivative CP70. The role of miRNAs and mRNA targets were tested using transfected miRNA mimics and siRNAs, respectively. CP70-derived extracellular vesicles were added to cisplatin sensitive A2780 cells, and the effect on drug resistance was measured. Delivery of miRNAs was assessed by qRT-PCR. *Results*: We identified several miRNAs that are increased in cisplatin-resistant cells. We show that most of these do not directly contribute to cisplatin resistance. Interestingly, miR-21-3p, the passenger strand of the known oncomiR, directed increased resistance to cisplatin in a range of ovarian cell lines. This effect was specific to the star strand, as miR-21-5p had the opposite effect and actually increased sensitivity of A2780 cells to cisplatin. We identify NAV3 as a potential target of miR-21-3p and show that knockdown of NAV3 increases resistance. Extracellular vesicles released by CP70 cells were also capable of increasing resistance in A2780 cells, which may be contributed by the delivery and increase in miR-21-3p. Finally, we use publically available transcriptomic data to demonstrate that miR-21-3p is raised, whilst NAV3 is reduced, in ovarian tumours that are resistant to platinum treatment. *Summary/conclusion*: Our data suggest that miR-21-3p can induce cisplatin resistance in ovarian tumours, potentially by targeting the NAV3 gene, which could be promoted by the localized release of drug-resistant cell-derived extracellular vesicles.

P-XII-5

Curcumin induces selective packaging of miR-21 in exosomes released by chronic myelogenous leukaemia cells

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Introduction: Chronic myeloid leukaemia (CML) is characterized by the clonal expansion of myeloid precursors. Exosomes are nanovesicles able to modulate intercellular communication and tumour microenvironment. Exosomes contain miRNAs that can influence gene expression in target cells. The miRNAs, such as miR-21, with tumour-suppressor functions are often lost in cancer. Some observation indicate a possible cellular disposal of miRNAs by exosomes. Curcumin affects the expression of microRNAs in CML cells and according to our data may play a role in this process. *Methods*: Exosomes were collected by K562 and LAMA84 conditioned medium by ultracentrifugation. CML cells were treated with Curcumin. The miR-21 expression, PTEN and VEGF mRNA were assessed by real time PCR. VEGF secretion and pAKT were evaluated by ELISA. The anti-cancer effects of Curcumin, in vivo, were evaluated with a xenograft CML tumour model. *Results*: Nanovesicles of CML cells treated with curcumin were characterized by physical and biochemical methods. DLS analyses indicated that isolated exosomes had an average size of 80 nm and contained Alix and TSG 101. Curcumin treatment caused miR-21 decrease in CML cells, but a greater amount was observed in exosomes. In order to support our hypothesis that decrease of miR-21 was determined by a selective enrichment of this miRNA in CML exosomes, we treated CML cells with GW4869, an inhibitor of exosome release. GW4869 treatment induced an increase of miR-21 in CML cells compared with curcumin-treated cells. The addition of curcumin, to CML cells, caused a dose-dependent increase in PTEN, well-known target of miR-21, at mRNA and protein level. Curcumin treatment decreased AKT phosphorylation and VEGF expression. The effects of curcumin on a xenograft CML tumour model, confirmed the in vitro results and the anticancer effects of curcumin. *Summary/conclusion*: Our data suggest that curcumin caused a decrease of miR-21 in CML cells through a selective packaging of miR-21 in exosomes.

P-XII-6

Identification of specific miRNA expression pattern in exosomes of invasive urinary bladder cancer cell lines

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Introduction: Interaction of tumour cells and tumour microenvironment (TME) plays an important role in tumourigenesis and progression. Thereby, exosomal microRNAs (miRNAs) can affect cell–cell communication at the site of origin as well as the TME. The aim of the project is the identification of a specific miRNA expression pattern from in-vitro obtained tumour-derived exosomes of urinary bladder cancer (UBC) cell lines in correlation to their malignant potential. Furthermore, we want to analyze the effect of these exosomal miRNAs on tumour-associated fibroblasts (TAFs). *Methods*: Exosomes were isolated from invasive (T-24, 253J-BV and J82) and non-invasive (RT-112, 5637) UBC cell lines. The number and size of vesicles were measured by NTA. Exosomal and contamination markers were analyzed by Western blotting. Total RNA was isolated from cells and their exosomes (upon treatment with RNase). MiRNA expression pattern of UBC cells and exosomes was analyzed using miRNA microarray and qPCR. Exosome-mediated miRNA transfer between cancer cells and TAFs was verified by 1) transfection of donor UBC cells with *non-human* miRNA, cel-miR-39, 2) Exosome isolation and RNAse treatment, 3) Transfer to recipient TAFs and 4) qPCR analysis using total RNA from the recipient TAFs. *Results*: The isolated exosomes exhibited a high amount of exosomal markers (CD63, CD81, syntenin). Sixteen miRNAs were identified, which distinguish invasive UBC cells from non-invasive cells. Exosomes secreted by invasive UBC cells are characterized by a specific miRNA signature of 25 miRNAs. Six differently expressed miRNAs were validated by qPCR. After successful transfection of RT-112 and T-24 with cel-miR-39, cel-miR-39 was detected in RT-112 and T-24 exosomes as well as in recipient TAFs cultivated in the presence of these exosomes. *Summary/conclusion*: Exosomes secreted by UBC cells exhibit a specific miRNA signature depending on the invasive

potential of the originating cells. We could prove an exosome-mediated transfer of miRNAs between tumour cells and TAF. These results emphasize the role of exosomal miRNAs for the interaction between tumour cells and the tumour microenvironment. Further studies have to show the functional relevance of selected exosomal miRNAs.

P-XII-7

Characterization of the functional role of rhabdomyosarcoma-derived exosomes in tumour cell biology and investigation of their miRNA cargo

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P-XII-8

Identification of optimal culturing conditions for studying exosomes from normoxic and hypoxic colorectal cancer cell lines

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P-XII-9

Delivery of functional protein using cell-engineered nanovesicle

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Introduction: Delivering a functional protein to the cytoplasm of recipient cells is a major goal of many drug delivery-related fields. Although liposomes and polymeric vesicles have widely been used for this purpose, some characteristics such as use of organic solvents in manufacturing process and absence of membrane-bound proteins were often problematic in scientific and medical applications. Recently, as extracellular vesicles become famous, techniques that utilize exosome as delivery vehicle were also developed. However, their application is also limited because of the low productivity of natural exosomes. To overcome this limitations, we assessed the protein delivery ability of cell engineered nanovesicles we had developed. As a result, they not only can be produced in a large quantity but also capable of delivering functional cytoplasmic proteins. *Methods*: B16Bl6 melanoma cell line was transfected with pMSCV-puro-IRES-GFP plasmid, and stable cell line was established with puromycin selection. Cultured wild type B16 (B16 WT) and transfected B16 (B16-PIG) cells were extruded through micro porous membranes to generate nanovesicles. Nanovesicles in extruded solution were separated using density gradient ultra-centrifugation and quantified using Bradford protein assay. B16 WT and B16-PIG nanovesicles were then treated to NIH-3T3 cells for 1 day, and puromycin resistance was assessed by measuring survival rates under puromycin condition. Survival rate was measured using CCK-8 cell viability assay kit. *Results*: Treatment of nanovesicles produced using puromycin resistance cells (B16-PIG) significantly increased the survival rates of treated cells when it compared to the B16 WT vesicle treated control groups ($\sim$2-fold higher survival rate, $p < 0.01$). *Summary/conclusion*: We confirmed the functional protein delivery ability of cell-engineered nanovesicle which also can be produced in a large quantity (in this study, puromycin resistance protein). Nanovesicles produced using B16-PIG cells almost doubled the survival of recipient cell compare to nanovesicles produced using B16 WT cells. Based on this result, we can deliver protein of interest to recipient cells by generating nanovesicles using cells that transfected with the protein of interest.

P-XII-10

Impact of extracellular vesicles from mesenchymal stromal cell-based microenvironment on chronic lymphocytic leukaemia B-cells

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Introduction: The interactions between chronic lymphocytic leukaemia (CLL) cells and the microenvironment (primarily composed by mesenchymal stromal cells – MSC) play an important role in promoting the increased survival of leukaemic B cells. Extracellular vesicles (EVs) produced by leukaemic cells and the microenvironment may be implicated in this cross-talk. EVs, including microparticles and exosomes, are small plasma membrane fragments with sizes ranging from 0.01 to 1 μm, and contain products specific to the original cell, such as microRNA, mRNA and proteins. Our objective is to assess the role of EVs in the cross-talk between malignant cells and their microenvironment. *Methods*: Ultracentrifugation at 150,000 $\times$ g during 1 hour was applied to isolate EVs from supernatant of MSC culture. Protein concentration was measured by BCA kit and Nanodrop. Different concentrations of EVs were added to CLL-B-cells to evaluate their impact on cell survival. PKH67 labelling and qRTPCR were performed to prove the inclusion of EVs in CLL B-cells ($n = 18$). *Results*: We first demonstrated that EVs from MSCs are able to enter in CLL B-cells. By flow cytometry with PKH67-labelled EVs, we observed that 44.2, 93.8 and 100% of CLL B-cells had integrated fluorescent EVs after 1, 3 and 24 hours, respectively. A total of 2 highly expressed mRNA (collagen and fibronectin) in MSC, also detected in MSC-derived EVs by qRTPCR, were increased in CLL-B cells after 24 hours of incubation with EVs confirming EV-mediated mRNA transfer to target cells. Further analysis of apoptosis in CLL cells were assessed by flow cytometry using an annexin/7AAD staining: addition of increasing concentrations of EVs showed a protective effect on CLL B-cells from cell death (mean decrease of 15.02% of apoptotic cells, $n = 13$/ p-value $= 0.0002$). *Summary/conclusion*: We demonstrated, by two methods, that MSC-derived-EVs enter into CLL B-cells. These vesicles protect CLL cells from spontaneous apoptosis and affect mRNA expression involved in CLL cell functions. This study provides evidence of the critical role played by EVs in the interactions between leukaemic cells and their microenvironment.

P-XII-11

Tumour cells and macrophages: can mutant p53 be the matchmaker using exosomes?

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P-XII-12

Identification of specific exosome-associated markers as early detectors of malignant transformation from neurofibroma to MPNST

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Introduction: Neurofibromatosis type 1 (NF1) is a hereditary syndrome characterized by the development of benign nerve-sheath tumours. These neoplasms represent a major risk factor for further transformation to malignant peripheral nerve sheath tumours (MPNST). There is an urgent need to develop non-invasive methods for the screening and early detection of NF to MPNST transition, as well as for monitoring of the disease and therapeutic outcome. Exosome-based diagnostics represents a cutting-edge frontier of cancer research and clinical management. Increasing body of evidence confirms the correlation of exosomes release and molecular composition with fundamental mechanisms driving cancer onset and progression, making them early sentinels of tissue alterations and appealing source for identification of novel disease-relevant biomarkers. *Methods*: We performed comparative analysis of candidate protein biomarkers through FACS, WB and ELISA assays using exosomes purified from MPNST, plexiform NF and dermal NF cell lines conditioned supernatants. RNA and miRNA markers were identified through qRT-PCR and miRNA array upon exosomes immunocapture from cell media and healthy plasma. Bio-luminescent MPNST cells injected in nude mice provided an in vivo model for best candidate's validation in plasma obtained by endpoint retro-orbital bleeding. *Results*: Exosomes originating from different cell models displayed differential exosome associated protein, RNA and miRNA markers clearly distinguishing MPNST and benign NFs in vitro. In vivo models confirmed that our assays can specifically detect exosome associated tumour markers in complex biological fluids. *Summary/conclusion*: Candidate exosome markers identified in in vitro and in vivo MPNST models pave the way to their validation in NF clinical samples with the aim of developing a non-invasive, sensitive and specific multi-marker assay for timely identification of NF1 patients with risk of MPNST transition.

P-XII-15

Functional analysis of extracellular vesicles as a novel regulatory agent of scirrhous type gastric cancer microenvironment

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P-XII-16

Melanoma-derived exosomes stimulate endothelial sprouts and metastatic invasion in a dose-dependent manner

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Introduction: Melanoma-derived exosomes are known as important players in the formation of tumour microenvironment by triggering angiogenic processes and enhancing metastatic evolution. However, it has not been studied if exosomes are able to determine a different cell fate for tumourigenesis. Here, we show that melanoma-derived exosomes can determine the fate of HUVECs to develop a tumour in a dose-dependent manner. *Methods*: Circular and perfusable collagen channel was fabricated by an array of microneedles placed into a collagen gel as a template. Isolated exosomes from mouse melanoma or human skin melanoma cell lines were injected to the collagen channel composed of HUVECs. Various amounts of exosomes containing 2.5–50 μg/ml of proteins were flowed through an artificial blood vessel and metastatic effects were monitored over time. Directional flow was continuously triggered by a hydraulic head to mimic interstitial flow on endothelial cells. *Results*: HUVECs were cultured on a collagen channel mimicking blood vessel. HUVECs internalized by melanoma-derived exosomes were observed for their cell fate over time. At low concentration of exosomes, some of HUVECs were converted into tip cells, which are leading cells of the vascular sprouts. Within 3 days, HUVECs proliferated into stalk cells, which are the initial cell fate to make lumen for angiogenensis. On the other hand, high concentration of exosomes induced HUVECs to develop into cancerous mesenchymal cells following the endothelial to mesenchymal transition (EMT) as enhanced malignancy induced by exosomes. EMT single cells derived from HUVECs acquired motility to migrate into the collagen gel. *Summary/conclusion*: Recent studies have shown that melanoma-derived exosomes internalized by endothelial cells promote angiogenesis and metastasis. Here, we show that melanoma-derived exosomes induce the different cell fate of endothelial cells in a dose-dependent manner: vascular formation or EMT.

P-XII-17

Investigating the role of exosomes in ovarian cancer metastasis

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Introduction: Ovarian cancer is accountable for more deaths than all other gynaecological cancers combined. Cancer metastasis involves breakaway of tumour cells from the primary site and spread to other organs of the body. Metastasis is one of the main causes of death in ovarian cancer; hence, elucidation of this mechanism, at least in part, will support development of effective therapies to improve the morbidity and mortality of ovarian cancer sufferers. Exosomes are released into the organ microenvironment or directly into the blood stream by both normal cells and tumour cells. Cancer cell-derived exosomes are selectively loaded with specific proteins and RNA molecules; upon delivery, these exosomes convey signals that advocate oncogenesis and promote cancer progression. The project aim is to determine how exosomes derived from different ovarian cancer cell lines contribute to metastasis. *Methods*: We established the invasive potential of 9 ovarian cancer cell lines using the scratch motility assay. SKOV-3 cells are the most motile; OVCAR-5 cells are intermediately motile and IGROV-1 cells are the least motile. Exosomes were extracted from SKOV-3, OVCAR-5 and IGROV-1 cells, and their ability to increase motile capacity of OVCAR-5 cells was investigated. *Results*: OVCAR-5 cell motility was increased by ovarian cancer exosome treatment, independent of the motile capacity of the cells from which they were derived. Despite their slower migratory phenotype, IGROV-1 and OVCAR-5 cell-derived exosomes were able to increase motility of OVCAR-5 cells to a similar extent as SKOV-3 cell-derived exosomes. *Summary/conclusion*: These results suggest that ovarian cancer cell-derived exosomes have motility increasing capabilities in recipient cells, irrespective of the motile phenotype of the cell of origin. This indicates that ovarian cancer cells can transfer motility promoting signals via exosomes regardless of the cancer cell of origin. This work provides new insight into the role of exosomes in ovarian cancer metastasis.

P-XII-18

Role of extracellular vesicles derived from mesenchymal stem/stromal cells in breast cancer progression and metastasis

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P-XII-19

Role of extracellular vesicles on multidrug resistance transfer between leukaemia cells

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Introduction: Chemoresistance of tumour cells is a major issue for the treatment of acute myeloid leukaemia (AML). This chemotherapeutics resistance is established by clonal selection of resistant leukaemic cells. However, a horizontal transfer of chemoresistance has recently been discovered between tumour cells via extracellular vesicles (EVs). Several researches demonstrated a transfer of multidrug resistance proteins, miRNA and mRNA from multiresistant cells to sensitive ones via EVs. The aim of this research was to investigate the role of EVs in chemoresistance transfer in AML. For this purpose, the promyelocytic leukaemia HL60 cell line have been compared to its multiresistant strain: HL60/AR. The latter overexpresses multidrug resistance-associated protein 1 (MRP1) conferring resistance to anthracyclins including daunorubicin. *Methods*: The production of EVs by HL60/AR (EVs/AR) was first investigated by transmission electron microscopy, and the expression of MRP1 was evaluated by a flow cytometry drug retention assay. The miRNA content of EVs/AR was also analyzed by qRT-PCR. The chemoresistance transfer via EVs/AR was then analyzed by treating HL60 with EVs/AR isolated by ultracentrifugation. Viability of HL60 after daunorubicin treatment was then assessed by a MTT cytotoxicity assay. *Results*: At the highest doses of daunorubicin investigated (0.5 to 1 μM), EVs/AR treatment confers a benefit to HL60, 48 and 72 hours after treatment. Indeed, the viability of EVs/AR-treated cells was significantly higher than viability of non-treated cells at these concentrations. *Summary/conclusion*: This study demonstrates a real benefit of EVs from resistant cells on sensitive cells viability. A potential increase of MRP1 expression via EVs/AR will be investigated in EVs/AR-treated HL60 by flow cytometry, and the contribution of a miRNA transfer will be analyzed.

P-XII-20

Exosomes released by chronic myelogenous leukaemia cells modulate bone marrow stromal cells (BMSC) phenotype by EGFR activation

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Introduction: Chronic myelogenous leukaemia (CML) is a myeloproliferative disorder characterized by Bcr-Abl oncoprotein endowed with a constitutive tyr-kinase activity. Exosomes (exo) are nanovesicles secreted from different cell types, including cancer cells. Exo shed by cancer cells are considered messengers in intercellular communication and can affect tumour microenvironment. We previously demonstrated that CML-derived exo modulate bone marrow microenvironment by stimulating IL8 secretion in BMSC. EGFR, as well as IL8, regulates cell proliferation and survival. Exo expressing EGFR ligands can initiate signalling that affect cancer progression. We hypothesized that the release of IL8 from BMSC is mediated by CML-exo through EGFR pathway. *Methods*: Human cell lines used were LAMA84 (CML cells) and HS5 (BM stromal cells). Exosomes were collected by LAMA84-conditioned medium and CML patients' serum by ultracentrifugation. Gene expression analysis was performed by real-time-PCR, and western blot assay used antibodies to EGFR, pEGFR, Snail and AREG. IL8 secretion was evaluated by ELISA. *Results*: We demonstrated that exo mediate the activation of EGFR signalling pathway in BM stromal cells in a dose- and time- dependent manner. The EGFR inhibitor, gefitinib, reverts the effects mediated by exo on IL8 mRNA expression and IL8 secretion by BMSC, suggesting a role of EGFR in this pathway. Exo derived in vitro from LAMA84 cells and in vivo from CML patients have exposed on their surface the EGFT ligand amphiregulin (AREG). Since IL8 is a direct downstream target of snail, we studied in BMSC the effects of exo treatment on the expression of snail, slug and their downstream targets. We observed an increase of snail and slug levels after exo treatment, and this effect was reverted by gefitinib. *Summary/conclusion*: In conclusion, we demonstrated that CML-exo displayed EGFR ligands and are able to activate EGFR signalling pathways in BMSC leading to IL8 release.

P-XII-21

Procoagulant activity of extracellular vesicles from patient with acute leukaemia has a predictive value for thrombotic events

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Introduction: Thrombosis induced by a hypercoagulable state is a common complication of patients with malignancies. Recent studies suggest that extracellular vesicles (EVs) procoagulant activity (PCA) may have prognostic value in identifying cancer patients with increased risk of developing venous thromboembolism (VTE) or disseminated intravascular coagulation (DIC). The aim of this study was to find a link between procoagulant state in patient with acute leukaemia and EVs-PCA. The other aim was to assess if EVs-PCA could be a good biomarker to predict thrombotic events. *Methods*: A total of 27 patients with acute leukaemia (AL) newly diagnosed were taking off at Day-0 (without treatment), D-3 and D-7 (3 and 7 days after treatment). All patients signed the informed consent following the requirements of local ethical committee (number B039201212954). The platelet-poor plasma was obtained from the supernatant fraction of the blood tubes after a double centrifugation for 15 minutes at 2,500 g. EVs from patient were isolated by ultracentrifugation for one and half hours at 100,000 g. EVs were concentrated 6.6-fold. Procoagulant activity of EVs derived from blood patient was studied by thrombin generation with an increased sensitivity to tissue factor (TF) positive EVs. EVs were used as inductor of coagulation, and the proportion of TF activity was assessed by the use of an antibody against TF. *Results*: Among 27 AL patients, 3 patients have an increased EVs-PCA at D-0 in comparison to healthy subjects. Among these 3 patients, 2 have a thrombotic event (DIC and clot in pyelon). In these 2 patients, a significant PCA came from TF. The patient with an increased PCA without thrombotic event has a minder TF activity in comparison to patients with thrombotic event. At D-0, the others 24 patients who have no increased PCA in their EVs, did not develop any thrombotic event. Moreover, the study of EVs-PCA shows an increased activity in 1 patient at D-3. And this patient developed an induced DIC at D-5. *Summary/conclusion*: This study shows the link between thrombotic events and EVs-PCA and suggests the role of EVs derived from leukaemic blast and other cells in procoagulant state in AL. Moreover, EVs-PCA could have a predicting value for VTE and DIC in patients with AL and could inform haematologists for the thrombosis prophylaxis.

Poster session XIII - Late breaker, Advances in isolation and characterization of EVs and EV subpopulations
Chairs: *Bernd Giebel and Anna Banizs*

P-XIII-1

Isolation and characterization of *Citrus limon* L. derived nanovesicles: potential use as antineoplastic agent

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Please see Oral with poster A

P-XIII-2

Characterization of oncosomes isolated from gastric cancer cell lines and their potential impact on autologous tumour cells

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Introduction: The discovery of extracellular vesicles (EV) has revised the interpretation of intercellular communication. It is now well established that EV play a significant role in coagulation, inflammation and cancer. Several techniques are available for EV isolation, however, the most widely used method is differential centrifugation. An alternative to this technique is a polymeric precipitation method that features the formation of mesh-like web, enabling EV capture, and their later pelleting at low centrifugal speeds. *Methods*: In this study, the tumour-derived EV (oncosomes) isolated from supernatants of newly established gastric cancer cell lines (GC1401,1415,1436) by differential centrifugation (DC) and polymeric precipitation (PP) were compared. Their average size (mean), the most frequent oncosome population (mode) and concentration were assessed by nanoparticle tracking analysis (NTA). Presence of several surface markers was evaluated by antibody staining and flow cytometry analysis. Additionally, mRNA and protein expression for chosen neoantigens were assessed by qRT-PCR and western blotting, respectively. *Results*: The mean size was higher for oncosomes isolated by PP, while the mode size was lower in oncosomes isolated by DC. Concentration was comparable for both types of isolated oncosomes. CCR6 and CD63 surface expression was higher in oncosomes isolated by PP, while CD44v6 and HER-2/neu expression was higher in oncosomes isolated by DC. The HER-2/neu and MUC1 mRNA expression was higher in tumour cells exposed to their respective autologous oncosomes isolated by DC. MAGE1 mRNA expression was slightly higher in GC1415 and 1436 tumour cells exposed to oncosomes isolated by PP. Western blot analyses revealed the presence of EMMPRIN, panCEA, MAGE1 and CD63 proteins in all three cell lines, whereas their respective oncosomes showed only EMMPRIN and CD63 protein expression. *Summary/Conclusion*: NTA analyses of the isolated oncosomes revealed a substantial difference in the mode values. The smaller mode values for the oncosomes isolated by DC may suggest that this method enables the isolation of the whole oncosome population (sizewise) or that the consecutive centrifugation steps may cause rapture of the larger oncosomes. Higher expression of CD63 and CCR6 on oncosomes isolated by PP may suggest the presence of a dominant exosome population capable of transporting CCR6 chemokine receptor to the targeted cell(s), which in turn, may lead to their increased chemotactic capabilities. All three kinds of oncosomes isolated by DC showed higher CD44v6 and HER-2/neu expression compared to the ones isolated by PP. Elevated HER2/neu, MUC1 and MAGE1 mRNA expression were observed in GC1401,1415,1436 tumour cells after their exposure to isolated oncosomes suggesting that these mRNAs maybe transported by oncosomes and maybe transcribed in the targeted tumour cells, thus, increasing their metastatic potential.

P-XIII-3

A new flow cytometer particle size calibration method: refractive index compensated light scatter

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Introduction: The complex relationship between particle size and the amount of light scattered at different collection angles makes it difficult to infer particle size from a flow cytometer's light scatter data. A population may be described as scattering an amount of light equal to a reference particle (e.g. a latex or silica bead of known size) but same sized particles of different refractive index can give very different signal strengths. When comparing data between flow cytometers the difficulties are compounded by differences in light scatter collection angles. Ideally it would be possible to produce stable reference particles of known size and of a refractive index and structure similar to the bacteria or microvesicles of interest. Submicron latex or silica beads of known size are widely available but stable particles of a refractive index more similar to bacteria or microvesicles are not commercially available. *Methods*: Apogee has developed a range of samples containing a continuum of particle size and of known refractive index capable of giving a ''snapshot'' of a flow cytometer's light scatter performance. From this data Apogee has developed a method (patent pending) to perform a refractive index compensated particle size calibration. The method requires a flow cytometer with sufficient sensitivity to measure sub-micron particles (e.g. small bacteria and extracellular vesicles) by more than one light scatter detector (e.g. the large angle (SSC) and small angle (FSC) light scatter detectors). The method does not require detailed knowledge of the flow cytometer's design and it may be applied to particles of a size similar to bacteria and extracellular vesicles. *Results*: This method predicts the position on a light scatter datagram of particles of a known size and refractive index. Due to their different refractive indexes, a quick and easy validation of the method has been performed by analysing a mixture of latex and silica bead populations of known size. Further work is needed to validate the method for biological samples. *Summary/Conclusion*: This new method allows particle size assessment from a flow cytometer's light scatter data and therefore standardisation of light scatter data between different flow cytometers.

P-XIII-4

Changes of microvesicle distribution in whole blood in dependence of storage time, temperature and anticoagulants

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Introduction: Microvesicles (MV) are cell-derived vesicles in the size of 100 nm up to 1000 nm. They are produced by a variety of cells into

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body fluids to interfere in coagulation, inflammation, communication and transport. It is known that the storage and preparation of plasma samples significantly influence the analysis of MVs. Thus, this study evaluated the microparticle release in whole blood samples under the influence of different anticoagulants, storage time and various temperature conditions. *Methods*: Samples were collected from healthy probands (n = 7 for each anti-coagulant) and subsequently stored for 0, 4, 8, 24 and 48 hours at room temperature (RT) or 4°C. Samples were centrifuged in two steps to harvest platelet-free plasma (PFP) which was immediately stored at −80°C until analysis. To identify MVs, PFP was stained with Annexin V (Pacific Blue) and calcein (FITC channel). To detect MV subpopulations, PFP was stained for MVs from red blood cell origin with an anti-CD235a (APC Alexa Fluor 750), for platelet-derived MVs with an anti-CD41 (APC) and for myelomonocytic cell (MMV) source with an anti-CD15 (Krome Orange). Measurement was performed on a CytoFLEX (Beckman Coulter) flow cytometer. In addition MVs were quantified with TruCount beads to determine their absolute number. To show the procoagulatory effect of the PFP, a tissue factor MV-activity (TF-MV) assay was performed. *Results*: Without prior storage, sodium citrate showed the lowest MV count compared to heparin and EDTA. Interestingly, EDTA showed a significant release of MMVs compared to sodium citrate. In addition, sodium citrate and heparin showed a stable MV count at RT or 4°C in the first 8 hours after blood collection. Overall, the MV count massively increased after 24 hours of storage independent of storage condition or anticoagulant which was related to all subpopulations. In addition, the procoagulatory (TFMV) potential increased significantly after 8-hour storage. *Summary/Conclusion*: Based on both, this work and literature data, sodium citrate seems to be the best working anticoagulants to analyse MV count and function. Motionless storage did not significantly influence MV count for 8 hours indicating a potential longer storage time prior to analysis. Large-scale studies are needed to evaluate the precise influence of storage time on MV count and, even more interesting, on MV subpopulations.

P-XIII-5

Improved criteria of MVs size discrimination allow better characteristics of patients with controlled and uncontrolled diabetes mellitus

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Introduction: Cell released microvesicles (MVs) vary in size and other parameters (i.e. surface markers, molecular properties, RNA and DNA content). MVs may reflect the diverse functional and dysfunctional states of the releasing cells and tissues along the complete individual pathways in health and disease and hyperglycemic conditions are considered to be important. C-reactive protein and triglyceride (TG) concentrations are commonly considered as secondary cardiovascular risk factors. We tested the hypothesis that hyperglycemia stimulates the formation of MVs diverse in size and nanoparticle tracking analysis (NTA) might be a useful tool for their detection. *Methods*: Type 2 diabetes mellitus patients T2DM uncontrolled (n = 3), T2DM controlled (n = 3) and healthy controls HC (n = 3) were enrolled to the study. T2DM group was divided according to haemoglobin A1c criteria. Citrate blood was centrifuged twice at 2500 × g for 15 minutes to obtain platelet poor plasma (PPP). Next PPP was centrifuged at 16,000 × g for 90 minutes at 4°C to obtain MV-enriched fraction and MV-depleted fraction. The size of plasma MVs was determined by the NTA method (Nanosight Ltd., Amesbury, Great Britain). Before analysis samples were diluted 1 000 times in buffer. The time of NTA analysis for a single sample was 30 seconds and the images were captured every 30 ms by EMCCD camera. Number of MVs was presented in 6 MV-classes according to their diameter: 100, 200, 300, 400, 500, 600 nm. The analysis was performed at room temperature (23.5°C). The two-sample variance test and r-Pearson correlation coefficient (r-PCC) were calculated using Origin Pro 9.0 64 bit program (OriginLab Co., Northampton, MA, USA). *Results*: The distribution of MVs size into two main MVs ranges (1–299 nm and 300–600 nm) indicated increased levels of smaller MVs in UD T2DM patients (452.29*E6/ml, $p < 0.1$) compared to control group (261.24*E6/ml, $p < 0.1$) and CD T2DM (257.41*E6/ml, $p < 0.1$). The distribution of MV size revealed that UD T2DM patients had two times higher concentration of 100 nm MVs than CD T2DM ones (2.71 *E6/ml vs. 1.28*E6/ml, $p < 0.1$). In control subjects we also found decreased number of 100 nm MVs (1.19*E6/ml, $p < 0.1$). Additionally, we observed the positive correlation between hsCRP levels and 100 nm MV concentrations in all groups (r-PCC for UD was 0.99, $p < 0.05$; CD − 0.97, $p < 0.05$ and controls − 0.68, $p < 0.05$), whereas TG levels were positively correlated in the case of UD patients only (r-PCC 0.91, $p < 0.05$). *Summary/Conclusion*: Our preliminary results demonstrate the role of size discrimination of MVs in better characteristics of diabetes mellitus patients. Proposed analysis showed that 100 nm MVs may be important in evaluation of dysfunctional or physiological state in patients with UD. Positive correlation between MV levels and hsCRP concentrations may suggest the relationship between inflammatory state and the number of small sized MVs.

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P-XIII-6

Characterization of vesicles in human cerebrospinal fluid

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Introduction: The extracellular RNAs (exRNAs) within extracellular vesicles (EVs) in the cerebrospinal fluid (CSF) can be interrogated to provide diagnostic and prognostic biomarkers of neurologic diagnoses. We present the first systematic quantitative and qualitative examination of CSF microvesicles in normal individuals. *Methods*: We examined exRNA in CSF obtained from normal donors using the Izon qNano analysis system. To establish a "signature" of normal CSF, we characterized vesicle size and concentration using tunable resistive pulse sensing (TRPS). CSF pooled and individual samples, both unpurified and purified using qEV columns, were evaluated. *Results*: EVs in pooled CSF samples evaluated by TRPS showed bimodal distribution of ~100 nm and ~200 nm diameter vesicles. The ~100 nm EVs may be in addition to peaks with possible dimeric or trimeric forms of particles, while the ~200 nm EVs did not represent contaminants, nor "dimers." The individual, non-pooled CSF specimens also had the ~100 nm EVs, but had lower concentrations of the ~200 nm EVs. Mean diameters for the two major peaks identified in normal CSF (113 nm and 251 nm) and Zeta potentials were obtained, and will be presented. *Summary/Conclusion*: TRPS qNano measures of EV serve the communities evaluating CSF analytics in normal as well as neurodegenerative and acquired brain diseases. Funded by NIH grants TR000903 (JAS, JFQ), NIH TR000931 (BSC, FHH), and TR000891 (KJ, MH).

P-XIII-8

Examination of extracellular RNAs in cerebrospinal fluid

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Introduction: The validation of a diagnostic or prognostic biomarker of human disease involves comparative studies of extracellular RNA (exRNA) yields and isolation which emerge from preparative techniques. *Methods*: We compared exRNA expression in cerebrospinal fluid (CSF) from six major classes of neurologic diseases (Alzheimer's disease, Parkinson's disease, low-grade glioma, glioblastoma, aneurysmal subarachnoid haemorrhage, and normal subjects). Uniform CSF pools from each disorder were evaluated using four RNA isolation kits: Total Exosome Isolation (Life Technologies), ExoRNeasy (Qiagen), miRCURY (Exiqon), and miRVana PARIS (Life Technologies), in each of three laboratories. We evaluated exRNA yields using Ribogreen Assay and Agilent Bioanalyzer chips. *Results*: We used Agilent small RNA bioanlyzer chips for exRNA sizing and ribogreen assay for exRNA yields. We present RNA yields and profiles resulting from the four compared kits across five neurologic diseases and controls. We will also present preliminary sequencing and qRT-PCR results for these fluids. *Summary/Conclusion*: This is the first comprehensive study of exRNAs in CSF across neurologic diagnoses. We provide data on the types of exRNA present in CSF in control subjects and patients with neurologic diseases.

P-XIII-9

Vesicle flow cytometry of extracellular vesicles in cerebral spinal fluid

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P-XIII-10

Plasma exosome profile from dairy cows with divergent fertility phenotypes

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Introduction: Placental exosomes have been recently recognised to have a role in human reproduction, being involved in maternal immune tolerance to foetal development; however, their role in dairy cow reproduction has not been established. The aim of this study was to characterise the exosome profile in plasma of dairy cows differing in their fertility phenotype. *Methods*: Plasma was obtained post-parturition (between 50 to 80 days) from cows characterised on the basis of genetic ancestry as fertile (New Zealand: NZ, $n = 24$) or subfertile (North American: NA, $n = 24$). Exosomes were isolated by differential and buoyant density centrifugation and characterised by size distribution (NTA, NanoSight NS500), the presence of CD63 (western blot), and their morphology (electron microscopy). The total number of exosomes was determined by quantifying the immunoreactive CD63 (ExoELISA kit, System Biosciences). The exosome pellet was resuspended in PBS and protein content was established by mass spectrometry (MS). *Results*: Enriched exosome fractions were identified as cup-shape vesicles with diameters around 100 nm and positive for CD63 marker. Before the exosome purification, the extracellular vesicles (EVs) (i.e. 100,000 $\times$ g pellet) were quantified. EVs protein concentration in NZ and NA cows averaged 3146 $\pm$ 675-and 3046 $\pm$ 967-g protein/ml ($p > 0.05$, Student's t-test). After exosome enrichment, the number of exosomes in NZ was greater than the NA cows ($p < 0.01$, $1.8 \times 10^9 \pm 1.2 \times 10^8$ versus $1.2 \times 10^9 \pm 4.4 \times 10^7$ exosomes vesicles/ml plasma). Bioinformatics analysis identified over 130 different proteins in exosomes involved in immunomodulatory pathways and cell-to-cell communication. *Summary/Conclusion*: While the role of exosomes in dairy cow reproduction remains to be established, their quantification and/or content in models with divergent fertility phenotypes could provide novel information to improve dairy cow fertility.

P-XIII-11

Analysis of sugar chain and extracellular vesicles for evaluation of complication in multiple myeloma

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Introduction: Multiple myeloma (MM) is often associated with monoclonal immunoglobulin (M protein), and the complication of MM such as osteolytic bone disease, anaemia and extramedullary plasmacytoma comes to the whole body. To reveal the relationship between extracellular vesicles (EVs) and the MM complication, we examined sugar chain on M-protein, cell surface and EVs in MM cell lines. *Methods*: Two cell lines of human myeloma (RPMI8226, KMM-1) were cultured in RPMI1640 medium with EVs-free FBS or FBS-free medium. EVs were isolated using ultracentrifugation method or ExoQuick kit. EVs maker proteins were detected by SDS-PAGE, western blot (WB), and proteins expressed on the surface of EVs were detected by immunoelectron microscopy or flow cytometry. Protein binding sugar chains were detected using lectins. *Results*: In RPMI8226 and KMM-1 cells, HSP70 was detected in the WB method. Further, in the KMM-1 cells, CD63 was also detected. There results suggested that EVs were secreted in both MM cell lines. Protein amount of EVs was almost identical in the two separation method, ultracentrifugation and ExoQuick. FBS in culture medium increased protein content of EVs, however, sugar chains in the FBS interfered analysis of M-protein sugar chain. Therefore, FBS-free medium were used for cell culture of MM cell lines. MM cells were stimulated with a growth factor, IL-6, and change of sugar chains were examined. As a result, in cell surface sugar chain of the KMM-1 cells, the reactivity with SSA lectin increased, suggesting that sialic acid binding sugar chain increased in M protein and cell surface proteins. Because sialic acids are known to be involved in tumour metastasis, the increased sugar chains may be involved in various complications of MM. Effects in EVs are under consideration. *Summary/Conclusion*: EVs could be effectively purified from two MM cell lines by culture in serum-free medium, and isolation by ultracentrifugation or ExoQuick method. In analysis of sugar chain on cell surface or M-protein, content of sialic acid associated to metastasis in the sugar chain increased after IL-6 stimulation.

P-XIII-12

Exosome release and bioactivity is associated with the invasiveness of ovarian cancer cell lines

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Introduction: Ovarian cancer is the most lethal gynaecological cancer with most patients (75%) diagnosed at an advance stage when the tumour has spread well beyond the ovaries. Recently, exosomes has received great attention as a potential biomarker. They are actively released by tumours into the peripheral circulation, playing roles in tumour progression and metastasis. The aim of this study was to characterize exosomes from ovarian cancer cell lines and determine their effect on target cells. *Methods*: High (SKOV-3) and low (OVCAR-3) invasive ovarian cancer cell lines were used to characterize their exosome release. SKOV-3 and OVCAR-3 cells were cultured (RPMI Media 1640, 10% exosome-free FBS) under an atmosphere of 8% O_2 for 24 hour. Cell-conditioned media were collected and exosomes (exo-SKOV3 and exo-OVCAR) were isolated by differential and buoyant density centrifugation and characterized by their density, presence of exosome-specific markers (CD63 and TSG101, western blot), size distribution (Nanosight™) and shape (electron microscopy). The effect of exo-SKOV-3 and exo-OVCAR-3 on human vein endothelial cell (HUVEC) migration, proliferation and tube formation were established using an Incucyte™ live-cell imaging system. *Results*: Exosomes were identified as vesicles with a typical cup-shape as observed under electron microscopy, diameters ranging from 50 to 100 nm with the presence of endocytic markers. The release of exosomes from SKOV-3 and OVCAR-3 cells incubated *in vitro* was 1.38 μg/10^6 cells and 0.59 μg/10^6 cells. Exosome release was 2.3 fold higher in SKOV-3 than OVCAR-3 cells. Exo-SKOV-3 and exo-OVCAR-3 increased HUVEC migration by 1.4- and 1.8-fold compared to the control, without exosomes ($p < 0.05$) respectively. Exosome effect on migration was associated with increased HUVEC proliferation. Sonication completely abolished the effect of exosomes on HUVEC. *Summary/Conclusion*: The results of this study show that exosomes could play a role in cancer progression through the promotion of angiogenesis.

P-XIII-13

Characterization of circulating microparticles in colorectal cancer patients

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Introduction: One of the major plausible roles of extracellular vesicles (EVs) and their large subset, microparticles (MPs) shed by cancer cells is to facilitate disease progression. Recent studies demonstrated that cancer cell–derived MPs or cargo enclosed in such vesicles offer new diagnostic possibilities. This work provides preliminary characterization of circulating MPs isolated from blood of colorectal cancer patients (CRC) and healthy controls using diverse analytical techniques. *Methods*: Blood samples (~ 5 ml) were collected from five CRC patients and five healthy controls for preliminary characterization. MPs were enriched by sequential centrifugation of platelet-poor plasma. Two dimensional polyacrylamide gel electrophoresis (2-D PAGE)-based proteomic analysis: MPs were batch-produced, pelleted and pooled to generate a 50 g of protein. 2-D PAGE gel spots were cut, destained from Coomassie Blue, trypsin digested, and subjected to liquid chromatography coupled with tandem spectroscopy (LC-MS/MS). NanoLC-MS-based proteomic profiling: platelet free plasma was filtered through 0.8 μm filters and MPs were enriched using size exclusion chromatography. MPs isolates were dilapidated and digested with trypsin following nanoLC-MS/MS. Flow cytometry (FCM): MPs suspension aliquots were labelled using calcein AM (lipid marker) and Alexa 647-Annexin V (phosphatidylserine marker). The number of MPs per mL of plasma was calculated based on the number of the events (N) in the gate between 0.3 and 1.0 μm beads and using the Countbright beads ratio. *Results*: We analyzed MPs isolated from blood samples of CRC patients and healthy controls using FCM and proteomic methods. Both types of analytical techniques confirmed the adequate efficiency of MP isolation. Our preliminary experiments revealed that the FCM techniques were able to quantitate MPs sized between 300 and 1000 nm and provided preliminary characterization of MP proteomes. 2-D PAGE and nLC-MS/MS-based comparative proteomic profiling indicated differences in protein composition of patient and control MP samples that have to be further validated using larger cohorts of donors. *Summary/Conclusion*: The described combination of analytical techniques demonstrated the potential to generate new knowledge about functions of MPs in cancer.

P-XIII-14

Size exclusion chromatography-preclearing of plasma from patient samples facilitates magnetic bead-based isolation of CD9 or CD81 expressing circulating EVs

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Introduction: Plasma is a complex mixture with a diversity of extracellular vesicles (EVs) derived from different cells, and a large number of macromolecules. Direct isolation of EVs from plasma by magnetic beads, based on surface protein expression, is difficult when compared with other body fluids, probably due to the high levels of proteins and lipids. Pretreatment of plasma, by size exclusion chromatography (SEC), may reduce interfering substances and may allow for the detection of specific EVs. Our aim has been to capture specific circulating EVs in plasma by combining SEC-preclearing and target specific magnetic bead isolation. *Methods*: Plasma samples (1 ml, EDTA, citrate), from fasting colorectal cancer (CRC) patients and healthy individuals, were precleared by SEC using PBS-washed Sepharose CL-2B (GE Healthcare) in custom made 10 ml columns. EVs were eluted in 0.5 ml fractions, concentrated (2:1) and fractions were screened for proteins and lipids. Separate SEC-fractions (6–13) and a joint fraction (8–10), depleted of proteins and lipids, were used to isolate CD9- and CD81-positive EVs with Dynabeads® (Thermo Fisher Scientific). Bead bound EVs were detected by flow cytometry (BD Accuri C6) using PE-labelled Ab against CD9 and CD81, read out as signal to noise ratio (S/N) by median fluorescence intensity to matched isotype controls. Results were supported by NTA (NS500, Malvern), Western blot and Transmission electron microscopy. *Results*: The effect of SEC-preclearing opens for reproducible bead binding of plasma EVs, from both citrate and EDTA, in a range of fractions. While flow cytometry showed that a major amount of CD9 and CD81-positive EVs were detected in fractions 7 to 12, the joint fraction (8–10) represented the most concentrated in EVs and simultaneously low in proteins and lipids. Precleared EDTA-plasma from CRC-patients ($n = 4$) showed S/N for CD9-positive EVs ranging from 9.8 to 18.1 and for CD81-positive EVs from 1.4 to 2.2 in the joint fraction (8–10). Healthy individuals demonstrated low S/N for both CD9 and CD81-positive EVs. *Summary/conclusion*: Preclearing of plasma samples by SEC sufficiently reduces interfering non-vesicular components to facilitate capture of CD9 of CD81 positive EVs with magnetic beads. This combined purification and isolation protocol may aid in obtaining specific circulating EVs from plasma patient samples, for further downstream characterization.

P-XIII-15

Glioblastoma cells exposed to 5-ALA release protoporphyrin IX containing microparticles detectable by high-resolution flow cytometry

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Introduction: 5-Aminolevulinic acid (5-ALA) is used for fluorescence-guided neurosurgery in glioblastoma multiforme (GBM) patients. 5-ALA is part of the heme synthesis pathway and after oral intake, fluorescent protoporphyrin IX (PpIX) accumulation is observed in GBMs, providing the neurosurgeon with visual feedback of the tumour. We hypothesized that after 5-ALA intake, microparticles derived from GBMs contain PpIX and thus become fluorescent, which allows tumour-derived microparticle detection by high-resolution flow cytometry. *Methods*: U87-MG/EGFRvIII (GBM) cells were exposed to 500 M 5-ALA and cultured for 24 hours. After 24 hours microparticles were isolated by sequential (ultra)centrifugation and studied using high-resolution flow cytometry. Fluorescent microparticles were added to platelet free plasma (obtained by centrifugation at 1,500 $\times$ g for 10 minutes, followed by 13,000 $\times$ g for 10 minutes) or cerebrospinal fluid (CSF) at different concentrations and visualized using high-resolution flow cytometry. *Results*: Fluorescent microparticles released by 5-ALA treated GBM cells could be detected by high-resolution flow cytometry (488 nm excitation; 630/22 nm band pass filter). Spiked into platelet-free plasma or CSF, these fluorescent microparticles could be reliably quantified, even at extremely low concentrations (4e6/ml). *Summary/conclusion*: High-resolution flow cytometry of PpIX positive microparticles provides a novel method to identify tumour-derived particles. Further research should focus on detection of these particles in GBM patients and their potential relation to tumour volume and response to therapy.

P-XIII-16

Functional analysis of exosome-mediated cellular motility in cancer

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Introduction: Metastasis is the leading cause of mortality in all cancers. A key process in metastasis is stimulating cellular motility, which allows cancer cells to migrate from the tumour to a distal site. Previous research from our lab determined that one method of stimulation involved interactions between cancer cells and exosomes produced by cancer-associated fibroblasts (CAFs). The cancer cells modified the CAF-exosomes by loading them with Wnt11. These modified exosomes can then activate non-canonical Wnt signalling within the cancer cells, resulting in increased cellular motility and metastasis *in vivo*. Additionally, this function was dependent on the tetraspanin Cd81 presence on the exosomes. Although extensive work was done to characterize this phenotype, the exact mechanisms involved in these interactions are unknown. I plan to further investigate the function of the CAF-exosome by examining the role of the exosome proteome in motility stimulation. *Methods*: Although the addition of Wnt11 is known, further modifications of the CAF-exosomes are currently uncharacterized. Through use of mass spectrometry, I am developing an in-depth proteomic map of the CAF-exosomes before and after cancer cell interactions. By modifying CAF-exosomes to express tagged proteins, I can isolate specific populations of exosomes through chromatography and immunoprecipitation. Furthermore, I am currently optimizing a high-throughput screen to investigate the role of each exosome protein in its motility-inducing function. CAFs will be treated with a shRNA library to produce CAF-exosomes lacking specific proteins. Cancer cells will be treated with these exosomes and automatically tracked to monitor their motility response. Variations in cellular motility will determine which components will regulate exosome function. *Results*: I am optimizing my methodology to produce a functional proteomic map of the CAF-exosomes. I am utilizing a size-exclusion column coupled to immunoprecipitation against exosome-specific proteins to purify and isolate whole CAF-exosomes for mass spectrometry analysis. Currently, I am utilizing Cd81 to isolate the motility-inducing CAF-exosomes prior to cancer cell interactions and a tagged Wnt11 protein to isolate the exosomes after cancer cell modifications. MDA-MB-231 expressing Wnt11-HA can successfully modified CAF-exosomes with the tagged Wnt11 protein. To investigate the motility-inducing function of the CAF-exosomes, I have produced an MDA-MB-231 cell line with fluorescent nuclei through H2B-Clover expression. Utilizing manual tracking, I have determined that these cells can be used to determine changes in cellular motility when exposed to CAF-exosomes. I am now developing a successful automated tracking algorithm. *Summary/conclusion*: Through these approaches, I aim to elucidate the CAF-exosome proteome and determine its necessary functional components. I hope to identify new mechanisms of exosome interaction in cancer.

P-XIII-17

Label-free single exosome detection using frequency-locked microtoroid optical resonators

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Please see Oral with poster A

P-XIII-18

Characterization of exosomes from mammalian circadian clock

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Introduction: Mammalian suprachiasmatic nuclei (SCN) are autonomous clocks that generate coordinated rhythms and drive oscillations in other peripheral tissues. We have identified that the conditioned media (CM) of SCN2.2 cells confer molecular rhythmicity to co-cultured fibroblasts via some diffusible factors. However, the type of signal that SCN cells use to coordinate circadian rhythmicity in fibroblast cells is currently unknown. Exosomes are extracellular nanoparticles that contain distinct subsets of RNAs and proteins. They play important roles in cell signalling, and intercellular communication. One potential mechanism of diffusible factors transfer from the SCN2.2 cells to the other cells is through exosomes. Therefore, studies were conducted to characterize SCN2.2 cell-derived exosomes. *Methods*: Exosomes were isolated and purified from CM of SCN 2.2 cells using a differential ultracentrifugation method. The transmission electron microscopy and Western blot analysis were employed to confirmed SCN-derived exosomes. Exosomal RNA and protein contents were analysed by a Bioanalyzer and 2-dimensional polyacrylamide gel electrophoresis (2-D GE). *Results*: The purified exosomes were disc-shaped vesicles with lipid bilayer membranes, and ranged from 30 to 150 nm in diameter. The exosomes were positive for exosomal marker CD63 by Western blot analysis. The exosomal RNA profile was different to those found in SCN2.2 cells, and revealed the presence of large amounts of small RNAs.There were approximately 50 proteins present in SCN-derived exosomes analysed by 2-D GE. *Summary/conclusion*: These studies demonstrated that exosomes were released from SCN2.2 cells. The characterization of SCN-derived exosomes is essential in furthering our understanding of the biological role of exosomes in circadian clock.

P-XIII-19

Comparison of the traditional ultracentrifugation techniques and PEG precipitation for isolating exosomes from ACS (autologous conditioned serum): a kinetic study

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Introduction: In the past few years, there is a renewed interest in the exosomes after it was known that they play a major role in cellular communication, not only in normal physiology but also in pathological situations and serve as cargoes of proteins, lipids and nucleic acids. Various techniques have been utilized for the isolation of exosomes such as ultracentrifugation, ultrafiltration, size exclusion chromatography, immuno-magnetic bead utilization and precipitation. In this study we demonstrate the advantage of the PEG precipitation technique over the traditional ultracentrifugation specifically considering the kinetics of ACS incubation at 37°C. *Methods*: In our study the production of ACS involves the incubation of the venous blood in the presence of medical glass beads at 37°C during an increasing time scale: 3, 6, 9 and 24 hours. We utilized a slightly modified polyethylene glycol precipitation method to isolate exosomes in combination with ultracentrifugation as mentioned by (1). Visualization and quantification was performed using transmission electron microscopy (TEM) and the nanoparticle tracking analysis (NTA). The protein content was revealed via SDS gel analysis. *Results*: The TEM analysis of the exosome isolated per ultracentrifugation showed the highest amount of intact exosomes ($2.89*10^8$ particles/ml) with a mean size between 100 and 150 nm after an incubation time of 6 hours, for the samples isolated per PEG precipitation the highest amount was also found to be after 6 hours incubation but with a much higher concentration with $8.22*10^9$ particles per ml and the mean size been 92.6 nm. In both cases there was an increase in size of the vesicles at 24 hours incubation as shown by TEM and NTA measurements. TEM could demonstrate the presence of aggregates up to 180 nm in the ultra centrifuged sample but only vesicles with a mean value of 100 nm after PEG precipitation. While the particle number decreased in the original ACS sample from $7.74*10^4$ at 0 hour incubation to $2.26*10^{10}$ after 24 hours, there is an increase in vesicle number in the exosome fraction from $7.48*10^9$ at 0 hour incubation up to $8.22*10^9$ after 6 hours and then decreasing again down to $3.50*10^9$ after 24 hours incubation. Such high amount of particles could not be observed after ultracentrifugation. The SDS analysis revealed the same band pattern with both isolation *Methods*. The intensity of some bands (e.g. above and below 17 kDa) was increased with increasing incubation time, as well as the protein concentration, supporting the hypothesis that the incubation time affects the protein content. *Summary/conclusion*: The PEG method of exosome isolation takes a longer period of time for isolation, with 2 overnight incubations, but provides a large volume of exosome containing fluid as compared to the ultracentrifugation. The method provides high yield of proteins and Exosomes facilitating the following analysis, as enough material is available for each incubation time.

Reference

1. Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, et al. Leukemia. 2014;28(4):970–3.

P-XIII-20

Size and shape characterization of hydrated and desiccated exosomes

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Introduction: Exosomes can be differentiated from other circulating vesicles by the markers of the endosomal pathway and their small size. Though an important differentiator, the size of exosomes is often reported with substantial variability. Several factors likely contribute to this variability, including biological origin, isolation technique and sizing method. *Methods*: In this work, we quantify the influence of different analytical techniques on exosome sizing by re-analyzing the same serum exosomes samples, isolated by ExoQuick precipitation, by SEM, cryo-TEM and particle mobility. *Results*: For hydrated exosomes, our results indicate a surprisingly large degree by which the measured hydrodynamic sizes exceed their geometric sizes. As expected, the geometric size of desiccated exosomes was found to be smaller than their hydrated size. However, the manner in which desiccation occurs has an influence on the sizing results. Specifically, exosomes desiccated on a surface (imaged by SEM) appear larger than exosomes desiccated in aerosol (characterized by their electrical mobility in the gas phase). Reminiscent of the coffee ring effect, the surface desiccation was observed to cause the particle size segregation in different spatial locations within the perimeter of the dried sample. *Discussion*: Cryo-TEM images are formed by electrons transmitted through vitrified sample and give 2-dimensional projections of 3-dimensional exosomes in their native hydrated state. Our results show that these projections are close to circular. This finding is consistent with only a near spherical shape of exosomes. The shape distortion away from spherical (e.g. their cup-shaped morphology, still reported commonly) is likely caused by the sample preparation steps, such as electrostatic immobilization of hydrated exosomes on a charged substrate prior to atomic force microscopy (AFM) imaging. Surface desiccation also leads to shape distortion, caused by non-isotropic drying front and thus unbalanced capillary forces. We find that airborne desiccation, on the other hand, which progresses isotropically, preserves close to spherical shape of exosomes and results in the smallest measured sizes among all techniques. Overall, we found that sizing results change significantly when different analytical techniques are used to characterize the size of the exosomes. The size variability between different methods is significant and consistent with the variability in sizes reported in literature for the cases when the types of cells that secreted the exosomes, the body fluids from which they were isolated, and exosome isolation methods were also the contributing factors. We attribute the observed large difference in hydrodynamic and geometric sizes to the presence of membrane-conjugated macromolecules that impede hydrodynamic mobility of exosomes. The difference between geometric and hydrodynamic sizes may therefore be useful in analyzing the conjugation of macromolecules to the surface of exosomes.

P-XIII-21

Evaluation of a practical, highly efficient and specific method for single-vesicle fluorescent labelling using nanoparticle tracking analysis (NTA) to characterize canine extracellular vesicles

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Introduction: A reliable method for characterizing the molecular phenotype of individual extracellular vesicles (EV) utilizing fluorescence immunolabelling is needed. Current methods are limited to bulk labelling of vesicles or complex, and inefficient antibody labelling. We evaluated the efficiency of 2 methods for single-vesicle immunolabeling and detection using commercially available reagents. *Methods*: EV were isolated by differential ultracentrifugation from serum-free conditioned medium of canine placental mesenchymal stromal cells from multiple donors. We compared the efficiency of CD9 immunolabelling of EV (1×10^{11} particles) between 2 Methods. (a) direct labelling using Qdot conjugated CD9 (1 g) versus (b) indirect labelling using biotinylated CD9 antibody or isotype (1g) and secondary

strepavidin-Qdot655. Biotinylated bead incubation, centrifugation, and filtration were used for removal of bead-unbound Qdot655 complexes. Direct antibody labelling (Life Technologies SiteClick Qdot655) was performed per manufacturer instructions. NTA in both light-scatter mode (LSM) and fluorescence mode (FM) was performed in replicates of 5 using a NS300 machine with a 488 nm laser and 500 nm long-pass filter with Nanosight NTA3.0 software. *Results*: Fluorescence was readily visible and distinct for labelled EV and free Qdots using NTA. Detection of CD9 was highly efficient using indirect labelling (FM:LSM ratio from 88–93.8% for particles >55 nm); FM and LSM generated similar size-density histogram profiles. Indirect isotype labelling showed disparate size profiles in LSM and FM with 99–100% particles >55 nm in LSM and 80–86% particles <55 nm in FM. Unstained EV incubated with streptavidin Qdot655 showed 99–100% particles >55 nm in LSM and 72–87% particles <55 nm in FM. In contrast, there was poor CD9 detection on EV using direct labelling (4–5.2% FM:LSM for particles >55 nm). Most (94%) particles in FM were <45 nm corresponding to unbound Qdots-CD9 complexes. Bead-facilitated removal of free Qdots decreases EV concentration without improving the FM:LSM ratio. There is minimal evidence of non-specific antibody or free Qdot binding to EV. *Summary/conclusion*: Direct labelling of EV underperforms compared to indirect labelling. Direct labelling of antibodies with Qdots may damage antibody-protein interactions. Indirect labelling and strategic removal of unbound complexes coupled with NTA permits efficiency surface phenotyping of EV.

P-XIII-22

Diurnal variations of circulating extracellular vesicles

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Introduction: The identification of extracellular vesicles (EVs) as intercellular conveyors of biological information has recently emerged as a novel paradigm in paracrine signalling. However, whether there are diurnal variations in the size, number, and tissue of origin of blood EVs is currently not known, and could have significant implications when using EVs as biomarkers for disease progression. *Methods*: To address this important question, we examined the relative number and size distribution of EVs at 3 different time points during the day from cell-free plasma of 6 healthy donors using a specialized flow cytometer, MoFlo XDP equipped with NanoView technology, that allows detection and relative quantification of particles as small as 100 nm in diameter. *Results*: Our results suggest that diurnal variation of biological systems could impact the number and size distribution of circulating EV populations. This could further extend to protein and nucleic acid composition of these particles and is something that warrants further investigation. Our findings lay the foundation for future explorations of the functional implications of these diurnal variations in EVs as well as their use as markers for disease progression and treatment efficacy. *Summary/conclusion*: In this study, we have used the MoFlo XDP equipped with NanoView technology to analyse the EVs size and number distribution in the blood of healthy donors at different time-points throughout the day. We demonstrated, using beads and liposomes, that the NanoView can accurately detect events down to a size of a 100 nm and be a more suitable instrument for detection and characterization of plasma EVs than certain commercially available Flow Cytometers. While using the NanoView, we discovered that in healthy donors, the quantity and size distribution of EVs in blood varies over the course of a day.

P-XIII-23

An in vivo fluorescent sensor for the detection of exRNA

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Introduction: MicroRNAs (miRNAs) are a class of short non-coding RNAs that downregulate gene expression by binding to complementary sequences in the 3'UTR of their target genes, thereby destabilizing the mRNA transcript and repressing protein translation. By this mechanism, numerous miRNAs have been shown to play a key role in many biological decisions including cell fate and cancer progression. Moreover, the recent discovery that some of these miRNAs are found in human body fluids gives rise to the possibility that circulating miRNAs could act as endocrine signals, affecting gene expression in neighboring cells, and perhaps even distant target organs and cells. Because of their small size, the detection of miRNAs remains a challenge. Although many systems allow for the detection of miRNA in cell populations or in fixed tissues, these standard protocols are highly invasive and lack the sensitivity for detecting smaller quantities of miRNAs. *Methods*: Here we introduce a highly responsive system for determining the expression of miRNAs in living single cells using an artificial genetic circuit, producing a positive readout for miRNA expression. To create a positive miRNA reporter, we created a novel system that produces a positive signal when an miRNA enters a cell and is active. *Results*: Our novel positive miRNA sensor circuit detects both moderately and highly expressed miRNAs, giving a proportional change in the EGFP expression level. Moreover, changes in miRNA expression is detected rapidly and tracked by fluorescent analysis by flow cytometry and microscopy in an *in vitro* cell culture system. The system is robust in that it produces a readily detectable signal in an *in vivo* xenograft model system that can be tracked during tumour progression. *Summary/conclusion*: In conclusion, our positive miRNA sensor system allows for the detection of changes in a single miRNA both in cellular *in vitro* and *in vivo* models, constituting a formidable tool for the study of extracellular miRNAs functionality and miRNA expression changes in mammalian development and disease.

Scientific Program ISEV 2015 meeting
Saturday April 25, 2015
Oral Presentations

Registration	08:00-09:00
Setting up posters (Poster sessions XIV, XV, XVI, XVII, XVIII, XIX, XX)	08:00-09:00
Ballroom D Meet the experts IV: EVs in biotechnology and therapeutics Chair: *Raymond Schiffelers* Speakers: *Janusz Rak and Samir El-Andaloussi*	08:00-08:45
Ballroom E Meet the experts V: Bacterial and parasite EVs Chair: *Pamela Wearsch* Speakers: *Yong Song Gho and Antonio Marcilla*	08:00-08:45
Ballroom F-H Meet the experts VI: EVs in inflammation and immunity Chair: *Esther Nolte-'t Hoen* Speakers: *Edit Buzás and Stefano Pluchino*	08:00-08:45
Sponsor Exhibition	10:00-18:00

Ballroom D
Symposium session 6A - EVs in blood
Chairs: *Uta Erdbruegger and Leonid Margolis* 09:00-10:00

O-6A-1

Determination of serum microRNA profiles in human stroke patients

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Introduction: Stroke is the third leading cause of death in the United Kingdom and the leading cause of long-term adult disability. There is only one licensed pharmacological intervention, intravenous delivery of thrombolytic recombinant tissue plasminogen activator within 4.5 hours of stroke, beyond which time there are significant haemorrhagic risks. An alternative intervention is needed and through their ability to alter the expression of multiple genes involved in stroke pathophysiology, miRNAs offer a novel therapeutic intervention. miRNA expression is altered in experimental stroke and in patients with stroke. Recently, active transport of miRNA in extracellular vesicles (EV), such as exosomes, has been demonstrated pre-clinically between cells in atherosclerosis. We hypothesized that miRNAs packaged in EV would differ between patients with stroke and patients without stroke, raising the potential for novel miRNAs to be used as biomarkers or therapeutic agents for modulation. *Methods*: We recruited 169 patients with suspected stroke and a blood sample was taken at 48 hours post-stroke. All participants gave full informed consent and the study was approved by the Scotland A Research Ethics Committee. A miRNA microarray was performed (Openarray™ platform) on samples from 39 patients (n = 10 non-stroke, n = 29 with stroke – further subdivided by TOAST classification into large artery (n = 9), cardioembolic (n = 10) or small vessel disease (n = 10) stroke). Validation of results was performed using samples from 169 patients. EVs were isolated from 200 μl serum before RNA was extracted and the concentration determined using nanodrop spectrophotometry. Taqman™ real-time quantitative polymerase chain reaction was used to determine the expression levels of specific miRNA(s). *Results*: The microarray identified 26 miRNAs that were significantly dysregulated between stroke versus non-stroke patients or between specific TOAST subtypes and non-stroke control. Of these, changes in 17 miRNA were validated in the larger cohort: levels of miRNAs-17 (relative quantification, RQ, vs. non-stroke = 1.74*), 20b (RQ, vs. non-stroke = 1.95*), 27 (RQ, vs. non-stroke = 1.83*), 30a-5p (RQ, vs. non-stroke = 1.67*), 93 (RQ, vs. non-stroke = 1.80*) and 199a-3p (RQ, vs. non-stroke = 1.91*) were significantly increased in stroke versus non-stroke patients (*$p < 0.05$ by unpaired Student's t-test). Furthermore, differences between TOAST subtypes were shown with small vessel disease consistently having the highest levels of miRNA. Bioinformatics analysis highlighted a number of important target genes implicated in stroke pathophysiology for each miRNA including genes involved in the regulation of apoptosis, angiogenesis and cell migration. *Summary/conclusion*: We have identified and validated changes in EV packaged miRNA expression in patients with stroke across differing stroke subtypes. This will direct future studies looking into paracrine signalling in the setting of stroke and the modulation of specific miRNAs as a novel therapy in the setting of experimental stroke.

O-6A-2

Platelet-derived extracellular vesicles in blood of patients with acute coronary syndrome

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Introduction: Platelets and endothelium release extracellular vesicles (EVs) in blood and it has been reported that the pattern of vesicle repertoire is altered in patients with acute coronary syndrome. However, the majority of these data come from the analysis of large particles that constitute less than 10% of total EVs. Flow cytometers, which are designed to analyze cells, are unable to analyze the majority of the released EVs, which have a diameter below 300 nm. Recently, we developed a nanotechnology-based assay that permits the analysis of the antigenic composition of individual extracellular vesicles. Here, we used this technology to analyze EV composition in blood of patients with acute coronary syndrome (ACS). *Methods*: EVs from platelet-poor plasma from 15 healthy donors and 15 patients with ACS were captured with 15 nm magnetic nanoparticles (MNPs) coupled with mouse antibodies specific for 1 of the 3 antigens CD31, CD41 and CD63. Captured EVs were stained for the other 2 antigens and analyzed with a flow cytometer set to trigger on fluorescence. *Results*: Plasma from ACS patients contained more EVs captured with anti-CD31, anti-CD41 or anti-CD63 MNPs than plasma from control individuals. This increase was not equal for EVs of different antigenic composition and seems to be the largest among CD41+ vesicles: There was a two-fold increase in the number of CD41-captured EVs (predominantly of CD31+/CD63+ phenotype). Also, a two-fold increase in the number of CD63-captured EVs as well as 1.5-fold increase in CD63-captured EVs was predominantly restricted to CD41+ vesicles. *Summary/conclusion*: Our results show that ACS is associated with an increase in plasma concentration of EVs predominantly of the phenotype CD41+, which is in agreement with their platelet origin. Further analysis of the antigenic composition of individual EVs with our nanotechnology-based assay may lead to the development of new diagnostic and prognostic tools.

O-6A-3

Assessment of cardiovascular status in rats using nanovesicle flow cytometry

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Introduction: Extracellular vesicles (EVs) – membranous particles shed and/or secreted from cells, are thought to play a role in normal physiology and disease. EVs are generally 100–200 nm in diameter and can be shed from the cell surface (ectosomes) and secreted from internal compartments (exosomes) of many different cell types.

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The combination of small size and heterogeneous origins make EVs extremely difficult to measure. We have developed a Nanoparticle Flow Cytometer and associated reagents and protocols to better detect these small and dim particles. *Methods*: EVs were obtained from supernatants of rat aortic endothelial cells (RAEC) cultures or rat plasma. EVs were separated from cell debris and soluble protein by low (2 cycles of 2.5K $\times$ g, 5 minutes) and high (20K $\times$ g, 4 hours) speed centrifugation, respectively, and resuspended in 0.1 μm filtered dPBS. Purified EVs were stained with an optimized concentration (determined by titration) of the fluorogenic lipid probe, di-8ANEPPS (red fluorescence), and DyLight 488-labelled cell surface markers and analyzed using the NFC with 488 nm excitation (150 mW) triggered on red fluorescence. Intensity-calibrated reference beads were used for calibration of fluorescence measurements. Liposomes and EVs were also analyzed using nanoparticle tracking analysis, providing an independent estimate of nanoparticle size that can be used to calibrate NFC measurements. *Results*: Fluorescence triggered detection of the membrane-bound lipid probe enabled measurement of individual liposomes and EVs less than 80 nm in diameter. Dilution experiments show an expected decrease in detected events with no change in intensity, indicating these measurements are not compromised by coincidental occurrence of multiple EVs in the measurement volume. We labelled annexin V and antibodies against cell surface markers with an F/P of 2–4 to maximize the brightness of these reagents and were able to detect their binding to EVs with a limit of resolution of $\sim$800 FITC MESF. We find that only a fraction of EVs from RAEC cultures are annexin V positive and that neither the number of EVs nor the AnnV+ fraction changes significantly with 24-hour treatment with TNF α or IL-1β. By contrast, LPS treatment of rats did result in a significant increase in plasma EVs. *Summary/conclusion*: Fluorescence-triggered detection of EVs stained with a fluorogenic lipid probe enabled robust and quantitative enumeration and sizing of individual EVs. Serial dilution of the sample can be used to ensure that the pervasive artefact of coincidence is not affecting the data, and the surface area staining of the lipid probe gives results that are comparable to the diameter estimates provided by NTA. Brightness-optimized reagents can greatly improve the detection of low abundance surface markers. We are now working to implement additional fluorescence channels to allow more parameters to be measured on individual EVs.

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O-6A-4

Microvesicles released from aged red blood cells are damaging for human endothelial cells in vitro

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Introduction: Blood for transfusion can be stored for up to 42 days, however, during storage a number of biochemical changes occur leading to release of extracellular vesicles with increased storage time. A number of serious complications may occur in patients post transfusion, including transfusion related acute lung injury. The aim of this study was to test the hypothesis that as red blood cells age during storage they release increasing numbers of microvesicles that are potentially damaging to the endothelium, causing oxidative stress and potentially causing cell death. *Methods*: Blood was purchased from National Blood Service and aliquoted before storage at 4°C for up to 42 days. At each time point plasma was prepared by centrifugation at 2,500 $\times$ g and MV were measured by flow cytometry using enumeration beads and 1.1 μm sizing beads, after labelling of particles with annexin V. MV were enriched by centrifugation at 17,000 $\times$ g for 15 minutes followed by measurement of haemoglobin in the pellet (MV fraction) and the supernatant, using Drabkin's reagent. For analysis of MV effect on endothelial cell function MV were isolated as above and resuspended in cell culture medium. Human umbilical vein endothelial cells (HUVEC) were preloaded with dihydrorhodamine-1, 2 and 3 and then incubated with MV for up to 24 hours with measurement of ROS production over time using a fluorescent plate reader. At the end of the experiment Alamar Blue was added to measure cell viability. *Results*: Release of AnnexinV$^+$ >1 μmMV peaked at day 14 of storage ($p < 0.001$ two-way ANOVA) whilst release of annexinV$^-$ MV peaked at day 28 ($p = 0.0028$ two-way ANOVA). Hb released in MV pellets also peaked at day 28 ($p < 0.0001$ two-way ANOVA) whilst Hb in the plasma rose steadily over time of storage until day 42 ($p < 0.0001$). Incubation of HUVEC with MV from blood stored for 42 days caused a significant increase in ROS production ($p < 0.001$) and a significant decrease in cell viability ($p < 0.01$) compared to untreated HUVEC or those treated with MV isolated from blood stored for 3 days. *Summary/conclusion*: These data suggest that the MV released during storage of blood for transfusion may have directly damaging effects on EC, possibly by delivering Hb directly onto the cells. Further studies are underway to determine whether RBC-derived MV activate endothelial cell pro-inflammatory gene expression and whether the inflammatory status of the endothelium prior to contact with RBC-derived MV alters their responses.

Ballroom E
Symposium session 6B - EVs in inflammation
Chairs: *Edit Buzás and Susanne Gabrielsson* 09:00-10:00

O-6B-1

Toxic lipids induce release of proinflammatory extracellular vesicles from hepatocytes

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Introduction: Hepatocyte lipotoxicity (cell stress and death induced by lipids) and macrophage-driven inflammation are key features of non-alcoholic steatohepatitis (NASH). Although toxic lipids activate hepatocyte-expressed death receptor 5 causing lipotoxicity, how this process is linked to inflammation during NASH is unclear. We posited that toxic lipids trigger hepatocyte release of extracellular vesicles (EV) which induce the proinflammatory phenotype of macrophages. *Methods*: Primary mouse and human hepatocytes and Huh7 cells were treated with lysophosphatidylcholine, a toxic metabolite of palmitate and released EV were purified by differential ultracentrifugation, quantified by nanoparticle tracking analysis, and were employed for macrophage treatment. C57BL/6 mice were placed on chow or NASH-inducing FFC (high saturated fats, fructose and cholesterol) diet. Two weeks prior to killing, the mice were treated with a ROCK1 inhibitor fasudil. *Results*: Lipotoxic treatment of primary hepatocytes and Huh7 cells induced EV release by 3-fold and 400-fold, respectively, with no effect on cell viability. The lipotoxicity-induced release of EV was prevented by pharmacologic inhibition or genetic deletion of components of the death receptor 5 signalling cascade mediating hepatocyte lipotoxicity such as death receptor 5, caspase 8, caspase 3 and ROCK1, a kinase activated by caspase cleavage. Treatment of mouse bone marrow-derived macrophages with lipotoxic hepatocyte-derived EV induced mRNA levels of cytokines IL-1beta and IL-6, suggesting that these macrophages acquired a proinflammatory phenotype. Similarly, EV derived from hepatocytes isolated from mice with NASH, but not control mice, also activated mouse macrophages. Finally, in the same mouse model of NASH, treatment with a ROCK1 inhibitor decreased NASH-induced serum levels of EV, which was associated with a reduction in liver injury as assessed by serum ALT levels. Consistent with a reduction in tissue injury, ROCK1 inhibitor normalized induced hepatic mRNA levels of TNF-alpha and a variety of macrophage markers to the levels of control mice and prevented accumulation of galectin-3 (a macrophage marker) positive cells in the liver. *Summary/conclusion*: In conclusion, lipotoxicity induces release of hepatocyte EV via death receptor 5 signalling pathway, which activates macrophages towards a proinflammatory phenotype. The inhibition of ROCK1-dependent release of EV by hepatocytes appears to be salutary in NASH.

O-6B-2

Cigarette smoke extract induces the release of extracellular vesicles by airway epithelial cells via cellular carbonyl stress

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Introduction: The airway epithelium of smokers is repeatedly exposed to cigarette smoke (CS), the major cause of chronic obstructive pulmonary disease (COPD). Exposure to CS causes oxidative cell stress via 2 mechanisms: (a) Reactive oxygen species (e.g. H_2O_2) within CSE directly exert oxidative stress. (b) Thiol-reactive carbonyl compounds (e.g. acrolein) indirectly cause oxidative stress by depleting thiol-group bearing antioxidants like glutathione (GSH). Compelling evidence has shown that several types of cell stress induce the release of extracellular vesicles (EVs). Therefore, we determined whether exposure of airway epithelial cells (AEC) to cigarette smoke extract (CSE) results in increased EV release. Using H_2O_2 and acrolein, we determined whether reactive oxygen species or reactive carbonyl compounds accounted for CSE-induced EV release. *Methods*: AEC were exposed for 6 or 24 hours to different concentrations of CSE, H_2O_2 or acrolein and in some experiments in the presence of the thiol-group bearing antioxidant N-acetylcysteine (NAC). Relative levels of CD63+CD81+ EVs in conditioned media were measured by bead-coupled flow cytometry. Oxidized and total GSH were assessed using a GSH reductase cycling assay. *Results*: Both basal and CSE-induced EV-release increased over time. At 24 hours, CSE induced EV release in a concentration-dependent manner up to 2.3-fold at 1.5% CSE. This was paralleled by increases in oxidized cellular GSH (3.1-fold) and total GSH expression (5.8-fold). Incubation of 25% CSE with 50 μM GSH for 24 hours resulted in complete GSH oxidation, confirming direct thiol reactivity of CSE. In line with this, NAC prevented CSE-induced EV-release likely by scavenging thiol-reactive components of CSE. Similar to CSE, acrolein induced EV release in a NAC-reversible manner. No EV induction was observed for H_2O_2. The majority of control and CSE-induced EVs was smaller than 200 nm, as signal recovery after 200 nm-filtration was 78±26%. *Summary/conclusion*: AEC release an increased quantity of small, CD63+CD81+ EVs when exposed to CSE. This is likely mediated by reactive carbonyl compounds. Currently, we are characterizing the composition and functionality of the CSE-induced EVs, focusing on immune regulatory properties that may be relevant to the pathogenesis of COPD.

O-6B-3

Altered glycosylation of proteins associated with syncytiotrophoblast-derived extracellular vesicles in pre-eclampsia

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Introduction: Pre-eclampsia (PE) complicates ~3% of pregnancies and is a leading cause of maternal mortality. One or more placental factors, released into the maternal circulation, trigger maternal raised blood pressure, proteinuria and inflammation. In PE, significantly more placental syncytiotrophoblast extracellular vesicles (STBEV) are released into the maternal circulation, with altered phenotype, cargo and biological activity. Our proteomic analysis has shown increased glycosylation of the sialic acid binding protein Siglec 6 in PE STBEV. The aim of this study was to further investigate STBEV-associated protein glycosylation in PE. *Methods*: Lysates and sections were prepared from 6 normal (N) and 6 PE placentas. STBEV were prepared using ex vivo dual placental perfusion and differential centrifugation (N, n = 10 and PE, n = 10). Size, purity and phenotype of STBEV were measured using NTA, flow cytometry (FC) and Western blotting (WB). Increased glycosylated Siglec 6 in PE derived placenta and STBEV lysates was confirmed by WB and deglycosylation analysis. Global normal and PE placental glycosylation levels were investigated by immunohistochemistry (IHC) using a panel of FITC conjugated lectins. Those lectins showing altered PE placenta staining were then used to

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analyse N and PE STBEV by WB and FC. *Results*: Levels of glycosylated Siglec 6 were significantly higher in PE placenta ($p < 0.0001$) and STBEV ($p < 0.05$). PE placenta expressed both unglycosylated (50 kDa) and glycosylated Siglec 6 (~70 kDa) whereas STBEV only contained glycosylated Siglec 6 (~60 and ~70 kDa forms). IHC results showed increased staining of PE placenta with lectins *Sambucus nigra* (SNA; binds sialic acid) and *Ricinus communis* agglutinin I (RCA 120; binds galactose), which was confirmed by STBEV FC (SNA: $p = 0.001$ and RCA 120: $p < 0.01$) but no clear difference in total levels were seen by WB. *Summary/conclusion*: As with cell–cell interactions, surface glycans may also play a significant role in vesicle-cell interactions. In PE, altered surface glycans and glycosylated protein enrichment in STBEV may affect functions such as cell targeting, clearance and immune activity. Possible triggers of altered glycosylation are oxidative and inflammatory stress, therefore, STBEV surface glycans may also have potential use as markers of placental stress.

O-6B-4

Extracellular vesicles as possible conveyors of tight junction protein to leukocytes in neuroinflammation

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Introduction: Leukocyte transendothelial migration (TEM) across the blood-brain barrier (BBB) is a hallmark of neuroinflammatory disease. Leukocytes are thought to negotiate the tight junctions (TJs) of the BBB that restricts the passage of soluble and cellular elements into the central nervous system (CNS). Our recent work shows leukocytes invading the CNS early in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), are coated with TJ protein claudin-5 (CLN-5), a BBB determinant. As circulating leukocytes in MS patients show elevated CLN-5 protein in exacerbation, and "microparticles" released by cultured brain microvascular endothelial cells (BMEC) stimulate leukocyte TEM, we hypothesize, *extracellular vesicles (EVs) released from BMEC in neuroinflammation transfer CLN-5 to leukocytes for TEM. Methods*: High-resolution 3D confocal imaging was used to detect CLN-5^+ leukocytes *in vivo* during EAE, and the binding of CLN-5^+ EVs from EAE mice to leukocytes *in vitro*. EVs were FACS purified from plasma or TNF-α-treated BMEC cultures of mice expressing eGFP-CLN-5 in endothelial cells (Tie-2-eGFP-CLN-5). All EVs were co-labelled with PKH-26 dye to determine the CLN-5^+ fraction. EV subtypes were isolated by differential centrifugation and validated by nanoparticle tracking analysis. Western Blot confirmed CLN-5 expression in EV subtypes. *Results*: eGFP-CLN-5^+ leukocytes were seen in the CNS of Tie-2-eGFP-CLN-5 mice by day 9 EAE, consistent with the transfer of endothelial CLN-5 to leukocytes. eGFP-CLN-5^+ EVs were also obtained from plasma and cultured BMEC of these mice. FACS purified, eGFP-CLN-5^+ EVs bound to leukocytes *in vitro*. CLN-5 protein was expressed by exosomes and microvesicles. *Summary/Conclusion*: Results show CLN-5^+ endothelial EVs are released *in vitro* and *in vivo* in neuroinflammatory conditions, and can bind to leukocytes. Focal transfer of endothelial- CLN-5^+ EVs to leukocytes might aid the latter in TEM across the BBB by forging transient interactions with TJs on BMEC.

Ballroom F-H
Symposium session 6C - Cellular uptake of EVs
Chairs: *Michiel Pegtel and Muller Fabbri* 09:00-10:00

O-6C-1

Super-resolution imaging of uptake and processing of prostate cancer-derived exosomes in living prostate epithelial cells

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Introduction: Prostate cancer (PCa) is the most common malignancy in men and 1 of the leading causes of cancer-related death in developed countries. While overall survival of patients with early-diagnosed localized PCa is high, metastasized PCa decreases survival dramatically. Tumour cells communicate with surrounding stromal and epithelial cells to enhance tumour progression and metastasis. Using quantitative imaging approaches, we will provide novel insight into the exosome-mediated mechanisms by which prostate cancer cells influence their microenvironment. *Methods*: Exosomes from a PCa cell line (DU145N) are isolated by differential ultracentrifugation and stained with a fluorescent membrane dye (e.g. PKH26 or PKH67). To provide more insight of its molecular mechanism, we have followed exosome uptake and further processed it by non-tumorigenic prostate epithelial cells on different time scales, from seconds to multiple hours, by live cell imaging using conventional confocal microscopy and, for high- speed imaging, spinning disk microscopy. Several stages of exosome uptake and processing are studied in more detail using super-resolution 4Pi and structured illumination microscopy (SIM). *Results*: Conventional time-lapse imaging shows a rapidly initiated and continuous cellular uptake of individual fluorescently labelled exosomes. High speed spinning disk microscopy shows binding and subsequent translocation of exosomes over the target cell membrane. Furthermore, it shows that internalized exosomes are transported via microtubules. Super-resolution co-localization studies between exosomes and specific subcellular compartments shows processing of internalized exosomes through the endocytic pathway. *Summary/conclusion*: The combination of several imaging approaches enabled us to visualize subsequent steps and dynamics of exosome uptake and further processing by target cells. Super-resolution imaging (SIM) allows us to unravel the molecular mechanisms of action of exosomes in PCa in more detail.

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O-6C-2

Syk is a target of lymphocyte-derived microparticles in the induction of apoptosis of retinoblastoma cells

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Introduction: Retinoblastoma (Rb) is an aggressive childhood cancer of the developing retina that is associated with epigenetic deregulation of several cancer pathways. In addition to the significantly upregulation of the proto-oncogene spleen tyrosine kinase (*SYK*), the angiogenic potential of Rb correlates with invasive growth and metastasis. Lymphocyte-derived microparticles (LMPs) possess strong antiangiogenic effect against pathological ocular angiogenesis and potent inhibitory effect on cell viability. This study is designed to elucidate the mechanisms underlying the anti-Rb effect of LMPs. *Methods*: LMPs were produced from human CEM T cells after 0.5 μg/ml actinomycin D stimulation. Rb cell line (Y-79) and primary cultured Rb cells (isolated from primary site intraocular retinoblastoma of Rb patients) were subjected to WST-1, apoptotic assay after treated with LMPs. Quantitative RT-PCR, immunohistostaining and Western blot were performed to detect interest gene expression and protein levels in LMPs and in Rb cells. To elucidate the signalling pathways mediating LMPs-induced cell death, inhibitory experiments using individual specific inhibitors or siRNA were performed in Rb cells. For example, SYK inhibitor, Pifithrin-α (PFT, inhibits p53-dependent transactivation) and siRNA against p21. *Results*: LMPs significantly reduced Rb cell viability and induced cell death of human primary Rb cells in a dose-dependent manner. LMPs treatment caused a significant decrease of SYK expression in Rb cells at both mRNA and protein levels. Inhibition of SYK activity reduced Rb cell viability but up-regulated the expressions of p53 and p21. More interestingly, inhibition of p53 and/or knockdown of p21 expression abrogated LMPs-induced cell death and LMPs-induced caspase-3 activity. *Summary/conclusion*: The strong proapoptotic effect of LMPs on Rb cells is associated with the reduction of SYK expression and up-regulated p53/p21 signalling pathway. These data may open unexpected avenues for the development of novel therapeutic strategies that are particularly useful and relevant for the treatment of Rb cancer.

O-6C-3

Hyaluronan-coated extracellular vesicles as novel biomarkers

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Introduction: Hyaluronan is the most abundant polysaccharide of the extracellular matrix. It is essential for maintenance of normal tissues but it also promotes cancer progression by creating a favourable microenvironment for the growth of tumour cells. Hyaluronan is detectable in normal body fluids like plasma and urine and highly concentrated in fluids with anti-friction properties like pleural, synovial and peritoneal fluids. In many pathological states like cancer and inflammation, the amount of HA increases around cells, in the extracellular matrix and in the body fluids. Accumulation of hyaluronan acts as a poor prognostic factor in many epithelial cancers and non-invasive methods to detect hyaluronan levels in cancer patients would be valuable in diagnostics. Hyaluronan is synthesized by specific plasma membrane-bound enzymes, hyaluronan synthases (HASs) that induce growth of extremely long filopodia coated with hyaluronan. Furthermore, HAS activity induces shedding of extracellular vesicles (EVs) originating from the tips of filopodia. Interestingly, EVs are suggested to interact with their target cells by utilizing receptors on the vesicular surface, such as CD44, which is the most common receptor for hyaluronan. Hyaluronan-CD44 interactions may act as universal mechanism facilitating cellular binding and uptake of EVs. *Methods*: These hyaluronan-coated EVs secreted by different cell types with HAS overexpression were characterized by NTA, immunoblotting and qPCR. Their binding and effects on target cell properties like morphology and hyaluronan staining were studied by using live cell confocal microscopy and correlative light and electron microscopy. Hyaluronan secretion by target cells was measured with ELISA-like assay. *Results*: EVs were found to contain both HAS proteins and mRNA. EV uptake regulated the level of hyaluronan secretion in target cells. Furthermore, the interaction of

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hyaluronan with CD44 facilitated both EV binding and uptake into target cells. *Summary/conclusion*: Hyaluronan-induced EV shedding and uptake is a universal process regulating progression of cancer, inflammation and healing of tissues. EVs act as carriers for hyaluronan on their surface and are potential vehicles in preparing the premetastatic niches. Furthermore, hyaluronan-EVs could be utilized as diagnostic tools and targets of therapy.

O-6C-4

Functional RNAi screening identifies a novel and druggable chemokine receptor that mediates EV uptake by tumor cells

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Introduction: Extracellular vesicles (EVs) are gaining increasing attention as stimulants of tumour growth, including for glioblastoma (GBM), thereby positioning EVs and their putative receptors as potential therapeutic targets. However, so far, no specific EV receptors have been identified that allow inhibition by clinically relevant small molecule drugs. *Methods*: To identify putative EV receptors, we performed a siRNA screen against 700 druggable GPCRs to evaluate inhibition of PKH67-labelled EV uptake. Results were confirmed in primary stem-like GBM cells. Expression and uptake analysis were performed by fluorescence microscopy and/or FACS. A cre-mTmG model was generated by lentiviral transduction and selection using standard techniques. *Results*: siRNA screen identified several EV receptors. Among them, a chemokine receptor (CR) was selected as the most potent EV receptor. A genetic Cre-mTmG-based reporter model for EV uptake was developed resulting in fluorescent colour – a switch upon EV uptake by recipient GBM cells, allowing monitoring of physiological relevant EV uptake by GBM cells in vitro and in orthotopic GBM mouse models in vivo. GBM EVs stimulated proliferation of GBM cells. The effect of genetic and pharmacological inhibition of the newly identified EV receptor in counteracting EV-induced cell growth was evaluated. In addition, we discovered that the CR ligand was directly exposed on EVs via coupling of the ligand to vesicular glycan groups. Displacement of glycan-chemokine interactions as well as incubation with ligand neutralizing antibodies inhibited EV uptake, similar as observed when blocking the chemokine receptor. Currently in vivo studies using Cre-mTmG reporter mouse models are ongoing to determine the effect of the pharmacological CR inhibitor on EV uptake and GBM growth, which will be reported at the ISEV meeting. *Summary/conclusion*: We have identified a CR involved in EV uptake. Our results also indicate that EV-mediated tumour crosstalk can be blocked by receptor-specific targeting.

Networking coffee 10:00-10:30

Poster viewing sessions XIV, XV, XVI, XVII, XVIII, XIX, XX
Posters not attended by authors 10:00-10:30

Ballroom D
Symposium session 7A - EVs as cancer biomarkers II
Chairs: *Koji Ueda and Evo K.L. Soendergaard* 10:30-12:00

O-7A-1

Large-scale proteome profiling of cell-specific extracellular vesicles and identification of specific markers by multiplex proximity assays

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Introduction: Extracellular vesicles (EVs), present in various biological fluids such as plasma, urine, cerebrospinal fluid (CSF), etc., hold great promise as diagnostic biomarkers in a broad range of diseases. The molecular composition of EVs depends on their cellular origin and can reflect the condition of the tissues they derive from. To validate large sets of candidate markers in EVs, multiplex and sensitive detection technologies with low sample consumption are required. *Methods*: Proximity extension assay (PEA) is a homogenous immunoassay with minute sample requirement. The assay uses sets of 92 pairs of matched antibodies equipped with DNA oligonucleotides that give rise to amplifiable reporter molecules upon binding of the antibody pairs to target molecules. *Results*: The results demonstrate strong variations in the proteome profile between EVs from different sources, including proteins not previously known to be associated with EVs. *Summary/conclusion*: By using the PEA technology we have been able to screen for the presence of large numbers of proteins located on the surfaces or internally in EVs isolated from different source.

O-7A-2

Blood-derived extracellular vesicle mRNA analysis of glioblastoma and healthy controls

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Introduction: Extracellular vesicles (EVs) are dynamic lipid structure released by all cells in vivo and in vitro and, frequently, closely reflect the content of the cell they are released from. We have previously described enrichment of retrotransposon elements in EVs from several tumour cell lines, including human glioblastoma (GBM), medulloblastoma and melanoma cells. Specifically, we determined that human endogenous retroviruses (HERVS mRNAs for HERV-H, HERV- C, HERVK-6 and HERV-W) were enriched up to 16-fold in EVs compared to donor tumour cells. These sequences are remnants of infections of ancient germ line cells by retroviruses resulting in insertions that have been transmitted down through many generations. We now sought to determine if elevated levels of these sequences are reflected in the blood of GBM patients as compared to healthy controls. *Methods*: In an initial pilot study, we tested serum samples from GBM patients ($n=6$) and healthy controls ($n=8$), and more recently we have analyzed 20 more GBM samples and 10 more healthy controls. These serum samples were initially filtered through an 0.8 μm filter and ultracentrifuged at 100,000 × g for 90 minutes (ML-55 fixed angle rotor). RNA was extracted using the miRNeasy kit and analyzed using the Agilent Bioanalyzer. 1.6 ng of RNA was reverse transcribed into cDNA using the VILO Superscript (Invitrogen) and L1, HERV-K and Alu levels were analyzed by qRT-PCR. All data is normalized to the housekeeping gene GAPDH. *Results*: We successfully detected these genes in the serum derived EVs from both GBM and healthy controls blood samples. Alu sequences are the most abundant repetitive elements in humans, followed by L1 and HERV-K and this was clearly confirmed in the samples that we analyzed. Importantly, the levels of each gene were significantly higher in GBM samples compared to the healthy control groups ($p \leq 0.001$). This was reflected in the pilot study and confirmed on the larger samples cohort. *Summary/conclusion*: Taken together, these findings suggest that we have a few new powerful biomarker candidates for brain tumour detection, diagnosis as well as follow-up care, which should encourage the use of these markers in future trials and clinical care.

O-7A-3

Biomarker-containing extracellular vesicle release from tumours into blood using ultrasound and nanodroplets

Robert Paproski, John Lewis and Roger Zemp
University of Alberta, Edmonton, Canada

Introduction: The diagnosis and prognosis of cancer typically involves analysis of physical biopsies. These procedures can significantly increase the risk of lethal infections, limiting the number of biopsies that can be taken and preventing the analysis of serial cancer samples throughout disease progression. Extracellular vesicles (EV) shed by solid tumours contain informative biomarkers such as proteins and nucleic acids and have been under recent investigation as a source of diagnostic biomarkers. Our goal was to develop an ultrasound (US)-based technique to stimulate the release of tumour EVs into the bloodstream which could be acquired through blood draws. *Methods*: Nanodroplets are ~200 nm perfluorobutane particles that, when exposed to US, can phase change into microbubbles which undergo energetic reactions. To determine if US ± nanodroplets could stimulate EV release, human fibrosarcoma HT1080 ± GFP cells in culture were incubated with or without nanodroplets and exposed to focused US (30 MPa pk-pk pressure, 10–10,000 cycles). Green fluorescent (GFP+) EVs were quantified in the supernatant after centrifugation (2,000 × g for 5 minutes) using the Apogee A50 micro flow cytometer. In vivo experiments involved injecting nanodroplets intravenously in chicken embryos bearing HT1080 ± GFP tumours, applying focused US to tumours, acquiring serum samples and analyzing GFP+ particle levels using the Apogee A50. miR-21 (miRNA overexpressed in many cancers), GFP and RAC1 mRNA levels were assessed in serum inside and outside of EV. *Results*: Only medium from HT1080 + GFP cells (and not HT1080 no GFP cells) contained GFP + EV (which were 180–240 nm). When US was applied to cultured HT1080 + GFP cells, GFP+ particle levels in the medium increased 17-fold compared to cells without US. In the presence of nanodroplets, US-mediated release of GFP+ EVs was further enhanced by 3-fold. When the blood from chicken embryos bearing HT1080 + GFP tumours was assessed for EVs, very few GFP+ particles were detected (< 100 GFP+ particles/ μL). However, after i.v. injection of nanodroplets and exposure of the tumours to US, GFP+ particles in the serum increased dramatically (~ 10,000 GFP+ particles/ μL). This increase in serum GFP+ particles was not observed when US was applied away from tumours. When assessing the impact of US and nanodroplets on the release of relevant biomarkers in the bloodstream, serum miR-21 and GFP mRNA levels were significantly increased, with the majority of the tumour-specific mRNA present within EV. Tumour-derived EV in the blood also contained RAC1 mRNA which, when sequenced, revealed the N92I mutation known to exist in HT1080 cells. *Summary/conclusion*: We have developed a new minimally invasive technique that increases blood levels of tumour-derived biomarker-rich EV. Focused US can be used to ensure only specific regions of tumours release EV into the blood. Tumour-derived EVs in the blood contain a variety of potentially useful biomarkers which could facilitate the delivery of personalized medicine approaches.

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O-7A-4

Perturbations in extracellular vesicle emission and phosphoproteome following exposure to oncogene-directed therapeutics – implications for targeting Epidermal Growth Factor Receptor in cancer

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Introduction: Cancer cells emit extracellular vesicles (EVs) containing active oncoproteins, the abundance and functional state of which could be biologically and clinically informative in the context of targeted therapy. For example, oncogenic forms of the epidermal growth factor receptor (EGFR/EGFRvIII) trigger changes in the phosphoproteome of cancer cells and in the corresponding cargo of tumour-derived EVs, both in vitro and in vivo. *Methods*: To explore how these changes may be affected by different classes of EGFR-targeting anticancer agents we compared the effects of the EGFR neutralizing antibody (Cetuximab), and 2 different, irreversible, second generation EGFR/panERBB kinase inhibitors (EKIs): canertinib (CI-1033) and dacomitinib (PF-00299804) in EGFR driven A431 cancer cells. We assessed numerical changes in EV output using NTA approach and analysed the cargo by Western, ELISA and antibody arrays. *Results*: We observed that while these different classes of targeted agents markedly reduced EGFR activity in the parental cells, as measured by the levels of phosphorylated EGFR (P-EGFR), they also induced distinctive (class-specific) changes in numbers, size distributions, protein and nucleic acid contents and EGFR isoform levels. These agents also affected P-EGFR content and that of EGFR signalling intermediates (MAPK, AKT) in the cargo of exosome-like EVs emitted following the drug exposure. *Summary/conclusion*: Overall, our findings suggest that the EV emission rates, oncoprotein content and their functional states may reflect the diverse activities of oncogene-directed therapeutics, and we suggest a possibility of exploring these changes as companion diagnostics in the course of targeted therapy.

O-7A-5

Use of exosomes as surrogate markers of plexiform neurofibroma and malignant peripheral nerve-sheath tumour transformation

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Introduction: The development of neurofibromas and neurofibroma transformation into MPNST are serious complication of Neurofibromatosis type 1 (NF1). Although it is known that the NF1 microenvironment contributes to the initiation and progression of these tumours, relevant tumour-stromal interactions remain incompletely understood. We have recently demonstrated that tumour-derived exosomes (30–100 nm vesicles) can support primary tumour growth and metastatic progression through bone marrow-derived dendritic cells (BMDCs) education. Here, we report for the first time evidences that circulating exosomes may be useful to monitor neurofibroma and/or MPNST. *Methods*: To determine the presence of circulating exosomes in neurofibroma and MPNST, we prospectively isolated and characterized exosomes from human and murine MPNST cell lines, *Nf1fl/fl;DhhCre* neurofibroma-bearing mice and littermate controls, and from *Nf1;Tp53* (NPCis) mice bearing GEM-PNST, and plasma from 17 patients with NF1, median age 16 years (range 5–54 years, 13 males). Twelve of Seventeen patients had plexiform neurofibroma (PN), and among them 3 were associated with MPNST. Sample collection was approved by Institutional IRBs. We isolated exosomes using standard serial ultracentrifugation methods and confirmed their number/size distribution by NanoSight. We further analyzed protein content by bicinchoninic-acid assay and BMDCs by flow cytometry. *Results*: The number and the protein content of exosomes produced by MPNST cell lines was significantly increased when compared with medium from immortalized human Schwann cells. The exosome protein cargo was also increased in plasma from *Nf1fl/fl;DhhCre* mice harbouring neurofibromas, and from NPCis mice with MPNST, compared with non-tumour-bearing controls. We also compared 2 subgroups, NF1 patients (with PN, with or without MPNST) and NF1 patients (without PN/MPNST) to healthy controls. We found that PN patients (with or without MPNST) have increased exosome protein concentration compared to controls and to NF1 patients without PN ($p = 0.030$). *Summary/conclusion*: Our data suggests that analysis of circulating exosomes in NF1 patients may be useful for early detection of subjects developing PN and/or MPNST. Furthermore, analysis of tumour-derived exosomes could be important to understand the role of the NF1 microenvironment in tumoural transformation.

Funding source: Our work is supported by grants from National Institutes of Health, Melanoma Research Alliance, Sohn Conference Foundation, the Mathew Larson Foundation, the Feldstein Foundation, the Children's Cancer and Blood Foundation, The Nancy C and Daniel P Paduano Foundation, Pediatric Oncology Experimental Therapeutic Investigator Consortium (POETIC), James Paduano Foundation, the Starr Cancer Consortium and Nuovo-Soldati Foundation (AG). Grants from the NIH, DOD Program on Neurofibromatosis, NTAP and the Children's Tumour Foundation support JQ and NR.

O-7A-6

Extracellular vesicles in human bile from malignant biliary stenoses contain overexpressed cancer-associated proteins

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Introduction: The differential diagnosis of biliary stenosis is critically challenging for gastroenterologists. The diagnostic value of all currently available methods (e.g. imaging technics, standard serum biomarkers) is limited by relatively poor accuracy and negative predictive value. New biomarkers are thus needed to enable rapid and accurate discrimination of malignant lesions. Extracellular vesicles (EVs) released in bile from surrounding cancer cells could represent a rich source of new potential biomarkers. Li et al., recently established the presence of EVs in human bile from cholangiocarcinoma (CC) patients and characterized their microRNA content. However, no data is still available on their protein counterpart. Here we present the first proteomic study of human bile EVs aimed at identifying new candidate biomarkers for malignant biliary stenoses. *Methods*: Bile samples were obtained from 40 patients presenting with a biliary stenosis due to pancreatic cancer (PAC), CC and chronic pancreatitis (CP). The study was approved by Ethics Committee of Geneva and Erasme Hospitals, and written informed consent was obtained for each patient. EVs were isolated from 3 pools of 4 samples by using differential centrifugation, inspected by electron microscopy (EM) and nanoparticle tracking analysis (NTA), and probed by western blotting (WB) for exosome markers. EVs proteins were subjected to proteomic analysis by GeLC-MS/MS and identified against the

SwissProt database. Selected cancer-related proteins were finally verified by WB in the whole cohort. *Results*: The presence, size, shape and number of EVs from bile collected from malignant and non-malignant biliary stenosis were established by EM and NTA. WB analysis for TSG101, Alix, HSP70 and CD9 confirmed the enrichment of EVs populations. The subsequent proteomic analysis of bile EVs allowed the identification of 521 proteins with at least 2 unique peptides. Among them, all the most known EVs markers stood out. A number of cancer-associated proteins were also identified and the overexpression of 3 of them (CD133, c-SRC, RAC-1) was finally verified in malignant samples. *Summary/conclusion*: This study highlights bile EVs proteins as new ''star players'' in the differentiation of malignant biliary stenoses.

Acknowledgements: Work supported by the ''Fondation Ernst et Lucie Schmidheiny''.

Ballroom E
Symposium session 7B - EVs and microorganisms
Chairs: *Amy Buck and Yong Song Gho* 10:30-12:00

O-7B-1

Bacterial membrane vesicles mediate the secretion of immunomodulatory factors during Mycobacterium tuberculosis infection

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Introduction: *Mycobacterium tuberculosis* (Mtb) is an intracellular pathogen that infects lung macrophages and employs immune evasion strategies to create a niche for survival within modified phagosomes. Mtb secretes lipoglycans and lipoproteins that disseminate throughout the endocytic network of infected cells and modulate macrophage effector functions such as phagosome maturation, MHC-II antigen presentation, and cytokine production. These Mtb molecules also escape from infected cells in extracellular vesicles (EVs) that can regulate the functions of uninfected immune cells in vitro and may shape the immune response to Mtb in vivo. Current models predict that Mtb molecules are secreted via exosomes, but a mechanism for their trafficking into EVs has not been addressed. *Methods*: The aim of this study was to investigate the biogenesis of EVs released by Mtb-infected cells. Murine macrophages were infected with Mtb in vitro for 24 hours and EVs were purified by differential ultracentrifugation. *Results*: IZON qNano and Western blotting analysis confirmed the isolation of 80–250 nm EVs that contain exosome markers (e.g. CD63, CD9) and Mtb lipoglycans/lipoproteins (e.g. LAM, LpqH), consistent with previous reports. However, electron microscopy with immunogold labelling indicated that exosome and Mtb molecules were present in distinct subsets of EVs. This finding was validated using a novel method that we developed to quantitate EV heterogeneity by immunofluorescence microscopy (“Single vesicle IF”). Exosomes and Mtb+ EVs were separated on sucrose gradients based on their distinct densities and only the gradient fractions that contained Mtb+ EVs stimulated cytokine production by uninfected macrophages. A microbial origin of Mtb+ EVs released from infected macrophages was suggested by similarities in size, composition and biological activity to bacterial membrane vesicles (MVs) produced by Mtb in broth culture. Consistent with this conclusion, the release of Mtb lipoglycans in EVs was dependent on bacterial viability. *Summary/conclusion*: Our data provides evidence for MV-based secretion of lipoproteins/lipoglycans during Mtb infection as well as the release of Mtb MVs into the extracellular environment. We therefore propose that Mtb MVs modulate the effector functions of infected macrophages and also circulate Mtb components beyond the site of infection to further regulate immune responses to Mtb. These mechanisms may promote the survival of Mtb and the pathogenesis of TB infection.

O-7B-2

A novel mechanism of bacterial toxin transfer within host blood cell-derived microvesicles

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Introduction: Shiga toxin (Stx) is the main virulence factor of enterohemorrhagic *Escherichia coli* (EHEC), which are non-invasive strains that can lead to hemolytic uremic syndrome (HUS), associated with renal failure and death. Although bacteremia does not occur, bacterial virulence factors gain access to the circulation and are thereafter presumed to cause target organ damage. Stx was previously shown to circulate bound to blood cells but the mechanism by which it could potentially transfer to target organ cells has not been elucidated. *Methods*: Stx within blood cell-derived microvesicles was detected by flow cytometry in blood samples from EHEC-infected patients (n = 18, of which 13 had HUS) and mice (n = 15) as well as in blood samples stimulated with toxin in vitro. Toxin containing microvesicles were also studied by electron microscopy in the kidney of a patient with HUS, in infected mice and in glomerular endothelial cells stimulated with microvesicles in vitro. The effect of toxin-containing microvesicles on glomerular endothelial cell viabilty was assayed by a viability assay and inhibition of protein synthesis. *Results*: Blood cell-derived microvesicles, shed during HUS, contain Stx and are found within patient renal cortical cells. The finding was reproduced in mice infected with Stx-producing *Escherichia coli* exhibiting Stx-containing blood cell-derived microvesicles in the circulation that reached the kidney where they were transferred into glomerular and peritubular capillary endothelial cells and further through their basement membranes followed by podocytes and tubular epithelial cells, respectively. In vitro studies demonstrated that blood cell-derived microvesicles containing Stx undergo endocytosis in glomerular endothelial cells leading to cell death secondary to inhibited protein synthesis. *Summary/conclusion*: This study demonstrates a novel virulence mechanism whereby bacterial toxin is transferred within host blood cell-derived microvesicles in which it may evade the host immune system.

O-7B-3

Vesicles from different Trypanosoma cruzi strains trigger differential chronic immune responses by C57BL/6 splenocytes

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Introduction: Trypomastigote forms of *Trypanosoma cruzi* vesicles that are enriched with *trans*-sialidase (TS)/gp85 glycoproteins and other alpha-galactosyl (alpha-Gal)-containing glycoconjugates, like mucins. Previous data from our group have shown that *T. cruzi* vesicles are potent agonists of TLR2 in murine macrophages having an important role during the initial steps of infection especially in the strains Yu-Yu and CL-14. Here, we evaluated the role of vesicles from different *T. cruzi* strains (Y, YuYu, CL-14 and Colombiana) in the modulation of chronic immune response in splenocytes from C57BL/6 mice. *Methods*: C57BL/6 Mice (10 per group) were infected with 50 parasites from each strain and positive animals were followed for 180 days. Spleen was removed and splenocytes (1×10^6 cells) were incubated with vesicles (5 mg/ml) for 72 hours. Supernatants were removed and the nitrite was determined by Griess reaction. Cytokines (IFN-gamma, TNF-alpha, IL-2, IL-4, IL-5, IL-6 and IL-10) were determined using the CBA kit (BD Biosciences). *Results*: No production of IL-2, IL-4 and IL-5 was detected after incubation with vesicles from all strains by chronic spleen cells. Regardless the type of strain used for in vivo infection, in chronic infected mice, Colombiana and Y strains vesicles exhibited a

more pro-inflammatory role than Yu-Yu and CL-14 strains. Interestingly, vesicles purified from Colombian strains have differences in the expression of α-galactosyl residues compared to Y, YuYu and CL-14. *Summary/conclusion*: Those data indicate that polymorphisms in the vesicles surface are determinant in the immunopathologic events not only in the early steps of infection but also in the chronic phase.

O-7B-4

Extracellular vesicles secreted by *Opisthorchis viverrini* promote tumourigenic changes in cholangiocytes

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Introduction: *Opisthorchis viverrini* is a parasitic liver fluke that causes major health problems in several Southeast Asian countries such as Thailand, Cambodia and Laos. This parasite induces chronic biliary inflammation, often culminating in cancer of the bile ducts, or cholangiocarcinoma (CCA). Indeed, *O. viverrini* is the single major cause of liver cancer in regions of NE Thailand where the fluke is endemic. The excretory/secretory products (ESP) of *O. viverrini* have been characterized using proteomic approaches, but substantially less is known about the mechanisms by which these ESP interact with the host and promote tumourigenesis. *Methods*: We combined cell biology and proteomics (including xCELLigence®) approaches to observe the internalization of *O. viverrini* secreted extracellular vesicles (EVs) by human cholangiocytes and assess the impact of their uptake on host cell protein expression. *Results*: *O. viverrini* adult flukes secrete EVs containing proteins that are diagnostic of exosomes, including tetraspanin transmembrane proteins. Internalization of EVs resulted in cholangiocyte proliferation and secretion of IL-6, and induced major changes in expression of proteins associated with processes such as phagocytosis, wound healing and cancer. We showed that antibodies to a recombinant *O. viverrini* surface tetraspanin blocked the uptake of *O. viverrini* EVs by cholangiocytes, highlighting a novel potential approach to vaccine development for this chronic infectious cancer. *Summary/conclusion*: These findings are the first to implicate parasitic helminth EVs in the disease process, and reveal novel molecular mechanisms of immunopathogenesis and tumourigenesis.

O-7B-5

Fungi extracellular vesicles transriptome

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Introduction: Extracellular vesicles (EVs) play an important role in the biology of various organisms, including fungi, in which they are required for the trafficking of molecules across the cell wall. Fungal EVs contain a complex combination of macromolecules, including proteins, lipids and glycans. In this work, we aimed to describe and characterize RNA in EV preparations from the human pathogens *Cryptococcus neoformans*, *Paracoccidiodes brasiliensis* and *Candida albicans*, and from the model yeast *Saccharomyces cerevisiae*. *Methods*: EVs were isolated from cell-free culture supernatants were recovered by centrifugation at 4,000 × *g* for 15 minutes at 4°C and the resulting supernatants were recentrifuged at 15,000 × *g* for 30 minutes, to remove smaller debris. The final supernatants were concentrated by a factor of 20 with an Amicon ultrafiltration system (100-kDa cutoff, Millipore). Concentrated supernatants were centrifuged at 15,000 × *g* for 30 minutes, to ensure the removal of aggregates, and the resulting supernatant was then ultracentrifuged at 100,000 × *g* for 1 hour to precipitate vesicles. The RNA was isolated with the RNeasy mini kit, and RNA-seq 100 ng of purified RNA from 3 independent biological replicates was used. The RNA-seq was performed in a SOLiD 4 platform and the sequencing data obtained were analyzed with CLC Genomics Workbench v 5.5.1. *Results*: We characterized EV RNA content, which consisted mostly of molecules less than 250 nt long. The reads obtained aligned with intergenic and intronic regions or specific positions within the mRNA. In *C. neoformans,* 90% of the reads mapped to intronic regions, the remaining 10% mapping to exons. In *P. brasiliensis*, 17% of the reads mapped to intronic regions, 82% to exons and 21% to exon-intron regions. In *S. cerevisiae* and *C. albicans*, which have few introns, 90% of the reads mapped to exons. We identified 114 ncRNAs, among them, 6 small nucleolar (snoRNA), 2 small nuclear (snRNA), 2 ribosomal (rRNA) and 1 transfer (tRNA) common to all the species considered, together with 20 sequences with features consistent with miRNAs. We also observed some copurified mRNAs, as suggested by reads covering entire transcripts, including those involved in vesicle-mediated transport and metabolic pathways. *Summary/conclusion*: We characterized for the first time RNA molecules present in EVs produced by fungi. Our results suggest that RNA-containing vesicles may be determinant for various biological processes, including cell communication and pathogenesis.

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O-7B-6

Production rates of extracellular vesicles among marine bacteria

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Introduction: Extracellular vesicles are abundant in the oceans, where they likely play a variety of roles within microbial communities. The concentration of vesicles varies from place to place in ocean ecosystems, reflecting the balance between species-specific production rates, degradation rates, and consumption rates by the marine food web. To gain insights into the forces influencing these patterns, we have measured vesicle production rates in a variety of cultured marine bacterial strains as a function of growth rate. Here, we describe the impact of light intensity on vesicle release in 3 strains of *Prochlorococcus* – the most abundant cyanobacterium in the oceans. We compare these results to measurements of vesicle release by 5 diverse marine heterotrophs, and investigate the amount of DNA each strain exports within the vesicles. *Methods*: Batch cultures of each microbe were grown under a range of environmentally relevant light intensities and temperatures. Vesicle production rates, measured on a per generation basis, were determined by following the concentration of vesicles (by nanoparticle tracking analysis) and cells (by flow cytometry) over time. Variation in DNA export was investigated using dye-based assays, BioAnalyzer, and visual quantitation of SYBR-stained vesicles. *Results*: We find that average vesicle production rates can vary by multiple orders of magnitude among cultured marine bacteria growing at their maximal growth rate.

Within *Prochlorococcus* strains, we find that vesicle production rates vary as a function of light limited growth. The average amount of DNA released in vesicles, and its fragment size distribution, also differs among strains. Only a small fraction of vesicles contain sufficient DNA to be visualized with fluorescent DNA stains, suggesting heterogeneous DNA packaging. *Summary/conclusion*: We have shown that there is significant variability in vesicle production rates per generation among model marine bacteria. Thus a microbe's relative abundance within a community may not dictate its contribution to the local vesicle population, or the relative frequency of its DNA within vesicle metagenomes. These data provide a first step toward improving our understanding of the distribution and biological roles of vesicles in the marine environment.

Ballroom F-H
Symposium session 7C - EV transcriptomics
Chairs: *Lorraine O'Driscoll and Esther Nolte-'t Hoen* 10:30-12:00

O-7C-1

Development of a high-throughput workflow for small RNA profiling of neuronal-cell-derived exosomes

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Introduction: Extracellular vesicles (EVs) include microvesicles, exosomes and other membrane-bound particles released by cells. EVs can be isolated from the majority of bodily fluids including blood and CSF and provide a rich source of protein and RNA biomarkers for the diagnosis of disease. We have applied the analysis of exosomal RNA to develop biomarkers for neurodegenerative disorders such as Alzheimer's and prion diseases. We sought to determine the influence of exosome isolation method on the profile of small non-protein coding RNA (ncRNA) obtained from the vesicle preparations. Common exosome isolation methods involve differential ultracentrifugation and ultracentrifugation coupled with density gradient separation. The question of whether a higher purity of exosomes is required for small ncRNA profiling, especially for diagnostic and biomarker purposes, has not yet been addressed. *Methods*: We investigated using deep sequencing whether there are any differences in the ncRNA profiles using vesicles isolated from neuronal cells using different techniques. We also established an automated bioinformatics pipeline to comprehensively characterize small ncRNA species from cells and exosomes prepared by the 2 exosome isolation methods. *Results*: By comparing the ncRNA content in exosomes, we identified an enrichment of miRNAs and a diverse range of ncRNAs including ribosomal RNA, transfer RNA, small nuclear RNA, small nucleolar RNA and Piwi-interacting RNA as compared to their cellular counterparts. We show that ultracentrifugation-based methods provide a suitable approach to identify ncRNA biomarkers in neurological diseases without requiring additional separation. The use of the high throughput workflow revealed a wealth of information on the different biotypes of small ncRNAs in exosomes prepared by density gradients and ultracentrifugation methods. *Summary/conclusion*: By comparing the overall small RNA profiles and their abundance level of individual RNA species, we found that both exosome isolation methods provided an efficient approach for exosomal small RNA profiling. Additionally, our findings present a high coverage approach for determining the optimal exosome isolation method for small RNA profiling.

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O-7C-2

Diurnal dynamics of small RNAs in plasma and plasma-derived exosomes of healthy human subjects

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Introduction: Extracellular small RNAs (ex-sRNAs) consist of microRNAs (miRNAs) and specific fragments of other RNA classes. Little is known about the diurnal dynamics in relation to ex-sRNAs in plasma and plasma-derived exosomes of healthy humans. *Methods*: To determine conserved patterns of RNA levels in plasma, we collected plasma samples from 10 healthy individuals. Additionally, in a time course experiment (36 hours; total 8 time points), we obtained plasma samples from 4 healthy individuals. We next isolated exosomes fractions from all plasma samples obtained in the time-course experiment. A total of 74 RNA-seq libraries were prepared from both plasma and plasma-derived exosomes – these libraries were analyzed using a newly developed in-house bioinformatics pipeline. *Results*: Selective patterns were found for specific miRNAs, several Y RNA fragments and few transfer RNA fragments in the plasma from 10 healthy individuals. In the time-course samples, the levels of these ex-sRNAs showed considerable intra- and inter-individual variability in both plasma and exosomal fractions. Three clusters of correlated ex-sRNAs were identified in plasma. Ex-sRNA levels in 1 cluster exhibit strong correlations between plasma and exosomes. Peak levels of this cluster in plasma were observed at mid-morning, while the other 2 clusters both peaked at noon. Inter-individual variability of ex-sRNA levels in plasma steadily increased between morning and noon but dropped towards evening. In contrast, the inter-individual variability of ex-sRNA levels in the exosomal fractions peaked at mid-morning but was lowest at noon and evening. *Summary/conclusion*: Our findings indicate that diurnal signatures and variability of ex-sRNA levels may reflect different physiological states of healthy humans during the course of a day. Our results further highlight that ex-sRNA concentrations in both plasma and exosomes are variable over time and between healthy individuals, which should be taken into account when assessing the potential of ex-sRNAs as biomarkers.

O-7C-3

Short and long RNA deep sequencing of two sub-populations of nano-sized exosome-like extracellular vesicles

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Introduction: Several different extracellular vesicles can be released by cells into the surrounding environment. We have recently identified 2 distinct nano-sized, exosome-like subpopulations of vesicles that both express common "exosome-markers," including CD63, but differ in size and density. The aim of this study was to evaluate the RNA content of these sub-populations of exosome-like vesicles. *Methods*: Exosomes were analysed from the human mast cell line, HMC-1. Cell, debris and larger vesicles were eliminated by a 300 × g and a 16,500 × g centrifugation. The remaining vesicles were then separated by different ultracentrifugation, filtration and density gradient protocols. Different pellets were evident after this separation and the vesicles in the different pellets were named exosome-like vesicles 1 (ELV-1) and ELV-2 and they were analysed for their RNA content by using both microRNA and whole transcriptome sequencing. Also the cellular RNA content was analysed for comparison. *Results*: Samples were analysed in 2 biological replicates with good reproducibility between them (Pearson 0.91–0.99). The ELV-2 mRNA correlated better with the cellular mRNA (Pearson 0.95; ELV-2 vs. cells), than the ELV-1 mRNA (Pearson 0.78; ELV-1 vs. cells).

The correlation between ELV-1 and ELV-2 was relatively weak (Pearson 0.75). miRNAs and mitochondrial tRNA were more prominent in the ELV-2 vesicles, while vault RNA, Y RNA, snoRNA and snRNA were more prominent in the ELV-1 vesicles. *Summary/conclusion*: The RNA cargo in subsets of exosomes-like EVs is significantly different. It seems to be more of a random sample of cellular RNA in 1 of the subsets, while the RNA cargo in the other subset seems to be more specifically loaded. Distinguishing diverse RNA cargo in subpopulations of EVs may have the capacity to further distinguish EV diversity, biogenesis and function.

O-7C-4

miRNA in extracellular vesicles derived from cerebrospinal fluid of glioblastoma patients

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Introduction: Extracellular Vesicles (EVs) are cell-secreted vesicles that range 30–2,000 nm in size. These vesicles are secreted by both normal and neoplastic cells. Because EVs contain genetic and proteomic contents that reflect the cell of origin, it is possible to detect tumour-specific material in EVs secreted by cancer cells. Importantly, EVs secreted by cancer cells transgress anatomic compartments and can be detected in the blood, cerebrospinal fluid (CSF), and other bio-fluids of cancer patients. There is a growing interest in analyzing EVs from the bio-fluids of cancer patients as for disease diagnosis and therapeutic monitoring. We explored the miRNA content of EVs isolated from the plasma and CSF of patients with glioblastoma, the most common form of primary brain cancer. *Methods*: EVs were isolated from plasma and CSF of glioblastoma patients. The microvesicle subpopulation was isolated by pelleting at 10,000 $\times$ g for 30 minutes after cellular debris was cleared by a 2,000 $\times$ g (20 minutes) spin. The exosome subpopulation was isolated by pelleting the microvesicle supernatant at 120,000 $\times$ g (120 minutes). qRT-PCR was performed to examine the distribution of miR-21, miR-103, miR-24, and miR-125. *Results*: We found that irrespective of the source or methods for EV assessment (Izon, Nanosight, or Electron Microscopy), there is less than 1 miRNA per 150–85,000 EVs based on qPCR quantitation. These results suggest that most EVs in clinical CSF samples carry little or no miRNAs and raise questions pertaining to the biologic plausibility of cell-to-cell communication through EV miRNA. Moreover, the reference transcripts commonly used as references for quantitative PCR (including miR-103, miR-24, and miR-125) and assumed to be invariant between EVs exhibit significant variability in EVs isolated from clinical CSF. When sub-fractionated into exosomes and microvesicles by differential centrifugation, we found that miRNAs reside in the exosome fraction. *Summary/conclusion*: Despite their relative rarity, CSF EV miRNAs, when quantitated in absolute levels, reliably discriminate between glioblastoma patients and patients with non-oncologic conditions. Our results demonstrate the potential and challenges of CSF EV as a platform for clinical biomarker development.

O-7C-5

A comprehensive method for the analysis of extracellular small RNA-seq data, including characterization based on cellular expression profiles and exogenous sequence detection

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Introduction: Analysis of extracellular RNA (exRNA) is a very promising approach to clinical diagnostics and therapy. However, due mainly to low yields, wet-lab and biochemical methods for extraction, purification, and sequencing of exRNAs are much more vulnerable to contamination and artefact than cellular RNA preparations. Furthermore, it has been suggested that exogenous-exRNAs may be present at detectible levels in some biofluids, but extreme care is required to ensure these sequences are not in fact derived from endogenous RNA molecules. To accommodate these considerations, we present the extra-cellular RNA processing tool (exceRpt), a software package optimized for the analysis of small-RNA-seq datasets obtained from exRNA preparations. *Methods*: We extend beyond existing tools used to assess cytosolic micro-RNAs (miRNAs) by deliberately addressing experimental considerations specific to exRNA profiling. The operation of the tool falls into 3 categories, 1) ''pre-processing'', 2) ''endogenous alignment'', and 3) ''exogenous alignment'': 1a) Alignment against synthetic spike-in sequences for calibration or titration; 1b) Explicit removal of common laboratory contaminants and ribosomal RNAs; 2a) Alignment to the full set of annotated, potentially spliced, endogenous RNA transcripts including all known miRNAs, tRNAs, piRNAs, snoRNAs, lincRNAs, mRNAs, retrotransposons, and circular RNAs; 2b) Alignment to the endogenous genome of the ''host'' organism; 3a) Alignment to all annotated exogenous miRNAs in miRBase; 3b) Efficient alignment to the full genomes of all sequenced bacteria, viruses, plants, fungi, protists, and select ''food'' vertebrates. *Results*: Our approach to exogenous RNA detection is deliberately conservative, due to the explicit removal of likely contaminants and prior alignment to endogenous sequences. However we present a re-analysis of published exRNA-seq data in which we confidently detect the presence of specific plant species. Using this software we have also created the first human cellular small-RNA reference atlas by uniformly re-processing 6.8 billion reads from 528 samples corresponding to 16 healthy tissues. We assess tissue-specific small-RNA expression profiles that can be used to elucidate exRNA analyses by identifying a single tissue, or even decompose contributions from multiple tissues, from which extracellular RNAs may be derived. We use qPCR miRNA expression data to corroborate these RNA-seq expression profiles and create a ''gold-standard'' set of tissue-specific miRNAs. This has been included in exceRpt in the form of a statistical classifier which, when given expression data of unknown tissue-of-origin, will predict the most likely percent contributions of each tissue-type. *Summary/conclusion*: The exceRpt software package is freely available to the community in the Genboree Workbench (www.genboree.org) where a user may upload and perform comparative/integrative analysis of their samples and store results using only a web browser.

O-7C-6

Whole transcriptome analysis suggests a cytosolic origin and enhanced shedding during S-phase for human EVs

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Introduction: Extracellular vesicles (EVs) are detectable in body fluids and contain functionally active macromolecules from the cells of origin, thus are potentially useful as clinical biomarkers and as analytical disease surrogates. While several reports have analyzed the

miRNA profile of EVs, the mRNA content of EV populations remains poorly characterized. *Methods*: We performed RNA sequencing (RNA-seq) of paired cells and EVs, collected by ultracentrifugation from cell media and of EVs collected from human plasma from cancer patients and healthy controls, followed by ad hoc computational analysis. *Results*: We found that the mRNA profile of large and nano-sized EVs from U87 cells differs markedly from the mRNA profile of the source cells. We show that, in comparison to whole cells, (1) EVs contain a significantly lower fraction of unspliced mRNA, (2) are enriched for shorter transcripts, and (3) are depleted for short half-life mRNA. These differences in mRNA between EVs and cells were strikingly similar to the difference seen comparing the mRNA of cytosol to whole cells, suggesting a cytosolic origin for EVs. Further, we show that mRNAs bearing a signal peptide are depleted from EVs, suggesting that EV formation in the cytosol may largely exclude the endoplasmic reticulum. We also show that EVs are enriched for histone and E2F target mRNAs, both of which are most highly transcribed during the S-phase of the cell cycle, suggesting that EV shedding is likely enhanced during S-phase. Lastly, we demonstrate that we can detect similar RNA enrichment patterns in EVs isolated from the blood of patients with invasive lobular breast carcinoma, suggesting the use of circulating EVs as biomarkers. *Summary/conclusion*: We have shown that mRNA profiles of U87 EVs are consistent with a cytosolic origin, and that their shedding may be cell cycle-regulated. Further, these same signals can be seen in circulating EVs from breast cancer patients.

This study was supported by grants from the National Institutes of Health (NCI NIH R00 CA131472 (to D.D.V.); NIH UCLA SPORE in Prostate Cancer award P50 CA092131 (to D.D.V.); Avon Foundation Fund 02-2013-043 (D.D.V.)).

Networking lunch 12:00-13:00

Poster viewing sessions XIV, XV, XVI, XVII, XVIII, XIX, XX
Posters attended by authors 12:30-13:00

Poster walk by chairperson, XIV, XV, XVI, XVII, XVIII, XIX, XX
Posters attended by authors 13:00-14:00

Ballroom D
Symposium session 8A - EVs in the tumour microenvironment
Chairs: *Hector Peinado and Dolores Di Vizio* 14:00-15:30

O-8A-1

Glioma-derived extracellular vesicles selectively suppress immune responses

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Introduction: Glioma-related immunosuppression has been well documented for many years; however, mechanisms by which this suppression occurs are not fully understood. Here we explore a role for extracellular vesicles (EVs) released from glioma cells as a means of immune modulation. *Methods*: Healthy donor peripheral blood mononuclear cells (PBMCs) were incubated with mitogenic stimuli and various concentrations of glioma-derived EVs. Intracellular signalling and cytokine output were determined by antibody microarrays and phenotypic changes were assessed by flow cytometry. Recall antigen testing, mixed lymphocyte reactions and migration assays were utilized to analyze functional capacity of PBMC and T cells. *Results*: Protein microarray data revealed induction of an immunosuppressive phenotype and cytokine output at high tumour vesicle concentrations, and an activated phenotype at low tumour EV concentrations. T cell activation antigen expression confirmed differential activation profiles between varying concentrations. Functional analyses revealed decreased migratory capacity of PBMCs after incubation with tumour EVs; however, upon wash-out of tumour EVs, recall antigen and mixed lymphocyte reactions indicate activation capacity is still retained in EV-treated cells. *Summary/conclusion*: The differential effects of high and low tumour EV concentrations dictate modulatory effects on PBMCs. These data provide a role for tumour EVs at high concentrations inducing selective tolerance of an immune response in a tumour setting. This suggests that lymphocytes in circulation of glioma patients may not irreparably impaired, as previously thought, but might be rescued to augment antitumour responses.

All use of human cells was approved by COMIRB protocols 95-100 and 13-3007. Funding was from the University of Colorado Cancer Center, UC Department of Neurosurgery, Cancer League of Colorado, and NIH/NIBIB 1R01 EB016378.

O-8A-2

Exosomal cargo to identify targets within the tumour microenvironment

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Introduction: Cancer-associated fibroblasts (CAFs) represent the most abundant cell type of the stroma and are a key component involved in regulating carcinogenesis. During the development of oral squamous cell carcinoma (OSCC), CAFs create a supporting niche by maintaining a bidirectional crosstalk with cancer cells, mediated by extracellular matrix, cell-cell contact, soluble factors and nanometre-sized membranous vesicles, such as exosomes. Recently, CAF-secreted exosomes were shown to be important mediators of paracrine signals that promote motility, and metastasis in breast cancer (1,2). Little is known about the molecular alteration in fibroblasts that determine their unique functional properties and induce the "CAF-like state." The elucidation of regulators of this cellular transition together with a better understanding of the role of CAF-derived exosomes on tumour progression, and metastasis can provide better opportunities for cancer treatment. *Methods*: To investigate stromal heterogeneity in OSCCs, we isolated matched pairs of human primary fibroblasts from resected tumours (CAFs) and adjacent tissue (AFs) and characterized them according to established CAF markers such as, morphology, α-SMA expression and the ability to degrade extracellular matrix components. We employed a quantitative shotgun proteomics approach to identify CAF-associated proteins in total lysate, conditioned media and exosomes. *Results*: Our comprehensive dataset of 4,160 proteins includes intracellular proteins, signalling factors and receptors associated with a pro-tumourigenic stroma. We applied quantitative proteomics to identify differentially expressed protein cargo in purified exosomes from matched pairs of CAFs and AFs resulting in a short list of 151 exosomal proteins specific to the CAF-like state. Interestingly, significant differences were observed in proteins involved in transmembrane transport, membrane trafficking and metabolic enzymes, suggesting that metabolic synergy between cancer cells and CAFs, could involve exosomal cargo. To assess the functional relevance of exosome-mediated crosstalk, we have shown a significant effect of CAF-derived exosomes on the motility of OSCC cells, when compared to control fibroblasts and exosome-free media. *Summary/conclusion*: Through a proteomics approach, we have identified a signature of CAF-enriched exosomal proteins potentially involved in pathways mediating tumour-stromal crosstalk. Our data will provide a solid foundation to mechanistically investigate how the tumour microenvironment supports tumour growth including the detection of novel exosomal cargo with functional relevance in tumour progression. Functional experiments in the context of migration, metabolic coupling and radiation response are currently in progress.

References

1. Shimoda M, Principe S, Jackson HW, Luga V, Fang H, Molyneux SD, et al. Loss of the Timp gene family is sufficient for the acquisition of the CAF-like cell state. Nat Cell Biol. 2014;16(9):889–901.

2. Luga V, Zhang L, Viloria-Petit AM, Ogunjimi AA, Inanlou MR, Chiu E, et al. Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell. 2012;151(7):1542–56.

O-8A-3

Breast cancer-derived extracellular vesicles promote brain metastasis via break-down of the BBB

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Introduction: A key event in brain metastasis is the invasion of cancer cells across blood-brain barrier (BBB). However, the molecular mechanism behind the passage of this natural barrier remains unclear. *Methods*: We established brain metastatic cell lines bone marrow-derived (BMD) from MDA-MB-231-D3H2LN breast cancer cell line. To assess the effect of extracellular vesicles (EVs) from BMD cell line, BBB model, which composes primary cultures of brain capillary endothelial cells, pericytes and astrocytes, was employed. Using this model, we investigated the association of the cancer derived-EVs in BBB break down and the invasiveness of the cancer cells. For EVs

preparation, the conditioned medium from cancer cell incubates in non-serum medium for 2 days was ultracentrifuged at 110,000 × g for 70 minutes at 4°C. *Results*: We showed that cancer-derived EVs trigger the disruption of BBB. The breakdown of BBB by the BMD cell-derived EVs was demonstrated by increased permeability of a fluorescent dye and decreased transendothelial electrical resistance (TEER) of BBB, an index of barrier integrity. Addition of BMD cell-derived EVs to the in vitro BBB model resulted in disruption of several tight junction proteins and adherence junction protein. However, it did not change the expression level of these proteins, suggesting that BMD cell-derived EVs may regulate the localization of tight junction proteins and adherence junction protein. In addition, EVs-specific microRNAs promoted the disruption of BBB through the abnormal localization of actin via the down-regulation of its target gene, PDPK1. PDPK1 degradation led to down-regulation of phosphorylated cofilin and the resultant activated cofilin-induced modulation of actin dynamics. Interestingly, BMD cell-derived EVs allowed the non-invasive breast cancer cells to invade across the BBB in vitro. Furthermore, BMD cell-derived EVs promoted brain metastasis of non-BMD cancer cells in vivo. *Summary/conclusion*: In conclusion, these results provide new insights regarding the role of cancer-derived EVs in brain metastasis of breast cancer.

O-8A-4

Extracellular vesicles containing mutant β-catenin activate Wnt signalling pathway in the tumour microenvironment

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Introduction: Colorectal cancer (CRC) arises as a result of accumulated mutations in key proteins that regulate cell proliferation, differentiation and death. Large scale sequencing studies have established the predominance of mutations in proteins involved in Wnt signalling pathway in a large number of CRC patients. It was also shown that over 90% of sporadic CRC cases, harboured mutation in APC and/or β-catenin gene both of which are involved in Wnt signalling pathway. One of the proposed mechanisms of intercellular signalling involves extracellular vesicles (EVs) which act as messengers carrying oncoproteins from malignant to non-malignant target cells. *Methods*: Conditioned media from LIM1215 CRC cells were differentially centrifuged followed by ultracentrifugation at 100,000 × *g* for 1 hour to pellet EVs. OptiPrep™ density gradient centrifugation was utilized to isolate pure population of EVs. Exome sequencing was performed in LIM1215 cells. The isolated exosomes were subjected to a high resolution mass spectrometry-based proteomics analysis. A proteogenomics analysis was conducted to identify the mutant proteins secreted via EVs. The Wnt signalling activity was measured using TOP/FOP luciferase assay. Further, the cell proliferation was measured using MTS assay. β-catenin knockout and knockdown LIM1215 cells were generated using TALENs and RNAi, respectively. Dimethyl labelling-based quantitative phosphoproteomics analysis was carried out using TiO_2 columns, and the eluted phosphopeptides were later analyzed by LC-MS/MS. *Results*: In this study, an integrative proteogenomic analysis identified the presence of mutated β-catenin in EVs secreted by CRC cells. Follow up experiments established that EVs released from LIM1215 CRC cells stimulated Wnt signalling pathway in a variety of recipient cells in the tumour microenvironment. Additionally, EVs derived from LIM1215 cells promoted proliferation in recipient cells. SILAC-based quantitative proteomics analysis confirmed the transfer of mutant β-catenin to the nucleus of the recipient cells. To understand the role of EVs in the tumour microenvironment, variety of cells including NK, T cells, cancer associated fibroblasts, endothelial, monocytes and pericytes were treated with EVs and analyzed by quantitative proteomics and phosphoproteomics. Even though the same EVs were used, the analysis highlighted the activation of different signalling pathways based on the recipient cell type. *Summary/conclusion*: The role of EVs bearing mutant β-catenin in context of Wnt signalling pathway has not been extensively studied yet. This is the first study highlighting the role of EVs carrying mutant β-catenin in increasing Wnt signalling pathway and proliferation in recipient cells. Here, we also show how EVs can induce various signalling pathways based on the cell type in the tumour microenvironment.

O-8A-5

The biodistribution and immune suppressive effects of breast cancer-derived exosomes

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Introduction: Metastatic spread is the main cause of breast cancer-related mortality. Exosomes are important mediators of intercellular communication between cancer cells and non-cancerous stroma, promoting tumour progression and metastatic spread. This study investigated the tissue distribution and immune-modulatory role of breast cancer exosomes. *Methods*: Optical imaging was used for the longitudinal tracking of fluorescently-labelled exosomes derived from a highly aggressive murine breast cancer cell line (E0771) in syngeneic, immune-competent mice. Specific uptake of fluorescently-labelled exosomes by various immune cell lineages in different tissues was assessed by flow cytometry. The effect of cancer-derived exosomes on T-cell proliferation and Natural Killer (NK) cell cytotoxicity was further confirmed by in vitro co-culture experiments. *Results*: Intravenously injected exosomes accumulated predominately in the lung, spleen and bone marrow. Specific uptake of fluorescently-labelled exosomes was observed in $CD11b^+/Gr^+$ myeloid-derived suppressor cells (MDSCs) in the lung and in $CD4^+$ and $CD8^+$ T-cells in various tissues. Importantly, injection of cancer-derived exosomes significantly reduced $CD4^+$ and $CD8^+$ T-cell abundance in mice. This was accompanied by an increase of $CD11b^+/Gr^+$ MDSCs. Proteomic analysis of tumour-derived exosomes uncovered an enrichment of TRAIL, B7-H4 and galectin 9 proteins, all of which are capable of promoting T cell apoptosis and decreased proliferation. Additionally, tumour-derived exosomes had a direct inhibitory effect on NK cell cytotoxicity as well as T-cell proliferation and function. *Summary/conclusion*: Our findings provide a first insight into the tissue-specific distribution of breast cancer-derived exosomes and their contribution to immune suppression by limiting T-cell and NK cell function and increasing MDSC abundance. This data suggest that immune-suppressive functions of exosomes could be potential targets of novel anti-cancer therapeutics in breast cancer.

O-8A-6

Senescence-associated EVs act as tumour suppressor at the tumour microenvironment

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Introduction: Extracellular vesicles (EVs) including exosomes released into the extracellular environment from a variety of cells and can be used for cell-to-cell communication in vivo. Interestingly, we found that significant level of EVs was secreted from senescent fibroblast. The senescence-associated secretory phenotype (SASP) implies cellular communication through SASP factors such as inflammatory cytokine and chemokine's, but little is known about the function of senescence associated EVs (SA-EVs) secreted from senescent fibroblasts in tumour environments. *Methods*: Characteristics of EVs are examined with qNano (iZON) and SP6800 (SONY corporation). EVs from culture media of young and senescence fibroblasts are purified with ultracentrifuge (HITACHI), and pelleted EVs are suspended with cultured medium and add to recipient cells. MicroRNA analysis was purified with MicroRNA Easy kit (Qiagen) and was performed with

next generation sequencing (IonPGM and Hiseq), microarray (Toray 3Dgene) and qRT-PCR (Exiqon). *Results*: By adding SA-EVs to cancer cell lines, SiHa and MDA-231MA, we found that SA-EVs significantly inhibited cell proliferation, colony formation and invasion of cancer cell lines. This data suggest that SA-EVs have tumour suppressive activity in tumour microenvironment. To understanding the function of SA-EVs, we analyzed microRNAs in EVs from young and senescent fibroblast, and found that several microRNAs are enriched in SA-EVs. Furthermore, these miRNAs from SA-EVs showed tumour suppressive function in cancers. *Summary/conclusion*: We found that SA-EVs contained several significant tumour suppressive microRNA implicating in tumour invasion, EMT and cell growth. These results suggest that SA-EVs play a major role of tumour suppression in tumour microenvironments.

Ballroom E
Symposium session 8B - EV therapeutics II: nucleic acid delivery
Chairs: *Elena Batrakova and Steven M. Jay* 14:00-15:30

O-8B-1

Identification and screening of exosome-tropic RNA by Selex method and its application in loading therapeutic RNA to exosomes

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Introduction: To efficiently load therapeutic RNAs to exosomes, exosome-tropic RNAs were screened by using a systematic evolution of ligands by exponential enrichment (SELEX) method. Then, the identified exosome-tropic RNA was used to deliver siRNA to exosomes. *Methods*: Pooled single stranded 80-base DNAs, each of which contains a randomized 40 base sequence, were used to transcribe the initial RNA pool. Obtained RNA pool was transfected into B16-BL6 murine melanoma cells, and exosomes were collected from the culture medium by ultracentrifugation method. RNAs were collected from the exosomes and subjected to the next SELEX round. After 12 rounds of screening, the RNA pools were subjected to cloning and sequencing. Then, the identified exosome-tropic RNA, named as ExoApt, was conjugated to the sense strand of siRNA targeting firefly luciferase (siLuc) to obtain ExoApt-siLuc. B16-BL6 cells were transfected with firefly luciferase-expressing plasmid and siLuc or ExoApt-siLuc, and the luciferase activity of the cells was measured. *Results*: The cloning and sequencing of the RNAs in the RNA pools after SELEX showed that 29 out of 56 clones had an identical RNA sequence, which was selected as an exosome-tropic RNA and named as ExoApt. Transfection of ExoApt resulted in high recovery of the RNA from exosomes compared with other RNAs found in the screened RNA pools. Transfection of ExoApt-siLuc as well as siLuc resulted in reducing the luciferase activity in B16-BL6 cells cotransfected with firefly luciferase-expressing plasmid. Moreover, ExoApt-siLuc was delivered to exosomes more efficiently than siLuc after the transfection to B16-BL6 cells. *Summary/conclusion*: These results indicate that the exosome-tropic RNA, ExoApt, obtained by the SELEX-based screening can be used for the delivery of siRNA and other therapeutic RNAs to exosomes.

O-8B-2

A comparative analysis between extracellular vesicles and liposomes as delivery vehicle for small interfering RNAs

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Introduction: As it is becoming more and more apparent that extracellular vesicles (EVs) play an important role in intercellular communication, interest in using them as a drug delivery vehicle is rising. For this reason, the potential of these vesicles as a new siRNA delivery approach was evaluated and compared with a classic, fusogenic liposome with similar physicochemical characteristics. *Methods*: EVs were purified from conditioned cell culture medium derived from a B16F10 melanoma cell line by iodixanol based density gradient ultracentrifugation and characterized in accordance with ISEV guidelines. SiRNA-EV association was evaluated using an array of methods based on gel electrophoreses, antigen coated beads for flow cytometry and density gradient colocalization. Uptake and functional siRNA delivery was evaluated in a monocyte/DC cell line using flow cytometry and RT-PCR. *Results*: In previous work, we showed that the aforementioned electroporation for intravesicular loading of siRNA in EVs is not feasible. For this reason, a new loading approach, in which siRNA modified with a cholesterol moiety, was used to ally siRNA to the EV lipid membrane. Here, we also show the importance of using chemically stabilized siRNA and high purity vesicles, as strong nuclease activity was found in vesicular samples purified by traditional UC protocols. Next, we assessed the functional siRNA delivery capacity in vitro and compared this with a negatively charged, fusogenic liposome. Although the EV-siRNA complex showed efficient cell uptake, no gene silencing effect could be observed (even using high EV concentrations). Surprisingly, the same amount of liposomes induced a strong target downregulation. *Summary/conclusion*: To conclude, we can state that electroporation, in contrast to previous reports, is not a feasible technique for loading siRNA in isolated EVs. Instead, we developed a new approach based on a cholesterol modified siRNA to efficiently and reproducibly load EVs with exogenous small nucleic acids. Despite efficient siRNA loading, in the here presented experimental setup the liposomes clearly outperformed the EVs in functional siRNA delivery.

O-8B-3

Exosome-mediated delivery of hydrophobically modified siRNA for Huntingtin mRNA silencing

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Introduction: Neurodegenerative disorders are one of the major classes of disease with significant unmet medical need, with many of them, including Huntington, being genetically defined and thus representing ideal targets for oligonucleotide therapeutics. Huntington's disease (HD) is caused by the autosomal dominant inheritance of the mutant *Huntingtin* (HTT) allele, with direct siRNA based targeting of huntingtin mRNA being considered a valid therapeutic intervention strategy. However, delivery of oligonucleotides to brain is challenging and represents the most serious limitation to realize the full potential of oligonucleotide-based therapies (ONTs) and advancement of treatments to clinic. Exosomes have been recently implemented in the trafficking of native small RNAs across cellular boundaries and are considered as a vehicle for transfer of therapeutic RNAs. *Methods*: Here, we develop a novel simple method enabling efficient loading of therapeutic RNAs into exosomes through hydrophobic modifications (hydrophobically modified siRNAs, hisRNA). *Results*: We demonstrated that exosome loaded with hsiRNA efficiently silence huntingtin in primary neurons in vitro, promote wide distribution in vivo upon brain infusion and cause silencing in both treated and non-treated side of the brains. *Summary/conclusion*: This novel approach opens an opportunity to use exosomes for delivery of oligonucleotides therapeutics for treatment of wide ranges of human disease.

O-8B-4

Exosome-associated AAV as a novel platform for gene therapy of hearing loss

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Introduction: In recent gene therapy trials, adeno-associated virus (AAV) vectors for diseases such as blindness and haemophilia were found to be safe and effective. Gene therapy for hearing and balance disorders is not as advanced, because gene delivery into the cochlea (particularly to sensory hair cells) is generally inefficient. Here, we show that exosome-associated AAV vectors (vexosomes) are highly effective carriers of transgenes to hair cells. *Methods*: Vexosomes from media of AAV-producing cells (293T) were harvested by ultracentrifugation. For in vitro cochlear transductions, we explanted organs of Corti from P1 CD1 mice. Conventional AAV1 vectors or AAV1 vexosomes, encoding for GFP, were added to the culture medium to determine the extent of transgene delivery and expression. For in vivo studies, we injected vectors at P1 into the scala media through cochleostomy or into the scala tympani through the round window membrane (RWM). To study whether vexosomes can rescue a disease phenotype in vitro, we explanted Corti organs from *Tmhs* (*Lhfpl5*) knock-out mice, which lack mechanotransduction (and hearing and balance) beyond P5. Cultures were transduced with vexosomes encoding TMHS (tetraspan membrane protein of hair cell stereocilia) and restoration of function was assessed by FM1-43 dye uptake, which is trapped inside functional hair cells. *Results*: In vitro, AAV1-vexosomes led to almost 100% transduction of inner (IHCs) and outer hair cells (OHCs), while regular AAV1 was able to transduce only up to 30% of IHCs and OHCs at equivalent genome copies per cell. In vivo, vexosomes also outperformed regular AAV. Delivered by cochleostomy, AAV1-vexosomes transduced 63.7 ± 6.5 and 30.0 ± 9.8% of IHCs and OHCs, respectively, whereas AAV1 transduced only 35.8 ± 0.7 and 16.7 ± 1.9% (mean fraction of transduced cells from 2 experiments with 10 animals in each group). Delivered by RWM, AAV1-vexosomes transduced 88.0 ± 2.2 and 25.2 ± 10% of IHCs and OHCs, whereas AAV1 transduced 75.0 ± 4.4 and 15.6 ± 0.4% (2 experiments). In vitro, AAV1-vexosomes encoding TMHS were able to restore FM1-43 accumulation in TMHS KO hair cells, apparently rescuing mechanotransduction. *Summary/conclusion*: Exosome-associated AAV is a powerful gene delivery system to the mammalian cochlea in vitro and in vivo. Vexosomes outperform all currently used techniques in the delivery of nucleic acids into hair cells, therefore they may be utilized to study hair cell physiology in vitro, and—in the future—for in vivo gene therapy.

O-8B-5

Engineering extracellular vesicles for DNA delivery

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Introduction: Extracellular vesicles (EVs) hold immense promise for utilization as drug delivery vehicles for a variety of reasons. EVs can potentially be procured from a patient's own cells, overcoming problems of immunogenicity associated with synthetic drug delivery systems. EVs may also directly introduce their cargo into the cytoplasm of a recipient cell via fusion with its membrane. This latter property would be especially beneficial for nucleic acid delivery, as many nanoparticle systems are taken up via endocytosis into endosomes, where RNA and DNA can be degraded. Recent reports show that small RNAs (siRNA and miRNA) can be loaded into EVs and targeted to specific tissues, however the potential of EVs to deliver DNA is not well known. Our goal was to determine whether EVs could be utilized for delivery of large nucleic acids such as linear or circular DNA that might be useful in numerous gene therapy applications. *Methods*: EVs were harvested from HEK293T cells via differential ultracentrifugation, and size and concentration were evaluated using nanotracking analysis (NTA) with a NanoSight LM10. dsDNA ranging from 250 bp to 10 kb in size were loaded into EVs by electroporation. DNA was labelled with picoGreen stain either before or after electroporation, and quantification was determined by fluorescence. EV-mediated transfer of DNA into cells was confirmed by PCR. *Results*: We optimized electroporation conditions for maximum delivery of DNA molecules into EVs and were able to load, on average, 1000s of copies of DNA per EV. Loading of DNA into EVs can be modulated based on the initial amount of DNA used, and DNA loading appears to be saturable. Additionally, the efficiency of DNA loading into EVs was found to be dependent on DNA size. Linear DNA is incorporated into EVs at much higher levels than circular DNA, and linear dsDNA [coding for the tRNA-Ser(CGA) gene from yeast] can be introduced into recipient cells via EV delivery. *Summary/conclusion*: The novel capability that EVs can be employed to transfer nucleic acids larger than siRNA or miRNA into cells were demonstrated. These results significantly broaden the potential use of EVs as non-viral gene delivery vehicles and thus have implications for therapeutic approaches in a multitude of conditions, including systemic protein deficiencies, autoimmune disorders and many more.

O-8B-6

G-force loading of virus vectors into vesicles for enhanced gene therapy vehicles

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Introduction: Adeno-associated virus (AAV) vectors have shown remarkable efficiency in a number of preclinical models of disease in several organs including the eye, brain, muscle, heart and liver. A major limitation to long term transduction, especially via the systemic route, is pre-existing immunity (humoral and cell-mediated) as well as the high vector load required to achieve high levels of gene expression in the desired organ. Natural nanoparticles released from all cells, called extracellular vesicles (EVs), may have utility in creating better AAV vectors for human gene therapy. We have previously shown that harvesting AAV vectors from the media of 293T producer cells contains AAV vectors endogenously associated with EVs (called ev-AAV). We have gone on to demonstrate that 293T-derived ev-AAV can evade neutralizing anti-AAV antibodies and greatly increase transduction in mice. An alternative strategy would be to load purified AAV vectors into separately purified EVs. This would have the benefit of using EVs from a variety of cellular sources with desired biological activity and also to use donor-derived autologous EVs. Here, we demonstrate a simple method for loading EVs with AAV vectors using high speed centrifugation forces. *Methods*: Iodixanol density gradient-purified AAV was mixed with conditioned media containing EVs from 293T cells and also primary human peripheral blood mononuclear cells (PBMCs) and then ultracentrifuged to co-pellet EVs and AAV. The pellet was resuspended in media and used in transduction assays and anti-AAV antibody neutralization assays. *Results*: Strikingly, we found that this process bestowed greatly enhanced transduction of cells in culture ($\sim$36-fold) as well as resistance to neutralizing anti-AAV antibodies. To demonstrate that EVs were essential to this enhancement, we mixed AAV with plain media (no EVs) or depleted conditioned media of EVs before addition of AAV using either Triton-X-100 EV lysis or ultracentrifugation. In all cases, there was a large decrease in the transduction efficiency and antibody evasion compared to conditioned EV media mixed with AAV. *Summary/conclusion*: Together, these data suggest that g-force loading of AAV into EVs represents a promising method to increase performance of the vector. We will continue to optimize the protocol and test the gene delivery functions in vivo.

Ballroom F-H
Symposium session 8C - EVs in tissue injury and repair
Chairs: *Ewa Stepien and Dimitrios Kapogiannis* 14:00-15:30

O-8C-1

Microparticles derived from human podocytes induce pro-fibrotic signalling in cultured human proximal tubule epithelial cells

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Introduction: Diabetic nephropathy (DN) is a serious complication arising from longstanding type I and II diabetes and is the leading cause of end stage kidney disease. While early DN is characterized by glomerular injury, notably to podocytes (specialized epithelial cells critical to filtering), progression to renal failure more closely correlates with development of tubulointerstitial fibrosis. However, mechanisms linking glomerular injury to tubular fibrosis are not clear. Recently, we reported that podocytes produce microparticles in response to diabetic stress conditions. In the present study, we examined whether podocyte microparticles interact with proximal tubular cells and influence cell function. *Methods*: Podocyte MPs were isolated from the media of cultured human conditionally-immortalized podocytes, and human proximal tubular epithelial cells were exposed to podocyte MPs. MP-cell interaction were examined by fluorescence microscopy. Effects of MPs on intracellular signalling responses (p38, ERK1/2, JNK) were examined by Western blot analysis. Expression of the extracellular matrix proteins fibronectin and collagen type IV as well as hypertension-related calcium regulated gene (hCaRG, a small intracellular protein involved in tubular repair) were examined by Western blot analysis. Reactive oxygen species (ROS) production was assessed by lucigenin chemiluminescence. *Results*: Fluorescence microscopy showed cell surface binding of podocyte MPs, suggesting a paracrine effect. Treatment with podocyte MPs increased p38 phosphorylation ($\sim$3-fold) after 30 minutes ($p < 0.05$, $n = 4$), while JNK and ERK phosphorylation were not altered over 24 hours ($p > 0.05$, $n = 4$). Expression of fibronectin and collagen type IV were significantly increased following 72 hours treatment ($\sim$3- and 5-fold respectively, $p < 0.01$, $n = 4$–6), while expression of hCaRG was significant decreased ($\sim$75%, $p < 0.01$, $n = 3$). Proximal tubular ROS production was increased by podocyte MP treatment at 16 and 24 hours ($p < 0.01$, $n = 4$). *Summary/conclusion*: Our results suggest that podocyte MP interact with proximal tubule epithelial cells and induce intracellular signalling, ROS production and fibrosis while impairing reparative processes. Such effects may play a role in the development of tubular injury in glomerular disease.

O-8C-2

Fibronectin-dependent exosome adhesion triggers sphingosine 1-phosphate-dependent hepatic stellate cell chemotaxis

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Introduction: Exosomes are cell derived extracellular vesicles (EVs) thought to promote intercellular communication by delivering specific content to target cells. However, exosomal contents and mechanisms of action on target cells remain undefined. The aim of this study was to determine whether endothelial cell (EC) derived exosomes could regulate the phenotype of neighbouring pericytes (hepatic stellate cells; HSC) and if so, then how. *Methods*: Exosomes were isolated by differential ultracentrifugation and characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis and western blotting (WB). WB was used to detected HSC Akt phosphorylation levels and transwell assay tested HSC chemotaxis. Exosome adhesion to HSC was detected by scanning electron microscopy (SEM). *Results*: Initial microarray studies showed that the canonical EC growth factor, FGF2 induced a 2.4-fold increase in mRNA levels of sphingosine kinase1 (SK1), an enzyme that produces sphingosine-1 phosphate (S1P). Exosomes derived from an SK1 overexpressing EC line increased HSC migration (3.2-fold, $n = 4$, $p < 0.05$). Incubation of HSC with serum-derived exosomes was associated with increased Akt phosphorylation (8.3-fold, $n = 6$, $p < 0.05$) and increased chemotaxis (2.5-fold, $n = 3$, $p < 0.05$). Exosomes were found to express the matrix protein and integrin ligand fibronectin by WB and TEM. Blockage of FN-integrin interaction through CD29 neutralizing antibody or RGD peptide attenuated exosome induced HSC Akt phosphorylation by 53 and 56%, respectively ($n = 3$, $p < 0.05$) and chemotaxis by 41 and 38%, respectively ($n = 3$, $p < 0.05$). SEM showed that CD29 antibody and RGD peptide decreased exosome adhesion by 14 and 38%, respectively ($n = 6$, $p < 0.05$). *Summary/conclusion*: EC-derived SK1 containing exosomes regulate HSC chemotaxis through FN-integrin dependent exosome adherence. These findings advance our understanding of liver pathobiology and identify exosomes as a potential target for fibrolytic therapy.

O-8C-3

Antithymocyte globulin-induced platelet prothrombinase activity is a consequence of complement activation and microvesicle release

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Introduction: T cell depletion with antithymocyte globulins (ATG) during hematopoietic stem cell or kidney transplantation can be complicated by thrombopenia and hypercoagulability. The underlying mechanism is still unclear. *Methods*: Platelet-rich plasma and full blood of healthy donors were incubated with ATG in the presence of normal human serum or plasma. Platelet aggregation was measured by light transmission aggregometry, platelet activation and degranulation was assessed by phosphatidylserine (PS) exposure and p-selectin expression, respectively. Platelet microvesicles (MV) released during ATG stimulation in vitro or isolated from plasma of transplant patients receiving ATG were phenotyped and counted by flow cytometry. Blocking antibodies against FcγRII, complement factors (C5, C7, C8, C9) and complement-depleted sera were used to implicate or exclude complement involvement. Thrombin generation by platelet MV was measured by a chromogenic thrombin assay. *Results*: We found that binding of ATG to platelets caused platelet aggregation, alpha-granule release, membrane PS-exposure and the rapid release of procoagulant platelet MV. Platelet activation and MV release were complement-dependent and required membrane insertion of C5b-8 but not stable lytic pore formation by C5b-9. Platelet MV had high prothrombinase activity, expressed clotting factors Va and Xa, but no tissue factor. Although FcγRII contributed to platelet aggregation and activation by ATG, MV release was FcγRII-independent. Moreover, the blocking of C5 complement factor inhibited ATG-induced MV release and consequently thrombin generation.

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In 10 hematopoietic stem cell and 9 kidney transplant patients, ATG treatment resulted in thrombopenia, elevated plasma levels of D-Dimer, thrombin-anti-thrombin-complexes and soluble C5b-9 indicating a state of heightened coagulation and complement activation. Flow cytometric analysis of complement fragments on platelet MV in patient plasma confirmed dose-dependent complement activation and MV release by ATG. *Summary/conclusion*: These data suggest that platelet MV release is a clinically relevant link between complement activation and thrombin generation and offer a potential mechanism underlying ATG-induced hypercoagulability.

O-8C-4

Repeated doses of cardiosphere-derived cell extracellular vesicles are hypo-immunogenic

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Introduction: Data from early clinical studies indicate that, while allogeneic cells can be used in any recipient without adverse safety effects, some recipients develop low-level immune responses, which may undermine the efficacy of subsequent administrations of identical biomaterial. Allogeneic cardiosphere-derived cells (CDCs) are therapeutic candidates in clinical development for cardiac repair. CDCs exert their beneficial effects via the release of extracellular vesicles (EVs), which can be utilized as a distinct regenerative therapy. EV-based therapy can potentially circumvent some of the limitations associated with cell transplantation, including concerns over immune sensitization with repeat dosing. Here, we evaluated the immunogenicity of repeated doses of xenogeneic (human) and allogeneic CDCs and CDC-derived EVs (CDC-EVs). *Methods*: Subcutaneous injections of CDCs (10^6 cells) and CDC-EVs (700 μg) were delivered in male rats as a general model from which data could be extrapolated to multiple indications. Doses were delivered every 2 weeks for up to 3 repeats. Sera were collected weekly, and in vitro and in vivo measures of immune sensitization to CDCs and CDC-EVs were performed. *Results*: As expected, repeated human (xenogeneic) CDC injections resulted in rapid humoral and cell-mediated immune responses. Delivery of human CDC-EVs did not induce any response after the first dose. Repeated dosing led to progressively increased immunogenicity, however, the response was diminished compared to that observed with human CDCs. Importantly, delivery of rat (allogeneic) CDC-EVs did not elicit any significant immune response even after repeated dosing. *Summary/conclusion*: Allogeneic CDC-EV delivery without immunosuppression elicits no overt immunogenicity after repeated dosing. These results support the use of a repeated dose treatment strategy with CDC-EVs, which provides great flexibility in the further development of this novel cell-free regenerative therapy. The lack of a robust immune response may enable safe and effective repeated dosing of CDC-EV, which is desirable in the treatment of chronic diseases.

O-8C-5

Physical and functional analyses of airway epithelial-derived exosomes and their role in the airway biology

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Introduction: Exosomes are small membranous vesicles (40–100 nm) released by various cell types. Our previous studies indicated that exosome-like vesicles from HTBE cell culture secretions carried specific cargo with and had innate protective properties. In this study, we physically characterized and compared vesicles isolated from 2 different cultured human airway epithelial cells, HTBE and Calu-3, DLS, and a multi-angle laser light scattering combined with size exclusion chromatography (SEC-MALLS). We further elucidated their organization, their cargo, and assessed their role in intercellular communication and airway biology. *Methods*: Mucus secretions were obtained from well-differentiated HTBE and Calu3 cells by 1 ml PBS washes. Washings were subjected to differential sedimentation to isolate exosomes. Exosomes were characterized by NTA, DLS, SEC-MALLS, EM and proteomic analysis. For cross-treatment experiments, HTBE cells were treated with Calu3 isolated exosomes, and Calu3 cells were treated with of HTBE isolated exosomes for 3 days. The apical secretions of the HTBE and Calu3 cells were collected for the following 3 days. Isolated exosomes and mucus from the apical secretions were subjected to biochemical and biophysical characterization including proteome and light scattering analysis. *Results*: The results indicated that epithelial cells release vesicles with distinct physical properties and sizes. HTBE vesicles have a 340 nm hydrodynamic radius (Rh) and a radius of gyration (Rg) 200 nm. Calu-3 derived vesicles have an Rh 165 nm and Rg 145 nm. EM analysis revealed that the spherical component of both vesicles has a radius of 40–100 nm, and that they carry filamentous entangled membrane mucins on their surface that increases their overall radius. Proteomic showed that the exosomal cross-transfer from one cell system to another significantly altered the protein content inside both cell's secretomes and vesicles, while the presence of common proteins such as the Lamp-1, CD59, Annexins, GTPases and heat shock proteins remained the same. This corresponds with an overall change of 5–10% in the proteome of both secretions, a change mediated by exosomes. In addition, the profile of mucins, which is important in the innate immune protection of the epithelial cells, was altered. *Summary/conclusion*: The mucin composition on the surface of exosomes defines the size and charge of exosome like vesicles as measured by light scattering techniques, and their surface properties mirror the properties of their cells of origin. Our data suggest that cellular information can be carried between the airway epithelial cells via exosomes and modulate the protein expression. Biophysical and biochemical characterization of exosomes may provide a unique tool for investigators to analyze exosome properties and address unanswered questions regarding normal airway biology, innate immunity, as well as airway remodelling during infection, inflammation, cancer and metastasis.

O-8C-6

Platelet exosomes, plexosomes, are targeted to platelet alpha-granules and can modulate canonical WNT signalling in target cells

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Introduction: Upon activation platelets release the contents of their preformed granules, the platelet ''releasate'' into the external milieu, which includes a myriad of soluble factors as well as exosomal vesicles. It is well known that the platelet acts as a courier of soluble granular proteins such as platelet factor 4 and thrombospondin from megakaryocytes in the bone marrow in to the blood stream and also whilst circulating, that platelets constantly endocytose soluble factors such as albumin from the plasma. However, it is currently unknown where platelet exosomes or ''plexosomes'' are derived, the specific factors that they contain and the effects of plexosomes on target cells following platelet activation. *Methods*: *Nbeal2*-knockout mice display the characteristics of human grey platelet syndrome, a rare bleeding disorder characterized by macrothrombocytopenia, with platelets lacking the contents of α-granules. Here we utilized *Nbeal2*-deficient mice to establish that plexosomes are specifically targeted to platelet alpha-granules in megakaryocytes in the bone marrow. Next, following extensive quality control analysis, we undertook a multiomics approach and characterized the proteomic and miRNomic contents of human platelet exosomes, isolated by ultracentrifugation following platelet activation, in a population of healthy donors. The physiologic effects of these secreted human plexosomes on target cells such as the endothelium were also

determined. *Results*: Utilizing *Nbeal2*-knockout mice, we established that plexosomes are specifically packaged into alpha-granules in the bone marrow, with *Nbeal2*$^{-/-}$ platelets containing less than 25% of the exosomes of wildtype platelets. Next, high mass accuracy quantitative proteomics of secreted plexosomes uncovered a specific marker of human plexosomes, glycoprotein V, which was confirmed by immunogold-electron microscopy. Interestingly, proteomic analysis also revealed that secreted plexosomes express active WNT glycoproteins on their surface. Furthermore, we quantified 300 miRNAs in human plexosomes by microarray profiling and a statistical comparison between these and the corresponding whole platelet miRNome revealed a cassette of 32 miRNAs preferentially secreted in human plexosomes. Interestingly, pathway analysis identified the WNT signalling network as the top pathway targeted by these 32 miRNAs. Finally, we demonstrated these secreted human plexosomes were readily taken up by EA.hy926 endothelial cells, where they could accelerate wound healing by modulating canonical WNT signalling, in a manner similar to the canonical agonist WNT3a. *Summary/conclusion*: Our in-depth analysis has revealed that secreted plexosomes are packaged specifically into platelet alpha-granules in megakaryocytes in the bone marrow. These plexosomes, released following platelet activation, can serve as powerful modulators of signalling networks within target cells.

Networking coffee	15:30-16:00
Poster viewing sessions XIV, XV, XVI, XVII, XVIII, XIX, XX Posters not attended by authors	15:30-16:00
ISEV General Assembly	16:00-17:00
J Extracell Vesicles: Editorial Board meeting	17:00-18:00
Kick-off meeting, EV-flow cytometry working group (ISEV-ISAC-ISTH), hosted by Marca Wauben and Jennifer Jones	18:00-19:30
Evening poster viewing A cash bar will be available for drinks and snacks	19:00-21:00

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Poster Presentations

Poster session XIV - EVs in tissue repair and injury
Chairs: *Andrew Hill and Jan Simak*

P-XIV-1

Role of Thy-1 in the uptake of extracellular vesicles by lung fibroblasts

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Introduction: Thy-1/CD90, a glycophosphatidylinositol (GPI)-anchored membrane protein that contains an integrin-binding RGD-like motif (RLD), regulates cell adhesion, migration and differentiation. In this study, we aim to examine uptake of Thy-1(+) fibroblast-derived extracellular vesicles (fEVs) by lung fibroblast. We speculate that this process is associated with functional presence of Thy-1 and activation/conformations of integrin located in raft-like structure. *Methods*: After 24-hour stimulation with TNF-α and IL-1β, fEVs were isolated/purified from rat lung fibroblasts (RFL-6) stabling expressing either mouse Thy-1 (CD90.2) or empty vector pcDNA3.1 Zeo and stained with deepRed (649/666 nm). CFSE-stained normal human lung fibroblasts (CCL-210, 1×10^3) were cultured with deepRed-stained fEVs (8 μl) for 30 minutes, fixed with 3.7% PFA and examined using a Leica SR GSD Microscope (150X). *Results*: In response to profibrotic IL-1β/TNF-α stimulation activated fibroblasts released Thy-1(+)fEVs, as indicated by flow cytometry analysis of fEVs labelled with FITC-Lactadherin and APC anti-Thy-1 Ab. Also we demonstrated that Thy-1(+)fEV$^{RLF\text{-}6(Thy\text{-}1)}$ did increasingly bind to CCL-210 cognate cell surface when compared to fEV$^{RLF\text{-}6(empty\ vector)}$ (9.81-fold, $p < 0.05$). It suggests that this interaction is likely involved in fEVs uptake and/or associated downstream effects. Further studies to identify molecular interaction and involvement of lipid rafts are in the process of being conducted. *Summary/conclusion*: These data, along with our previous studies, indicate that Thy-1 is of importance in cell-to-cell communication via interaction with cognate cell surface molecules thus affecting transfer of EV molecular signals (supported by NIH #HL082818 & Pulmonary Fibrosis Foundation).

P-XIV-2

Exosomes secreted during adipogenic differentiation of human adipose-derived stem cells induce adipogenesis of human adipose-derived stem cells

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Introduction: Loss or damage of adipose tissue that result from traumatic injury and tumour resection need reconstructive approaches, such as cell/tissue transplantation or tissue engineering. Although stem cell-based therapies have clear beneficial effects on adipose tissue regeneration, there are still a number of concerns, such as limited survival and the reduced regenerative capacity of engrafted stem cells, as well as immune-mediated rejection. Differentiating stem cells during adipogenesis secrete extracellular vesicles containing various adipokines and small molecules, which could act as critical signals of adipogenesis, homeostasis, and lipid metabolism. In this study, we hypothesized that exosomes secreted during adipogenesis may contain specific biochemical cues that promote and regulate the differentiation of human adipose-derived stem cells (hASCs) with adipogenic potential. We propose that exosomes may provide a cell-free therapeutic approach for regenerative medicine and for adipose tissue regeneration in particular. *Methods*: Exosomes were isolated from conditioned media during adipogenic differentiation by ultracentrifugation, concentration and purification. The isolated exosomes were analyzed by transmission electron microscopy (TEM), dynamic light scattering (DLS), western blot and growth factor array. Adipogenesis of hASCs was monitored in the presence of exosome-containing medium (Ex-medium) for 14 days. The differentiation potency of exosomes was analyzed by quantitative analysis of accumulated lipid droplets in adipogenic-differentiation medium and Ex-medium. *Results*: The exosomes were approximately 60 nm in diameter and expressed exosomal markers such as CD63 and CD81. The exosomes also contained various soluble factors related to adipose tissue development, such as ACRP30, ANGPT1, TNF-α, leptin and MCSF. In the presence of exosomes, hASCs differentiated into mature adipocyte, accumulating lipid droplets. Overall results suggest that the adipogenic differentiating hASC-derived exosomes could act as a biochemical cue for control of hASC fate toward adipogenic lineage. *Summary/conclusion*: Our findings indicate that adipogenic differentiating hASC-derived exosomes may act as a biochemical cue directing adipogenesis of stem cell.

P-XIV-3

Exosome-mimetic nanovesicles derived from insulinoma induce differentiation of lineage-negative bone marrow mononuclear cells into insulin-producing cells

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Introduction: Exosome-mimetic nanovesicles are bioinspired cell-derived nanocarriers produced by subjecting cells to serial extrusion through filters with decreasing pore sizes. Here, we used exosome-mimetic nanovesicles derived from insulinoma cells (INVs) to induce the differentiation of stem cells into insulin-producing β cells (IPCs) for the treatment of diabetes, a well-known metabolic disorder characterized by autoimmune destruction of IPCs. *Methods*: INVs were produced by a serial extrusion of MIN-6 cells and further characterized by electron transmission microscopy, dynamic light scattering and nanoparticle tracking analysis. Lineage-negative bone marrow mononuclear cells (Lin^- BMNCs) were isolated from mice by magnetic-activated cell sorting. To confirm the differentiation of stem cells into IPCs in vitro and in vivo, insulin and PDX-1 levels were measured by qRT-PCR, western blot and confocal microscopy. *Results*: INVs have the shape of spherical bilayered proteolipids with an average diameter of $\sim$200 nm. After Lin BMNCs were treated with INVs in vitro and in vivo, their morphologies were changed into the spread shape. Not only the protein and mRNA levels of insulin and PDX-1 but also the number of insulin or PDX-1-positive cells were increased significantly in the INVs-treated Lin^- BMNCs. Moreover, most of the differentiated cells co-expressed insulin and PDX-1,

which infers that our protocol succeeded in efficiently inducing stems cells into IPCs. *Summary/conclusion*: In this study, we showed that INVs could induce the differentiation of Lin BMNCs into IPCs, in vitro and in vivo. Considering that there are many hurdles to treat diabetes due to the donor shortage and immune rejection problems of islet transplantations, these results imply that exosome-mimetic nanovesicles can be applied for regenerative medicine as therapeutic options.

P-XIV-4

Ionizing radiation exposure stimulates microparticle production: bystander effect

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Introduction: Irradiated cells can communicate with each other by releasing soluble factors into the surrounding medium. Microparticles are also known to mediate communication between cells. The main aim of this study was to establish whether microparticles are involved in radiation-induced bystander signalling by investigating the direct effect of radiation exposure on MP release from several cell types. *Methods*: Primary human microvasculature endothelial cells from dermis (HMVEC-d) and lymphatic vessels (HMVEC-L), primary human keratinocytes (NHEK), as well as human monocytic (U937), fibroblastic (HLF1) and epithelial (T84) cell lines were used in this study. Confluent cultures of cells were exposed to 0 (control), 10, 20 or 40 Gy gamma rays (^{60}Co source), and culture medium was collected at 3, 6, 24 and 72 hours after irradiation. Culture supernatants were centrifuged for 5 minutes at 400 g to remove cell debris. Annexin V^+ MPs were counted by flow cytometry. *Results*: All cell types under study produced Annexin V^+ MP levels above detection threshold in all conditions, with highest and lowest basal levels being produced by NHEK cells and HMVECs, respectively (200 vs. 30 MPs/μl). More importantly, kinetic analysis of MP numbers revealed some variability according to cell type and time. Interestingly, we observed a time and dose-effect of irradiation on MP levels released from monocytic cells at day 1 and day 3 after irradiation. Main MP number changes were observed in 10 and 40 Gy-irradiated cultures of HMVEC-d (3-fold increase after 1 and 3 days, respectively) and 40 Gy-irradiated cultures of HMVEC-L and U937 cells (4- and 2.6-fold increase after 1 day, respectively) as compared to non-irradiated cells. No significant differences were obtained in MP production for the other cells. Although most cells displayed increased MP levels, regardless of irradiation dose, after 1 and 3 days of culture, NHEK cultures showed decreased MP numbers, while they were stable in HMVEC-d cultures (excepted at 40 Gy). *Summary/conclusion*: Taken together, these results suggest that irradiation increases MP production from monocytes in a dose-dependent manner, while endothelial cells, and to a lesser extent fibroblasts, produce increased numbers of MPs upon exposure to high dose ionizing radiation in vitro, that may mediate radiation-induced bystander effect.

P-XIV-5

In vitro assessment of primary hepatocyte-derived extracellular vesicles and their role in drug-induced liver injury

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Introduction: Hepatocytes release extracellular vesicles (EV) that can diffuse into peripheral blood through the fenestrations within the sinusoidal endothelium. Evidence suggests that the abundance and cargo of hepatocyte-derived extracellular vesicles (HDVs) change during pathological conditions, prior to the elevation of traditional markers of liver injury. The early release and dynamic content of HDVs during cell stress indicate a potential biological role in propagating drug-induced liver injury (DILI) reactions. The objective of this work is to evaluate the role of HDVs in DILI using cultures of primary rat and human hepatocytes. *Methods*: The present work utilized cultures of primary rat and human hepatocytes to explore EV content and release from control and drug-treated cells. Briefly, HDVs were isolated from conditioned culture medium via polymer precipitation. Western blots were performed to test HDV fractions for enrichment of exosomal proteins. Nanoparticle tracking analysis was conducted to confirm the size distribution of vesicles in HDV preparations. Total RNA was extracted from HDVs and hepatocytes for copy number analysis using absolute qPCR. Cytotoxicity was assessed by measuring leakage of cellular contents into culture media. *Results*: HDV enrichment methods yielded a population of small vesicles (median of 81.3 and 100.8 nm from human and rat hepatocytes, respectively) that are enriched for the exosomal protein markers flotillin and CD81. Under basal conditions, human and rat HDV fractions contained detectable levels of 9 liver-enriched transcripts including albumin. The absolute quantity of HDV transcripts varied across human hepatocyte donors. In response to the prototypical hepatotoxicant acetaminophen, albumin mRNA in rat HDV fractions exhibited significant dose-dependent increases while cellular albumin mRNA remained unchanged. These results suggest that HDV-associated albumin transcript may be actively released in greater quantities during cell stress. *Summary/Conclusion*: An improved understanding of HDV release and content is necessary to evaluate their role in the signalling mechanisms that underlie DILI. We present novel analysis of primary rat and human HDVs in vitro, which will provide insight into HDV in human DILI, and will help evaluate the translational value of HDV studies in rats, a key species in pre-clinical testing. To our knowledge, this is the first evaluation of the transcriptional content of exosomes released by cultured primary human hepatocytes. Our results suggest that HDV secretion by primary hepatocytes is altered in response to toxicant-induced injury.

P-XIV-6

Cardiac surgery induces coordinated plasma changes in cardiac enriched microRNAs and exosomes: possibilities for new biomarkers and targets for therapeutic interventions

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Introduction: Cardiac surgery is associated with ischaemia/reperfusion injury, which is difficult to grade using the available biomarkers (troponins and cardiac enzymes). Moreover, surgical patients can develop acute complications, whose mechanisms are not fully understood and that are difficult to predict, making therapeutic treatment unsatisfactory. For these reasons, new mechanistic understanding and circulating biomarkers of the response of the myocardium (and distant organs) to cardiac surgery are intensively searched. MicroRNAs (miRNAs, miRs) are small, non-coding RNAs that are key regulators of gene expression. Exosomes are nanovesicles (30–100 nm) which can be released by cells in response to certain stimuli, including stress. Exosome-mediated miRNA transfer may be a mechanism of intercellular signalling and exosome-associated miRNA in biofluids are potential biomarkers. We performed a translational study comparing the expression of cardiac-related miRNAs and plasma exosomes in patients pre- and post-coronary artery bypass grafting (CABG). *Methods*: As part of a prospective cohort study of over 2,000 cardiac surgery cases, peripheral blood was taken from non-diabetic patients (n = 20) undergoing CABG both immediately before anaesthesia and after grafting (after heparin reversal). Plasma was extracted using a standardised protocol. Exosome concentration

was determined using nanoparticle tracking analysis (Nanosight) and relative miRNA expression was quantified using qPCR. *Results*: There was a significant increase in post-op expression of miR-1, -24, -133a, -208b, -210 and -223 ($p < 0.05$ vs. pre-op for all, Wilcoxon test). The mean fold-change increases ranged from 3 (miR-24) to 50 (miR-133a vs. pre-op). Similarly, the concentration of exosomes in the plasma increased postoperatively (mean 3.2×10^{13}/ml pre-op vs. 4.4×10^{13}/ml post-op, $p = 0.03$, paired *t*-test, $n = 11$). *Summary/conclusion*: CABG is associated with an increase in plasma exosome concentration. The corresponding increase in both exosome concentration and cardiac-associated miRNAs post-CABG suggest that these miRNAs may be transported within the exosomes. Further work is underway to determine if this is the case and whether these miRNAs have a potential functional role in mediating the postoperative complications of cardiac surgery.

P-XIV-7

Extracellular vesicles promote an EMT-like phenotype underlying fibrosis and skeletal muscle degeneration in NEU1-deficient mice

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Introduction: Lysosomal sialidase NEU1 is the enzyme responsible for the removal of terminal sialic acids from sialo-glycoproteins. The degradation/processing of target NEU1 substrates is pivotal for the homeostatic control of basic cellular processes, as demonstrated by the severe systemic effects of NEU1 deficiency characteristic of the lysosomal disease sialidosis. Studies of the molecular mechanisms of pathogenesis in *Neu1*-KO mice, a faithful model of the disease, have identified NEU1 as negative regulator of lysosomal exocytosis. Deficiency of Neu1 results in excessive exocytosis of lysosomal contents and extracellular vesicles (EVs) with deleterious consequences for ECM and PM integrity, and tissue homeostasis. In muscle, excessive lysosomal exocytosis promotes progressive infiltration/invasion of the muscle bed by abnormally expanded connective tissue with characteristics of severe fibrosis. Altered composition of the connective tissue and muscle microenvironment ultimately leads to muscle degeneration. The scope of this study was to investigate the molecular cues downstream of Neu1 deficiency that provoke this fibrotic phenotype, focusing on the excessive release of EVs by *Neu1*-KO myo-fibroblasts. *Methods*: Primary myo-fibroblasts, isolated from gastrocnemius (GA) muscles, were used for proliferation, invasion/migration assays and for EVs purification. EVs from WT and *Neu1*-KO myo-fibroblasts were subjected to microarray and proteomic analyses and used in co-culture experiments with primary murine and human fibroblasts. Gene expression assays were performed on total RNAs from EV-treated cells and muscle tissue. IF and IHC analyses were carried out on WT and *Neu1*-KO GA's using markers for fibrosis and epithelial-mesenchymal transition (EMT). *Results*: Our results show that primary *Neu1*-KO fibroblasts had higher proliferation rate that WT cells were capable to migrate through and invade ex vivo basement membranes and had increased exocytosis of EVs. Microarray and proteomic analyses on WT and *Neu1*-KO GA muscle RNA's and EVs revealed upregulation of genes/proteins specifying the TGFβ and β-catenin/WNT signalling pathways and markers of EMT. Induction of EMT may explain the increased proliferation of the *Neu1*-KO myo-fibroblasts and the abnormal expression of ECM components. We further demonstrate that both human and murine WT fibroblasts acquired *Neu1*-KO proliferative and invasive characteristics when co-cultured with Neu1-KO EVs and showed increased expression of genes involved in fibrosis and EMT. *Summary/conclusion*: We present new evidence that *Neu1*-KO EVs from primary myo-fibroblasts are loaded with high levels of signalling molecules, TGFβ and WNT/β-catenin, capable of inducing an EMT phenotype to primary human and murine WT fibroblasts. Thus, an EMT-like process likely underlies the fibrosis and myopathy in the *Neu1*-KO mice. This unusual fibrotic process may resemble that occurring in idiopathic pulmonary fibrosis, opening new possibilities for diagnosis and therapy in this patient population.

P-XIV-8

Effect of oxygen-stop decompression on bubble formation and microvesicles release after an open-sea air dive

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Introduction: Decompression sickness (DCS) is a systemic pathophysiological process with a wide variety of signs and symptoms related to dissolved gas. DCS has been associated with endothelial dysfunction, platelet activation and alterations in coagulation pathways. Extracellular vesicles levels increase in association with the shearing stress caused by bubbles in the blood. Decompression protocol using oxygen-stop accelerates the washout of nitrogen loaded in tissues. We hypothesized that the beneficial effect of oxygen-stop decompression on intravascular bubbles might involve decreased levels of circulating microvesicles (MV) after SCUBA diving. *Methods*: Healthy experienced divers performed 2 protocols in a randomised order: (a) firstly, an open-sea air dive (sea temperature $17 \pm 2^{\circ}C$) at 400 kPa (30 msw, metres of sea water) for 30 minutes with a 9-minute air-stop decompression at 3 msw (French Navy MN90 shedule) ($n = 10$); (b) secondly, the same open-sea air dive with a 9-minute oxygen-stop decompression ($n = 15$). Bubble grades were monitored with the KISS score. We evaluated the plasma levels of platelet-derived MV (defined as AnnexinV + CD41 +) and endothelial cells-derived MV (defined as AnnexinV + CD31 + CD41-) by flow cytometry 1 hour before and after each dive. *Results*: Mean KISS bubble score was significantly lower after the dive with oxygen-decompression stop compared to the dive with air-decompression stop (4.3 ± 7.3 vs. 32.7 ± 19.9, $p < 0.001$). Flow cytometry analysis demonstrated that, after the dive, the circulating levels of platelet-derived MV significantly increased in the air-stop decompression group ($753/\mu l +/- 245$ vs. $381/\mu l +/- 191$, $p = 0.003$) whereas there are no significant changes in the oxygen-stop decompression group ($329/\mu l +/- 215$ vs. $381/\mu l +/- 191$, $p = 0.2$). Moreover, the circulating levels of endothelial cells-derived MV trended to increase in the air-stop decompression group ($43/\mu l \pm 44$ vs. $25/\mu l \pm 14$, $p = 0.065$) whereas no changes were observed in oxygen-stop decompression group ($21/\mu l \pm 13$ vs. $25/\mu l \pm 14$, $p = 0.27$). *Summary/conclusion*: Unlike air-stop decompression, oxygen-stop decompression does not induce air bubble formation, platelet or endothelial activation. Oxygen breathing during decompression stops could prevent the thrombotic events after stressful decompression and, therefore, may be beneficial in reducing the development of DCS.

P-XIV-9

Size distribution and thrombin generation activity of membrane vesicles in cryopreserved blood platelets

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Introduction: Platelets (PLTs) for transfusion can only be stored for 5 days at $22–24^{\circ}C$. Freezing was proposed as a promising but as yet unproven approach to extended PLT storage for transfusion medicine. The current state of the art method is 6% DMSO cryopreserved platelets (CPP). Here we investigate platelet membrane changes in CPP, the size distribution of generated platelet membrane vesicles (PMVs) and their contribution to procoagulant activity of CPP products. *Methods*: PMVs in CPP and liquid stored PLTs (LSP, paired control) were characterized by flow cytometry (FC), nanoparticle tracking analysis (NTA), laser scanning confocal microscopy (LSCM), field emission scanning electron microscopy (FESEM),

transmission electron microscopy (TEM), atomic force microscopy (AFM), thrombin generation (TG) assay and light transmission platelet aggregometry (LTA). *Results*: FESEM and TEM showed marked disintegration and vesiculation of platelet plasma membrane in CPP compared to almost resting phenotype of corresponding LSP. FC revealed that FC counts of $CD41a^+$ PMVs and annexin V-binding PMVs in CPP were about 20 × and 70 × higher, respectively, compared to LSP. Similarly, the NTA showed about 5 × higher counts of PMVs compared to LSP processing control. NTA size distribution histogram of PMVs in CPP indicated a peak of about 100 nm of particle hydrodynamic diameter, corresponding to the size range of exosome-like vesicles. The TG assay showed that CPP possessed about 5 × higher TG activity (thrombin peak height/10^6 PLTs), compared to LSP. Interestingly, the exosome size PMVs were responsible for most of the TG activity of CPP product since the activity remained in the 20,000 *g* supernatant of CPP but it was substantially decreased after spinning at 100,000 *g*. *Summary/conclusion*: The CPP exhibit dramatic platelet membrane vesiculation and release of exosome size PMVs responsible for a substantial part of procoagulant activity of the CPP product. These results warrant further investigation on the content, phenotypes and activities of PMVs in CPP products to achieve standardization of in vitro potency of these products and optimize their safety and haemostatic efficacy profile in vivo. These findings and conclusions have not been formally disseminated by FDA and should not be construed to represent any Agency determination or policy.

P-XIV-10

Microparticles are increased in human endotoxemia and promote coagulation via the intrinsic pathway

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Introduction: Human endotoxemia is characterized by acute inflammation and activation of coagulation as well as increased circulating microparticles (MP). Much emphasis has been placed on potential MP procoagulant effects mediated through tissue factor (TF) and the extrinsic pathway. However, little data exist regarding the procoagulant properties of MP in endotoxemia. Herein, we sought to further characterize MP and their procoagulant properties using a model of human endotoxemia, including the relative contributions of the extrinsic and intrinsic pathways in MP-mediated procoagulant activity. *Methods*: LPS 2 ng/kg was infused into 17 healthy subjects with blood samples obtained at 4 time points (baseline, 3, 6, 24 hours after infusion). Total MP, platelet MP (PMP), red cell MP (RMP), monocyte MP (MMP) and endothelial MP (EMP) were enumerated in plasma using high sensitivity flow cytometry. MP procoagulant activity was measured with the Zymuphen MP-Activity assay. MP-dependent clot time was assessed by a re-calcification time. MP-dependent thrombin generation was measured by calibrated automated thrombogram (CAT). Relative contributions of the extrinsic and intrinsic pathways in MP-mediated procoagulant activity were assessed using plasmas deficient in FVII or FXI or with blocking antibodies to TF or FXIa. *Results*: Total MPs and PMPs were significantly elevated at 6 hours ($p < 0.05$) with a trend towards a significant increase in RMPs ($p = 0.09$). In general, MPs obtained at 3 hours following endotoxin infusion were more procoagulant than baseline MPs, with increased prothrombinase activity ($p < 0.0001$), decreased clotting time ($p < 0.0001$), and shortened lag time ($p < 0.05$) and time to peak thrombin ($p < 0.05$) by CAT. The use of FVII deficient plasma or blocking antibody to TF did not significantly affect MP-mediated procoagulant effects. In contrast, MP-mediated clotting time was prolonged in FXI deficient plasma ($p < 0.0001$) and a FXIa-blocking antibody inhibited all parameters in the CAT assay ($p < 0.0001$). *Summary/conclusion*: MP numbers, particularly those from platelets and red cells, are increased in endotoxemia, and the intrinsic pathway appears to play a more dominant role in the MP-mediated procoagulant effects in plasma.

P-XIV-11

How can we determine the nanoparticles haemocompatibility with human blood?

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Introduction: Nanosciences and nanotechnologies are in constant evolution. Development of new therapeutic and diagnostic agents using nanotechnologies to reach their pharmaceutical target require the knowledge of biocompatibility of nanoparticles with the blood compounds. Haemostasis is the ensemble of physiological phenomena which causes bleeding to stop. It also maintains vascular integrity. A dysfunction of the haemostasis can lead to slow down or even to completely stop the circulation of the blood. It is, therefore, primordial to study the haemocompatibility of nanoparticles NPs. The aim of this study is to evaluate the biocompatibility of manufactured NPs on haemolysis, platelet function and coagulation. *Methods*: Various techniques assessing erythrocytes integrity, activation and aggregation of the platelets or the impact of NPs on coagulation cascade were investigated. An approach in transmission and in scanning electronic microscopy was also accomplished. Five NPs (carbon nanotubes, carbon black, silicon dioxide, copper oxide and silicon carbide) with different physicochemical properties were studied. *Results*: The Impact-R® with scanning electronic microscopy support and the calibration thrombin generation tests were the reference method to investigate the potential impact of NPs on platelet function and the procoagulant activity of NPs, respectively. *Summary/conclusion*: We suggest guidelines for testing NP haemocompatibility which responds to a request of scientific community due to lack of recommendations for the evaluation of nanomaterial haemocompatibility.

P-XIV-12

Microparticles, plasminogen activator inhibitor (PAI-1), and antithrombin: key players of veno-occlusive disease

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Introduction: Veno-occlusive disease (VOD) is a severe complication of haematopoietic stem cell transplantation (HSCT) and chemotherapy treatment. Early VOD prompt recognition and treatment are imperative to improve patient outcome. The increase of plasminogen activator inhibitor-1 (PAI-1) has been reported as an early marker of VOD in patients undergoing HSCT or during chemotherapy for Wilms tumour. Cell derived microparticles (MP) are involved in the pathophysiology of endothelial damage but their role in the pathogenesis of VOD has never been investigated. *Methods*: We retrospectively analysed plasma samples from 12 paediatric patients with malignancies and 6 of them developed VOD after HSCT or after chemotherapy. We studied MP cells origin (red cells-, platelets-, white cells- and endothelium-derived MP) and MP functional markers (tissue factor and adhesion molecules such as CD144). We correlated the new MP

data with clotting and fibrinolytic parameters. *Results*: A statistically significant difference was found for antithrombin (AT) ($p = 0.021$), D-Dimer ($p = 0.029$) and platelets ($p < 0.001$). MP were higher on early sampling, but were reduced in VOD. However, MP staining for CD144+ (an endothelial gap junction) were significantly higher in patients who developed VOD than in those who did not (55 vs. 13; $p = 0.016$). VOD. PAI-1: act showed an inverse association with MP CD144+ (-0.251, $p = 0.043$), MP CD31+/CD41+ (-0.004, $p = 0.026$) and antithrombin (-0.470, $p = 0.038$). *Summary/conclusion*: These findings suggest a clear procoagulant status in VOD, with AT-, PMP- and PLT-consumption over time. The consistent finding of elevated MP CD144+ before VOD suggest that this marker may become a useful sign of early VOD. Our group is currently performing a large prospective study to further clarify these findings.

P-XIV-13

Characterization of different populations of platelet membrane microvesicles released from TRAP-activated platelets

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Introduction: Platelet membrane microvesicles (PMVs) circulate in blood and their elevated counts are associated with various diseases. A potential role of PMVs in adverse events following platelet (PLT) transfusion is also of concern, since PMVs are released from PLTs during processing and storage of PLT transfusion products. Here we characterized different populations of PMVs released from thrombin receptor-activating peptide (TRAP)-activated PLTs. *Methods*: PMVs were characterized by flow cytometry (FC), nanoparticle tracking analysis (NTA), confocal microscopy (CM), electron microscopy (SEM and TEM) and atomic force microscopy (AFM). Proteome and lipid composition were analyzed by mass spectrometry (MS), and PMVs activity was assayed by thrombin generation (TG). *Results*: CM showed that PMVs were formed in TRAP-activated PLTs from both plasma as well as internal membranes. The mitotracker labelling detected released mitochondria within MPV populations. NTA showed that PMV sizes range from 80 nm to 700 nm. The larger PMVs (PMV20) were sedimented at 20,000 *g* followed by 100,000 *g* centrifugation for isolation of small exosome-like PMVs (PMV100); PMV20 and PMV100 contained 61 and 94% of PMVs < 300 nm, respectively. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) content in PMV100 was approximately twice higher than in PMV20; the cholesterol ester (CE)/phospholipid ratio was 2.7 times higher in PMV100 compared to PMV20. Proteome analysis revealed the presence of PLT marker CD41 and exosome markers CD9 and Hsp70 in both PMV20 and PMV100; the amount of mitochondria-related proteins was more than twice higher in the PMV20. Phosphatases and membrane trafficking proteins were found only in PMV20. Both PMV populations were pro-coagulant in vitro; TG activity (thrombin peak height/10^9 PMVs) was 1.8 times higher for PMV20 compared to PMV100. *Summary/conclusion*: Activated PLTs release heterogeneous populations of PMVs differing in size, protein and phospholipid composition. TG activity of PMVs was observed, including small exosome-like PMV population, rich in PS. In addition to FC, reliable analysis of PMVs requires high resolution methods capable to detect and quantify PMVs < 300 nm. These findings and conclusions have not been formally disseminated by FDA and should not be construed to represent any agency determination or policy.

P-XIV-14

Revisited role of microvesicles in haemostasis

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Introduction: Microvesicles (MVs) represent a heterogeneous population of submicronic vesicles that are released in response to cell activation or apoptosis. MPs harbour a large repertoire of cell surface receptors, mRNA and biological activities representative of their parent cells and related to their involvement in many biological functions such as haemostasis. The aim of these presentations is to discuss a new vision of MVs as complex and ambivalent structures that express both activators and inhibitors of coagulation but also convey fibrinolytic properties. *Methods*: This vision emerges from the work of our team during the past 10 years on the procoagulant and proteolytic properties of microvesicles. We propose to briefly review some key results integrated with the literature and our most recent unpublished data on the fibrinolytic potential of some MV subpopulations as arguments to support our new concept on the role of MVs in haemostasis. *Results*: Beyond their well-described procoagulant property, accumulating data show that specific endothelial cell-, leukocyte-, tumour-derived MVs bind plasminogen and vectorise plasminogen activators, leading to an efficient plasmin generation and matrix metalloproteinases activation. The existence of a measurable MV-dependent plasmin generation capacity (MV-PGC) in the circulation or in body fluids raises the question about the pathophysiological relevance of this activity. We recently test the hypothesise that MV-PGC would be conveyed by MVs in septic shock patients and would determine the disease outcome by counterbalancing the risk of microthrombosis. An original assay was developed and validated to reproducibly and selectively measure MV-PGC in human plasma samples. Interestingly, circulating leukocyte-derived MVs from septic patients can generate plasmin via the urokinase system. Moreover, this activity is associated with a better survival and inversely correlates with coagulopathy, hypoxia and organ failure. We also demonstrated that MVs generated in inflammatory conditions or from septic patients contribute to clot dissolution *in vitro*. *Summary/conclusion*: Although, *in vivo* demonstration is still lacking, accumulating data suggest this MV-dependent fibrinolytic activity may counterbalance the systemic pro-coagulant state. Thus, specific MV subpopulations emerge as a new actor in fibrinolysis. These evidences changed our procoagulant vision of the role of MVs in haemostasis into systems regulating the balance between coagulation and fibrinolysis.

P-XIV-15

Isolation, characterization and procoagulant role of platelet-derived extracellular vesicles

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Please see Oral with poster C

Poster session XV - Cellular targeting and uptake of EVs
Chairs: *Raymond Schiffelers and Samir El-Andaloussi*

P-XV-1

Effects of macrophage-derived extracellular vesicles on cellular migration are specific to both source and target cells

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Introduction: Others and we have previously shown that macrophages secrete mRNA-, micro RNA- and protein-containing extracellular vesicles (EVs) that target other cells, impacting on a large span of functions, from differentiation to motility. However, most of the studies to date are done in the context of cancer or other pathologies. Here, we sought to identify functional effects of the proteins that are present in EV derived from THP1 monocytes, and compare them with EVs produced by peripheral blood monocyte-derived macrophages (MDM), in terms of composition and effects upon several types of target cells. *Methods*: A preliminary proteomic analysis revealed within these EVs, a plethora of proteins involved in the cytoskeleton control, therefore conceivably affecting the migratory behaviour of target cells. To test this hypothesis, we isolated EVs from (a) PMA-differentiated and naïve THP1 cells, (b) MDM and (c) monocytes differentiated in vitro into M1 or M2 macrophages. EVs were quantified by flow cytometry and physiological amounts were added to target cells. Starting either immediately after EV addition or after 24 hours of incubation, we performed migration assays in the Radius 96-well plates, in the continuous presence of the same EVs. *Results*: EVs from differentiated THP1 cells enhanced the migration of lung fibroblasts and pulmonary artery endothelial cells to 120 and 114%, respectively, but reduced the in vitro wound closure in lung epithelial cells to 75%. Similarly, MDM-derived EV increased fibroblasts migration to 156%. When we used EV derived from M1 (M1-EV) or M2 (M2-EV) in vitro differentiated macrophages, the effects were more nuanced. M2-EV significantly decreased MCF 10A epithelial cells motility to 47%, while M1-EV significantly increased their motility to 108%. However, when EVs targeted the transformed MDA-MB-231 epithelial cells, the effects were opposite: M1-EV reduced their migration to 75%, while M2-EV had no effect. *Summary/conclusion*: Our data indicate that EV actions are specific to both the status of source cells and the type of target cells. These findings are relevant for the EV-mediated intercellular communication of macrophages with their tissue microenvironment during normal and pathological processes.

P-XV-2

Preferential uptake of mantle cell lymphoma exosomes by B-lymphocytes

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Introduction: Mantle Cell Lymphoma (MCL) is an incurable mature B cell neoplasm. It is relatively rare and aggressive disease with short median survival. MCL is characterized by the overexpression of cyclin D1, which is a key regulator of the cell cycle progression. Currently all treatment regimens are based on chemotherapeutic regimens, which in most cases ends with tumour resistance and relapse. Therefore, further understanding of disease pathology and development of novel therapeutic and diagnostic modalities are needed. Exosomes are small natural extracellular vesicles, which play an important role in intercellular communication and in various physiological and pathological processes. We aimed to characterize MCL exosomes, decipher their cell specificity and internalization mechanism. *Methods*: We have isolated MCL-derived exosomes from the conditioned media of Jeko1 and Mino, MCL cell lines and MCL patients' cells by deferential ultracentrifugation. Exosomes were characterized by immune electron microscopy, Nanoparticle tracking analysis, Western blot and flow cytometry. Exosomes uptake by subsets of leukocytes was analyzed by confocal microscopy and flow cytometry. *Results*: Internalization of MCL-derived exosomes by MCL cells was visualized 10 minutes post administration and increased thereafter. Only minor fraction of exosomes internalized into Jurkat, T-cell leukaemia cell line, when these cells were co-cultured with Jeko1 or Mino cells. MCL Patients' derived exosomes were preferentially internalized by both healthy and malignant B-lymphocytes with no apparent internalization to T lymphocytes. MCL derived exosomes internalization was not inhibited by specific knock down using siRNA against Caveolin1 and Clathrin suggesting on a non-classical endocytosis mechanism. Nevertheless, the internalization of MCL exosomes found to be mediated by lipid raft/cholesterol-dependent pathway using specific inhibitors to various endocytosis mechanisms. *Summary/conclusion*: Our findings demonstrate B-lymphocytes specificity of MCL exosomes and emphasize the potential of harnessing MCL exosomes for the delivery of therapeutic payloads while exploiting their natural specifically towards MCL cells.

P-XV-3

Studies of paclitaxel-loaded EVs as drug delivery vehicles

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Introduction: The poor penetration of anticancer drugs is a challenge for oncological treatments. Extracellular vesicles (EVs) have gained interest as drug vehicles due to their natural capacity for cargo delivery. In this study, prostate cancer (PCa) cell line-derived EVs were studied as carries of paclitaxel, an anti-mitotic cancer drug. *Methods*: EV populations from LNCaP and PC3 cell lines were isolated by differential centrifugations. EVs were characterized by TEM, NTA and Western blotting. The importance of EV surface proteins was studied by trypsin treated control EVs. Uptake of DiD-labelled EVs to PCa cells was determined in time by confocal microscopy and quantified by flow cytometry and a novel fluorescence image analysis (FIA). EVs were loaded with 5–50 nM paclitaxel, measured by spectrophotometry and confirmed with UPLC-MS. The cytotoxic effect of the paclitaxel-loaded EVs was assessed by AlamarBlue viability assay after 24 and 48 hours. The cellular localization of the drug and the EVs was examined by live cell confocal microscopy using Oregon green-labelled paclitaxel and DiD-labelled EVs. *Results*: The 3 different methods (microscopy, flow cytometry and FIA) showed similar efficacy of EV uptake during 24 hours irrespective of the used EV population or the cell line. Significant uptake was observed already by 9 hours. EVs on their own EVs increased the viability of the PCa cells, whereas the paclitaxel-loaded EVs had a cytotoxic effect at 48 hours. This effect was reduced by trypsin digestion of the EVs to remove surface proteins as shown by Western blotting. Interestingly, the localization of the OG-labelled paclitaxel in the recipient cells differed depending on whether the drug was introduced by the EVs or by media. *Summary/conclusion*: Uptake of multiple EV subtypes and origins can be quickly compared with FIA. EVs may be used as an efficient drug delivery system to tumour cells, which is influenced by the EV surface receptors.

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P-XV-5

Towards endothelial cell-specific extracellular vesicle isolation

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Introduction: Extracellular vesicles (EV) in blood are derived from a heterogeneous collection of cell types. Using EVs and their contents as biomarkers is potentially challenging as the cell type of interest – tumour cell, inflammatory cell, etc. – may contribute only a small percentage of the wider population of EVs. To improve the use of EVs as biomarkers, EVs or subtypes such as exosomes could be isolated from a particular cell type based on cell markers. Elevated levels of EVs from endothelial cells origin were found in plasma from patients with vascular diseases, where they serve as a surrogate marker of endothelial function. *Methods*: We cultured human umbilical vein endothelial cells (HUVEC). Cell culture media was collected and exosome-enriched EVs were isolated using differential ultracentrifugation (UC) alone, poly(ethylene glycol) (PEG) precipitation alone or PEG-based concentration followed by dilution and UC. EVs obtained from the PEG/UC method were used for flow cytometry studies. We bound the EVs to 0.22 μm magnetic beads designed for endothelial origin EV isolation and labelled with an endothelial cell marker. EVs attached to beads were then co-labelled with fluorescently tagged anti-CD81, a putative exosomal marker. *Results*: We compared the particle populations obtained by the 3 methods using nanoparticle tracking analysis. PEG or PEG/UC resulted in 3 to 6-fold more particles than UC alone, as well as slightly larger apparent particle size. We note that not all of these particles are necessarily EVs. Flow cytometry identified a $CD81^+$ labelled population of magnetic beads, apparently larger vesicle sizes and a particle concentration of 1.6×10^6 per ml. Microscopy identified marked clumping of exosomes bound to or free of the beads. NTA of this same fraction returned a particle concentration as 3.4×10^{11} particles/ml, suggesting significant clumping of vesicles. *Summary/conclusion*: Enriching for EVs by cell of origin is challenging but certain to improve biomarker specificity. UC alone was superior to PEG or PEG/UC. The use of PEG to concentrate EVs tended to clump and suffer from antigen masking resulted impractical for flow cytometry, although perhaps a dilution strategy could overcome this problem.

P-XV-6

Uptake pathways of endothelial extracellular vesicles into endothelial cells

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Introduction: Endothelium long has been a key target of molecular therapy. Many different types of extracellular vesicles (EVs) are known to communicate with endothelium, and the interest is growing toward the use of EVs as delivery vectors. In order to achieve that goal a better understanding of the cellular uptake mechanism of EVs is important since it determines the intracellular fate of the internalized particle. Cellular uptake of EVs occurs almost entirely through endocytosis. The endocytotic pathway used by EVs depends on many factors, that is, origin of the EVs, the type and cell cycle of recipient cells etc. We previously characterized EVs isolated from endothelial cells. Here, we explored the uptake kinetics and endocytosis of the endothelial EVs into parent cells. *Methods*: DiO-labelled endothelial EVs were characterized by TEM and quantified by NanoSight particle size analyzer. Uptake of DiO-EVs into endothelial cells was investigated under 37°C and 4°C temperatures by imaging flow cytometry. The effect of pathway specific blocking agents – bafilomycin A1, chlorpromazine and nystatin – on the endocytosis of DiO-EVs into endothelial cells was tested by imaging flow cytometry. *Results*: According to our data, the uptake of endothelial EVs was energy dependent and the kinetics showed a saturable pattern. Among the pathway inhibiting agents, chlorpromazine exerted the highest blocking effect, reducing the uptake of EVs by 89%, while bafilomycin A1 and nystatin had significantly lower effect. *Summary/conclusion*: Our results indicate that all examined pathways play a role in the energy dependent uptake of EVs, but the receptor mediated clathrin-dependent pathway is the dominant route. Surface epitopes participating in the uptake remain to be identified.

P-XV-7

Targeting and sub-cellular localization of cell-derived microparticles to macrophages

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Introduction: Extracellular vesicles can form microparticles (MPs) capable of acting as intercellular communication vectors. Cell-derived MPs are thus being explored as potential vehicles for vaccines. MPs reportedly interact with cells via both active and passive transport mechanisms including fusion, endocytosis and receptor binding. This study seeks to clarify the mechanism by which MPs interact with macrophages and monitor their uptake and internal localization. *Methods*: MPs were isolated from culture supernatants of the follow cell lines: RAW264.7 (macrophage-like), Meg01 (megakaryocytes), E.G7 (T cell) and NIH3T3 (fibroblast). These MPs were incubated with RAW264.7 in the presence of inhibitors targeting active transport, clathrin-coat mediated endocytosis or scavenger receptor mediated uptake to assess the contribution of each mechanism to vesicle internalization over time. To track the subcellular localization following internalization, MPs were incubated with HEK293T cells transiently transfected with plasmids encoding markers of each compartment in the endocytic pathway (RAB 5, 9, 11 and CD63 proteins). *Results*: MPs derived from fibroblasts but not T cells were readily taken up by macrophages. This internalization required the formation of clathrin-coated pits on the surface of target cells. Scavenger receptor blockage abolished 40% of the internalization of RAW264.7 MPs, which were the most negatively charged vesicles as determined using a Zeta-Nanosizer. In contrast, megakaryocyte derived MPs were unique in being uptaken by macrophages via passive transport mechanisms. Confocal microscopy showed that internalized MPs localized to CD63+ compartments, suggesting that the particles accumulated in late endosomes and did not reach early endosomes. *Summary/conclusion*: MPs derived from different cell types gained entry to macrophages via different routes, with clathrin-coat mediated endocytosis being the primary mechanism for most MP. In all cases the internalized MPs homed to the late endosomes. Ongoing studies are designed to optimize the targeting of MPs to macrophages and establish whether delivery to their late endosomal compartment will be effective for loading and processing encapsulated antigens, including vaccines.

P-XV-8

Haemocompatibility of estetrol and estradiol cyclodextrins complexes

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Introduction: In order to prevent damages to premature babies, we want to develop liposomes based estetrol and estradiol to increase their concentration into the brain. These liposomes will be administered in vivo by iv injection. The study of their haemocompatibility if of major importance. In fact, a dysfunction of the haemostasis can lead to slow down or even to completely stop the circulation of the blood. It is therefore primordial to study the haemocompatibility of nanoparticles NPs. The aim of this study is the evaluation of the haemocompatibility of estetrol and estradiol-based liposomes on haemolysis, platelet function and coagulation. *Methods*: Crysmeb® and hydroxypropyl-β-cyclodextrin (substitution degrees of 0.63) HEPES buffer solutions were prepared at different concentrations. An excess of estradiol or estetrol was added to the cyclodextrin solutions, and the dispersion was stirred at 140 rpm at 25°C for 24 hours. Then the unsolubilized hormone was separated by filtration. Various techniques assessing erythrocytes integrity, activation and aggregation of the platelets or the impact of NPs on coagulation cascade were investigated on human blood. *Results*: Estetrol and estradiol alone or coupled with cyclodextrines induced no haemolysis. Estetrol, with or without clyclodextins had no impact on platelet function and coagulation. Complexes cyclodextrin-estradiol avoid platelet aggregation and extend the blood coagulation. *Summary/conclusion*: Estetrol and complexes cyclodextrin-estetrol are haemocompatible. Complexes cyclodextrin-estradiol show anti-aggregating and anti-coagulant properties.

P-XV-9

Changes in plasma microvesicle origins and concentrations over time following traumatic injury

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Introduction: Elevated levels of circulating plasma microvesicles (MV) have been described in a number of clinical conditions including severe traumatic injury. The source of these MV populations and changes in their release patterns is unknown following traumatic injury. Hence, this study aimed to quantify and determine the origins of plasma MV in severe trauma patients over time. *Methods*: In total, 19 severe traumatic injury patients with an injury severity score of >15 were studied along with 10 healthy controls. MV were isolated by double centrifugation (2,000 *g* 20 min; 13,000 *g* 2 min) from blood collected from day 1–28 after initial injury. Plasma concentrations of MV were compared using nanoparticle tracking analysis (NTA size range 100–1,000 nm) and flow cytometry (FACS, sizes >500 nm). Cellular origins of plasma MV were determined using cell specific markers by FACS. *Results*: PMV number measured by FACS was <1% of the number counted by NTA, but both methods gave values that were higher in trauma patients than controls. Furthermore, patients with trauma had higher number of MV derived from platelets(CD41+), RBCs(CD235a+) endothelial cells(CD144+) and leukocytes(CD45+). The increases were evident throughout the 28 day follow-up. *Summary/conclusion*: Severe traumatic injury was associated with elevated levels of MV of several different origins. Their continual elevation over time suggests an ongoing process of release that may be related to complications that develop in traumatic injury. However, a larger study will be required to test whether they are useful biomarkers for outcome.

P-XV-10

The role of platelet-derived microvesicles in neutrophil recruitment to endothelium during inflammation

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Please see Oral with poster C

P-XV-11

Unique expression of long non-coding RNAs in the liver and plasma in acetaminophen-induced liver injury

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Introduction: Long non-coding RNAs (lncRNAs) are emerging as new regulators of genes, as biomarkers and therapeutic targets for various diseases. lncRNAs can be associated with extracellular vesicles (EVs) and can be detected in circulation. Acetaminophen (APAP)-induced liver injury is a significant clinical problem and is a major cause of acute liver failure. Identification of reliable biomarkers is the foremost step for early diagnosis of the disease and for potential new treatments. In this study, we hypothesized that APAP treatment causes differential expression profile of lncRNAs that could be exploited as novel biomarkers. *Methods*: C57BL/6 mice received either saline or 300 mg/kg APAP for 6 hours. Total RNA from the liver and plasma were subjected to RT^2 lncRNA PCR array. APAP overdose patient samples were obtained from Conquering Diseases Biorepository at the University of Massachusetts Medical School. EVs were measured and identified from plasma by Nanosight and electron microscopy. *Results*: A significant increase in the number of circulating EVs was found in patients with APAP overdose. Interestingly, the number of microvesicles (150–800 nm) was higher than exosomes (50–150 nm) in these patients. Similarly, circulating EVs were increased in mice treated with APAP. Out of 84 lncRNAs tested, majority of them were decreased after APAP treatment. The hepatic expression of lncRNAs such as Crnde, Dio3os, Neat1 and Snhg4 was increased while Meg3, Rian, Dleu2 and Pidi were decreased significantly after APAP treatment. Pathway analyses of these lncRNAs suggest their role in various cellular processes such as apoptosis, cell proliferation, angiogenesis, autophagy and differentiation. A differential expression of lncRNAs was found in the liver and plasma after APAP treatment. Interestingly, Neat1 was increased in the liver but found to be decreased in plasma. In contrast, Dleu2 was decreased in the liver where as it was increased in plasma. lncRNA Malat1 was decreased while Firre was increased in plasma after APAP treatment. These lncRNAs are associated with cell death, apoptosis, angiogenesis and cell cycle. *Summary/conclusion*: Our results suggest that lncRNAs have potential roles in liver injury caused by acetaminophen overdose and injury is reflected in EVs distribution. Further, extracellular lncRNAs could be exploited as new biomarkers for the diagnosis of drug-induced liver injury.

P-XV-12

Adipogenic RNAs are transferred in osteoblasts via bone marrow adipocytes-derived extracellular vesicles

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Introduction: In osteoporosis, bone loss is accompanied by increased marrow adiposity. Given their proximity in the bone marrow and their shared origin, a dialogue between adipocytes and osteoblasts could be one of the ways occurring in the competition between human Mesenchymal Stem Cells (hMSC) differentiation routes, leading to adipocyte differentiation at the expense of osteoblast differentiation. The adipocyte/osteoblast balance is highly regulated at the level of gene transcription. In our work, we focused on PPARγ, CEBPα and CEBPd, as these transcription factors are seen as the master regulators

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of adipogenesis and are expressed precociously, and on leptin and adiponectin, considered as adipocyte marker genes. In 2010, our group has demonstrated, thanks to a coculture model, that in the presence of hMSC-derived adipocytes (hMSC-Adi), hMSC-derived osteoblasts (hMSC-Ost) express less amounts of osteogenic markers but exhibit expression of typical adipogenic genes. Nevertheless, the mechanisms underlying this modulation of gene expression are not clarified. Recently, adipocytes were described as releasing EVs, containing and transferring adipocyte specific transcripts, like PPARγ, leptin and adiponectin. Here, we wondered if EVs could be the way by which adipocytes transfer adipogenic RNAs in our coculture model. *Methods*: hMSC-Ost were incubated in conditioned medium obtained from hMSC-Adi (hAdi-CM) cultures, and adipogenic and osteoblastic mRNAs and miRNAs were quantified. EVs were isolated from conditioned media collected from cultures of hMSC at different stages of adipocyte differentiation and specific adipogenic transcripts were detected inside. Inter-species conditioned media exposition were performed, following PCR using species specific primers for adipogenic transcripts detection. *Results*: We observed in hMSC-Ost incubated in hAdi-CM an increase in the adipogenic PPARγ, leptin, CEBPα and CEBPd transcripts, dependent on their amount in the cells of origin, as well as the anti-osteoblastic miR-138, miR30c, miR125a, miR-125b, miR-31 miRNAs, probably implicated in the observed osteocalcin (OC) and osteopontin (OP) expression decrease. Moreover, EVs were isolated from conditioned media collected from cultures of hMSC at different stages of adipocyte differentiation, and these specific adipogenic transcripts were detected. Finally, thanks to inter-species conditioned media exposition, and using species specific primers, we could highlight for the first time a horizontal transfer of adipogenic transcripts from medullary adipocytes to osteoblasts. *Summary/conclusion*: Here was shown for the first time a RNA transfer between hMSC-derived adipocytes and osteoblasts through EVs. Additional studies are needed to clarify if this mechanism has a role in the adipocytic switch driven on osteoblasts by adipocytes inside bone marrow and if EVs could be a target component to regulate the competition between osteoblasts and adipocytes in the prevention or the therapy of osteoporosis and other osteopenia.

P-XV-13

Extracellular vesicles as inductive signals, programmers of kidney development and diagnostic disease markers

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Introduction: Research on extracellular vesicles (EVs) gave rise to the hypothesis that EVs serve as a novel, but evolutionary ancient signalling system in embryonic induction and morphoregulation. EVs are very good candidates for serving as key developmental programming units as they typically contain several RNA species, proteins/enzymes and probably also metabolites. In this study, we use the mammalian kidney as a model system to investigate the role of EV, characterize them and analyze their potential as diagnostic disease markers. *Methods*: EVs are purified from ureteric bud (UBtip and UBstalk) cell lines and metanephric mesenchyme (MM)-derived cell lines (mK3, mK4) by sequential ultracentrifugation and sucrose gradient centrifugation. Vesicle fractions are analyzed by nanoparticle tracking analysis (NTA), electron microscopy (EM and anti CD63 IEM), Western Blotting using EV-specific markers (CD81, CD63, Hsp70) and mass spectrometry. The induction of EVs is done by serum starvation for 3–4 hours before medium collection. MM cells are dissected from E11.5 mice embryos. The primary MM cells are isolated from the ureteric bud and dissociated into single cell suspension. They are re-aggregated to form kidney structures following induction with BIO, UB cells or conditioned – EV rich medium. Obtained kidney-like pellets are analyzed using immunofluorescence and confocal microscopy. *Results*: First preliminary results on the purity, size distribution and composition of EV harvested from embryonic kidney cells have been obtained, and differences between the cell lines can be observed. To follow labelled EV during nephrogenesis, we are employing a dissociation/re-aggregation assay which was developed in our lab. Results showed that it is possible to induce nephrogenesis in mouse MM with ureteric bud recombination. *Summary/conclusion*: This study will lead to major openings in the key question of how organ specific shape and cell differentiation patterns are influenced by EV-mediated cell–cell interactions during kidney development. Additionally, the obtained results could have major influences on and open new possibilities for the early detection of, for example, in utero anomalies in humans.

P-XV-14

Ligand-independent NOTCH signalling via ARRDC1-mediated microvesicles

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Introduction: Mammalian cells are capable of secreting nano-scale vesicles (50–200 nm) such as ARMMs (arrestin domain-containing protein 1 [ARRDC1]-mediated microvesicles) into extracellular space. ARMMs have the capacity to carry signalling molecules and thus may represent a new way of intercellular communication. *Methods*: Sucrose gradients ultracentrifugation; mass spectrometry; CRISPR knock-out. *Results*: Secreted ARMMs contain NOTCH2 protein and mediate a non-canonical intercellular NOTCH signalling. Mass-spectrometry of purified ARMMs from multiple types of mammalian cell lines identified NOTCH2 as a protein component of the vesicles. The NOTCH protein in ARMMs was not full length but contains the critical intracellular region (NICD) that is required for its transcriptional activity in the nucleus. The incorporation of NOTCH2 into ARMMs is likely facilitated by the ITCH E3 ligase, which is also secreted into ARMMs. ARRDC1 overexpression increased the short form of NOTCH2 incorporation into ARMMs without affecting the cellular NOTCH2 protein level. Conversely, CRISPR-mediated knockout of ARRDC1 dramatically reduced the amount of short form of NOTCH2 in secreted ARMMs. Re-expression of ARRDC1 in ARRDC1-knockout cells recovered NOTCH2 in ARMMs to normal level. Using transwell experiment, we further showed that GFP-tagged NOTCH2 can be delivered into recipient cells to regulate NOTCH2 target genes. *Summary/conclusion*: We identified a ligand independent NOTCH2 signal pathway that is dependent on ARMMs. Our data show that NOTCH2 can go through extracellular vesicle ARMMs and move from donor cells to recipient cells in distance without cell–cell interaction and ligand binding.

P-XV-15

Studying the influence of extracellular vesicles on mouse kidney development using the dissociation re-aggregation model

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Introduction: The fast progress in extracellular vesicle (EV) research has shown the importance of vesicles in cell–cell communication. Here, we present our techniques and advances in studying how different cell types communicate via vesicles influencing morphogenesis. *Methods*: EVs were purified from Renca renal carcinoma and mK4 metanephric mesenchyme derived cell culture media by sequential and sucrose gradient ultracentrifugation and analyzed by electron microscopy (EM), nanoparticle tracking analysis (NTA) and Western Blotting using EV-specific markers (Hsc70, CD81 CD[AS1] 63). Renca cells were cultured either under normoxia or hypoxia (1% oxygen) for 24 hours before medium collection. Metanephric mesenchyme (MM) cells were isolated from E11.5 mice embryos by micro-dissection and cultures as a monolayer. Morphol-

ogy, proliferation and viability of monolayer MM cells treated with Renca EVs were assayed by immunofluorescent staining and live cell imaging using IncuCyte ZOOM (Essen BioScience). Also dissociated MM cells were re-aggregated in co-culture with different cell types, such as mK4 cells [AS1]. *Results*: We found that conditioned media from Renca and mK4 cells influences growth of MM cells in culture. EM and NTA analysis showed hypoxia treatment to greatly increase the EV count in cultured media. Hypoxia also changes the size distribution of EVs. In addition, we have generated cell lines (mK4-Wnt4 FLAG and mK4-Wnt4 TM FLAG) stably expressing a FLAG-tagged Wnt4 protein and a transmembrane version of it. Wnt4 was found in EVs isolated from mK4-Wnt4 Flag cells but not in those from mK4-Wnt4 TM Flag cells. To test potential effect of these cell lines, we have introduced them into the embryonic kidneys using dissociation/re-aggregation assay. We observed that the parental cell line (mK4) have negative effects on the kidney development in vitro while mK4-Wnt4 cell lines rescued the phenotype. *Summary/conclusion*: The in vitro cell and organ culture models have proven to be useful tools in finding out the roles of EV during inductive events and organogenesis. Signalling between different cell types and the effects of vesicles coming from outside sources can be systematically investigated in our setup.

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Poster session XVI - EVs in biofluids and reproduction
Chairs: *Rebecca Dragovic and Christina Beltrami*

P-XVI-1

Maternal serum-derived exosomes evaluated early in the second trimester of pregnancy provide predictive biomarkers of risk for spontaneous preterm birth (SPTB)

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Introduction: This study characterizes proteomic differences found in circulating serum exosomes during the early second trimester of pregnancy as potential biomarkers of spontaneous preterm birth (SPTB). The proteomic content may reflect subtle changes in the maternal-foetal interface and serve as early risk predictors of SPTB. *Methods*: Frozen serum samples were obtained from healthy pregnant women (weeks 15–17 gestation) without prior history. Exosomes were isolated using size exclusion chromatography (SEC)/gel filtration methods, and proteins were TRIzol extracted and analyzed in 2 separate LC/MS studies, first using an open proteomic platform ($n = 48$) and then a targeted MRM panel ($n = 60$). Peptide features were blindly analyzed in patient cohorts of full term ≥ 37 weeks and SPTB at ≤ 34 weeks. *Results*: In the open proteome learning set (study 1; $n = 48$ patients), unique expression pattern was observed amongst 99 proteins statistically identifying 2 cohorts ($p < 0.05$) with the potential to serve as biomarkers. Eighteen proteins contained a minimum of 2 significant peptide fragments differentiated between the preterm and term populations (12 preterm, 6 term) in both first and second pregnancy groups. Area under the ROC curve analysis indicates that each of these 18 proteins of interest had robust values of >0.73. The protein biomarkers identifying SPTB outcome map to inflammatory and cell injury pathways that differ from the term profile. These proteins of interest were included in a targeted MRM assessment of >100 exploratory proteins using a gel filtration isolation mini-SEC kit (study 2; $n = 60$ patients). Area under the ROC curve for numerous targeted proteomic 8-plex biomarker combinations reveal a robust AUC >0.8 suggesting the potential for high sensitivity and specificity for these exosomal markers. *Summary/conclusion*: A novel library of exosomal biomarkers with statistical significance has been identified with potential clinical usefulness for the early identification of women at risk for preterm birth. The proteins of interest demonstrate a functional involvement of the NF-kB inflammatory pathway detected early in gestation in the SPTB population. These observations may lead to an improved understanding and future management the underlying events leading to preterm birth.

P-XVI-2

Exosomes isolated from trophoblast cells modulate the response of skeletal muscle cells to insulin under diabetic conditions

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Please see Oral with poster C

P-XVI-3

Follicular fluid extracellular vesicles regulate cumulus-oocyte-complex expansion through preferential uptake by cumulus cells

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Please see Oral with poster C

P-XVI-4

Diversity of extracellular vesicles in human ejaculates identified by cryo-electron microscopy and tomography

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Please see Oral with poster A

P-XVI-5

Transfer of colostral exosomes during early calf development

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Introduction: Colostrum is a nutrient-dense biofluid high in immunological factors, bioactive molecules and extracellular vesicles (EVs), such as exosomes (exo). It is well-known that EVs are mediators of intercellular communication; therefore, we herein looked into the potential transfer of EVs, more specifically exo, from colostrum to neonatal calves. *Methods*: Blood was drawn from calves pre colostrum feeding and at various postprandial points. Exo were isolated from colostrum and plasma by differential ultracentrifugation and density gradient centrifugation and further analyzed by western blotting for CD63 and MFGE8 as a general and milk-specific marker, respectively. Particle count and diameter in distinct gradient fractions were evaluated by NTA. Additionally, protein quantification (BCA assay) and RNA analysis (RT-qPCR) were also performed in Exo fractions. *Results*: While most Exo in bovine plasma are found in fractions of $\leq 30\%$ sucrose (≤ 1.13 g/ml), colostral Exo feature a higher density, floating in 40–50% sucrose (1.18–1.23 g/ml). After the initial colostrum feeding post-partum, the calf's particle count in 40% sucrose, but not 30% sucrose, increases significantly. This indicates a transfer of colostral Exo to the calf's circulation. Consistently, total Exo protein in calf plasma increases linearly over the first days post-feeding. These findings are supported by immunoblotting, while plasma Exo from adult animals and neonatal calves are MFGE8-deficient, and the milk-specific MFGE8 signal is found in calf plasma Exo as early as 6 hours post-feeding. Furthermore, RT-qPCR shows a significant increase of milk-specific micro RNAs (e.g. miR-200b, miR-148a and miR-30a-5p) in calf plasma post colostrum feeding, also suggesting an uptake from milk. *Summary/conclusion*: In summary, we found that exo from bovine plasma and colostrum feature distinct flotation densities. Both protein and RNA markers as well as particle density in the calf's circulation indicate that exo from colostrum are taken up in early calf development.

P-XVI-6

Lipid content of extracellular vesicles isolated from bovine ovarian follicles according to oocyte competence

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Introduction: Three cellular types isolated by a basement membrane compose ovarian follicles: granulosa, cumulus and an oocyte. Part of the crosstalk among these cell types occurs in a cavity filled by follicular fluid (FF) called antrum. Intercellular communication within the follicle is crucial to induce cell proliferation, differentiation and to dictate the oocyte competence to sustain embryo and foetal development. Recently, extracellular vesicles such as exosomes and microvesicles were identified within the follicular fluid and suggested as mediators of cell communication. Extracellular-vesicles can transfer bioactive molecules such as lipids, proteins, mRNA and miRNAs. Our hypothesis is that extracellular vesicles from bovine FF present different lipid composition associated to oocyte competence. *Methods*: In order to test this, hypothesis bovine ovaries were collected from slaughterhouses and follicles between 3–6 mm were individually dissected. Follicle contents were separated under a stereomicroscope to allow the collection of FF and the oocyte. Cells and cellular debris were removed by differential centrifugation before the storage of FF at −80°C. Oocytes were individually placed in a culture drop and 18 hours later were analyzed for maturation. Activation of the oocytes to generate parthenogenetic embryos started 26 hours after the beginning of maturation. After 7 days in culture, we accessed the development and grouped the follicular fluid according to developmental outcome. Developmental groups were formed according oocyte competence: 1) mature oocytes that did not start embryo development (non-competent); 2) cleaved embryos that blocked development prior to blastocyst stage and 3) blastocysts (competent). Based on the competence groups, we isolated extracellular vesicles from pools of 10 follicular fluids. Lipids were extracted from the pools of extracellular vesicles and analyzed by tandem mass spectrometry. *Results*: Our results demonstrated that our model to access oocyte competence individually is viable and produce at least 30% of parthenogenetic blastocysts. Based on lipids analysis, we identified different lipids enriched according to the development competence of the oocytes. We identified 6 lipids associated with non-competent oocytes; monoalkenyl diacylglycerol (MADAG 52:8+NH4 (−FA 18:1 (NH4)) and MADAG 48:8+NH4 (−FA 16:1 (NH4)) is an example of the lipids found in extracellular vesicles. Three lipids were associated with competent oocytes, for example, digalactosyldiacylglycerol (DGDG 36:2+NH4 (−DGDG (NH4))), which was identified to be present in extracellular vesicles. *Summary/conclusion*: Thus, our results demonstrated that lipids are differently expressed in extracellular vesicles and are associated with oocyte competence. Further experiments are necessary to explore the different lipid molecules present in the extracellular vesicles and their role during follicle growth and oocyte maturation. Supported by FAPESP GIFT-2012/50533-2; CEPID-CTC-2013/08135-2; BPD-2013/10473-3.

P-XVI-7

Primary human endothelial cells: targets for syncytiotrophoblast vesicles and reprogramming

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Introduction: Preeclampsia (PE) is a pregnancy-associated disorder defined by proteinuria and hypertension. The pathological hallmark is general endothelial dysfunction. In PE, there is increased release of the placenta specific syncytiotrophoblast extracellular vesicles (STBEVs), which trigger an inflammatory response. EVs can be internalized, and transfer proteins and RNAs to recipient cells, thereby altering gene expression. In this study, we investigated STBEV uptake by primary endothelial cells in-vitro, and effects on gene expression. *Methods*: Human placental cotyledons, from normal and PE pregnancies, were perfused using the dual ex-vivo perfusion method. Maternal perfusate was collected, and STBEVs were isolated by ultra-centrifugation at 110,000 × g. STBEVs size and concentration were determined using nanoparticle tracking analysis (NTA). STBEVs labelled with PKH67 were added to cultured primary human coronary artery endothelial cells (HCAECs). Uptake was evaluated at specific time points; 15, 30, 45, 60, 90 minutes, 6 and 24 hours, and cells were fixed in PFA and nuclei labelled with Hoechst 33342. Cells were visualized by fluorescence microscopy. Total RNA was extracted from cells treated with STBEVs for 24 hours and gene expression evaluated by RTqPCR using TaqMan Human eNOS array as templates. *Results*: STBEV uptake was observed in HCAECs after 45 minutes, followed by progressive increase throughout the time course. At 24 hours, the majority of cells appeared to have internalized the STBEVs. There was no difference in uptake between PE and normal STBEVs. After 24 hours treatment, several genes were down regulated in cells treated with PE STBEVs compared to normal. *Summary/conclusion*: This is the first demonstration of HCAECs as STBEV targets. Clearly, HCAEC internalize STBEVs, and our data suggests that STBEVs can affect recipient cell gene expression. This indicates that STBEVs may play a role in reprogramming endothelial cells and, thereby, contribute to hypertension seen in PE.

P-XVI-9

Isolation of protein and exRNA from patient plasma by novel size exclusion chromatography protocols reveals biosignature of vesicular and non-vesicular origin

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Introduction: Extracellular RNA (exRNA) and proteomic content within extracellular vesicles (EVs) are of substantial interest as a means of cell–cell communication and the provision of clinical biomarkers. However, exRNA may also circulate in non-vesicular protein and lipid complexes. This study evaluated novel approaches to plasma fractionation and EV dispersion that might affect exRNA and protein yield from both vesicular and non-vesicular compartments. *Methods*: Size exclusion chromatography (mini-SEC) columns containing 10 ml volumes of agarose CL-2B were used to fractionate 1 ml volumes of plasma with novel dispersion buffers varying in ionic and oncotic make up. Profiling of individual fractions eluted from mini-SEC was conducted by Western blot with total protein stain or western blot of canonical EV proteins. EV presence was also determined by transmission electron microscopy (TEM). RNA analysis was conducted following spin-filter concentration, TRIzol extraction and purification by RNeasy. *Results*: EVs were identified in effluent fractions 3 and 4 (peak 1) as defined by the presence of CD63, CD9 and CD81 as well as the observation of characteristic ultrastructure determined by TEM. Fractionation of plasma with low ionic strength buffers had the highest content of protein and RNA (>50 ng/ml plasma) and resulted in the co-elution of EVs with high abundant contaminating plasma proteins. Plasma fractionation using selected organic buffers resulted in a reduced background with clearer identification of EV-associated antigens (CD63). RNA was harvested in increased concentrations using low ionic strength buffers suggesting a possible role for protein aggregation in the prevention of hydrolysis. Large format exRNA was also isolated from non-particulate non-EV fractions (peak 2) suggesting non-vesicular origin. *Summary/conclusion*: The use of various elution buffers with mini-SEC warrants further investigation as a means of selective exRNA or vesicle protein enrichment. In particular, the isolation of vesicular and non-vesicular exRNA may have clinical value. We are currently investigating these protocols to determine patient categorisation in a variety of clinical settings that can support multiplexed proteomics and deep sequencing in an efficient manner.

P-XVI-10

Aggregation of extracellular vesicles induced by high-speed sedimentation and re-suspension: a combined cryo-electron microscopy and flow cytometry study

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Introduction: Extracellular vesicles (EVs) attract high interest due to their multiple physio-pathological implications. Intense efforts are devoted to their isolation, a first step towards a potential identification of biomarkers. Most studies on EVs from blood plasma, other body fluids or cell culture supernatants involve one or several steps of high-speed centrifugation, in order to separate EVs from proteins and other soluble components. However, sedimentation/re-suspension procedures may lead to co-isolation or aggregation of EVs (1) and, hence, to false results in EV phenotype or composition analysis. We have performed a detailed investigation of the influence of high-speed centrifugation/re-suspension procedures on the aggregation of EVs from platelet free plasma (PFP) samples. Cryo-electron microscopy (EM) and immuno-gold labelling were used for imaging EV aggregates, and flow cytometry (FCM) was used for quantifying them. *Methods*: For cryo-EM and FCM, PFP samples were analyzed either unprocessed or after one or several steps of centrifugation at 20,000 g for 30 minutes and re-suspension in a volume of buffer equal to the initial volume. For cryo-EM, samples were double labelled with 4-nm annexin-5 gold nanoparticles (Anx5-gold-NPs) and 10-nm-gold-NPs conjugated to either anti-CD41 or anti-CD235a antibodies (mAbs), in order to label phosphatidylserine-positive EVs, platelet and erythrocyte-derived EVs, respectively. For FCM, EVs were double labelled with Anx5-Cy5 and either anti-CD41-PE or anti-CD235a-PE mAb. EV concentrations were determined by triggering detection both on a fluorescence signal and on the forward scatter (FS) signal (2), using a Gallios flow cytometer. *Results*: By cryo-EM, we found that sedimentation/re-suspension procedures induce the formation of large clumps of EVs, of several μm in diameter. It is important to note that we have not observed such aggregates in PFP samples that have not been centrifuged, over more than 100 PFP samples so far. EV aggregates contained tens to hundreds of EVs, of either spherical or tubular morphology (3), and of various phenotypes. By FCM, we observed a large increase in the number of EVs detected by FS triggering, which must correspond to the μm-size aggregates observed by cryo-EM. In addition, the number of events double labelled with Anx5-Cy5 and anti-CD41-PE mAb, and to a lesser extent with anti-CD235a-PE mAb, increased significantly. The absolute amount of sedimentation-induced EV aggregates was variable between experiments. *Summary/conclusion*: We show here that sedimentation/re-suspension procedures used for EV isolation induce the formation of large EV aggregates. The presence of such aggregates may lead to erroneous conclusions concerning the existence of EVs bearing multiple antigens and also in the proteomic or genomic analysis of EV sub-populations.

References

1. Yuana Y, Levels J, Grootemaat A, Sturk A, Nieuwland R. Co-isolation of extracellular vesicles and high-density lipoproteins using density gradient ultracentrifugation. J Extracell Vesicles. 2014; 3:23262, doi: http://dx.doi.org/10.3402/jev.v3.23262
2. Arraud et al., J. Thromb. Haemost. doi: 10.1111/jth.12767.
3. Arraud et al., J. Thromb. Haemost. 2014;12:614–627.

P-XVI-11

Single-step size exclusion chromatography enriches for plasma vesicle-associated miRNAs that are directly applicable for cancer biomarker discovery using small RNAseq

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Introduction: Circulating microRNAs are generally believed to have biomarker potential for diagnosis, prognosis and monitoring of treatment response in multiple diseases, including cancer. Extracellular vesicles (EVs) are actively secreted by tumour cells and normal cells, and they encapsulate miRNAs, protecting them from degradation by RNases. Circulating miRNAs can also be associated with and protected by proteins and HDL; moreover, dying cells release biomolecules (i.e. RNA, DNA, protein) into circulation. The use of tumour-derived EV as biomarker has many advantages: 1) they reflect living tumour cells, 2) EV-associated miRNAs are stable in archived material, 3) EV-secreted miRNAs outnumber other types, 4) EV-associated miRNAs promote tumourigenic processes, 5) tumour vesicles carry proteins on their surface that trace them back to the tumour cells. Thus for circulating miRNA biomarker research it maybe of critical importance to isolate (tumour) EV from circulation. *Methods*: We performed size exclusion chromatography using sepharose CL-2B and commercially available qEV columns (iZON™) on 1.5 ml plasma to separate EV from protein/HDL. *Results*: Electron Microscopy showed the presence of EV in the vesicle fractions but not in the protein/HDL fractions. Particle analysis using qNano (iZON™) showed that the vesicle fractions are highly enriched in particles with a size-distribution that corresponds to the EM images. Moreover, western analysis showed the presence of exosome-marker CD63 in the vesicle fractions, but not the protein/HDL fractions. RNA was isolated using TRIzol from the EV and protein/HDL fractions separated by SEC, followed by RT-PCR. The EV fractions were highly enriched for vtRNA1-1, let7a and miR142-3p, whereas protein/HDL fractions are enriched for miR92a, miR21 and miR451. EV isolated using SEC contained sufficient RNA of suitable quality for screening using RNAseq. Classical Hodgkin lymphoma plasma EVs had a distinct smallRNA profile compared to healthy donor plasma EVs. We validated potential miRNA biomarker candidates by RT-PCR. *Summary/conclusion*: All together these results indicate that EVs isolated using SEC are useful for miRNA biomarker discovery in cancer patients.

P-XVI-12

Extracellular vesicles and small RNA of the cervicovaginal compartment in Macaques

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Please see Oral with poster A

P-XVI-14

Optimizing procedures for transport and processing of clinical cerebrospinal fluids to preserve extracellular vesicular (EVs) miRNAs

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Introduction: Tumour specific genetic material can be detected in Extracellular Vesicles (EVs) isolated from blood, cerebrospinal fluid (CSF) and other bio-fluids of glioblastoma patients. As such, EVs have emerged as a promising platform for biomarker discovery in patients with glioblastoma. Clinical translation of this platform will require trials necessitating transport of bio-specimens to centralized sites for molecular characterization. However, the optimal procedure to transport clinical EV samples remains poorly characterized. *Methods*: EVs were isolated from CSF by differential centrifugation, and subjected to lyophilization in a benchtop manifold freeze-drier. qRT-PCR was performed to determine the level of miR-21, miR-24, miR-103 and miR-125. *Results*: CSF EVs that were lyophilized and stored at room temperature (RT) for 7 days exhibited a 30–45% reduction in the number of EVs. This reduction was not associated with changes in EV morphology on transmission electron microscopy, suggesting that lyophilization induced EV disintegration. Consistent with this observation, the total RNA content of the lyophilized EVs was 30–45% lower than the input material. Similar reductions were observed when select miRNAs (miR-21, miR-24, miR-103 and miR-125)

were quantitated by qPCR. In contrast, the EV number and morphology remained largely unchanged if CSF were stored at RT. Total RNA, miR-21, miR-24, miR-103 and miR-125 levels were well preserved under this condition. Stability of miRNAs was observed for up to 7 days. A single cycle of freezing and thawing did not significantly alter EV number, morphology, RNA content or miRNA levels. However, incremental decreases in these parameters were observed after 2 cycles of freezing and thawing. *Summary/conclusion*: These results suggest that EVs in CSF are stable at room temperature for at least 7 days and can be transported as such. Repeated cycles of freezing and thawing should be avoided to minimize experimental artifacts.

P-XVI-15

F-NTA and plasmonic nanoparticles: new tool to visualize, measure, count and characterize exosomes in biological samples

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Introduction: We illustrate a novel method for characterization of size and immunophenotype of exosomes; this relies on a custom software based on multicolor and possibly fluorescent nanoparticle (NP) tracking analysis (F-NTA), which can provide size distribution and relative presence of differently labelled species visualized by optical microscopy. We exploited membrane fluorescent staining of exosomes, fluorescent antibodies and binding by functionalized metallic NPs; the last are detected via confocal microscopy (CM) thanks to the elastic scattering due to their strong plasmonic resonance peak, and their high mass also allows for the separation of specific biomarker-expressing exosomes from other biological entities by simple centrifugation. *Methods*: Engineered hexapeptide coating of $\sim$30 nm gold NPs allowed for the conjugation with antibodies (against paradigm exosome markers, e.g. CD63 and CD9, or specific tumour markers), while decreasing unspecific binding. Incubation was performed with either purified or spiked-in exosomes from human tumour cell cultures. Membrane staining of exosomes was included, and multiplexing analysis of isolated exosomes was done by mean of CM and F-NTA. *Results*: Analysis of exosomal biomarkers by combination of CM and F-NTA enabled quantitative analysis of extracellular vesicle populations in biological samples. Work flow and analysis of results are further facilitated by ad-hoc development of the dedicated software. We show that commonly used isolation protocols provided preparation of $\sim$60% CD9 or CD63+ exosomes, while our approach yielded more than 90% purity. *Summary/conclusion*: The presented method can provide more operator-independent results, high accuracy in vesicles sizing and fast profiling of their composition; moreover, the engineered NPs allow simultaneous purification of specific biomarker-expressing exosomes for any kind of further analysis.

P-XVI-16

Release, cellular origin, and fate of microvesicles during circulation of whole blood over adsorbents for lipid apheresis

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Introduction: The aim of this in vitro study was to assess the generation of microvesicles (MVs) during passage of whole blood over hemapheresis columns. We examined the cellular origin of released MVs as well as their interaction with adsorbent polymers and with blood cells. *Methods*: Freshly drawn blood anticoagulated with acid citrate dextrose-A (1:20) and 0.8 IU/ml heparin was circulated for 4 hours over columns containing methacrylate-based resins which are clinically applied in lipid apheresis to remove LDL cholesterol. A total volume of 50 ml of blood was used, and the adsorbent columns were downscaled equivalent to clinical use. Samples were taken every hour and cells were quantified using a blood cell counter. Flow cytometry was performed with a Gallios Flow Cytometer (Beckman Coulter) after calibration with fluorescent beads to cover the MVs (0.5 and 0.9 μm) and the cell size ranges (0.9 and 3 μm). MVs were defined as annexin V^+ (AV) events in the MVs gate, and their cellular origin was identified using the following markers: $CD45^+$, $CD14^+$ (monocytes), $CD45^-$, $CD41^+$ (platelets), $CD45^-$ and $CD235a^+$ (red blood cells). *Results*: In freshly drawn blood; red blood cells, platelets and leukocytes accounted for 94.9%, 5% and 0.1% of all blood cells, respectively, while 48% of all MVs were platelet-derived. Passage of blood over the adsorbents resulted in increased levels of red blood cell- and platelet-derived MVs. While only 0.2 and 2.3% of red blood cells and platelets exhibited AV reactivity, 56% of all leukocytes and 75% of all monocytes were AV^+, indicating their association with MVs. To specify the cellular origin of MVs adhering to monocytes, we analyzed aggregates of red blood cell-derived MVs with monocytes ($AV^+CD235a^+CD14^+CD45^+$ cells) as well as aggregates of platelet-derived MVs with monocytes ($AV^+CD41^+CD14^+CD45^+$ cells) and found that nearly 80% of MVs adhering to monocytes were derived from platelets. *Summary/conclusion*: MVs are markers for cellular activation in apheresis.

P-XVI-17

Radiolabeling extracellular vesicles

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Introduction: Extracellular vesicles (EVs), which carry abundant biomolecules such as lipids, proteins and RNAs, act as important messengers in intercellular communications and get involved in a variety of biological processes. EVs have emerged as potential therapeutic targets as well as vehicles for drug and gene delivery. Our objective of this project is to develop methods for radiolabeling EVs that would facilitate the understanding of in vivo distribution and pharmacokinetics. *Methods*: EVs were isolated from FBS and cell culture media by serial ultracentrifugations and characterized with size-exclusion high performance liquid chromatography (SE-HPLC), transmission electron microscopy (TEM), NanoSight, ELISA and total protein level measurement. Through chemical bond or lipid insertion, the purified EVs were conjugated with chelators such as DOTA and DTPA. Size distribution of EVs after modification with chelators was evaluated. Those modified EVs were incubated with In-111 or Cu-64 to form radioactive complexes like EV-DOTA-Cu64 and EV-DMPE-DTPA-In111. Stability of those radiolabeled EVs in PBS was tested under room temperature and 37°C. *Results*: EVs were successfully isolated from FBS and cell culture media. SE-HPLC showed single peak at 7.5 minutes, and all other characterizations gave supportive *Results*: After conjugation with DOTA or DTPA, size distribution of EVs did not show apparent change. The SE-HPLC chromatogram showed the success of radiolabeling with high yield, and radiolabeled EVs were stable in PBS under room temperature but not under 37°C. *Summary/conclusion*: EVs isolated from FBS or cell culture media were modified by conjugation with DOTA or DTPA and radiolabeled with Cu64 and In-111.

P-XVI-18

Postprandial increase in blood plasma concentration of tissue factor-bearing extracellular vesicles

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Introduction: Tissue factor (TF)-bearing extracellular vesicles (EVs) have been suggested to play an important role in both normal

hemostasis and thrombus formation (Zwicker et al., 2011, Arterioscler Thromb Vasc Biol). Postprandial activation of the coagulation system has been reported in various studies, and a number of underlying mechanisms have been suggested. Increased levels of circulating EVs (Tushuizen et al., Thromb Res, 2011) and TF antigen (Motton et al., Thromb Haemost, 2005) in the postprandial state have been observed separately. We aimed to investigate if TF-bearing EVs are released into the circulation following food intake. *Methods*: Platelet free blood plasma from 20 healthy persons in the fasting state and following a non-standardized meal was analyzed by flow cytometry (FC) to detect possible postprandial changes in the concentration of phosphatidylserine(PS)-exposing EVs and EV subgroups exposing CD41, CD146 and CD62E as markers of cellular origin and CD142 (TF). The applied flow cytometric method mainly detects EVs with a diameter of more than 200 nm. Furthermore, the samples were analyzed by the recently developed EV Array (Jørgensen et al., 2013, JEV) focusing on smaller EVs. For comparisons of fasting and postprandial results Wilcoxon matched-pairs signed-rank test was used. *Results*: Significantly increased plasma concentrations of PS-positive EVs, EV subgroups derived from platelets and endothelial cells as well as TF-bearing EVs were observed in the postprandial as compared to the fasting state using FC, the median increase of TF-bearing EVs being 98%. In contrast, no significant change in the fraction of TF-bearing EVs detected by EV Array was observed. *Summary/conclusion*: Food ingestion induces EV release from platelets and endothelial cells and increased concentrations of TF-bearing EVs measurable by FC. Though their effect on the coagulation system is not uncovered, the impact of prandial state on the concentration of TF-bearing EVs should be considered when designing studies on these particles' potential as biomarkers of risk of thrombosis.

P-XVI-19

Optimizing procedures for transport and processing of clinical cerebrospinal fluids to preserve extracellular vesicular (EVs) miRNAs

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Introduction: Reliable and time-efficient enumeration of microvesicles (MVs) remains a critical methodological challenge. Present counting strategies often combine bead based enumeration methods with gating strategies utilizing either beads or fluorescence markers. Here, we propose a novel fixed platelet strategy and compare its accuracy to established gating strategies and enumeration methodologies. *Methods*: Samples were analyzed on a BD Aria III flow cytometer with small particle detection module allowing MV enumeration via several strategies. Gating was guided via either: 1) fluorescent MV events, 2) scatter-based strategies using either polystyrene beads (300–1,000 nm) or 3) by excluding debris/instrument noise on the low end and purified fixed platelets on the upper end. MVs sampled from bronchoalveolar lavage fluid, plasma or cell culture were enumerated within these defined gates based on known concentrations of: 1) fluorescent beads, 2) fixed human platelets or 3) actual volume consumed during acquisition (MV/μl). *Results*: In comparison to MVs/μl assayed (volume determined using the sample weight pre and post analysis and the specific gravity of the analyte), the technique with the greatest agreement was the novel platelet based strategy, while bead based enumeration frequently under- or over-counted. Enumeration when gating by fluorescence or the expanded (platelet based) scatter gate yielded comparable results when using a sufficiently high-sensitivity cytometer. *Summary/conclusion*: The presented fixed platelet MV enumeration strategy allows for greater agreement than bead based strategies in relation to mass consumed per sample (MV/μl). Platelet based gating enables a larger MV analysis gate which closely matched fluorescent gating unlike conventional bead-based gates which may be advantageous in samples where fluorescent discrimination of MVs is not possible.

P-XVI-20

Surface plasmon enhanced fluorescence spectroscopy for EV analysis

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Introduction: Extracellular vesicles (EVs) are gaining interest in many fields of research, but still methods for their analysis are mostly complex and lack sensitivity or specificity. Recently plasmonic biosensors appeared as EV analysis tools making use of surface plasmon resonance for detection of EVs captured by specific antibodies to the sensor surface. Additionally, surface plasmons have the capability of coupling with fluorophores in their proximity and thus enhancing fluorescence emission. This attribute is employed in surface plasmon enhanced fluorescence spectroscopy (SPFS), on basis of which we want to develop a method for EV analysis. *Methods*: EVs from cell culture supernatant of the cell lines CaOV3, HCT8 and BJ-1 were purified by filtration and ultracentrifugation. Additionally, biological samples were used, i.e. ascites and plasma of ovarian cancer patients and plasma of healthy individuals. The vesicles were analyzed with flow cytometry in fluorescence mode examining the surface markers CD63, CD9 and PS as general EV makers and EpCAM and CD24 as markers for ovarian cancer cell derived EVs. A model system using SPFS for EV analysis was built, where annexin-V is used as capturing molecule on the sensor surface. *Results*: With the means of flow cytometry in fluorescence mode EVs purified by ultracentrifugation could be detected and analyzed. The general EV markers were, as expected, present on EVs in all samples. The ovarian cancer specific markers were only present on EVs from CaOV3 cell culture supernatant and ascites, but not from plasma of ovarian cancer patients, indicating that the concentration of cancer derived EVs in plasma might be below the detection limit. The SPFS model system could detect standard vesicles down to fM concentrations. *Summary/conclusion*: Combining the specificity of molecular binding, the sensitivity of SPFS and the simplicity of plasmonic sensors, SPFS can provide a cheap and simple platform for EV analysis for scientific and clinical application.

P-XVI-21

Multimodal reporter system for simultaneous tracking of intraperitoneal ovarian tumour growth and tumour-derived extracellular vesicle biodistribution in vivo

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Introduction: Extracellular vesicles (EVs) released by neoplastic cells can affect host cells and facilitate tumour growth. EVs have been shown to play a role in ovarian cancer progression using in vitro experimental set-ups. This study aims to establish in vivo model of ovarian cancer to longitudinally examine tumour derived-EV release and subsequent uptake by normal cells in a mouse xenograft model. *Methods*: Two human ovarian cancer cell lines, A2780 and OVCAR5,

have been stably transduced with firefly luciferase (Fluc) and a membrane-bound *Gaussia* luciferase (GlucB) reporter to investigate cell growth and EV biodistribution, respectively. To semi-quantitate EVs via the GlucB reporter, nanoparticle tracking anslysis was compared to GlucB activity. Both cell lines were injected intraperitoneally into nude mice (A2780 n = 3, OVCAR5 n = 3, no injection control n = 3). *Results*: The multimodal system was first tested in vitro to demonstrate that the Fluc signal reflects tumour cells growth. GlucB was confirmed to label vesicles by sucrose gradient fractionation of conditioned media followed by western blot analysis (GlucB and Alix) and GlucB activity. Tumour growth is traced once a week by Fluc signal. In parallel, serum and urine were collected to detect GlucB-labelled EVs in biofluids. Biodistribution of the EVs will also be evaluated via GlucB signal in collected omentum, spleen, kidneys and liver. *Summary/conclusion*: This study aims to elucidate EV biodistribution in the course of intraperitoneal ovarian cancer growth and its implication during disease progression. This work has been supported by NIH NCI P01CA069246 grant, NIH NCI U19 CA179563 supported by the NIH Common Fund, through the Office of Strategic Coordination/Office of the NIH Director.

P-XVI-22

Measurement of extracellular vesicles in the urine of patients with kidney diseases

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Introduction: A test that is minimally invasive and sensitive could improve early diagnosis of kidney diseases and facilitate early medical intervention, decreasing progression to end stage renal disease. Proteomic approaches to investigating kidney diseases have identified that urinary EVs (uEVs) are involved in kidney damage or kidney repair by affecting various aspects of kidney disease, including, podocyte injury, endothelial injury, tubulointerstitial damage, inflammation and repair. For example, uEVs containing Podocalyxin correlate with disease severity in patients with diabetic nephropathy while uEVs with Podocin are specific markers of podocyte injury. To date, detection and quantitation of these particular EVs in urine have not been done by flow cytometry. This study sought to develop methodology for the flow cytometric profiling of various EVs in urine to discern a biomarker pattern for the early detection of podocytopathies before they progress to severe disease. *Methods*: uEVs were assessed using a modification of methodology previously developed to measure plasma EVs. Briefly, urine collected from healthy subjects is centrifuged at 2,500 × g for 15 minutes followed by staining with a panel of fluorochrome-labelled cell-type-specific antibodies detecting CD3, CD31, CD41, CD144, Annexin, anti-Podocin and anti-Podocalyxin. Data was acquired on a modified Becton Dickinson FACSCanto A, and the pattern and number of uEVs was determined using FlowJo Software (Treestar, Inc). *Results*: In a proof of concept study, over 40,000 uEVs/μl were isolated from normal human urine and cell-source profiled, revealing a distribution of 3% CD3, 1% CD144, 3% CD31 and 2% CD41. Optimization studies indicated that refrigeration diminished yield by an average of 86%. *Summary/conclusion*: Methodology for the flow cytometric detection of cell-source specific uEVs was demonstrated. This will allow identification of a signature pattern of uEVs that can serve as the basis for a clinical screen for kidney diseases.

Poster session XVII - EVs as cancer biomarkers
Chairs: *Lucia Languino and Hidetoshi Tahara*

P-XVII-2

Chip-based exosome RNA analysis for monitoring drug efficacy

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Introduction: Exosomes have emerged as a new class of circulating cancer biomarker. These vesicles abound in biofluids and harbour diverse molecular constituents, thereby offering a minimally invasive avenue for longitudinal monitoring. Despite such clinical potential, it remains challenging to utilize exosomes for objective evaluation of drug efficacy: 1) conventional methods for exosome analyses often involve time-consuming and labour-intensive processing, and 2) effective enrichment of cancer exosomes is necessary to unmask efficacy signature. *Methods*: We herein report the development of a new microfluidic device for on-chip exosome RNA analysis. The system enables direct isolation and real-time evaluation of total RNA. Employing this technology, we compared the mRNA profiles of glioblastoma-derived exosomes against those of parental cells. *Results*: We identified key exosomal markers predictive of drug efficacy. Likewise, we analyzed the mRNA contents of cancer exosomes directly enriched from blood samples of glioblastoma patients. *Summary/conclusion*: Our study suggests that exosomal mRNA profile could be an early indicator of drug efficacy and a potential molecular stratifier for human clinical trials.

P-XVII-3

Extensive analysis of plasma EVs from small cell lung cancer patients of the RASTEN study compared with healthy controls

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Introduction: Lung cancer is a leading cause of cancer death worldwide with an increasing incidence, especially among women. Small cell lung cancer (SCLC) is associated with early metastasis and poor prognosis with 5-year survival rates of approx. 10 and 5% in limited and extensive disease (ED), respectively. It thus remains an important challenge to identify common biomarkers and targets for new therapies of SCLC. Here, we use an internationally unique study-based SCLC cohort (RASTEN: a randomized phase III study of standard treatment ± enoxaparin) to elucidate the potential role of EVs as non-invasive biomarkers of lung cancer. *Methods*: Plasma from 245 SCLC patients (108 with limited and 137 with ED) and 60 matched control subjects were subjected to analysis by the extracellular vesicle array (EV Array), encompassing 29 capturing antibodies targeting well known EV-associated as well as hypoxia and lung cancer-related proteins. The binding of EVs was visualised and scored with a cocktail of biotin-conjugated CD9, CD63 and CD81 antibodies. *Results*: We were able to detect and phenotype EVs in all samples from only 10 μL of plasma. In a multivariate classification model, a panel of 20 markers was found to distinguish diseased from controls with an area under the curve (AUC) of 0.81. The 20-marker model had a sensitivity of 0.74 and a specificity of 0.83, and it classified patients with 75.5% accuracy. CD106 and osteopontin were of particular interest as their expressions were significantly different ($P < 0.01$ and $P < 0.05$, respectively) between limited and ED. Further, the expression of osteopontin, CAIX, endoglin and CD276 was clearly correlated in the SCLC patients, but not in the healthy group, which may indicate that EVs carrying these markers are produced either by the same cells or as response to the same stimuli, e.g. hypoxia. *Summary/conclusion*: Plasma EVs from SCLC patients have a specific protein signature compared to healthy subjects. The EV Array technique states itself as a relatively simple, non-invasive tool with potential to identify and to provide prognostic information on SCLC patients.

P-XVII-4

Exosomal miRNAs as biomarkers of lung cancer

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Introduction: Prognosis of lung cancer still remains grim largely due to recurrence and aggressive metastasis of the disease. Exosomal microRNAs (miRNAs) have recently attracted major interest as potential prognostic and diagnostic biomarkers of cancer. The aim of this study was to 1) characterize the miRNA profiles of lung cancer cells and exosomes isolated from conditioned media and 2) establish primary and recurrent subcutaneous lung cancer xenografts and characterize serum exosomal miRNAs and to identify those that are altered in secondary/recurrent lung cancer using nude mouse xenograft model. *Methods*: Normal lung (Beas-2b) and lung cancer (H1299) cells were cultured and exosomes were isolated from conditioned medium by ultracentrifugation. Athymic nude mice ($n = 25$) were inoculated with H1299 cells. When tumours reached 300–400 mm^3, tumours were excised from 18/25 mice and observed for recurrence of disease; the primary tumours in 7 mice were allowed to grow. Primary tumours, recurrent tumours and serum from the animals were harvested at euthanasia. Exosomes were isolated from serum using Exoquick reagent. Exosomal miRNA profiles were determined by Human MicroRNA A Card containing 384 TaqMan Assays. *Results*: A total of 89 miRNAs were observed to be significantly modulated in H1299 lung cancer cells (52 miRNA were upregulated and 37 downregulated) compared to normal lung Beas-2b cells. The exosomes isolated from conditioned media indicated several miRNAs to be in agreement with cells of origin. A similarity was observed between miRNAs from serum exosomes and tumours, indicating their origin from the lung tumours. miRNAs such as miR-21, miR-155 were found to be significantly up regulated in recurrent tumours compared to primary tumours as well as in serum exosomes of recurrent tumour versus non-tumour or primary tumour-bearing animals. *Summary/conclusion*: Exosomal miRNA signatures appear to be true representation of pathological profile of lung cancer, suggesting that miRNAs could serve as promising biomarkers for non-invasive diagnosis of the disease.

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P-XVII-5

Exosomal proteins as diagnostic biomarkers in advanced lung adenocarcinoma

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Introduction: Reliable minimal-invasive methods are needed in order to facilitate better diagnosing of lung cancer. Exosomes are cell-derived vesicles displaying various proteins on their membrane surfaces. In addition, they are readily available in blood samples and hereby constitute potential minimal-invasive biomarkers of human diseases, such as cancer. Here, we examine the possibility of distinguishing advanced lung adenocarcinoma patients from control subjects based on the differential display of exosomal protein-markers in plasma samples. *Methods*: EDTA-plasma was isolated from 109 adenocarcinoma patients with advanced stage (IIIa-IV) disease and 110 matched control subjects. The extracellular vesicle array (EV Array) was used to phenotype exosomes from plasma samples without preliminary purification. The array contained 37 antibodies targeting well known exosome-associated as well as lung cancer-related proteins and was used for exosome-capture. The binding of exosomes was visualised with a cocktail of biotin-conjugated CD9, CD63 and CD81 antibodies. *Results*: The EV Array analysis was capable of detecting and phenotyping exosomes in all samples from only 10 μL of plasma. Multivariate analysis using the Random Forests method produced a combined 30-marker model separating the 2 patient groups with an area under the curve (AUC) of 0.83, CI: 0.77–0.90. The 30-marker model has a sensitivity of 0.75 and a specificity of 0.76, and it classifies patients with 75.3% accuracy. Interestingly, the expression of exosome-associated proteins was of high importance in distinguishing the 2 groups whereas only few of the selected lung cancer-related proteins contributed. *Summary/conclusion*: Our study reveals that plasma-exosomes from advanced stage lung adenocarcinoma patients have a specific protein signature. Hereby, the EV Array technique states itself as a simple, non-invasive tool with potential to identify lung cancer patients.

P-XVII-7

Procoagulant extracellular vesicles in patients with small cell lung carcinoma (SCLC) and their impact on the thrombogenic profile

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Introduction: Lung cancer is a growing epidemic with a high mortality rate. SCLC is the most aggressive subtype and patients with SCLC have an increased risk of venous thromboembolism (VTE), more prominent amongst patients with extensive than limited stage of the disease. The coagulation system may not only be involved in cancer associated hypercoagulability, but also in the biology of cancer. Several components have been suggested to be associated to the procoagulant state as extracellular vesicles (EVs), carriers of procoagulant factors, e.g. tissue factor (TF) and procoagulant phospholipids (PPL). Here, we use a unique study-based SCLC cohort (Rasten) to investigate whether EVs may serve as biomarkers of hypercoagulability in SCLC patients. *Methods*: Plasma samples from 245 SLCL patients, 105 with limited disease (LD) and 140 with extensive disease (ED), were collected and compared to 61 age-matched controls. To determine the concentration and the size distributions of EV's, Nanoparticle Tracking Analysis (NTA) was utilized. The endogenous thrombin potential (ETP) was analyzed by means of calibrated automated thrombogram (CAT) and PPL activity was measured using a chronometric method. TF was detected by an Olink Multiplex platform. *Results*: Using NTA, we observe an increase in the mean particle concentration and size, a 40% increase in ETP and a 50% increase in the PPL for the patients compared to the control persons, but independently of the LD/ED status. The levels of TF in SCLC shifted to a more diseased profile. *Summary/conclusion*: PPL activity correlates excellently with ETP indicative of an existing hypercoagulable state in these patients. Identifying EV subpopulations responsible for or contributing to the procoagulant state observed in SCLC patients may be important to improve chances of preventing thrombotic events at an earlier state. Thus, these technologies holds potential in revealing associations between coagulation deviations, EV size and concentrations.

P-XVII-8

Exosomes and their potential for detection of lung cancer in early-stage disease

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Introduction: A recent study performed by our group showed that advanced lung adenocarcinoma patients have a distinct exosomal protein profile compared to a matched group without cancer. To improve overall survival (OS), it is crucial to develop tools capable of detecting early stage lung cancer as well. In addition, it is unsettled if different histologic subclasses result in distinct exosomal protein profiles. The aim of this study is to explore the potential of using exosomal proteins as biomarkers in lung cancer of all stages and histology. *Methods*: A recent study by our group showed that advanced lung adenocarcinoma patients have a distinct exosomal protein profile compared to a matched group without cancer. To improve OS, it is crucial to develop tools capable of detecting early stage lung cancer as well. In addition, it is unsettled if different histologic subclasses result in distinct exosomal protein profiles. The aim of this study is to explore the potential of using exosomal proteins as biomarkers in lung cancer of all stages and histology. *Results*: In a pilot study, 109 patients with adenocarcinoma lung cancer locally advanced or advanced disease (28 stage IIIA, 20 stage IIIB and 61 stage IV) were matched with 110 control patients. A significant difference was found in relative intensity between the control group and each stage individually (IIIA $p < 0.001$, IIIB and IV $p < 0.0001$). The study group was then expanded to include all stages and histology: 543 patients; 157 control patients and 386 patients with NSCLC (adenocarcinoma 68%, squamous cell 25% other 7%). 65% had locally advanced or advanced disease as to 35% having local disease. Results from the EV array analysis will be presented at the conference. *Summary/conclusion*: To determine the EV Arrays' stability and usefulness in a clinical setting, establishing variance between histology and stages is highly important. We demonstrate that the EV array detects lung cancer in advanced stages and will now determine further clinically utility.

P-XVII-9

Gastric cancer circulating exosomes: detection and relevance for disease progression

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Introduction: Gastric cancer is one of the leading causes of cancer-related mortality. New prognostic and predictive biomarkers are urgently needed. In this study we have evaluated the role of circulating exosomes in a prospectively recruited cohort of advanced gastric cancer (AGC) patients and their potential role as biomarkers in response to first-line therapy, including a subgroup of patients

treated with the new anti-membrane receptor antibody onartuzumab. *Methods*: Fifty-six patients with AGC were recruited between January 2013 and March 2014: median age 66y, male/female (40/16; 70%/30%), ECOG 0-1/2 (73%/27%), irresectable locally advanced/metastatic (40%/60%), intestinal/ diffuse/ mixed (50%/46%/4%), HER2 status +/unknown (14%/83%/3%). Thirteen patients with AGC were recruited from January–December 2013: median age 66y, male/female 10/3 (77%/23%), ECOG 0-1 100%. Treatment schedule was mFOLFOX6 combined with Placebo (8 patients; 60%) or onartuzumab (5 patients; 40%). Median time to progression (TTP) and median overall survival (OS) of the entire cohort were 263 days and 428 days, respectively. Survival outcome as per treatment schedule are summarized in this table:

Chemo + Placebo n = 8: TTP 243 (range 43–495); OS 428 (range 311–598)

Chemo + Onartuzumab n = 5: TTP 300 (range 168–377); OS 452 (range 169–967)

We have evaluated the use of circulating exosomes to predict the efficiency of standard chemotherapy versus the combination with therapies anti-MET (onartuzumab). Samples were collected at basal time (in the 24 hours previous to initiate chemotherapy) and previously to each chemotherapy cycle every 2 weeks. We evaluated number of particles present in the plasma by NTA, total exosomal protein levels, hepatocyte growth factor (HGF) level in the samples soluble fraction was measured by ELISA and MET and p-MET in the exosomes using Mesoscale. *Results*: Comparing placebo with onartuzumab group (n = 13), we observed an increase in the HGF level in the plasma soluble factor from patients under treatment compared with the Placebo group in a time-dependent fashion. We observed a trend to increase in the exosomal protein amount per ml of plasma in onartuzumab versus placebo. Similarly, the total protein detected in the exosomes was increased in the onartuzumab-treated patients, showing a trend versus the Placebo treated group. MET and phospho MET levels in circulating exosomes decreased after the first week in patients treated with onartuzumab. Importantly, patients with higher number of particles at the beginning of the treatment present higher OS and TTP regardless of the group of treatment. *Summary/conclusion*: Basal levels of circulating exosomal MET and phospho MET, or HGF in plasma might be used as biomarkers of survival outcome of AGC patients treated with systemic therapies. Basal levels of circulating particles could be predictive of AGC patient survival outcome. Further studies are required to verify this hypothesis and determine their functional role and cargo associated.

P-XVII-10

Malignant cell-derived extracellular vesicles express different chromogranin epitopes compared to prostasomes

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Introduction: Prostasomes are EVs exocytosed by prostate epithelial cells. They have been assigned many roles propitious to sperm in favour of fertilization. Prostatic cancer cells can also produce and release EVs. *Methods*: We assessed, using ELISA, the surface expression of chromogranin proproteins on prostasomes and malignant extracellular vesicles (EVs) of 4 different prostate cancer cell-lines, 2 hormone sensitive and 2 hormone refractory. We used a panel of chromogranin A and chromogranin B polyclonal antibodies against peptides in-between hypothetical cleavage sites along the proproteins. *Results*: A diverging pattern of chromogranin peptides was apparent when comparing prostasomes and malignant EVs indicating a phenotypical change. We also compared western blot patterns (prostasomes and malignant EVs) for selected antibodies that displayed high absorbances in the ELISA. Western blot analyses revealed various cleavage patterns of those proproteins that were analyzed in prostasomes and malignant EVs. *Summary/conclusion*: We conclude that chromogranins are constituents of not only prostasomes but also of malignant prostate cell-derived EVs with different amino acid sequences exposed at the membrane surface giving rise to a mosaic pattern. These findings may be of relevance for designing new assays for detection or even possible treatments of prostate cancers.

P-XVII-11

Vn96-captured urinary extracellular vesicles contain biomarkers for diagnosis and prognosis of prostate cancer

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Introduction: Prostate cancer (PCa) is one of the most commonly diagnosed malignant diseases in men worldwide. Although the serum PSA test has improved early detection of PCa, its lack of specificity and the inability to distinguish aggressive from indolent disease has led to overdiagnosis and over treatment of PCa. There is a great demand for new biomarkers for accurate PCa diagnosis, prognosis and monitoring. Urinary extracellular vesicles (EVs) have valuable potential as a novel, minimally invasive and enriched source of biomarkers. Conventional means of EV isolation involve time-consuming ultracentrifugation (UCF) or expensive commercial methods. We have developed a fast, simple and cost-effective method for enrichment of EVs from urine using a peptide (Vn96) with affinity for heat shock proteins. *Methods*: Post digital rectal exam (DRE) urine samples were collected from patients scheduled for prostate biopsy (with informed consent and regional ethics board approval). EVs were isolated in parallel using UCF and Vn96 peptide techniques. Western blotting was used to assess and compare the presence of canonical EV markers (e.g. CD9, ALIX and HSP/C70) and prostate-specific markers (e.g. FOLH1 and PSA). Total RNA was extracted from UCF and Vn96 EVs and urine sediments. RT-qPCR was used to assess the expression of known urinary PCa markers (e.g. PSA and PCA3) and a number of mRNAs and miRNAs previously identified by others in plasma and urine sediments, as having an association with PCa. *Results*: Western blots of Vn96 and UCF enriched urinary EVs showed the presence of canonical EV and prostate-specific markers, often more abundant in the Vn96 preparations than their UCF counterparts. RT-qPCR demonstrated the majority of transcripts and miRNAs of interest could be measured in both UCF and Vn96 enriched EVs. A five-member mRNA biomarker panel resulted in a test with better accuracy for PCa diagnosis (based on biopsy results) using Vn96 EV RNA compared to UCF EV RNA (ROC curve AUC = 0.768 vs. AUC = 0.676, respectively). Continued Vn96 EV biomarker analysis led to a combined group of 6 mRNAs and 4 miRNAs with 90% specificity and 75% sensitivity for PCa diagnosis. This EV panel score increased with PCa aggressiveness, yielding a sensitivity approaching 100% where Gleason 7 or greater disease was identified on biopsy. Measurement of an established PCa urine biomarker, PCA3, using Vn96 EVs versus urine sediments resulted in improved test specificity and accuracy. At a fixed sensitivity of 76%, urine sediment PCA3/PSA ratio specificity was 55% with AUC = 0.709, while Vn96 EV PCA3/PSA ratio specificity was 69% with an AUC = 0.803. *Summary/conclusion*: Vn96-captured urinary EVs provide an enriched source of prostate-specific protein markers and diagnostically informative mRNAs and miRNAs. A quick and simple EV enrichment method using Vn96 can significantly aid the discovery and clinical application of PCa biomarkers in urine.

P-XVII-12

Potential use of stromal cell-derived exosomal mRNAs as markers of aggressive prostate cancer

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Introduction: The stroma surrounding carcinomas is aberrantly altered, consisting of myofibroblast-like cells. This is a rate limiting step in tumour progression, associated with treatment-resistant cancer and poor prognosis, making the stroma an attractive source for biomarkers of aggressive disease. Cells within the tumour environment secrete exosomes which are detectable within the patient's circulatory system. We have, therefore, analyzed exosomal mRNA from normal and tumour-associated stromal cells and have identified several targets that may serve as future biomarkers of aggressive prostate cancer. *Methods*: Patient-matched normal and tumour stroma were cultured from needle biopsies of radical prostatectomy specimens, taken from disease-free or cancerous regions respectively. Exosomes were isolated by serial filtration and ultracentrifugation, prior to determining exosome size and concentration by nanoparticle tracking analysis. Exosome concentration was normalized to cell number before RNA extraction and subsequent RT-PCR. *Results*: Tumour-associated stromal cells secrete twice as much exosomes compared to matched normal stromal cells. Furthermore, the absolute mRNA content was increased in exosomes from the tumour stroma. Of the 84 genes tested, more than 30 were elevated in tumour-associated stromal exosomes compared to exosomes from the normal stroma. These targets include Gremlin-1, TGFβ1, STAT1, HGF and VEGF. Incidentally, mRNA targets elevated in exosomes did not necessarily match those mRNA targets that were elevated within the cellular mRNA, suggesting that there may be selected enrichment of specific mRNA targets within the exosome. *Summary/conclusion*: This study not only highlights the potential of using exosome-derived mRNA targets as potential markers of aggressive prostate cancer but also specifically mRNA targets present within stromal cell-derived exosomes. Future work shall focus on isolation of exosomes from patient serum and validation of key mRNA targets identified within the current study.

P-XVII-13

Metabolomics of urinary EVs – a pilot study

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Introduction: Urinary extracellular vesicles (EVs) are regarded as a promising non-invasive source of biomarkers for urological diseases. While RNA and proteins have been the focus of many studies, we conducted a pilot experiment to clarify whether metabolites within urinary EVs can be quantified and whether they can provide information about prostate cancer. *Methods*: We purified EVs from 3 prostate cancer patient urine samples before and after radical prostatectomy and 3 controls and subjected them together with their source urine samples to analysis of 102 targeted metabolites by UPLC-MS-MS. Since a standard protocol for normalization of the EV-derived data does not exist, we tested several possibilities including urine creatinine, particle number (NTA) and the quantity of EV enriched protein markers (WB). *Results*: The analysis revealed that 32–55% of the screened metabolites were present in EVs derived from 10–60 ml urine samples. While most metabolites were more abundant in urine samples, a few were better detected in the purified EV preps. Normalization of the results by NTA and WB gave highly similar results, while normalization to creatinine did not correlate well with other methods. By normalizing to the amount of EVs (NTA and WB), we detected 4 metabolites that were systematically lower in cancer than in controls or in samples after radical prostatectomy. *Summary/conclusion*: Since we successfully detected similar numbers of metabolites across all samples, we conclude that relatively small urine volumes are sufficient for detection of EV associated metabolites. The data suggests that a few metabolites are more readily found from purified EVs than urine and that the levels of some EV metabolites may differ between cancer and non-cancer cases. However, it is also evident that the variability between individuals is high and that the selected normalization method has a large effect on the results.

P-XVII-14

Advanced profiling of miRNAs in EVs derived from 13 ovarian cancer cell lines

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Introduction: Most ovarian cancer patients are diagnosed at an advanced stage because of a lack of obvious symptoms and the current limitations in detection techniques. For resolving those clinical issues, detecting cancer-specific microRNAs (miRNAs) is one of the crucial factors. Extracellular vesicles (EVs) carry bioactive shipments like miRNAs and these cancer-derived EVs are circulating in patients' blood and other body fluids like ascites and they can be a biomarker. The aim of this study was to investigate a novel potential biomarker for diagnosis of ovarian cancer. *Methods*: EVs were isolated from serum free culture supernatants of 13 ovarian cancer cell lines and 3 human ovarian surface epithelial (HOSE) cell lines by ultracentrifugation at $>100{,}000 \times g$ for 70 minutes at 4°C. The particle number was measured by the nanoparticle tracking assay (NanoSight). The protein concentration was determined using a Qubit assay kit. A miRNAs microarray was performed on both exosomal and cellular miRNAs. The results of microarray were validated in the cell lines, patients' serum and ascites by qRT-PCR. *Results*: Although the particle number and RNA concentration per cell were various, there was a tendency between RNA concentration and protein level. Microarray analysis revealed that the characteristic of cellular miRNAs compared between the cancer and HOSE cell lines were clearly different. However, the characteristic of exosomal miRNAs did not have any tendency. From the results of microarray, 25 exosomal miRNAs were selected because they were found in only cancer cell lines. After validation by using qRT-PCR, we selected 12 exosomal miRNAs which met the criteria that half of the cancer cell lines highly expressed in comparison to HOSE cell lines. We are now validating the selected miRNAs in ovarian cancer patients' serum and ascites. *Summary/conclusion*: In this study, we demonstrated the advanced profiling of EVs in ovarian cancer cell lines. This data can be the first step for the development of novel biomarkers which are applicable for clinical sites.

P-XVII-15

Del-1 on exosomes is involved in breast cancer cell invasion and provides a biomarker for early detection

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Introduction: Cancer cell-derived exosomes containing proteins are linked to the disease pathogenesis in various cancers. *Methods*: To understand the mechanism of BC metastasis and simultaneously identify early biomarkers of this cancer, we studied exosomes secreted from BC cells. We focused on the Del-1 protein based on the fact that this protein is highly expressed in MDA-MB-231 exosomes, located on extracellular matrix and involved in the integrin signalling pathway as well as cancer progression. Enzyme-linked immunosorbent assays (ELISAs) were used to measure Del-1 in plasma samples from healthy controls, patients with BC, BC patients after surgical resection, patients with benign breast tumours and patients with non-cancerous diseases, in 2 cohorts. *Results*: Here, we report Del-1 protein on exosomes, which are sufficient for enhancement of cancer cell invasion and for acceleration of lung metastasis at the initial stage of cancer mouse models. This invasion is most

likely mediated via the integrin-FAK signaling cascade in cancer cells. However, these effects are significantly suppressed when Del-1 is inactivated, providing evidence for a critical role of Del-1 in development of cancer. In human patients with breast cancer, the levels of Del-1 on exosomes are significantly elevated at the early stage of breast cancer, as determined by ELISA with high sensitivity and specificity, but return to almost normal after removal of the tumour. *Summary/conclusion*: Taken together, these results identify a new function of Del-1 on exosomes in cancer cell invasion and its utility as an early diagnostic biomarker of breast cancer.

P-XVII-16

Chloride intracellular channel protein 4 (CLIC4) is a serological cancer biomarker released from tumor epithelial and/or stromal cells via exosomes and microvesicles

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Introduction: CLIC4 is a member of the highly conserved 7 member family of metamorphic CLIC proteins. Cytoplasmic CLIC4 translocates to the nucleus where it is an integral component of TGF-beta signalling. When expressed in cancer cells, CLIC4 is a tumour suppressor; in contrast, when expressed in the tumour stroma, CLIC4 enhances tumour growth. In multiple human cancers, CLIC4 protein is excluded from the nucleus and lost from the cytoplasm of tumour cells resulting in a reduced tumour response to growth inhibition by TGF-beta. The progressive reduction of CLIC4 in tumour cells correlates with increased tumour progression. In the tumour stromal compartment, CLIC4 is upregulated by TGF-beta secreted by tumour epithelial cells, in conjunction with expression of alpha-smooth muscle actin, a hallmark of fibroblast-to-myofibroblast conversion. Recently, CLIC4 has been validated as a serological biomarker for human ovarian cancer. Since CLIC4 has no target signal sequence for the secretory pathway, we evaluated whether it can be released into circulation via exosomes and/or microvesicles from tumour epithelial and/or stromal cells. *Methods*: Serum from normal and tumour bearing mice, and cell culture supernatants of primary myofibroblasts and ovarian serous adenocarcinoma cells were examined for release of exosomes containing CLIC4. Exosomes were isolated by differential centrifugation and ultracentrifugation follow by density gradient. Exosomal markers (ALIX, TSG101 and CD9) and CLIC proteins were analyzed by immunoblots together with mass spectrometry analysis. *Results*: Immunohistochemical analysis of human ovarian and breast cancer tissue arrays established that CLIC4 is preferentially expressed in tumour stroma and in the majority of tumours, CLIC4 is almost absent in tumour epithelium with the exception of ovarian serous adenocarcinomas, where its expression is upregulated in both compartments. High levels of CLIC4 were detected in exosomes from cultured ovarian cancer and myofibroblasts. Furthermore, CLIC4 containing exosomes were isolated from serum of breast tumour bearing mice that increased with metastatic load. *Summary/conclusion*: Currently, we are developing an in vivo model to test how specifically circulating CLIC4 levels can correlate with tumour stage and evaluating if CLIC4 contributes to the biogenesis of exosomes.

P-XVII-17

Clinical and technical potentials and prospects of the EV array

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Introduction: The Extracellular Vesicle (EV) Array (1) facilitates the ability to detect and profile extracellular vesicles for the presence of multiple surface-exposed antigens simultaneously. The EV Array uses only small amounts (1–100 μl) of starting material and is run in a high-throughput manner. *Methods*: The possibilities of the technology have been tested in various technical and clinical correlations. Several body fluids have successfully been tested including plasma, saliva, urine, bone marrow, ascites, synovial and cerebral spinal fluids. Plasma and bone marrow from larger cohorts of patients with various cancer types (NSCLC, melanoma and osteosarcoma) and healthy individuals have been analyzed with respect to their contents of several known markers. *Results*: Surprisingly, these studies generally found that healthy persons has a higher content of the exosomal markers CD9 and CD81 in their plasma. Whereas, another known exosomal marker, CD63, showed an equal expression in both healthy and cancerous plasma. The analysis of plasma samples from lung-cancer patients, patients with lung symptoms and healthy controls revealed a pronounced diagnostic potential of the EV Array. Exosomes from plasma and bone marrow from patients with melanoma and osteosarcoma was analysed extensively. The correlations between the exosomal markers found in plasma versus bone marrow showed large variations between the 2 cancer types. For melanoma patients the contents of numerous of the exosomal markers from bone marrow was equivalent to the findings in plasma. Whereas, for osteosarcoma patients several immune markers (CD4, CD8, HLA ABC, CD49d, etc.) and CD63 was found at significantly higher levels in bone marrow versus plasma. This could indicate either a high local production of exosomes in the bone marrow or a high recruitment of exosomes from other locations. Summary/conclusion: Taken together these studies illustrate the numerous applications of the EV Array.

Reference

1. Jørgensen M, Bæk R, Pedersen S, Søndergaard EKL, Kristensen SR, Varming K. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. J Extracell Vesicles. 2013;2:20920, doi: http://dx.doi.org/10.3402/jev.v2i0.20920

P-XVII-18

mRNA-sequencing of tumour-educated platelets allows for multiclass liquid biopsy-based diagnosis of cancer

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Introduction: Cancer diagnosis and subsequent treatment is frequently hampered by limited access to adequate tissue of the primary tumour or of metastatic lesions. To overcome such limitations, the use of blood-based liquid biopsies has been suggested. Blood represents a biosource of tumour-educated platelets (TEPs) that sequester biomolecules during tumour growth, thereby altering the platelet mRNA profile. *Methods*: Blood platelet samples of 192 patients with cancer covering 6 tumour types (40 non-small cell lung cancer, 40 glioblastoma, 37 colorectal cancer, 37 pancreatic cancer, 13 liver cancer and 25 breast cancer) and of 52 healthy donors were isolated from whole blood by differential centrifugation. Subsequently, total RNA was isolated, subjected to SMARTer mRNA amplification and

submitted for whole transcriptome mRNA sequencing on the Illumina platform. Healthy donors, pan-cancer and individual cancer classes were distinguished by a self-learning support vector machine (SVM) algorithm, using a set of transcripts with moderate to high expression. *Results*: The 244 blood platelet samples were successfully sequenced and demonstrated a good intersample correlation of the detected mRNAs. Based on the mRNA profiles all tumour samples were clearly distinguished from healthy individuals, and the pan-cancer SVM-supported classification test classified both groups correctly with high sensitivity and specificity. Also, all patients without overt metastases were correctly predicted as cancer patients. Moreover, a multiclass cancer diagnostics TEP-test, to distinguish multiple tumour subclasses and healthy controls provided an overall accuracy of more than 70%, far exceeding random classification. In addition, we distinguished HER2-postive and mutant KRAS and EGFR tumour from their wild-type counterparts. Also, patients with metastatic tumours in lung, brain, and liver were accurately diagnosed according to the tumour in the tissue of origin. *Summary/conclusion*: Molecular interrogation of TEP-based liquid biopsies may leverage cancer diagnostics. Especially, this platform may allow for highly-sensitive early-stage cancer screening. Further evaluation of this diagnostic platform will guide for implementation in clinical diagnostics.

P-XVII-19

Finding prostate cancer biomarkers in platelets

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Introduction: In the development of individualized therapy, we need a window in to the tumour. It should be minimal invasive, being able to find the disease and follow how the tumour alters its behaviour to circumvent the treatment. Platelets could be one such window, with its ability to sequester tumour-derived material during its passage through the circulation. *Methods*: With conventional quantitative PCR and microarray techniques ''potential'' biomarkers were analyzed from WTA (whole transcriptome amplified) platelet-derived RNA isolated from cancer patients and healthy controls. *Results*: We demonstrate that platelets contain several cancer-associated RNA biomarkers. In addition, there was a distinct biomarker signature in platelets from cancer patients of different grades as compared to normal control subjects. *Summary/conclusion*: These findings are preliminary, and need to be validated in a larger patient cohort. However, they support our earlier findings in gliomas that platelets may carry the needle from the haystack that will give us a window into the cancer biology of individual patients.

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Poster session XVIII - EV transcriptomics
Chairs: *Olga Volpert and Tek Lamichhane*

P-XVIII-1

Extracellular vesicles as potential biomarkers for Type 1 diabetes (T1D)
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Introduction: Type 1 diabetes (T1D) results from autoimmune-mediated destruction of the pancreatic β cells and manifests as insulin deficiency and hyperglycaemia. At the time of clinical recognition, β cell mass is reduced by approximately 80%. Thus, there is a need for earlier markers of T1D onset and progression. Because extracellular vesicles (EVs) can be isolated from a variety of body fluids and may carry lineage markers and/or cargo of the cell of origin, we hypothesized that β-cell-derived EVs may serve as useful biomarkers for the diagnosis of T1D. *Methods*: To this end, the RNA cargo of EVs isolated from in vitro and in vivo models of T1D were analyzed. To mimic the inflammatory milieu of T1D, mouse insulinoma (MIN6) cells and human islets were incubated for 24 hours in the presence or absence of a cytokine mixture. Female NOD mice were used as an in vivo model of T1D, and human serum was obtained from healthy volunteers. EVs were isolated from cell and islet supernatants and serum using ExoQuick™. EVs were visualized by transmission electron microscopy (TEM). EV markers were assessed by western blot; RT-qPCR or droplet digital PCR (ddPCR) were used to define EV RNA and miRNA content. *Results*: β-cell-derived EVs displayed a prototypical cup-shaped morphology with size ranging from 30–250 nm. The EV markers CD63 and CD9 were identified in the vesicular fraction. Interestingly, expression profiling of EVs isolated from cell and islet supernatants and serum revealed the presence of insulin mRNA and the β-cell enriched miR-375. Expression of microRNAs involved in β-cell stress and apoptosis such as miR-146a, miR-29a and miR-25a was also confirmed. Interestingly, expression of miR-146 showed a statistically significant increase in EVs isolated from cytokine-treated MIN6 cells and human islets, while expression of EV miR-25a was significantly increased in pre-diabetic 10-weeks-old NOD mice compared to CD1 controls. *Summary/conclusion*: These results suggest that β-cell RNA species are potentially sorted into EVs and that β-cell-derived EVs may reach the bloodstream. The signature of EVs isolated from in vivo and in vitro models may help differentiate T1D from control conditions and may serve as early prognostic markers.

P-XVIII-3

Deconvolution of exRNA in biofluids
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Introduction: Cell-of-origin type heterogeneity of vesicles and extra cellular RNAs (exRNAs) in peripheral biofluids could be a significant complication when trying to assess disease relevant signals or monitor information from the central nervous system. The choice of biofluid to be tested for exRNA biomarkers arising from injuries to the brain depends upon the invasive nature of the sample collection, the amount of brain-related exRNA transcripts found in these biofluids, the background exRNA from other tissues amongst other considerations. Deconvolution of exRNA data from biofluids using bioinformatics techniques would help identify the proportion of exRNA from the various tissues in the body and help in the right choice of biofluid for detection of brain-related exRNA transcripts. *Methods*: RNAseq data (exRNA) from CSF, blood, urine and saliva will be examined for the amount of brain-related transcripts available for the detection of RNAs, which are characteristic of brain injuries. The exRNA RNASeq count data from biofluids are modelled as a weighted average of the RNA expression profiles of pure tissue samples (RNASeq ATLAS: contains RNASeq data from 95 human individuals and 27 different tissues). As many genes/transcripts are expressed at similar levels in all tissues, we seek to include only those genes/transcripts that are representative of each tissue in the analysis. The results from *baySeq*, an empirical Bayesian method and Random Forest machine learning algorithms help identify a list of genes that are unique or at least highly differentially expressed in each tissue with respect to the tissues, which can be used as the RNA signature for that particular tissue for the deconvolution. A subset of this list of genes where there is differential expression between the brain and other tissues would constitute the brain-related transcripts/genes. Finally, for the deconvolution, we have adopted the use of the package *DeconRNASeq*, which employs a globally optimized non-negative decomposition algorithm through quadratic programming to estimate the proportion of RNA arising from the various tissues. *Results*: The deconvolution model has been validated for predetermined in-silico mixtures of tissues. Preliminary results show the exRNA data to be indicative of the biofluid. exRNA from blood seems to consist of a high proportion of RNA from bone-marrow, and CSF contains a high proportion of brain related RNA. Also, CSF samples contaminated with blood show a high proportion of bone-marrow transcripts. *Summary/conclusion*: The deconvolution would enable us to compare the relative proportion of brain related genes/transcripts found in blood, urine, saliva and CSF to identify the most appropriate biofluid to seek an exRNA signature capable of predicting brain related injuries and a part of this information could potentially aid in isolation procedures to enrich for CNS markers.

P-XVIII-4

Quantification of microRNAs in small-volume samples using fast multiplex real time PCR
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Introduction: It has recently granted that a miRNA expression profile signified the developmental lineage and differential cellular states. Thus, the small fragments of RNAs became considered as a novel diagnostic parameter for significant diseases. Interestingly, exosomes have been investigated as cargos for miRNAs in intra/extra-cellular communication and an enriched source of miRNAs disease markers in bio-fluids. Expression profiles of miRNAs are generally determined with cDNA microarrays or Nanostring, which is not reliable for less abundant miRNAs. Thus, the quantification of individual miRNAs is carried out with real time PCR with respect to reference RNAs. Major limitation of real time PCR in profiling miRNAs is that only 5 kinds of RNAs can be quantified from single sample at a time. *Methods*: We propose a novel multiplex qPCR with encoded porous particles that can quantify more than 100 miRNAs in single sample of 10 μl at a time. Each particle contained specific primers for a miRNA and marked with a specific pattern. The encoded pattern could be varied to the level of thousands of species. The particle was very porous enabling delivery of 30 nm objects, which guaranteed effective enzyme activity throughout the volume of the particle. *Results*: For the first (1) demonstration of the particle-based real time PCR, 4 kinds of miRNAs (miR-9, miR-219, miR-132, U6) were incorporated in 500 μm hemispheric particles individually. PCR efficiency in the particle was 93%, and typical amplification of oligonucleotides was fluorescently represented by SYBR green. miRNAs were reverse transcribed to cDNAs simultaneously and analyzed by real time PCR in a single chamber of 10 μl without interference between individual particles. Ct values for each targets miRNAs were consistent among same type

of particles, quantifying to the concentration of sub pM. *Summary/conclusion*: Since the amplification in each particle occurred independently, additional expansion of target miRNAs is simply achievable by adding more particles containing specific primers. Based on the capability of different pattern codes, more than hundreds of miRNAs can be quantified in an exosome sample of tens of microlitre.

P-XVIII-5

Evaluation of messenger RNA transfer among extracellular RNA

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Introduction: There are increasing amounts of data that suggest that extracellular vesicles (EVs) contain mRNA that might be functional in recipient cells. Most of the studies have been based on PCR, microarray analyses or sequencing that identifies the presence of a small region of a specific mRNA in the exRNA fraction. In this study, we aimed at evaluating the possibility of functional mRNAs being transferred by EVs. *Methods*: We isolated RNA from 2 different EV fractions (10,000 and 100,000 g pelleted) released by U87, D384 and 293T cells. To detect specific mRNAs, we first used regular PCR in combination with reverse transcriptase. We used multiple primer pairs for each mRNA, so that different length products were amplified (fragments with different sizes and also the full length coding region of the same message). We looked at exogenous mRNAs (FLuc, GLuc, mCherry, Cre, 2 different Cas9 mRNAs) derived from transfected cells, and at endogenously expressed ones (c-myc, paxillin, EGFR and 28S RNA). We used the XRN-1 exonuclease to detect the presence of the 5′ cap. We also subjected mRNA samples to in vitro translation using the reticulocyte lysate system. *Results*: We confirmed the presence of all the analyzed mRNAs in EVs. Full length messages, were detected only in the case of relatively small messages (less than 2 kb), while fragments of these and fragments of larger messages were always detected in high amounts. Using in vitro translation with the same amount of RNA, we detected GLuc protein activity from RNA of GLuc-expressing 293T cells, but surprisingly not in the EV RNA from these cells, even though the full-length GLuc message was detectable in all EV fractions. Interestingly, the GLuc mRNA in cells and also EVs was resistant to XRN-1 cleavage suggesting the presence of a protective 5′ cap. *Summary/conclusion*: mRNA detected in vesicles is mostly fragmented, although capped full length messages are present up to 2 kb. It remains to be elucidated as to why GLuc message translates by cells (see abstract from Lai et al.) but not via the in vitro reticulocyte lysate system. This work has been supported by NIH NCI P01CA069246 grant, NIH NCI U19 CA179563 supported by the NIH Common Fund, through the Office of Strategic Coordination/ Office of the NIH Director and the Richard Floor Biorepository Fund.

P-XVIII-6

Characterization of miRNAs purified from rat serum extracellular vesicles submitted to low, moderate and high aerobic exercise

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Introduction: Extracellular vesicles (EVs) are vesicles released by cells and circulate in biological fluids. They carry proteins, lipids and nucleic acids that participate in the regulation of gene expression in the recipient cell. MicroRNAs are non-coding small RNA single stranded that regulates the level of mRNA in cells. Different studies show that aerobic exercise can change the pattern of some miRNAs in tissues and biological fluids such as plasma and serum. The work aims to characterize miRNAs purified from rats serum extracellular vesicles submitted to low, moderate and high aerobic exercise. *Methods*: Three groups of rats were exercised in a treadmill under different intensities [low intensity (L), moderate intensity (M), high intensity (H)]. Non-exercised group was used as control [non-exercised (NE)]. After the exercise, blood was collected and EVs were purified from 250 μl of serum using Exoquick (System Biosciences). Extracellular vesicles were characterized by Electron Microscopy, TRPS (qNANO), DLS, AFM and Western Blotting. Small RNAs were purified and send to SeqMatic facility for library preparation, and sequencing was performed by Illumina GAIIX. Reads were filtered by quality and trimmed, then aligned to *Rattus norvegicus* genome (Rnor_5.0) and mature miRNA database (miRBase). Differential presence of miRNAs were identified by Deseq2 package with PAdj <0.1. *Results*: Between non-exercised group and low exercised group, 2 miRNAs were significantly highly increased (rno-miR-486; 375-5p) and 8 miRNAs were significantly decreased (rno-miR-741-3p; 376a-3p; 770-5p; 770-3p; 191a-5p; 7578; 1247-5p; 874-5p). Between non-exercised group and moderate exercised group, none miRNAs were significantly increased or decreased. Between non-exercised group and high exercised group, 2 miRNAs were significantly increased (rno-miR-124-3p; 127-3p) and rno-miR-191a-5p were significantly decreased. *Summary/conclusion*: It is known in the literature that expression of miR-124 in the hippocampus of adult rats is increase after exercise. Low levels of miR-124 in the cortex were related to social dysfunction in Frontotemporal dementia while high levels of miR-124-3p works as a metastatic suppressor in Bladder cancer cells. Upregulation of miR-127 caused a downregulation of B-cell lymphoma 6 protein, a proto-oncogene that is usually hypermutated in diffuse large B-cell lymphoma (DLCL) while Inhibition of miR-127 expression is linked with Hepatocellular carcinoma. Levels of rno-miR-191a-3p were down regulated in plasma of Retinopathy of prematurity (ROP) model rat samples. All together, the results may contribute for the understanding of health benefits related to physical exercise and miRNAs.

P-XVIII-7

Reference profile of normal human plasma exosomal RNAs

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Introduction: Exosomes are cell-derived microvesicles containing selectively enriched nuclear acids and proteins. Exosomal RNAs and proteins may have great potential as disease biomarkers. So far, however, there is no systematic investigation of exosomal RNA distribution in normal human population. *Methods*: To thoroughly characterize exosomal RNA profiles, we isolated exosomes from 300 μl of plasma samples in 50 normal individuals and performed RNA sequencing analysis. The subjects included 25 females and 25 males with ages from 25 to 79. *Results*: From the 50 sequencing libraries, we received $\sim$12.6 million of raw reads per library, of which $\sim$6.5 million ($\sim$51%) were successfully mapped to known RNA databases. Among all mappable reads, a diverse collection of microRNA (miRNA) was the most abundant, contributing to $\sim$40%, followed by lncRNA (22%), piRNA (21%), mRNA (6%) and other RNAs (11%). The expression levels of 5 most common miRNAs (miR-99a-5p, miR-128, miR-129-5p, miR-22-3p and miR-181a-5p) collectively accounted for 38% of 270 known miRNAs with $\geq$5 reads per million. The top 5 abundant piRNAs accounted for 94% of 43 piRNAs with $\geq$5 reads per million. Top 5 lncRNAs accounted for 76% of 117 with $>$5 reads per million. We then performed the stability test for each of commonly expressed miRNAs ($\geq$32 reads per million) and found that miR-99a-5p, miR-30d-5p, miR-30a-3p, miR-222-3p and miR-124-3p were the top 5 most stably expressed among all of the 111 selected exosomal miRNAs. We also evaluated the effect of gender and age on miRNA expression level and found 4 and 1 miRNAs showing association with gender and age (FDR $\leq$0.1), respectively. Finally, we examined miRNA variants (isomiRs) and observed frequent isomiRs in all common miRNAs. *Summary/conclusion*: This study unravelled a wide variety of RNA species embedded in circulating exosomes and moreover identified

baseline invariant references for exosomal RNA profiles in non-diseased human plasma samples that could have utility as normalizers.

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P-XVIII-8

Integrated transcriptomic analysis of extracellular vesicles and their originating cells based on EVpedia database

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Introduction: Into the extracellular space, most mammalian cells secrete extracellular vesicles (EVs), which are spherical bilayered proteolipids with various bioactive molecules including RNAs. To provide a comprehensive view of the EV high-throughput studies, a community web portal for EVs research, EVpedia, has been developed. However, the integrated study using transcriptomic datasets in EVpedia has not been attempted yet due to the hurdles of different originating species and analysis platforms. Here, we used integrated analysis method for the systematic characterization of EV transcriptomes. *Methods*: Scatter plots and principal component analyses were used for the quality assessment of transcriptomes. For transcriptomic analysis of EVs and their originating cells in EVpedia, we developed and applied the integrated analysis method that covers various species and platforms, which stems from the concept of virtual arrays. *Results*: A total of 18 mRNA transcriptomes and 20 miRNA datasets with high quality were selected from EVpedia. We found that the overall RNA profiles of EVs and their originating cells have significant positive correlations, which imply that the mRNA contents of EVs reflect those of the originating cells. Especially, a group of RNAs such as PDGFB, INS, CD4, CDKN2A, MIR-122, MIR-218 and MIR-126 were commonly enriched in EVs and they contribute to sharing functions of EVs such as cell proliferation. Also, after integrating with public clinical datasets, we identified some EV RNA biomarker candidates such as CSTE and CEPBA for pancreatic and colorectal cancers, respectively. *Summary/conclusion*: In this study, our integrated method for EV transcriptomic analyses suggests a new strategy to understand EV RNAs by integrating transcriptomes of EVs, their originating cells and public clinical samples.

P-XVIII-10

Characterization of miRNAs purified from horse plasma extracellular vesicles during an endurance riding

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Introduction: Extracellular vesicles (EVs) are vesicles released by cells and circulate in biological fluids. They carry proteins, lipids and nucleic acids that participate in the regulation of gene expression in the recipient cell. MicroRNAs are non-coding, small, single-stranded RNAs that regulate the level of mRNA in cells. Different studies show that aerobic exercise can change the pattern of some miRNAs in tissues and biological fluids such as plasma and serum. The work aims to characterize miRNAs purified from horse plasma EVs during an endurance riding. *Methods*: Plasma samples were collected from 10 Arabian horses engaged in a 160 km race. All animals completed the race. Samples were taken pre-race at the night before (T0), at the second veterinarian-gate (66 km) (T1), at the end of the race (T2), 2 hours after the race (T3) and 15 hours after the race (T4). EVs were purified from 500 μl of plasma using Exoquick (System Biosciences). EVs were characterized by Electron Microscopy, TRPS (qNANO), DLS and western blotting. Small RNAs were purified using miRCury – Biofluids. RNAseq Libraries were constructed, and sequencing was performed by Illumina MiSeq. Reads were filtered by quality and trimmed, then aligned to *Equus caballus* genome (EquCab2) and mature miRNA database (miRBase). Differential presence of miRNAs were identified by Deseq2 package with PAdj <0.1. *Results*: Electron microscopy, DLS and TRPS showed particles with diameter and morphology compatible with exosomes and microvesicles. Protein markers currently identified in exosomes preparation were positive (CD9, CD81, TSG101 and flottilin) while calnexin (negative marker) was absent. Between before race (T0), second veterinarian-gate (66 km) (T1), end of the race (T2) and 15 hours after the race (T4) none miRNAs were statistically significant but between T0 and 2 hours after the race (T3), 6 miRNAs were significant found decrease (eca-miR-25; 423; 30d; 140; 342; 143). *Summary/conclusion*: MiR-25 was found upregulated in heart failure, both in mice and humans. Overexpression of miR-30d increased insulin gene expression, while inhibition of miR-30d abolished glucose-stimulated insulin gene transcription. MiR-25 and miR-30d directly target the 3′UTR of TP53 to downregulate p53 protein levels. MiR-423 was upregulated in hepatocellular carcinoma (HCC). MiR-423 exerts growth-promoting effects in hepatic carcinogenesis through the suppression of tumour suppressor p21Cip1/Waf1 expression. Also miR-423 was upregulated in breast cancer patients that went on to develop metastasis. MiR-342 was identified as biomarker in the diagnosis of acute myeloblastic leukemia (AML). The up-regulation of mir-143 was observed in a HCC model during tumour metastasis. In general, the results showed that many miRNAs found upregulated in cancer were found downregulated in EVs samples after exercise contributing to the knowledge that exercise is an important factor in the prevention of some kinds of cancer.

P-XVIII-11

Small RNA sequencing discriminates subsets of extracellular vesicles released by melanoma cells – evidence of unique microRNA cargos

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Introduction: Melanoma cells release different types of extracellular vesicles (EVs) into the extracellular milieu that are involved with communication and signalling in the tumour microenvironment. Subsets of EVs include exosomes, microvesicles and apoptotic bodies which carry protein and genetic (RNA) cargo. *Methods*: To define the contribution of the RNA cargo of melanoma cell-derived EVs, we performed small RNA sequencing to identify different small RNAs in the EV subsets. Using validated centrifugation protocols, we separated these EV subsets released by the melanoma cell line MML-1 and performed RNA sequencing with the Ion Torrent platform. *Results*: Various, but different, non-coding RNA were detected in the EV subsets, including microRNA, mitochondrial associated transfer RNA, small nucleolar RNA, small nuclear RNA, Ro associated Y-RNA, vault RNA and Y-RNA. We identified in total 1,041 miRNA in cells and EV subsets. Hierarchical clustering showed enrichment of specific miRNA in exosomes, including hsa-miR-214-3p, hsa-miR-199a-3p and hsa-miR-155-5p, all being associated with melanoma progression. Comparison of exosomal microRNAs with microRNAs in clinical melanoma samples indicate that multiple miRNAs in exosomes also are expressed in melanoma tissues, but not in benign naevi. *Summary/conclusion*: This study shows the presence of distinct small RNAs in subsets of EVs released by melanoma cells, with significant

similarities to clinical melanoma tissue and provides unique insights into the contribution of EV associated extracellular RNA in cancer.

P-XVIII-12

Mining the sorting machinery of extracellular miRNAs in neural stem/precursor cells

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Introduction: Neural stem/precursor cell (NPC) transplantation protects the central nervous system from inflammatory damage via cell-to-cell communication mechanisms. Recent works suggest that the exosome-mediated transfer of molecules such as microRNAs (miRNAs) might play an important role in mediating the protective effect of NPCs. Here, we aim to identify the machinery that sorts miRNAs to exosomes in murine NPCs. Our hypothesis is that such a mechanism might act (a) at the transcriptional level, with transcription factors (TFs) driving the transcription of exosomal miRNAs; or (b) at the post-transcriptional level, with carrier proteins that recognize specific miRNAs, bind to them and mediate their export to exosomes. *Methods*: We used RNA-Seq to identify miRNAs significantly more abundant in exosomes than parental cells. To address whether specific TFs drive the transcription of secreted miRNAs, we tested whether any TF binding site is enriched in their promoters. In parallel, we used a variety of motif enrichment tools available in R/Bioconductor (Cosmo, BCRANK, motifRG) to find short motifs enriched in secreted miRNAs. *Results*: We found that no specific TF binding site is enriched in the promoters of secreted miRNAs. However, we identified 2 short motifs over-represented in exosomal miRNAs, one of which matches the binding sequence of hnRNPA2B1, which previous works have shown to be involved in miRNA secretion. By western blot, we found that hnRNPA2B1 is not present within NPC-derived exosomes, suggesting that other proteins might be involved in miRNA secretion in NPCs. *Summary/conclusion*: Exosomal miRNAs do not seem to be regulated by specific TFs. We are currently investigating whether other proteins and/or other miRNA features (such as the pre-miRNA secondary structure) might be responsible for miRNA secretion in NPCs. Altogether, this work will help to shed light on the molecular mechanism behind miRNA trafficking and on its implication on the therapeutic effect of transplanted NPCs.

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Poster Session XIX - EVs and microbes
Chairs: *Lynn Pulliam and Michiel Pegtel*

P-XIX-1

Interspecies communication between plant and mouse gut host cells through edible plant-derived exosome-like nanoparticles

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Introduction: Exosomes, small vesicles participating in intercellular communication have been extensively studied recently. However, the role of edible plant-derived exosomes in interspecies communication has not been investigated. Here, we investigate the biological effects of edible plant-derived exosome-like nanoparticles (EPDEN) on mammalian cells. *Methods*: In this study, exosome-like nanoparticles from 4 edible plants were isolated and characterized. A standard protocol was used for isolating and charactering mammalian cell-derived exosomes. *Results*: We show that these EPDENs contain proteins, lipids and microRNA. EPDENs are taken up by intestinal macrophages and stem cells. The results generated from EPDEN-transfected macrophages indicate that ginger EPDENs preferentially induce the expression of the anti-oxidation gene, *heme oxygenase-1* (HO-1) and the anti-inflammatory cytokine, IL-10; whereas grapefruit, ginger and carrot EPDENs promote activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Furthermore, analysis of the intestines of canonical Wnt reporter mice, that is, B6.Cg-Tg(BAT-lacZ)3Picc/J mice, revealed that the numbers of b-galactosidase^{+} (b-Gal) intestinal crypts are increased, suggesting that EPDEN treatment of mice leads to Wnt-mediated activation of the Tcf4 transcription machinery in the crypts. *Summary/conclusion*: The data suggest a role for EPDEN mediated interspecies communication by inducing expression of genes for anti-inflammation cytokines, anti-oxidation and activation of Wnt signalling, which are crucial for maintaining intestinal homeostasis.

P-XIX-2

Humans absorb dietary microRNAs from chicken eggs, and the postprandial increase of plasma microRNAs includes a microRNA that humans cannot synthesize endogenously

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Introduction: Our previous studies suggest that (a) humans absorb vesicle-encapsulated microRNAs from cow's milk, (b) milk microRNAs are delivered to peripheral tissues where they alter the expression of human genes and (c) endogenous synthesis of microRNAs does not compensate for dietary microRNA depletion in mice. The following uncertainties remained: Can humans absorb microRNAs from foods of animal origin other than milk? Are the postprandial increases in plasma microRNAs truly caused by absorption of dietary microRNAs or does milk cause an increase in the expression of genes coding for microRNAs? *Methods*: Here, we addressed these questions by conducting an egg feeding study in humans (approved by the institutional IRB). Apparently healthy adults were fed 3 nutritionally relevant doses of hard-boiled chicken eggs (2, 3 and 4 eggs) in a randomized crossover design. The concentrations of 5 plasma microRNAs were assessed at timed intervals for up to 24 hours using qRT-PCR. *Results*: The analyses included a microRNA, *gga*-miR-1451, which humans cannot synthesize. Postprandial concentrations of plasma microRNAs were up to 150% higher than baseline and reached up to 80 fmol/L. Importantly, the plasma concentrations of *gga*-miR-1451-5p increased from below detection limit in time zero samples to 1 fmol/L 9 hours after egg consumption. A bioinformatics analysis was conducted and suggests that 66 of the 994 known chicken microRNAs have sequences identical to human microRNAs and target 69 KEGG pathways; the "non-human" *gga*-miR-1451-5p might target 10 KEGG pathways. *Summary/conclusion*: We conclude that humans absorb microRNAs from chicken eggs and that postprandial increases of microRNA levels in body fluids are caused by absorption of dietary microRNAs rather than increased expression of microRNA genes. Our observations add additional evidence in support of previous observations that food-borne microRNAs have biological activity in humans.

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P-XIX-3

Isolation and characterization of *Helicobacter pylori* CagA protein-containing exosome

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Introduction: Cells from various origins release vesicles with unique biophysical and biochemical properties. Particularly, exosomes are defined as lipid-bilayer membrane vesicles released into the extracellular environment on fusion of multivesicular bodies (MVB) with the plasma membrane. The composition of exosomes depends on the cell type of origin; therefore, they are known as ideal tools for intercellular communication. In this study, we isolated exosomes from the *Helicobacter pylori* CagA-expressing gastric epithelial cells and characterized their functions. *Methods*: AGS human gastric epithelial cells and MKN28 human gastric epithelial cell-derived WT-A10 cells that inducibly express CagA (WT-A10) were cultured. Exosomes were isolated by differential ultracentrifugation. The exosomes were characterized by immunoblotting, transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Cellular uptake and cell morphological changes by CagA-expressed exosomes were observed by confocal laser scanning microscope (CLSM). *Results*: We obtained 120-nm-sized nanoparticles with lipid bilayer structure from WT-A10 cells. CagA protein and exosome markers including CD9 and HSP70 were detected in the vesicles. Trypsin protection assay showed that CagA existed in the lipid bilayer of the inside of the exosomes. The CagA-containing exosomes were efficiently up-taken by AGS cells and the exosome-treated cells displayed an elongated cell-shape known as the hummingbird phenotype. These results indicate that *H. pylori* CagA proteins were delivered to the other cells in the form of exosomes. *Summary/conclusion*: *H. pylori* CagA is known as a risk factor of gastric diseases, while the relationship with extra gastric diseases has not been proven. This study raises the possibility that exsomes released from *H. pylori*-infected gastric epithelial cells could deliver CagA into non-gastrointestinal compartments, where CagA might invoke *H. pylori*-associated extra gastric disorders.

P-XIX-4

Non-typeable *Haemophilus influenzae* induces the release of pro-inflammatory extracellular vesicles by macrophages

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Introduction: Control over infections is mainly conferred by immune cells including dendritic cells and macrophages and involves a sound

intercellular communication. Over the years it has become clear that extracellular vesicles (EVs) fulfil an important function in this communication. Such EVs, for example, derived from macrophages infected with intracellular pathogens, have also been shown to induce pro-inflammatory responses. Here we investigated whether macrophages also release immunostimulatory EVs in response to non-typeable *Haemophilus influenzae* (NTHi), a gram-negative bacterium frequently associated with exacerbations in patients with chronic respiratory disease. *Methods*: EVs released by macrophages during NTHi-infection or stimulation with heat-inactivated (hi) NTHi were assessed by flow cytometry to determine release kinetics. Moreover, EVs were isolated by ultracentrifugation for characterization by electron microscopy (EM) and western blotting (WB). In addition, the functional activity of the crude EV pellet and purified EVs obtained by size exclusion chromatography (SEC) were determined. *Results*: Flow cytometric analysis revealed a dose- (MOI 0.1–10) and time-(2–8 hours) dependent release of CD63+/CD81+-EVs which peaked at 8 hours after NTHi (6-fold) or hi-NTHi (10-fold) challenge. EM and WB also confirmed that vesicles were released during infection. As compared to the control EVs, EVs from macrophages following 6 hours of infection (NTHi) or stimulation (hi-NTHi) significantly enhanced the release of TNF-α by naive macrophages. Similarly, these EVs induced the release of IL-8 by respiratory epithelial cells. Flow cytometric analysis of the fractions obtained after SEC of the crude vesicle pellet from NTHi-infected macrophages, demonstrated the presence of CD63+/CD81+-EVs in fractions 8–11. These were also the fractions that triggered the release of TNF-α from naive macrophages. *Summary/conclusion*: Both infection with NTHi and stimulation with hi-NTHi resulted in an enhanced release of EVs. In addition, these EVs possess a pro-inflammatory character as they trigger the release of pro-inflammatory cytokines from naive macrophages and airway epithelial cells. The presence of pro-inflammatory NTHi-derived outer membrane vesicles in the vesicle population from infected macrophages, however, cannot be excluded. Therefore, the pro-inflammatory response induced by this population cannot solely be attributed to EVs.

P-XIX-5

Effects of microvesicle-targeted treatment on EHEC infection in mice

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Introduction: Enterohemorrhagic *E. coli* (EHEC) infection may lead to haemolytic uremic syndrome (HUS) with acute kidney injury and death. The unique virulence factor associated with HUS is Shiga toxin (Stx). Previous studies have shown that microvesicles are released from blood cells during EHEC infection. These microvesicles possess prothrombotic and pro-inflammatory potential. A recent study from our group has shown that Stx reaches its target organs within microvesicles in humans and mice. Preliminary in vitro data has shown that the release of Stx-containing microvesicles from blood cells can be inhibited by certain substances. To study the importance of microvesicles bearing Stx for the development of full-blown disease in mice, we attempted to reduce microvesicle release and thus the transfer of Shiga toxin to its target organs using a calcium channel blocker and a purinergic receptor inhibitor. *Methods*: A well-established mouse model of *E. coli* O157:H7 infection mimicking certain aspects of human HUS was used. BALB/c or C57BL/6 were orally infected with *E. coli* O157:H7 and treated with nifedipine, an L-type Ca^{2+}-channel-blocker (5 mg/kg/day via subcutaneous pump) or suramin, a purinergic receptor inhibitor (20 mg/kg on day 1, 3, 5 i.p.). Mice were followed for the development of weight loss and symptoms. Mircovesicle levels were analyzed by flow cytometry. *Results*: Treatment with nifedipine resulted in lower platelet-derived microvesicle levels in treated Balb/c mice (n = 5) compared to control mice (n = 5). However, these mice exhibited a more pronounced weight loss and a higher morbidity (100% in treated compared to 60% in untreated). Mice treated with suramin (n = 5) also exhibited a tendency to reduced microvesicle release. Suramin-treated mice were protected from weight loss during infection. *Summary/conclusion*: Mice treated with substances that reduced microvesicle release in vitro showed a tendency to lower systemic microvesicle levels. The biological effects of suppression of microvesicle-release in EHEC-infected mice cannot be determined yet. Future results may offer a novel treatment for this infection.

P-XIX-6

Nifedipine and ouabain inhibit microvesicle release

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Introduction: Shiga toxin (Stx) is the main virulence factor of enterohemorrhagic *Escherichia coli*, which are non-invasive strains that can lead to haemolytic uremic syndrome (HUS), associated with acute renal failure, haemolytic anaemia, thrombocytopenia and the risk of death. Although bacteraemia does not occur, bacterial virulence factors gain access to the circulation whereby they cause target organ damage. Our group has shown that blood cell-derived microvesicles, shed during HUS, contain Stx and are found within patient renal cortical cells. In this project we aimed to test if the Ca^{2+} channel blocker nifedipine and the Na/K ATPase antagonist ouabain could inhibit microvesicle release in vitro. *Methods*: Human whole blood was incubated with nifedipine or ouabain for 20 minutes at 37°C. Samples were then stimulated with Stx2 (200 ng/ml) or left unstimulated for 40 minutes at 37°C. Blood cells were removed by centrifugation steps. Microvesicles were labelled with anti-CD42b-PE antibody for 30 minutes to detect platelet origin and analyzed by flow cytometry. *Results*: Stx2, incubated with human whole blood, induced the release of platelet-derived microvesicle in vitro and these microvesicles contained toxin. Nifedipine and ouabain inhibited Stx2-induced microvesicle release. *Summary/conclusion*: This study demonstrates 2 substances that inhibit the in vitro release of microvesicles from platelets. The ultimate therapeutic goal is to identify substances that can block microvesicle release and thus attempt to prevent Stx-mediated damage.

P-XIX-7

Secreted exosomes from *Heligmosomoides polygyrus* modulate cellular responses of the murine host

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Introduction: Exosomes are nanovesicles (50–100 nm), which facilitate cellular communication through the transfer of small RNAs, lipids and proteins. It has been shown that parasites' secretome can play a key role in both pathogenicity and host immunoregulation. Previous work has shown that exosomes derived from intracellular parasites, such as those discovered in *Trypansoma cruzi* and *Leishmania donovani*, are able to modulate the host inflammatory immune response, an adventitious strategy promoting parasite survival. Recent indications suggest that pathogens may use exosomes for cross-phylum communication. In this study, we demonstrate that secreted vesicles from the murine gastrointestinal nematode *Heligmosomoides polygyrus* exhibit a range of immunosuppressive and regulatory properties on murine cells and in vivo. This report adds to a growing number of studies showing that extracellular parasites release exosomes and we discuss how this contributes to their role in the host-helminth interactions. *Methods*: RNA analysis, ELISA, Tissue culture, flow cytometry and gene and immunization studies. *Results*: Through microarray of *H. polygyrus* exosome-treated small intestinal epithelial cells, we see significant gene changes, including those involved in the

regulation of signalling and the immune response. We have investigated a number of these genes in an attempt to clarify a signalling mechanism upon exosome interaction with cells. Furthermore, there is evidence that exosomes can affect the cytokine and gene signatures in both classical and alternatively activated bone-marrow-derived myeloid populations. Using a model of lung inflammation, in vivo studies demonstrate that in both prophylactic and co-administration experiments exosomes modulate the innate cellular response. This is represented by changes in number and function of innate lymphoid cells, bronchoalveolar lavage eosinophils and type-2 cytokine output. Finally, we are able to show that immunization of mice with exosomes can contribute to protection from a subsequent *H. polygyrus* infection. *Summary/conclusion*: This work suggests that exosomes secreted by nematodes could mediate the transfer and uptake of parasitic products into host cells, establishing cross-species communication to suppress the host ''danger'' or inflammatory response.

P-XIX-8

The splenocyte cellular immune responses from chronic mice after stimulation with Trypanosoma cruzi vesicles

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Introduction: Infection by *T. cruzi* induces a complex immune response that involves effectors and regulatory mechanisms. Previous study from our group demonstrated that vesicles from *T. cruzi* were able to differentially activate the innate immune compartment and previous exposure to those structures resulted in an increase in the parasite invasion in the heart muscles. However, little is known about the origin of the cytokines after stimulation by splenocytes during the chronic phase of Chagas disease. The aim of this study was to evaluate the impact of 2 *T. cruzi* strain vesicles on the intracellular cytokine profiles by chronic splenocyte mice infected with different *T. cruzi* strains. *Methods*: C57BL/6 Mice (5 per group) were infected with 50 parasites from each strain (Y, YuYu, CL and Colombiana) and positive animals were followed for 180 days. Control mice were not infected. Flow cytometry assays for IL-10, IFN-gamma and TNF-alpha cytokines were conducted on spleen chronic cells (1×10^6 cells) incubated with vesicles (5 microgram/ml, Yuyu and Colombiana strains) for 24 hours to short-term culture and immunostained with specific labelled antibody. The acquisition was performed using a FACSFortessa (BD Bioscience) and analyzed for frequencies and cell surface markers using FlowJo software package. Distinct gating strategies were used to analyze the cytokine-expressing leucocyte subpopulations from the innate (monocytes, dendritic cells and NK-cells) and adaptive immunity (lymphocytes). *Results*: Regardless the type of vesicle used, the activation was observed in splenocytes from all infected mice. Data analysis demonstrated that in the presence of vesicles an anti-inflammatory cytokine profile was observed in Yuyu strain stimulated culture with increased number of IL-10 in CD4 and CD8 T cells. In Colombiana strain, the data demonstrated that despite an increase of IL-10 in CD4, CD8 e B cells, a comparable number of TNF-alpha was observed in monocytes and dendritic cells. *Summary/conclusion*: Our results demonstrated that *T. cruzi* shed membrane components increasing inflammation by stimulation of IL-10 synthesis and thus may play a role in the pathogenesis of Chagas' disease during chronic phase. These results suggest that T cells are important source of IL-10 in chronic infection. This may be associated with protection of the host against the severe pathology induced by type 1 immune response.

P-XIX-9

Isolation and purification of extracellular vesicles from the parasite Schistosoma mansoni

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Introduction: *Schistosoma mansoni* is a blood fluke parasite responsible for schistosomiasis, 1 of the most chronic and debilitating parasitic diseases of low to middle income countries in the tropics. Approximately 200 million people are infected with different schistosome species, representing a major health problem and perpetuating poverty in endemic areas. Currently, chemotherapy is the major control strategy used, however, reduced efficacy of the only available drug, praziquantel, has been reported. Major research efforts are focussed on the development of new methods to identify proteins that could be used as novel vaccine candidates. *Methods*: The aim of this study was to isolate extracellular vesicles (EVs) secreted by *S. mansoni* larvae - a developmental stage of the parasite that migrates through the body before developing into adult worms in the hepatic veins. A combination of different methods including ultracentrifugation, filtration and sucrose gradient separation were employed for the isolation and purification of EVs. *Results*: The purified vesicles were analysed by transmission electron microscopy (TEM) and NanoSight® to confirm their presence, size and concentration prior to characterisation of their molecular profiles. *Summary/conclusion*: The characterisation of EVs from *S. mansoni* could be of importance to understand host-parasite relationships and aid in the design of new strategies to control this devastating neglected tropical disease.

P-XIX-11

The role of exosomes in *Chlamydia* pathogenesis

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Introduction: *Chlamydia trachomatis* genital infection is a major cause of sexually transmitted diseases worldwide. In women, infections are often asymptomatic and can lead to severe irreversible complications, including pelvic inflammatory disease, ectopic pregnancy and tubal factor infertility. Although, the mechanism of chlamydial disease pathogenesis is not fully understood, the chlamydial cryptic plasmid has been reported to play a role. While exosomes have been implicated in the pathogenesis of cancer, HIV and *Trichomonas*, their role in *Chlamydia* pathogenesis has not been previously investigated. In this preliminary study, we determined the ability of exosomes released from *Chlamydia* infected cells to package chlamydial proteins that may be involved in pathogenesis. *Methods*: To examine the potential role of exosomes in *Chlamydia* pathogenesis, an in vitro HeLa cell infection model was used. Thus, HeLa cells grown to confluence were infected with *C. trachomatis* serovar D at a multiplicity of infection of 5. Exosomes were collected and purified 72 hours post-infection by ultracentrifugation and analyzed using the Nanosight LM10 optical microscope together with the Nanoparticle Tracking Analysis software, and mass spectrometry. *Results*: Although the number of exosome particles released by infected cells was lower than that of uninfected controls, the total number of proteins incorporated in the former was higher. Of the total number of proteins incorporated in exosomes released from infected cells about 6% were chlamydial proteins. These included outer membrane associated proteins and proteins involved in signalling, metabolism and DNA/protein synthesis. These proteins are currently being characterized to identify those that may be involved in *Chlamydia* pathogenesis. *Summary/conclusion*: The results indicate that the exosomes released from

Chlamydia infected cells incorporate a large number of *Chlamydia* proteins, some of which may play a role in pathogenesis.

P-XIX-12

Immunomodulatory effect of extracellular vesicles secreted from parasitic trematodes in a murine host model

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Introduction: Helminths affect more than 2 billion people, mainly in developing countries. Recent studies have described the production of extracellular vesicles (EVs) by trematodes and nematodes, suggesting that they may play an important role in host-parasite relationship, including signalling events modifying the host immune response. We have studied the immunomodulatory role of EVs produced by parasitic trematodes in an experimental rodent model of infection. *Methods*: Two groups of Balb/c mice were immunized subcutaneously with either PBS (control) or EVs purified by differential ultracentrifugation from *E. caproni* adults. Both groups were challenged after immunization. The immune response was analyzed by ELISA and western blot. *Results*: Mice immunized with EVs presented a delay in the course of the infection, less severe symptoms and a high survival rate compared with control mice. Immunizations resulted in a systemic antibody response quite similar to the response reported for an infection, with high levels of IgM and IgG. After infection, IgG levels were statistically higher in the immunized group versus non-immunized mice. No differences were detected in the burden of adults isolated from both groups of animals. *Summary/conclusion*: Immunization with parasite EVs promotes a delay in the development of the parasite improving mice health status and consequently increasing the host survival rate. This provides evidence that EVs from parasites play a role in the modification of the host immune response although does not provide protection against an experimental infection.

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P-XIX-13

Trypomastigote vesicles from YuYu and y strains of *Trypanosoma cruzi* differ in their surface composition and promote parasite invasion

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Introduction: *Trypanosoma cruzi*, the etiologic agent of Chagas disease, releases vesicles containing a wide range of surface molecules including GPI-anchored glycoconjugates. Our group previously demonstrated that vesicles released by infective *T. cruzi* forms modulated inflammatory responses in host cells and during in vivo infection. Also, in vitro experiments showed that YuYu strain of *T. cruzi* release more vesicles than the Y strain. The objectives of this work were to characterize and define composition of the vesicles released by Y and YuYu strains of *T. cruzi* and to analyze parasite infection in host cells prior to vesicles exposure. *Methods*: *T. cruzi* trypomastigotes were cultured in LLC-MK_2 cells. After parasite recovery, vesicles were obtained from the supernatant for 2 hours. Macrophages and HeLa cells (10^4) were incubated with vesicles for 1 hour. After that, cells were exposed to parasites (MOI:10:1), fixed with methanol and stained with Hoescht. Parasite counts were performed using a digital video-imaging fluorescent inverted microscope. *Results*: Quantitatively, vesicles released by trypomastigote forms of YuYu strain contained more proteins than the Y strain. Furthermore, the Y strain vesicles containing larger amounts of alpha-galactose epitopes compared to the vesicles isolated from the YuYu strain. Finally, we found vesicles in the cytoplasm after their interaction with HeLa cells for 45 min and 2 hours of incubation, indicating incorporation by these cells. Pre-incubation with vesicles resulted in an increase of cell invasion by trypomastigotes from both strains. *Summary/conclusion*: In conclusion, vesicles differ in their pattern of liberation and composition and increase infection in the host cell.

P-XIX-14

Flow cytometric analysis of nano-sized extracellular vesicles and virions

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Introduction: Cells release nanosized particles, exosomes, membrane vesicles and virions that carry different antigens. Conventional flow cytometry, designed to analyze cells, cannot be applied to analysis of small particles with a diameter of less than 300 nm, which constitute the majority of extracellular vesicles (EVs) and small RNA viruses like HIV. Here, we report on new nanotechnology that permits high-throughput analysis of the antigenic composition of individual small EVs and virions. *Methods*: EVs or HIV virions were captured by 15-nm magnetic nanoparticles (MNPs) coupled to ''capture'' antibodies against surface proteins. Various antigens were revealed on these complexes with specific fluorescent antibodies. Unbound antibodies were separated in a magnetic field. Separated complexes were analyzed with a flow cytometer by triggering on fluorescence rather on light scattering. *Results*: 1) Both virions and EVs are efficiently captured by MNPs coupled to the antibodies against surface antigens: more than 95% of particles of interest are captured; 2) by choosing a proper antigen it is possible to capture and to analyze minor populations of virions or EVs that may be not identified when a total population of particles is analyzed; 3) conformation of surface proteins can be probed on individual particles, given the availability of specific antibodies; 4) the antigenic makeups of both EVs and virions are highly heterogeneous even if they are produced in the same cell populations and 5) it is possible to capture and analyze individual virions or EVs directly in biological fluids without preliminary isolation (e.g. with ExoQuick), which can change particle distribution. *Summary/conclusion*: The new nanotechnology permits analysis of individual antigenic makeup of EVs and virions and allows investigation of the distribution of the surface molecules that determine their functional properties, for example, the ability to interact with particular cells.

P-XIX-15

Comparison of methods of purification of extracellular vesicles

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Introduction: Extracellular vesicles (EVs) are attracting attention as intercellular communicators, cell-transforming agents, shelters for viruses and now as theranostic tools. They contain molecules (proteins, lipids, messenger RNA, microRNA) that indicate their producer cell type, physiological significance and impact on the functions of neighbouring cells. The characterization of EVs present in HIV-1-infected individuals provides insight into pathogenesis, inflammation and disease progression. However, their potential to become reliable research or diagnostic tools is currently limited due to the difficulty of distinguishing apoptotic and plasma membrane EVs, exosomes, and

virions. The aim of this study is to compare sucrose density gradient, iodixanol density gradient, velocity gradient and a commercial kit in terms of effectiveness at separating exosomes (acetylcholinesterase-positive EVs) from HIV-1 particles in cell culture supernatants and thereby evaluate their suitability for characterizing exosomes obtained from plasma of HIV-1-positive patients. *Methods*: Western blot analysis, ELISA, hydrodynamic size measurement and an assay for acetylcholinesterase were used to characterize EVs. *Results*: The commercial kit and the differential centrifugation methods precipitated both HIV-1 and exosomes. Protein p24 was found in the exosome-rich fractions of the sucrose density gradient but not in those of the iodixanol density or velocity gradient. Iodixanol velocity gradient provides the best method of separating HIV-1 from exosomes found in culture supernatants. *Summary/conclusion*: Although EVs and their contents provide helpful information about several key events in pathogenesis associated with HIV-1 and other agents of disease, their purification and characterization must be improved before they can be used reliably as biomarkers.

P-XIX-17

HIV-infected microglia and astrocyte cells secrete exosomes containing Nef

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Introduction: While combination anti-retroviral therapy has improved and saved the lives of millions of people living with HIV, the prevalence of HIV-associated neurocognitive disorder is increasing. Previous studies support the involvement of Nef in neuropathology of HIV, but the exact mechanism is unexplored. We hypothesize that Nef is exported from HIV infected microglia and astrocytes via exosomes, which then target and affect surrounding cells similar to what we have previously shown for HIV-infected T-cells. *Methods*: To test this, VSV-G pseudotyped HIV viruses (NL4-3, YU2 and NL4-3 Δnef) were produced in 293T cells and tested for infectivity using luciferase assay in TZM-bl cells, while western blot was used to check their composition. After infection of microglia and astrocytes with these viruses, culture media was filtered and exosomes/viruses pelleted by ultracentrifugation and later separated on OptiPrep gradient. Samples before and after gradient separation were analysed for AChE activity using enzyme assay, concentration of HIV p24 was determined by ELISA and the presence of p24, Nef, flotilin and AChE by western blot analysis. *Results*: In microglia, secretion of protein Nef and p24 was detected 3 days post infection with HIV virus. Furthermore, the level of Nef in microglia decreased after 5 days post infection, while p24 stayed constant. In astrocytes, viral infection resulted in secretion of p24 within 3 days and Nef after 5 days. According to these results samples used for further analysis were collected 5 days post infection for microglia and 7 days for astrocytes. Later OptiPrep gradient fractions efficiently separated exosomes and viruses from infected cells, as AChE activity was present in upper 6 fractions, whereas p24 was present in lower 5 fractions. In microglia, cells Nef protein was detected in medium fractions of OptiPrep gradient, while in astrocytes it was detected in most of the fractions. *Summary/conclusion*: We showed that infected microglia and astrocytes besides viruses secrete exosomes containing protein Nef. In further studies, we will focus on the functional role of Nef-exosomes on the other cells of the central nervous system.

P-XIX-18

Nef-transfected human microglia secrete Nef-containing exosomes

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Introduction: The main targets of HIV in the brain are microglia and astrocytes. Microglia are a type of glial cells that are involved in brain defence and immune system and constitute 10–15% of the brain cells. HIV protein Nef is one of the first and most abundant viral proteins, and it was previously detected in autopsied brain of HIV patients. We and others have shown that Nef stimulates its own export via exosomes, which affect viability of neighbouring cells. The aim of this study was to examine the effects of Nef-GFP expression on exosome release from human microglia. *Methods*: hTERT immortalised human microglia, transfected with Nef-GFP plasmid were grown in DMEM supplemented with exosome-depleted FBS. Exosomes were isolated from cell culture media after 1–4 days of incubation using one of the following methods: 1) pelleting by ultracentrifugation, washing with PBS and subsequent separation on sucrose gradient; 2) filtering through 0. 22 μm filter, purification 20% sucrose cushion and washing with PBS and 3) filtering through 0.22 μm filter and pelleting by ultracentrifugation. Exosomal proteins were extracted with RIPA buffer or TCA precipitation and analysed by western blot for different protein markers. Concentration of exosomes was determined by NTA analysis or by total exosome protein content. *Results*: Nef-GFP transfected microglial cells efficiently expressed Nef protein with maximum expression on the second day. Sucrose gradient analysis showed that Nef containing vesicles separate to fractions which correspond to exosomal fractions. The exosomal character of vesicles was additionally confirmed with electron microscopy. Kinetic studies showed that most of the Nef-containing exosomes are released on the third day after transfection, so we selected this day for all further analysis. Exosome samples contained specific exosomal protein markers like flotillin, Hsc70 and Tsg101. Furthermore, we were interested whether Nef increases the quantity of released exosomes. Quantification with NTA showed that Nef-expressing microglia release 4 times as many exosomes, while the total exosome protein content predicts 8 times as many exosomes, compared to control. *Summary/conclusion*: We conclude that Nef increases the release of Nef-containing exosomes. In future, the function of this exosomes will be studied.

Poster session XX - Late breaker, EV cargo: novel findings
Chairs: *Kenneth Witwer and Valentina Minciacchi*

P-XX-1

Role of extracellular vesicles and miRNA cargo in the control of epididymal functions important to sperm maturation

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Please see Oral with poster A

P-XX-2

Oxygen tension regulates the miRNA profile of extracellular vesicles secreted by human bone marrow-derived stem cells

Colleen Davis, Charan Balaji, Periyasamy-Thandavan Sudharsan, Sadanand Fulzele, Randy Ruark, Kelly Homlar, Raymond Corpe, Carlos Isales, Liu Yutao, William Hill and Mark Hamrick
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Introduction: Mesenchymal stem (stromal) cells (BMSCs) located in the bone marrow microenvironment are known to secrete extracellular vesicles (EVs), including both exosomes and microvesicles. These vesicles are also thought to be highly enriched in miRNAs, which can impact target cells through endocytosis and membrane fusion. The bone marrow "stem cell niche" is believed to be relatively hypoxic, with a gradient in oxygen tension existing within bone where oxygen tension is higher near the centre of the medullary cavity and lower along the peripheral endocortical surface. Here we tested the hypothesis that oxygen tension plays a key role in regulating the stem cell niche by altering the secretion of EVs and their miRNAs. *Methods*: To test this, human BMSCs were collected from bone marrow discarded from patients undergoing total knee and hip arthroplasty. BMSCs were isolated from hematopoietic lineage cells using magnetic bead sorting, and BMSCs were then cultured in either normoxic (37°C, 21% O_2) or hypoxic (37°C, 3% O_2) conditions and Evs harvested from conditioned medium. Relative size and concentration of EVs was performed using both transmission electron microscopy (TEM) and nanoparticle (Nanosight) tracking analysis. *Results*: Nanosight analysis quantitatively showed similar concentrations of exosomes collected under hypoxic conditions (17.21×10^8 particles/ml) and normoxic (16.77×10^8 particles/ml) conditions, although the mean particle size was greater under hypoxic conditions (185nm vs.129 nm). miRNA expression differences between normoxic and hypoxic samples were assessed using miRNA PCR array. Analysis showed that hypoxia increased the expression of only 2 miRNAs more than 2-fold (mir-125a, miR-101), whereas hypoxia downregulated 16 miRNAs more than 2 fold. Pathway and functional analysis of these miRNAs using miRSystem revealed that these miRNAs target 208 different genes in the human development reactome. These include genes involved in transcriptional regulation of adipogenesis and the differentiation of pluripotent stem cells. *Summary/conclusion*: Together, our results suggest that oxygen tension is likely to play an important role in regulating the microRNA content of exosomes secreted by BMSCs, and that oxidative stress may impact the differentiation of bone marrow cells via changes in the miRNAs transported by exosomes and microvesicles.

P-XX-3

Detection of DNA in extracellular vesicles Isolated from malaria-infected red blood cells

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Introduction: *Plasmodium falciparum* (Pf)-infected human red blood cells (RBCs) release extracellular vesicles (EV) at an elevated rate. Due to the absence of RBC machinery, EV generation likely takes advantage of parasite-derived trafficking machinery in the RBC cytoplasm. Laboratory studies have implicated EVs in the spread of drug resistance through the transfer of DNA between parasites but the details of this transfer remains unknown. *Methods*: EVs were isolated from Pf-infected RBCs using trans-well cultures. After incubation, released EVs were collected from the lower chamber and purified. Intact vesicles were treated with DNAse and lysed before DNA was isolated (EV-DNA). Digital droplet PCR (ddPCR) was then used to investigate the composition of EV-DNA. Using droplet formation, the ddPCR method allows the end point measurement of ~20,000 individual PCR amplifications with 0.1ng of DNA per sample. The ratio of positive droplets containing a Pf single-copy gene (S, seryltRNA synthetase) verses a Pf multicopy gene (M, dihydroorotate dehydrogenase) within the same reaction was calculated (M:S ratio). *Results*: To address whether EV-DNA was derived directly from the parasite genome, we compared the M:S ratio from genomic and EV-DNA. EV-derived human DNA was not efficiently amplified while parasite DNA was readily detected using this method. The average M:S ratio from EV-DNA was 2.8 (range of 2.3–3.3), indicating about 3 copies of the multicopy gene for every single copy gene. This was ~3-fold lower than the average ratio from genomic DNA (M:S of 6.9 (range of 6.4–7.3). *Summary/conclusion*: Our ddPCR studies show that EV-DNA is not simply fragmented genomic DNA. There is an apparent selection step that occurs as DNA travels from the nucleus to be packaged into EVs. In our model, which exploits the presence of a multicopy gene, differences could be due to the EV exclusion of putative extra-chromosomal DNA (ecDNA) that carries the extra copies of the dihydroorotate dehydrogenase gene. Preliminary analysis of the fragment sizes of EV-DNA indicate that packaged DNA is <2 kb, suggesting that the selection maybe based on fragment size (putative ecDNA is >50 kb). We are presently utilizing EM and flow cytometry to investigate the nature of purified EVs and the precise location of EV-DNA.

P-XX-4

Source cell microenvironment impacts extracellular vesicle cargo composition

Divya Patel, Tek Lamichhane and Steven Jay
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Please see Oral with poster B

P-XX-5

Acoustic microfluidic system for microvesicle purification

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Please see Oral with poster A

P-XX-6

Microfluidics exosome lysis and detection of microRNA for cancer study and diagnosis

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Introduction: Exosomal microRNA plays a critical role in cancer progression and metastasis and could serve as a reliable prognostic and diagnostic molecular marker. Current *in vitro* analysis of exosomal microRNA requires millilitres of cell media and cDNA amplification techniques such as RTPCR. *Methods*: In this work, a microfluidics-based approach to the analysis of exosomal microRNA is presented based on surface acoustic wave (SAW) exosome lysis and ion-exchange nanomembrane RNA sensing. *Results*: Using microRNA hsa-miR-550 as a model target and raw cell media from pancreatic cancer cell lines as a biological sample, SAW-based exosome lysis is shown to have a lysis rate of 38%, and an ion-exchange nanomembrane sensor is shown to have a limit of detection of 2 pM. Total analysis time requires approximately 1.5 hours, without the need for reverse transcription or amplification. The platform also requires much smaller sample volumes than existing technology ($\sim$100 μl cell media as opposed to $\sim$ml) and operates with minimal sample loss. *Summary/conclusion*: This approach for detection and quantification of exosomal microRNA is thus advantageous for studies involving mouse models or other situations where the working fluid is scarce. It may also prove suitable in the clinic for miRNA diagnostic and prognostic applications.

P-XX-7

Optimization of extracellular vesicle loading for microRNA-93 delivery

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Introduction: Extracellular vesicles (EVs) are natural microRNA (miRNA) carriers and thus have potential as miRNA delivery vehicles for therapeutic applications. However, as the concentration of miRNA naturally contained within EVs is relatively low, extrinsic loading methods may be required to facilitate physiologically relevant payloads. We compared external miRNA loading via electroporation with genetically induced overexpression of miRNAs in EV producing cells to determine the optimal approach. *Methods*: Human Embryonic Kidney cells (HEK293T) were used for EV production and miR-93, a known inducer of vascularization, was selected as the cargo molecule. HEK293T cells were transfected with plasmid precursor for miR-93 using lipofectamine and released EVs were isolated by differential centrifugation. RNA overexpression in cell and EV populations was confirmed with qRT-PCR. Additionally, EVs were isolated from non-transfected cells and loaded with miR-93 by electroporation. Loading was quantified using fluorescence measurements. *Results*: qRT-PCR measurements show increased miR-93 levels in both transfected HEK293T cells and the EVs they release. Although there is a 180-fold increase of miR-93 expression in the cells compared to control, EVs only show a 30-fold increase. In external loading studies, electroporation resulted in 3 pmol of miR-93 loaded into 10 μg of EVs. In comparison, EVs subjected to passive loading (no electroporation) contained less than 1 pmol of the target miR. *Summary/conclusion*: Both transfection and electroporation can be used to increase the amount of miR-93 present in EVs. The relative benefit of external loading compared to miRNA overexpression in EV producing cells remains unknown, and studies directly comparing the methods are necessary. Additionally, it remains to be determined how each loading method impacts cargo bioactivity. Overall, delivery of miR-93 via EVs can enable revascularization of ischemic tissues, and efficient loading will bring the approach closer to clinical translation.

P-XX-8

KRAS-dependent sorting of miRNAs and other small RNAs to secreted EVs

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Introduction: Although long thought to act cell autonomously, mutant *KRAS* colorectal cancer (CRC) cells release protein-laden exosomes that can alter the tumour microenvironment. We have previously shown that mutant KRAS induces EGFR-ligand trafficking to exosomes and drastically alters exosomal protein contents, leading to activities that contribute to neoplastic growth. *Methods*: We have performed small library RNAseq analysis on cells and matched exosomes from isogenic CRC cell lines differing only in *KRAS* status to determine whether mutant *KRAS* regulates the composition of secreted small RNAs. *Results*: Exosomal small RNA profiles were distinct from cellular profiles, with principle component analysis showing clusters of mutant *KRAS* cell-derived exosomes distinct from wild type *KRAS* cell-derived exosomes. Secreted RNA species encompassed several different classes of small RNAs, including ribosomal and tRNA fragments, as well as mature miRNA sequences. *miR-10b*, was selectively increased in wild type *KRAS*-derived exosomes, whereas *miR-100* was selectively increased in mutant *KRAS*-derived exosomes. Ceramide inhibition resulted in accumulation of *miR-100* in mutant *KRAS* cells, suggesting *KRAS*-dependent miRNA export. In Transwell cell culture experiments, mutant, but not wild type, *KRAS* donor cells conferred *miR-100*-mediated target repression in wild type *KRAS* recipient cells. *Summary/conclusion*: These findings suggest extracellular miRNAs can function in target cells and uncover a potential new mode of action for mutant *KRAS* in CRC. These results have implications for miRNA-driven tumour evolution directed by specific secreted miRNAs, as well as other new classes of secreted functional RNAs.

P-XX-9

Engineered glycosylation stabilizes exosome targeting peptides and enables targeted exosome delivery

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Please see Oral with poster C

P-XX-10

Embryonic stem cell exosomes-encapsulated curcumin: novel therapy for cerebral ischemia in Type-1 diabetes mellitus

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Introduction: Type-1 diabetes mellitus (T1DM) is a major stroke risk factor and is associated with poor recovery compared with non-diabetic stroke patients. The therapy available for stroke is tissue plasminogen activator (tPA) is not effective in diabetic stroke because it leads to excessive vasodilation and haemorrhagic transformations. Therefore, in the present study, we investigated the hypothesis that encapsulating curcumin to mouse embryonic stem cell (MESC) exosomes mitigates type-1 diabetic stroke injury. *Methods*: We employed 8–10 weeks old male genetic T1DM Ins2 + /- Akita mice and created ischemia for 40 min following reperfusion for 7 days. Exosomes were isolated from MESC culture conditioned media and encapsulated with curcumin (*cur-exo*^{5c}). The therapeutic *cur-exo*^{5c} nano-units (20 μg) were used for mice treatment through intranasal route for 7 days.

Following groups of mice were used for the study: (a) $Sham^{Akita}$, (b) $Sham^{Akita} + cur\text{-}exo^{Sc}$, (c) IR^{Akita}, (d) $IR^{Akita} + cur\text{-}exo^{Sc}$. Infarct area was measured by triphenyltetrazolium chloride. Brain cryo-sections were analysed for vascular (VE-cadherin), glial (GFAP), neuronal (NeuN) coupling in ipsilateral cortical area. Neuronal loss, neurodegeneration was analysed with NeuN, fluorojade-C staining. *Results*: We confirmed curcumin-encapsulation in MESC-derived exosomes and their targeted delivery to the mice brain through intra-nasal route. After 7 days treatment, we found improved neurological functions, reduced infarct volume and edema in $IR^{Akita} + cur\text{-}exo^{Sc}$ mice as compared to IR^{Akita} mice. *Cur-exo*^{Sc} treatment reduced vascular inflammation as determined by reduced ICAM level in cerebral vessels of $IR^{Akita} + cur\text{-}exo^{Sc}$ mice as compared to IR^{Akita} mice. Staining of brain coronal sections with NeuN and GFAP confirmed significant recovery of neurons and glia in $IR^{Akita} + cur\text{-}exo^{Sc}$ mice as compared to IR^{Akita} mice. Furthermore, *cur-exo*^{Sc} treatment significantly attenuated cell death in $IR^{Akita} + cur\text{-}exo^{Sc}$ mice as determined by Tunel staining ($p < 0.001$, $n = 6$). *Summary/conclusion*: Combination therapy using curcumin with MEEC exosome represent a novel treatment for stroke during diabetes.

This work was supported by National Institutes of Health grant HL107640 to NT.

P-XX-11

Characterization of extracellular nucleic acids inside and outside exosomes

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Please see Oral with poster C

P-XX-12

Characterization of induced pluripotent stem cell microvesicle genesis, morphology and pluripotent content

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Introduction: Microvesicles (MVs) are lipid bilayer-covered cell fragments that range in diameter from 30 nm to 1μM and are released from all cells and tissues. An increasing number of studies reveal that stem cell MVs contain microRNA, mRNA and protein that can be detected in the extracellular space. *Methods*: In this study, we characterized induced pluripotent stem cell (iPSC) MV genesis, content and fusion to retinal progenitor cells (RPCs) in vitro. Following MV isolation via ultracentrifugation, Nanosight particle tracking, transmission electron microscopy (TEM) and SEM were utilized to determine MV release rate, genesis and morphology. QPCR was used to determine the presence and level of mRNA species within MVs compared to iPSCs. Immunogold TEM was used to characterize MV protein content and labelled MVs were imaged fusing to RPCs using fluorescence microscopy. *Results*: Nanoparticle tracking revealed that iPSCs released approximately 2,200 MVs cell/hour in the first 12 hours with an average diameter of 122 nm. Electron microscopic analysis of iPSCs revealed cytoplasmic origin of MVs and release via lipid bilayer budding. The mRNA content of iPSC MVs was characterized and revealed the presence of the transcription factors Oct-3/4, Nanog, Klf4, and C-Myc. The protein content of iPSCs MVs, detected by immunogold electron microscopy, revealed the presence of Oct-3/4. Isolated iPSC MVs were shown to fuse with RPCs in vitro at multiple points on the plasma membrane. *Summary/conclusion*: These findings reveal that mRNA and protein cargo in iPSC-derived MVs have established roles in maintenance of pluripotency and plasticity. Building on this work, iPSC-derived MVs may be shown to be involved in maintaining cellular pluripotency and may have application in regenerative strategies for neural tissue.

P-XX-13

Proteomic, transcriptomic and genomic characterization of exosome-like vesicles in uterine aspirates

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Introduction: Extracellular vesicles (EVs) include microvesicles and exosome-like vesicles (ELVs), among others. ELVs are small membrane vesicles that may carry bioactive shipments that include proteins, lipids, mRNA, non-coding RNA and DNA, released from the cell of origin, to the microenvironment. ELVs can be found in a variety of body fluids including, blood, urine, saliva, breast milk and ascites; representing a possible reservoirs of biomarkers. A major bottleneck in this field is the lack of standardization for already challenging techniques to isolate and characterized ELVs. Most of ELV's biomarker studies have focused on common body fluids, however, ELVs in uterine aspirates have been poorly investigated but have great interest for the discovery of biomarkers of gynecological pathologies. *Methods*: This study obtained approval from the institutional review board. Written informed consent was obtained from all patients. EVs from uterine aspirates were obtained by 3 different methods of ultracentrifugation and characterized at nature level, by electron microscopy and NanoSight particle tracking; at protein level, by protein quantification and immunoblotting; at RNA and miRNA level, by BioAnalyzer and RTqPCR; and at DNA level, by mutational analysis. *Results*: Here, we standardized the protocol for EVs isolation from uterine aspirates by ultracentrifugation and further characterized their number, shape and their protein, RNA and miRNA and mutational cargo. *Summary/conclusion*: Our data demonstrate that isolation of EVs from uterine, as well as its characterization at proteomic, transcriptomic and genomic level, is feasible and promising. This study brings forward the identification of critical molecules in easy-to-access samples, which can be used as markers of gynecological diseases.

[†]These authors have contributed equally to the work.

Scientific Program ISEV 2015 meeting
Sunday April 26, 2015

Registration	08:00-09:00

Ballroom D
Meet the experts VII: EVs in the tumor microenvironment
Chair: *Aled Clayton*

Speakers: *David Lyden and Mattias Belting*	08:00-08:45

Ballroom E
Meet the experts VIII: EVs as diagnostics and prognostics
Chair: *Fred Hochberg*

Speakers: *Lorraine O'Driscoll and Clark Chen*	08:00-08:45

Ballroom F-H
Meet the experts IX: Possibilities and limitations of single EV-based analysis
Chair: *Jennifer Jones*

Speakers: *Marca Wauben and Alain Brisson*	08:00-08:45

Sponsor Exhibition	10:00-TBA

Ballroom D

Symposium session 9A - Advances in isolation and characterization of EVs and EV subpopulations

Chairs: *Olga Volpert and Cecilia Lässer* 08:45-10:15

O-9A-1

Curvature and lipid sensing using synthetic peptides: a paradigm shift in exosome capture strategy

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Introduction: Exosomes are cell-secreted, highly curved lipid nanovesicles that serve as intercellular carrier of oligonucleotides and proteins. These vesicles (diameter = 30–150 nm) also play important roles as mediators of intercellular communication and immune regulation, both in physiological and pathological conditions. The traditional imunoaffinity-based detection and isolation of exosomes using protein biomarkers is not fail-proof due to variability in protein expression levels. Meanwhile, ultracentrifugation is lengthy and tedious, and chemical precipitation is non-specific that could lead to impure exosome isolates. Therefore, a simple, direct, and global exosome capture strategy is desirable. *Methods*: We embarked to fill this gap by utilizing peptides with selective affinity for highly curved and anionic lipid nanovesicles as our design platform, with the goal of developing a generally applicable exosome-capture strategy based on peptide-lipid interactions. A mini-library of peptides was engineered and synthesized using microwave-assisted solid phase synthesis and characterized by mass spectrometry. Liposomes were used as model systems to mimic different vesicle sizes, composition and surface potential (30, 100, 400 nm; 0–20% phosphatidylserine). Confirmation of liposome morphology, size and charge were performed using electron microscopy, dynamic light scattering, and zeta potential analyses, respectively. *Results*: Our findings showed that the liposome models were morphologically spherical and mimic exosomes, with polydispersity index of 0.07–0.02. Zeta measurements indicated that the surface potential for each batch had the desired anionic strength of the particular liposome models. Our binding studies showed that the peptides recognized and bound to liposomes, were not only surface localized but also inserted into the membrane bilayer, with dissociation constants in the nanomolar range as measured by biolayer interferometry. Further, the peptides showed selection for liposome models that closely resembled exosomes in size and surface potential, while large liposomes and those that had neutral surface potential showed negligible binding. To test the ability of these peptides to capture exosomes, their biotinylated analogues were immobilized on magnetic beads, incubated with exosomes from HeLa and metastatic prostate cancer (PC3) cell cultures, and the amount of isolated exosomes captured was determined by measuring total proteins and oligonucleotides. This assay showed that our method exceeds the efficiency of a commercially available isolation reagent that relies on monoclonal antibody against exosome-bound CD63. *Summary/Conclusion*: Our method is sensitive to the ubiquitous phospholipid components of exosomes but is independent of protein biomarker cargoes, opening a new direction for a simple, robust and efficient capture of exosomes.

O-9A-2

Novel methods of accurate isolation and characterization of EVs for standardizable measurements

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Introduction: Progression from extracellular vesicle (EV) to clinical applications will be hindered by the lack of an effective, standardized method for isolation and characterization of EVs from different sample types. We have quantified the effect of sample preparation using size exclusion chromatography (SEC) columns, and other measurement variables on tunable resistive pulse sensing (TRPS) measurement of EVs from plasma and cerebrospinal fluid (CSF). TRPS systems are very effective but can be difficult to operate due to non-specific binding (NSB) in the pores. Reagents designed to overcome this NSB were tested, and the impact on sample measurement results quantified. *Methods*: CSF was collected via either lumbar puncture or brain surgery. Plasma samples were obtained from bovine blood. EVs were isolated from human and ovine CSF and bovine plasma using gravity flow SEC. TRPS was used to measure both diluted raw samples and SEC purified samples, with and without the NSB reagent. *Results*: The concentration of EVs in ovine CSF was found to be 5×10^{10}/ml, with a variation of up to 26% between individuals. Gravity flow SEC achieved purification of EVs in 10 minutes. The mode diameter of CSF EVs was 103 nm for unprocessed human CSF EVs and 108 nm for SEC purified EVs. Pre-treatment of measurement pores to prevent NSB gave approximately 90% reduction in pore clogging issues. Concentration of EVs in human CSF was found to be 8.68×10^{8}/ml and 9.48×10^{8}/ml from the same sample using a pre-treated and untreated pore respectively. Mean diameters were 118 and 122 nm. The use of SEC columns and the NSB reagent significantly reduced the standard deviation of multiple measurements of the same samples. The use of EV concentration within a defined size range rather than as a single number was shown to reduce the SD of one series of measurements from 52 to 37%. *Summary and conclusions*. When effective protocols are followed, the variation introduced by isolation and measurement can be minimized. A target CV of 30% across a range of different users is estimated to be achievable, but not yet achieved. Larger scale studies are required to definitively demonstrate the links between health states and EV concentrations. With sufficient confidence in the accuracy and consistency of the underlying measurements, such studies should significantly advance the level of understanding of the links between health states and EV levels. Standardized methods that allow comparison between results obtained on different samples and by different researchers can also provide a necessary pathway to gaining regulatory approvals for EV based diagnostic tests, prognostic tests and therapeutics.

P-IV-10

Analysis of extracellular vesicles using imaging flow cytometry

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Introduction: Extracellular vesicles (EVs) such as exosomes (70–160 nm) and microvesicles (100–1,000 nm) can be harvested from cell culture supernatants and from all body fluids. Current standard techniques to visualize, quantify and characterize EVs are electron microscopy, nanoparticle tracking analyses (NTA) and dynamic light scattering. To further characterize and discriminate EVs, it would be desirable to simultaneously analyze the presence of different molecules expressed on the surface of EVs using a high throughput technology. While standard flow cytometry due to the configuration

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of conventional flow cytometers is limited to measure particles down to approximately 300–500 nm, a new flow cytometry method called imaging flow cytometry potentially allows for the analysis of EVs smaller than 300 nm. The main drawbacks when using conventional flow cytometry are the high signal-to-noise ratios and the limitation to set the trigger only on one of the given channels, conventionally the forward scatter or a given fluorescence channel after fluorescence labelling for example with PKH dyes. In contrast, imaging flow cytometry allows simultaneous triggering in all given channels. *Methods*: Here, we investigated whether a more consistent characterization of EVs could be achieved, when the trigger was simultaneously set on the side scatter and the different fluorescence channels. By using the Amnis ImageStream^{x} MkII imaging flow cytometer, we comparably analyzed in a first set of experiments EVs harvested from different cell lines, either expressing CD63-eGFP or CD63-mCherry fusion proteins. Obtained samples were also analyzed by NTA. *Results*: By triggering simultaneously on the side scatter and the eGFP and mCherry fluorescence signals, we were able to visualize and enumerate individual EVs. Even though we were able to clearly discriminate eGFP, mCherry and unlabelled EVs, the concentration estimated by the imaging flow cytometer was a decade lower than the concentration determined by NTA. *Summary/conclusion*: We propose that imaging flow cytometry provides a feasible method for the field of EV research to perform high-throughput, multiparametric EV surface analyses.

O-9A-4

Label-free characterization of extracellular vesicles from human mesenchymal stem cells by Raman spectroscopy

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Introduction: Extracellular vesicles (EVs) from human mesenchymal stromal cells (hMSCs) have been extensively proposed as a powerful and versatile therapeutic tool for regenerative medicine. They are a valid alternative to cell therapy because of remarkable therapeutic potential and suitability for drug delivery, together with reduced risk of ectopic tissues formation and immune rejection compared to cells. Nonetheless, the heterogeneity of EVs makes often the comparison of experimental results biased. We propose herein Raman spectroscopy as a label-free non-destructive method suitable for EV characterization prior to their use in complex disease models and as an alternative to mass spectrometry or flow cytometry, both suggested as minimal requirements by ISEV (J Extracell Vesicles. 2014;3:26913). *Methods*: After the standard mesenchymal characterization procedure, hMSCs (2nd–4th passage) from bone marrow and adipose tissue were cultured until 90% confluency in medium containing 10% EV-deprived foetal bovine serum. hMSCs were then maintained 72 hours in serum-free conditions. Conditioned medium was centrifuged to remove possible cell debris and apoptotic bodies before being ultracentrifuged twice for 1 hour at 100,000 *g* (Bekman L7–65; Rotor 55.2 Ti). Freshly isolated EVs were analyzed by confocal Raman microspectroscopy with both 532 nm and 633 nm laser sources. *Results*: Raman spectra revealed the concomitant presence of phospholipids, cholesterol, ceramides, proteins, and nucleic acids in EVs from both cell sources. The general chemical composition of both samples shown by Raman microspectroscopy is in accordance with the reported ultrastructure of EVs, making it a suitable method for basic characterization of EV samples. Even more interestingly, beside the presence of common Raman peaks, the comparison of EVs from hMSCs of different tissues cultured under the same experimental conditions highlighted a specific Raman fingerprint dependent from the cell of origin. *Summary/Conclusion*: The preliminary results reported herein support the use of Raman spectroscopy as a valid tool for a fast EV characterization without vesicle damaging and chemical/physical fixation. Thanks to the avoidance of sample preparation and labelling, Raman spectroscopy can provide the overall EV biochemical fingerprint that facilitates EV applied research, overcoming difficulties in research data comparison. For these reasons, Raman-based *Methods* have the potentialities to become new tools for the routine inspection of EV samples before *in vitro* or *in vivo* use.

O-9A-5

Characterization of transplant tissue-specific exosomes in a xenoislet transplantation model

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Introduction: In the field of transplantation, there is a critical need for non-invasive biomarker platforms for monitoring transplant rejection. We propose that transplant tissues release exosomes into recipient plasma, and their characterization would constitute a novel biomarker platform. We tested this hypothesis in a mouse xenoislet transplantation model. *Methods*: Human islets were transplanted into diabetic, athymic mouse ($n = 20$). Animals were killed at variable time points (14 to 198 days). Islet graftectomy was performed in 6 animals and they were killed after 5 ($n = 3$) and 21 days ($n = 3$). Naive ($n = 5$) and allogeneic mouse transplants ($n = 5$) served as negative controls. Exosomes were isolated using size exclusion chromatography and ultracentrifugation. *Results*: Islet transplantation corrected diabetes during follow-up. On NanoSight fluorescence mode, human islet-specific exosome signal (anti-human major histocompatibility complex (MHC) antibody-quantum dot) was detected in the recipient mouse plasma total exosome pool at all tested time points, but was absent in the controls ($p < 0.0001$). These findings were confirmed on Western blot analysis. After donor islet-graftectomy, decreased human islet signal was detected on 5 days, but was absent on 21 days.

Transplanted islet-specific exosomes were purified from recipient plasma using affinity antibody conjugated bead technology (anti-human MHC antibody beads). Unlike whole plasma and antibody unbound exosomes, the protein and RNA cargo of human MHC bound fraction was enriched in *bona fide* islet-specific markers such as insulin, glucagon, and somatostatin on Western blot and RT-PCR analyses ($p < 0.0001$). The bound fractions in islet graftectomy samples showed absence of islet-specific markers.

Affymetrix microarray analysis performed on transplant islet-specific exosomes showed distinct microRNA and mRNA signals compared to the islet graft mass. *Summary/Conclusion*: Transplant islet-specific exosomes can be non-invasively tracked, purified, and characterized from recipient plasma. Understanding changes in transplant tissue-specific exosome profiles from recipient plasma/ bodily fluids under conditions of transplant organ maintenance versus rejection may constitute an important, novel biomarker platform in transplant diagnostics.

O-9A-6

Vastly diverse morphology of mast cell extracellular vesicles isolated by density flotation revealed by cryo-electron microscopy and tomography

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Introduction: Extracellular vesicles (EVs) in biological fluids can have many shapes and forms. To elucidate their functions, vesicles are

often purified using different density sorting *Methods* such as sucrose or optiprep gradients. We asked: "Do isolated EVs from a single cell type also have a diverse morphology?" *Methods*: Cryo-electron microscopy and tomography was used to examine morphologies of EVs isolated from human mast cells (HMC-1) by upward displacement in a density gradient after sequential centrifugations to remove apoptotic bodies. *Results*: A vast range of sizes and shapes of EVs was discovered. Single vesicles could contain one, two, three or even more smaller vesicles. Other membrane compartments such as tubules and vesicle sacs were also present. Some EVs were electron dense, some had a surface coat and yet others carried double membrane bilayers. The diversity of morphologies of the EVs is presented both as two-dimensional cryo-electron photographs as well as three dimensional cryo-electron tomography film clips. *Summary/Conclusion*: It is reasonable to assume that the different vesicle morphologies represent different functionalities, origin, destinations or combinations thereof. Thus, to understand the functional outcomes of EVs research, it is of utter importance to know what different types of EV the used isolate contain.

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Ballroom E

Symposium session 9B - EVs in infection, immunity, and inflammation

Chairs: *Juan Falcon-Perez and Stefania Raimondo* 08:45-10:15

O-9B-1

Enterobacteria-secreted particles induce production of exosome-like S1P-containing particles by intestinal epithelium to drive Th17-mediated tumourigenesis

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Introduction: The tumorigenic function of exosomes has become widely recognized. However, the potential role of intestinal mucosa-derived exosome-like nanoparticles (IDENs) in the regulation of the intestinal immune system of a colon tumour–bearing host has not been studied. Based on the characteristics of exosomes released from intestinal epithelial cell (IEC) lines, it has been suggested that IEC-derived exosomes influence antigen presentation and antimicrobial responses. However, because cell culture cannot completely mimic the microenvironment of the gut community of a host bearing a colon tumour, it has not been possible to determine whether the exosomes in the intestinal mucosa of a colon tumour–bearing host regulate the host immune response by communicating with intestinal bacteria. *Methods*: We used three well-established Th17-regulated colon cancer mouse models to determine whether IDENs under different pathophysiological conditions play a role in the induction of intestinal Th17 cells by communicating with enterobacteria (ET)-secreted particles (ET-BSPs). *Results*: We find that enteropathogenic bacteria-secreted particles (ET-BSPs) stimulate intestinal epithelium to produce IDENs (intestinal mucosa-derived exosome-like nanoparticles) containing elevated levels of sphingosine-1-phosphate, CCL20 and prostaglandin E2 (PGE2). CCL20 and PGE2 are required for the recruitment and proliferation, respectively, of Th17 cells, and these processes also involve the MyD88-mediated pathway. By influencing the recruitment and proliferation of Th17 cells in the intestine, IDENs promote colon cancer. We demonstrate the biological effect of sphingosine-1-phosphate contained in IDENs on tumour growth in spontaneous and transplanted colon cancer mouse models. *Summary/Conclusion*: Collectively, IDENs communicate with the established mammalian gut community and regulate gut immune homeostasis. Dysregulation of the composition of IDENs (for example, the overproduction of S1P) promotes colon tumour growth.

O-9B-2

The diplomonad flagellate *S. vortens* displays impaired proliferation and heightened oxygen sensitivity in EV-depleted media

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Introduction: Giardiasis is a disease caused by the intestinal parasite *Giardia lamblia*, a diplomonad flagellate. Approximately one billion humans are infected by *Giardia* symptomatically or asymptomatically in any given year. Closely related *Spironucleus* species are economically important parasites of fish and may serve as useful models for *Giardia* in axenic culture. In this study, we examined *S. vortens* in culture to assess release of extracellular vesicles (EVs) and dependence on EVs in the environment. *Methods*: *S. vortens* were obtained from ATCC and maintained in axenic culture in conditions ranging from 1% to atmospheric oxygen and from 22 to 25°C. Cultures were observed regularly for signs of stress and transition from trophozoite to encysted form. Cell counts were obtained by using ProtoSlo and haemocytometer. Motility was assessed visually and movies were obtained. Culture medium was depleted or not of serum EVs by standard ultracentrifugation procedures. EV abundance was assessed by nanoparticle tracking analysis. *Results*: *S. vortens* cultures were sensitive to high, atmospheric oxygen concentration, but also to EV-depleted medium. Trophozoites cultured in depleted media experienced an approximately one-log decrease in proliferation as well as reduced motility. Under these conditions, dose-dependent oxygen sensitivity was apparent. However, in depleted media, there were no significant differences in the number of vesicles released by *S. vortens* exposed to 1%, 2.5%, 5%, or atmospheric levels of oxygen. *Summary/Conclusion*: Our results indicate that EVs in culture media of the diplomonad flagellate *S. vortens* are vital for normal growth and behaviour. Ongoing research seeks to determine the molecular component(s) that contribute to this effect. Despite clear effects of oxygen on *S. vortens* state (trophozoite/cyst) and proliferation, it did not appear that EV release by the parasites was modulated by oxygen levels

O-9B-3

Detection of hepatitis C proteins in urinary exosomes of hepatitis C-infected patients

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Introduction: Since its appearance in the United States, the incidence of new cases of hepatitis C virus (HCV) has decreased, yet it accounts for more disease and death than Human Immunodeficiency Virus (HIV). In other parts of the world, especially in developing countries, the prevalence of HCV infection is even greater. There is therefore great need to develop novel diagnostics that would provide for highly sensitive, specific, non-invasive and early detection of hepatitis C infection. *Methods*: In this study, we developed a urine-based ELISA, capable of distinguishing hepatitis C-infected patients from negative controls. It is based on the ability of asparagine linked glycosylation homolog-6 (ALG6) antibody to reversibly bind urinary exosomes, thus allowing for detection of hepatitis C proteins using specific monoclonal antibodies. *Results*: The assay identified hepatitis C non-structural protein 3 (NS3) and non-structural protein 4 (NS4) as the predominant hepatitis proteins in urinary exosomes of Hepatitis C-infected patients. When used in combination with hepatitis C core antigen, as a diagnostic marker of infection, the assay had a sensitivity and specificity of 100%. Nanoparticle tracking analysis also revealed higher urinary exosome concentration in infected patients (9.86×10^8 particles per ml of urine) compared to healthy subjects (1.57×10^8 particles per ml of urine), implying that infection with the virus was sufficient to stimulate exosome production and release. *Conclusion*: These findings represent a novel technique, which has the potential for development into a point of care diagnostic. It is non-invasive, not requiring the use of blood, thus ensuring the safety of healthcare workers, and is acceptable in developing countries where blood collection is considered objectionable.

O-9B-4

Immunosuppressive exosomes persist in plasma of acute myeloid leukaemia patients in complete remission and may contribute to relapse

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Introduction: Tumour-derived exosomes are emerging as one of the key immunosuppressive mechanisms in cancer. We have previously shown that patients with newly diagnosed acute myeloid leukaemia (AML) prior to any therapy have higher levels of exosomes (in μg protein/mL plasma) than normal donors (NC). These exosomes carry an immunosuppressive cargo, including membrane-associated TGF-β1, MICA/MICB and markers of myeloid blasts. They also persist and are detectable in AML patients who achieve morphologic complete remission (CR) after initial therapy. We hypothesize that persistence of these exosomes may contribute to the high relapse rates seen in AML. *Methods*: Venous blood (20–50 mL) was obtained from patients newly diagnosed with AML prior to any treatment, after the completion of induction chemotherapy in patients who achieved CR, and from age-matched healthy volunteers (NC). Exosome fractions were isolated from plasma by size-exclusion chromatography on Sepharose 2A columns followed by ultracentrifugation. Exosome protein content (μg protein/mL plasma), size by qNano and morphology by TEM were determined. Exosomes were characterized by western blots for expression of classical exosomal markers and of novel myeloid cell surface markers associated with AML, interleukin-3 receptor alpha chain (CD123) and C-type lectin-like molecule-1 (CLL-1). Isolated normal human NK cells were co-incubated with AML exosomes and multiparameter flow cytometry was used to monitor changes in expression levels (mean fluorescence intensity or MFI) of NKG2D on NK cells. *Results*: The exosome fractions isolated from AML patients' plasma at diagnosis (n = 13) had higher ($p < 0.005$) mean protein content (81.5 ± 10.8 μg) than those obtained from NC plasma (13.1 ± 2.4 μg). Exosome protein levels in patients who achieved CR (n = 8) remained elevated, similar to the levels at the time of AML diagnosis (78.5 vs 77.5 μg). The high protein levels in exosomes of AML patients in CR were evident at the time when leukemic blasts were undetectable in the bone marrow by conventional haemato-pathological methods. These exosomes carried blast markers, CD123 and CLL-1 as well as TGF-β1. Co-incubation of the exosomes from AML patients in CR with NK cells resulted in down-regulation of NKG2D with a concomitant reduction of NK-cell cytotoxicity. *Summary/Conclusion*: Exosomes in plasma of AML patients in CR have a distinctive molecular profile associated with immune suppression. Persisting or elevated levels of biologically-active exosomes in AML patients' plasma impair anti-leukaemia immune responses and thus may contribute to leukaemia relapse.

O-9B-5

Transplantation of adult mesenchymal stem cells prolongs lifespan and healthspan in a mouse model of accelerated aging: a role for microvesicles

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Introduction: The accumulation of cellular damage, including DNA damage, contributes to aging and aging-related degenerative changes. Excision repair cross complementary group 1-xeroderma pigmentosum group F (ERCC1-XPF) is a structure-specific endonuclease that is required for multiple DNA repair pathways. Mutations affecting expression of ERCC1-XPF cause a severe progeroid syndrome in humans and in mice. Previously we have showed that intra-peritoneal (IP) injection of muscle derived stem/progenitor cells (MDSPCs) isolated from young wild-type mice into ERCC1-deficient mice confers significant lifespan extension. Here we examined the ability of bone marrow-derived mesenchymal stem cells (BM-MSCs) from young mice to prolong lifespan and the mechanism behind that extension. *Methods*: We used ERCC1-deficient mice as a model of accelerated aging to examine the effect of stem cells and exosomes on aging. BM-MSCs were isolated from young (4 weeks), old (> 2 years) and ERCC1-deficient (2 weeks) mice and cultured in low oxygen condition (3% O_2). Exosomes were harvested from the BM-MSC conditioned media by ultracentrifugation. *Results*: A single treatment with $1x10^6$ BM-MSCs from young mice subjected briefly to oxidative stress and injected IP was able to prolong lifespan of *Ercc1*$^{-/-}$ mice. Furthermore, the healthspan of the treated mice was improved. In contrast, BM-MSCs derived from aged mice or not subjected briefly to oxidative stress had no or only a limited effect on lifespan. Using BM-MSCs transduced with a vector expressing shrimp luciferase, we observed that BM-MSC do not migrate after 1 week post IP injection and that stressed cells survived longer *in vivo*. These results suggest that the extension of lifespan is due to a paracrine/endocrine mechanism. To identify factors released by young, functional stem cells important for extending lifespan and healthspan, we utilized assays that measure the ability of an agent to reduce senescence in ERCC1-deficient murine embryonic fibroblasts (MEFs) and aged BM-MSCs. Using these assays, we demonstrated that conditioned media from WT, but not *Ercc1*$^{-/-}$ BM-MSC was able to reduce senescence in MEFs and BM-MSCs in culture. Moreover, these activities co-purify with microvesicles released by stem cells. In preliminary experiments, IP injection of microvesicles derived from young BM-MSC extended lifespan and healthspan of the ERCC1-deficient mouse model of accelerated aging. *Summary/Conclusion*: These results demonstrate that treatment with young, but not old BM-MSCs can extend lifespan and healthspan in a mouse model of accelerated aging. Moreover, the results suggest that at least part of the therapeutic effect on lifespan/healthspan is mediated by exosomes derived from the young BM-MSCs.

P-X-13

Antigen-loaded exosomes and microvesicles induce different immune responses in vivo

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Introduction: Extracellular vesicles (EVs) including exosomes (exo) and surface-shed microvesicles (MV) transport proteins and nucleic acids, and mediate communication between cells. They are explored as therapeutic vehicles in several diseases including cancer and neurodegenerative disorders. MV are 100–1,000 nm in diameter and generated by direct budding from the cell surface; exo are 30–120 nm in diameter, formed in endosomes and are released extracellularly. Due to their differences in size and biogenesis, MV and exo are likely to function and transport cargo in different ways. Exo can present antigen via MHC and interact with immune cells, MV could have similar or complementing functions which makes them potentially suitable in therapeutic applications. Our group has specialized in investigating immune-regulating properties of exo, and we have now compared exo and MV side by side in vitro and in vivo. *Methods*: MV and exo from OVA-pulsed bone marrow-derived dendritic cells were analyzed using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and flow cytometry. Wild-type mice were immunized intravenously with antigen-loaded MV or exo, and immune responses were assessed 7 days post immunization. Splenocytes were examined for IFN gamma production by ELISPOT; cell phenotypes were assessed using flow cytometry and serum concentrations of Immunoglobulin subtypes were measured by ELISA. *Results*: Exo and MV displayed overlapping sizes and characteristics based on TEM and NTA, as well as similar setup of immune-relevant markers based on flow cytometry. Exo-based OVA delivery (exo-OVA) rendered strong IFN gamma responses from splenocytes restimulated with the CD8-specific OVA-peptide SIINFEKL but weak when restimulated with the whole OVA protein. MV-OVA, however, induced opposite effects. MV and exo also induced quantitative differences of immune cell subtypes in spleen, for example, higher numbers of T follicular helper cells after MV injection suggesting activation of different cells by the 2 vesicle types. *Summary/conclusion*: Exo induce qualitatively different immune responses compared to MV. The MV-induced involvement of T follicular helper cells and responsiveness to whole antigen restimulation suggest that MV-associated antigen is processed differently compared to exo-associated antigen. The understanding of how exo and MV can induce different immune responses may help to develop optimal vesicle-based tools in immunotherapy.

Ballroom F-H

Symposium session 9C - Novel findings in EV cargo

Chairs: *Mattias Belting and Paola de Candia* 08:45-10:15

O-9C-1

Label-free detection of binding kinetics of affinity reagents to subpopulations of extracellular vesicles

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Introduction: Extracellular vesicle detection by current *Methods* has to overcome several challenges including the lack of universally accepted molecular markers. Search for such markers is significantly delayed by currently available isolation and characterization *Methods*. The goal of this work was to use quartz crystal microbalance biosensor (QCM) technology for label free detection of binding of a panel of candidate affinity ligands to extracellular vesicle surfaces. *Methods*: Extracellular vesicles subpopulations (apoptotic bodies, microvesicles and exosomes) were isolated from the conditioned media of different human and mouse cell lines (including 5/4 murine T cell hybridoma line, U937 human monocytic cell line and THP-1 human monocytic cell line) by differential centrifugation and gravity driven filtration. Extracellular vesicles were characterized by tunable resistive pulse sensing and their quantity and quality were assessed by their protein to lipid ratio. Purified vesicles were tested using a QCM biosensor (Attana, Sweden) and a panel of commercially available antibodies, cholera toxin subunit B as well as lectins. *Results*: We found evidence for binding affinities in the nM range for the tested affinity reagents. Interestingly, the kinetics of binding were strikingly different in the case of antibodies and lectins. Also, the unique binding pattern of cholera toxin B subunit suggested complex interaction upon binding to vesicle surfaces. *Summary/Conclusion*: Taken together these data argue for the use of alternative, less expensive affinity reagents than antibodies for the detection and characterization of extracellular vesicles.

O-9A-3

Biotinylation proteomics to identify extramembraneous proteins associated with extracellular vesicles

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Introduction: To understand extracellular vesicle (EVs) structure, it is important to determine which EV proteins are present outside of EVs, versus inside of the EVs (membrane protected cargo). Identifying EV surface proteins can also help to understand their surface-to-surface interaction with recipient cells, and may predict pathways by which they are taken up by recipient cells. Also, EV surface proteins can putatively function as biomarkers in disease. The aim of this study was to identify which proteins in EV isolates are present outside versus inside of vesicles. *Methods*: EVs were isolated from human mast cell line (HMC1) using a sequential ultracentrifugation protocol followed by upward flotation. Collected samples were treated with biotin to tag membrane proteins or other proteins outside of the EV membrane. The proteome of the EV subsets were further analyzed using LC-MS/MS (duplicates), distinguishing proteins with biotin tags from proteins without biotin tags. *Results*: The number of all EV proteins detected was 1647, of which 195 were biotin tagged. Based on unique peptides, the top five proteins from the biotin-tagged list were moesin, neuroblast differentiation-associated protein, talin-1, long chain fatty acid-CoA ligase and alpha-enolase. The top five proteins which were not tagged with biotin were cytoplasmic dynein, HspA8, Flotillin-1, ATP-citrate synthase and mast/stem cell growth factor receptor KIT. Common surface markers for EV's such as CD63, CD9 and CD81 were detected in both the biotinylated and non-biotinylated protein lists. *Summary/Conclusion*: The percentage of biotin-tagged proteins was approximately 13% versus the non-biotin-tagged proteins. Several proteins considered to be cytosolic proteins, were found to be biotin tagged, which suggests either rupture of vesicles or, non-conventional presence of these proteins outside of the EVs. Biotinylation-based proteomics may be helpful to distinguish the location of proteins in extracellular vesicle isolates.

O-9C-2

Development of a single-step isolation platform to analyse exosomal RNA and cell-free DNA in plasma from cancer patients

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Introduction: Circulating nucleic acids in the bloodstream of cancer patients are of great interest to medical research because of their potential to yield information on a patient's disease status and guide treatment options without requiring a tissue biopsy. Any diagnostic test that seeks to utilize biofluids for mutation analysis needs a platform that can maximize the capture of tumour-derived mutations in circulation. Blood plasma contains at least two cell-free sources of nucleic acids: circulating cell-free DNA (cfDNA), generated from apoptotic or necrotic cells, and RNA enclosed in extracellular vesicles including exosomes (exoRNA), which are actively secreted by cells in the body. *Methods*: We devised a method to simultaneously isolate both exoRNA and cfDNA from blood plasma using a single isolation column, maximizing assay sensitivity and capturing molecular information from both biologically different sources. The isolation of high quality nucleic enables effective downstream processing by RT-qPCR and targeted re-sequencing with NGS for mutations in BRAF, NRAS, KRAS, PIK3CA, MEK1 and EGFR. Here we utilized our combined exosome and cfDNA platform to co-isolate and analyse exoRNA and cfDNA in blood plasma samples from colorectal cancer patients as well as malignant melanoma patients and compared it to the result of using cfDNA alone. *Results*: The combined exoRNA and cfDNA column always isolated more copies of the genes of interest compared to looking at cfDNA alone. The increased number of molecules available for rare mutation detection increased the detection sensitivity in both malignant melanoma and colorectal cancer patients. *Summary/Conclusion*: Efficient isolation of exoRNA and cfDNA is critical for maximizing mutation detection sensitivity. The mechanisms of exosome release and cfDNA release are biologically different. Analysing both of these entities allows you to monitor both the living and dying process of the tumour and increases the mutation detection sensitivity compared to analysing cfDNA alone.

O-9C-6

A novel mechanism for loading specific target proteins into exosomes

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Introduction: Exosomes have the capacity to transfer biological material from one cell to another. The outcome of this cell to cell communication depends on the specific cargo that is transferred. Here we show the ability to specifically load exosomes with proteins that allows for targeted cell to cell communication. Previously we have identified that Nedd4 ubiquitin ligases can be transported into exosomes under the control of the ubiquitin ligase adaptor protein Ndfip1. Based on this interaction we developed a novel mechanism to load-specific proteins into exosomes. *Methods*: We constructed fusion proteins that are able to interact with Ndfip1 and therefore potentially be transported into exosomes. After transfecting these fusion proteins into different cell types we used western blotting to confirm the presence in purified exosomes and different uptake experiments to investigate the transport of the active fusion protein to recipient cells. *Results*: We demonstrate that a targeted CRE fusion protein is transported into exosomes only in the presence of Ndfip1 compared to an untagged CRE protein which cannot be detected in exosomes. Furthermore we were able to show by using Cre reporter cells the transfer of active CRE fusion protein to recipient cells. Mutating the PPxY motive in the adaptor protein Ndfip1 prevented the interaction with the fusion protein and therefore resulted in a significant reduction of CRE transport into exosomes. The specific transport mechanism was confirmed using a tagged-mCherry fusion protein which could also be detected in exosomes. *Summary/Conclusion*: We have identified a mechanism to load-specific target proteins into exosomes that are functional in recipient cells. The ability to transport-specific proteins in exosomes may provide a method for the development of therapeutic exosomes in the future.

O-9C-4

A urine exosome gene signature predictive of aggressive prostate cancer (Gleason = 7)

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Introduction: Prostate cancer (PCa) is the second leading cause of cancer death among men in the United States, with an anticipated 233,000 new cases and nearly 29,480 deaths in 2014. The definitive diagnostic for PCa is the prostate needle biopsy, typically recommended for men with elevated serum PSA levels and/or a suspicious digital rectal exam (DRE) with added indication from family history, age and race. The majority of prostate cancers remain indolent, infrequently resulting in death. Thus, there is a major risk for detecting cancers that are clinically insignificant and do not require treatment. Unfortunately, due to the low positive predictive value (PPV) of PSA and the high prevalence of low risk PCa, approximately 70% to 80% of men will undergo an unnecessary biopsy. *Methods*: The study population consisted of men aged $=40$ years scheduled for an initial prostate needle biopsy, due to a suspicious DRE and/or PSA levels. First catch non-DRE urine specimens were collected at six sites in standard collection vessels, stored at 2–8°C (up to two weeks) and shipped on ice to a central laboratory (Exosome Diagnostics, St. Paul, MN). Upon receipt, samples were filtered (0.8 μm), and exosome isolation and RNA extraction performed. A previously defined gene panel of ERG, PCA3 and SPDEF were measured and the copy numbers of the genes converted to yield a score predictive of Gleason $=7$ prostate cancer. *Results*: Urine samples from 170 men (PSA 2–10 ng/ml, first biopsy; median age 62 years; median PSA 5.1 ng/ml; 70% negative DRE; 77% no family history; 82% Caucasian) were evaluated. A dichotomous gene signature demonstrated good clinical performance in predicting biopsy results. For GS7+, the NPV and PPV were 98.6% and 34.7%, respectively. A continuous score alone had an AUC of 0.76 for discriminating GS7+ from GS6 and benign disease, and the results were significantly better than the prostate cancer prevention trial risk calculator (PCPTRC), AUC 0.60. *Summary/Conclusion*: In summary, this study presents the first exosome-based, non-invasive molecular assay utilizing the exoRNA signature as a prognostic tool for predicting high-grade PCa among men presenting for an initial biopsy with serum PSA levels within an expanded grey zone (>2 and <10 ng/ml).

O-9C-5

MicroRNAs contained in extracellular vesicles play a functional role in cardiac remodelling

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Introduction: Plasma miR-30d is a theragnostic biomarker independently associated with cardiac remodelling in patients with advanced heart failure. We have previously shown that miR-30d is present in extracellular vesicles (EVs) and is enriched in the coronary sinus. EVs and their cargo have recently garnered attention in cardiovascular biology as mediators of intercellular communication. Here we describe paracrine signalling between cardiomyocytes (CMs) and fibroblasts, and describe a novel murine model to study CM-derived EVs. *Methods*: EVs in cell culture medium from primary neonatal CMs or from plasma were isolated using standard filtering and ultracentrifugation techniques and were characterised using electron microscopy and flow cytometry. CMs seeded on a silicon membrane were subjected to rotational stretch and miR levels were assessed using qPCR in the EV fraction and cell lysates. Transfer of fluorescently-tagged EVs was assessed by confocal microscopy using an Olympus BX62 microscope with Qimaging Emc2 EMCCD cooled camera. Rosa mTmG transgenic mice were bred with alpha-MHC driven, tamoxifen responsive Cre recombinase expressing mice (mer-Cre-mer) to specifically tag CM membranes with GFP, and EVs in the plasma of these mice were characterized further. *Results*: miR-30d is significantly increased in the EV fraction at 6 and 24 hours ($n = 4$–5, $p < 0.05$) but not in the cell lysate, suggesting specific release of miR-30d. Transfer of a *C. elegans* miRNA, cel-miR-39, and miR-30d from CMs to neonatal rat cardiac fibroblast cultures (CFs) via EVs was demonstrated by direct application of CM-derived EVs to culture and by co-culture of the two cell types separated by a 0.4 mm membrane. Furthermore, uptake of CM-derived EVs by CFs was visualised using an Olympus BX62 microscope with Qimaging Emc2 EMCCD cooled camera. miR-30d appears to function as a cardioprotective agent by suppressing TNF-α-induced apoptosis ($n = 4$, $p < 0.05$) in CMs through suppression of the target MAP4K4, and alters proliferation and expression of fibrosis genes in primary fibroblasts. The offspring of Rosa mT/mG mice crossed with mer-Cre-mer mice express membrane-targeted Tomato in all cell types and tissues until tamoxifen treatment causes loss of the mTomato cassette and expression of the mGFP in CMs only. Plasma isolated from tamoxifen treated mice contained both red and green vesicles. EVs were isolated by differential centrifugation and analysed and sorted using the Propel Labs' Nano-View forward scatter detector integrated onto a Beckman Coulter MoFlo Astrios EQ cell sorter. Vesicles <200 nm were detected and sorted based on colour for Tomato red, EGFP, and a small population that expressed both colours. GFP-expressing EVs derived from the heart contain miR-30d as well as other myomirs. *Summary/Conclusion*: MicroRNAs contained within CM-derived EVs appear to be dynamically regulated and play an important role in cardiac remodelling. The Rosa mT/mG murine model allows for the investigation of CM-derived EVs in health and disease.

Networking coffee 10:15-10:45

Ballroom D-E
Plenary Session
Chairs: *Marca Wauben and Andrew Hill* 10:45-11:45

Gary Ruvkun, Ph.D.
Department of Genetics, Massachusetts General Hospital,
Boston, MA, USA

The tiny RNA pathways of *C. elegans*

Ballroom D-E
Plenary closing ceremony, prizes & announcement of ISEV 2016 11:45-12:30

Networking/farewell lunch 12:30-13:30

Departure 13:30