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What is the blood concentration of extracellular vesicles? Implications for the use of extracellular vesicles as blood-borne biomarkers of cancer

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Abstract

Circulating biomarkers have a great potential in diagnosing cancer diseases at early stages, where curative treatment is a realistic possibility. In the recent years, using extracellular vesicles (EVs) derived from blood as biomarkers has gained widespread popularity, mainly because they are thought to be easy to isolate and carry a vast variety of biological cargos that can be analyzed for biomarker purposes. However, our current knowledge on the plasma EV concentration in normophysiological states is sparse. Here, we provide the very first mean estimate of the plasma EV concentration based on values obtained from a thorough literature review. The different estimates obtained from the literature are correlated to the isolation techniques used to obtain them, illustrating how some methodologies may over- or underestimate the plasma EV concentration. We also show that the estimated plasma EV concentration (approximately 10^{10} EVs per mL) defines EVs as a minority population compared to other colloidal particles of the systemic circulation, namely the lipoproteins, which are known contaminants in EV isolates and carry biomarker molecules themselves. Lastly, we introduce the possibility of regarding EVs and lipoproteins as a continuum of lipid-containing particles to which biomarker molecules can be associated. Using such a holistic approach, increased strength of plasma-derived cancer biomarkers may soon be revealed.

Introduction

Extracellular vesicles (EVs) have gained wide popularity within the past decade due to their role in intercellular communication and disease biology, and their potential in biomarker development and drug delivery [1-3]. The EVs are lipid bilayer particles composed of a range of different lipids and proteins (especially phospholipids, cholesterol and tetraspanin proteins), whereas they can carry proteins, RNA and DNA in their aqueous core (Figure 1A)[3]. They are produced by most cells of the body, also under pathological conditions such as cancer [4]. The EVs secreted by cancer cells can be used as a signature of disease progression, severity, and therapeutic efficacy [2]. In the recent years, there has been a strong focus on the use of EVs as such biomarkers of disease (Figure 1B)[5-7]. The progression of the research field is driven by collaborative efforts between clinicians with access to patients, and basic researchers with the analytical expertise to study the biological material obtained from the patients [6]. Due to easy access to blood sampling and the extent of the general information possessed in such samples, this type of biological material has been the choice for most of the biomarker studies focusing on EVs. From the original sample, EVs are isolated either from plasma or serum using standard isolation methods available in the EV community [4]. Published work in the recent years has highlighted that it is very difficult to obtain a pure sample of EVs from blood plasma or serum because several types of lipoproteins contaminate the EV samples [8-11]. Lipoproteins are biological particles responsible for the transportation of lipids throughout the body. The lipoprotein family is comprised of five subclasses including chylomicrons, very-low-density

lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL).

Here we discuss the reported EV levels in human blood plasma based on a literature survey and the proposed lipoprotein contamination as a function of the methods used to isolate and detect the EVs. The key problem discussed is the important but highly ignored fact that EVs are a minority population compared to other colloidal particles in blood (lipoproteins) – a fact which was recently called for information on [3]. The fact that EVs are also a minority population to red blood cells and platelets is not considered here [8]. We show based on this discussion that there are 6 orders of magnitude higher total lipoprotein particle concentration compared to the EV concentration in plasma. The combination of the physical properties of lipoproteins and EVs [9] and the much higher level of lipoproteins compared to EVs drives the lipoprotein contamination. Therefore, we stress the importance of considering the lipoprotein contamination when interpreting biologically relevant functions of EVs.

What is the EV concentration in human blood?

EVs are enumerated using various analytical procedures to obtain a concentration that can be correlated to the concentration of different biomarker molecules, e.g. DNAs, RNAs, proteins, and lipids, contained within the EVs (Figure 1A)[3]. Enumeration of EVs can also in itself be used as a biomarker for different cancers, since these diseases are characterized by a remarkable increase in total plasma levels of the EVs [7,10-15]. This is hypothesized to be mediated by pH reduction in the tumor microenvironment, which stimulates EV production [7,12-16]. Despite the large popularity of enumerating EVs in blood for different purposes, there is still no accepted baseline value that could be used as a setpoint for other studies. The reason for this is likely multifaceted but does include the differences in both isolation and quantification methodologies [17], and lack of reproducibility of findings [8]. This makes comparisons between different studies difficult.

We argue here that a baseline concentration of EVs in blood from healthy humans can be estimated, if data from many studies across different isolation and quantification methodologies are obtained. Through a literature survey including studies from 2013 – 2018 (the years of rapid growth in the publication frequency and of significant improvement in isolation and quantification methodologies, Figure 1B), we identified 38 original articles that provided 59 estimates of the EV concentration in blood from healthy human subjects [10,13,18-53]. Only studies that provided a particle concentration were included, whereas studies providing only EV protein concentration with no correlation to the absolute numbers of EVs were left out. EV concentrations in subjects with disease or obtained from other species were also not considered. Thematic analysis of all the studies published between 2013 and 2018 revealed that the vast majority of published studies used EVs as biomarkers in cancer, whereas disease groups like cardiovascular, infectious, and metabolic diseases were less frequently studied (Figure 1C).

Regardless of the methodology employed to isolate EVs from blood sample of healthy subjects, reported EV concentrations span more than seven orders of magnitude with a geometric mean of ~ 10^{10} EVs per mL (Figure 2A). This estimate is dependent upon the isolation protocol, although some outlier studies seem to be independent of this, e.g. some very low values measured after ultracentrifugation (Figure 2A). Those values could largely be explained by the quantification method used to determine the EV concentration, since these methods vary greatly both in their sensitivity and size threshold (Figure 2B).

The reason for the large variation in published data on EV concentrations in plasma is likely multifaceted. The methods used to assess the EV concentration in blood samples are neither specific nor sensitive enough to capture all EVs and/or distinguish EVs from non-EV entities [54]. Also, the EV samples used for enumerations have typically been exposed to purification steps prior to the concentration measurements – steps that likely introduce EV loss and are insufficient in removing all the lipoproteins (discussed below). An inherent large variation of EV concentration in plasma across healthy human individuals has also been reported [23] – a biological variability observed using identical isolation and

enumeration procedures [9,55]. The variations of several orders of magnitude of the reported EV concentrations in plasma may also be driven by the fact that large variations are easily introduced when studying low abundant entities from a biological specimen (Figure 2). Adding together all of these challenges, we start to understand why there is such a huge discrepancy between the reported EV concentrations in plasma and why we are currently not in a position to determine the exact concentrations of EVs in plasma or any other body fluids, as is possible for other blood components such as lipoproteins [9]. However, we argue that if we consider the majority of concentration measurements derived from nanoparticle tracking analysis (NTA), i.e. keeping the quantification parameter stable, we may close in on the mean EV concentration, hereby yielding an estimate of ~ $2 * 10^{10}$ EVs per mL (Figure 2C). The NTA tool is by far the most commonly used technique to determine EV concentrations and size distributions and is currently considered as the gold standard, at least when considering statements from the International Society for Extracellular Vesicles (ISEV) and the extent of use (Figure 2B)[4,54,56-58]. However, NTA does not have the required specificity to distinguish between EVs and the possible non-EV entities contaminating EV isolates from a blood sample, which should be remembered when interpreting this estimate.

Isolation methods and contamination as factors in EV enumeration

The type of isolation protocol employed to obtain an EV sample is of importance for the outcome of EV concentration measurements. When considering data derived from NTA measurements only (Figure 2C), it is apparent that the least varying data is obtained from ultracentrifugation procedures, providing values close to the geometric mean of all the measurements. Conversely, polyethylene glycol (PEG)-based purification (considered less pure than ultracentrifugation, e.g. ExoQuick®) and size-exclusion chromatography (SEC)-based purification (considered purer than ultracentrifugation) yield datasets with geometric means and variations many-fold higher than those measured for ultracentrifugation techniques. EV concentrations obtained from PEG precipitation is about ten-fold higher than the values based on ultracentrifugation and density gradient ultracentrifugation (Figure 2C). This could be due to the co-precipitation of apoB-containing particles such as chylomicrons and VLDL, IDL and LDL with the EVs [59]. PEG precipitation is actually a well-known method to remove apoB-particles from HDL in plasma samples (Figure 3A)[59,60]. SEC has become a popular method to isolate EVs. However, large apoB-containing lipoproteins (chylomicrons and VLDL, and likely also LDL) are co-eluting with EVs due to their overlapping size distributions (Figure 3B + Figure 4A). Thus, both PEG- and SEC-based isolation methods lead to a significant amount of lipoprotein-contamination in the 'purified' EV samples.

One of the limitations of NTA is that it is not capable of distinguishing between EVs and similar sized particles including large lipoproteins [56,61], which could lead to overestimation of the EV concentration in the PEG and SEC-isolated samples due to the significant proportion of large lipoproteins in the EV sample. It is worth mentioning that the biological function of chylomicrons is to deliver dietary lipids from the intestine to the muscle and adipose cells. The chylomicron level thus varies over the course of a day, with the maximum level in blood being reached four hours after a meal, whereby the level drops down after fasting [9]. VLDL levels change in the opposite direction [62]. The relationship between food uptake and chylomicron/VLDL levels may be the reason why reported EV concentrations based on SEC isolation vary so much, clearly stressing the concern about lipoprotein contamination in some of those samples (Figure 2C)[4,9,55,62,63].

The ultracentrifugation method is the most commonly used method to isolate EVs from plasma (or cell cultures)[17,63]. The procedure depends upon the sedimentation rate of differently sized particles with varying densities in a sample, which would suggest that EVs associate with the pellet, whereas the smaller lipoproteins would be present in the supernatant that is most often discarded [4]. The EV concentrations derived from studies employing this technique are much lower than those derived from PEG-

and SEC-based procedures. For example, there is almost a 15-fold difference between the geometric mean values obtained from SEC and ultracentrifugation, respectively (Figure 2C). While the NTA measurements from this method only fall within two orders of magnitude, there is still problems associated with the purity obtained from this technique (Figure 3C, further discussion below).

When isolating EVs according to their mass density (on a density gradient) instead of size, the contamination of the final EV sample may be reduced. This is because the potential lipoprotein contamination in this procedure (HDL) may theoretically already have been removed in preceding ultracentrifugation steps (according to the gold standard protocols)(Figure 3C+D). However, as we shall see later, the HDL is not likely to be removed completely in this preceding ultracentrifugation step, leaving it able to co-isolate with EVs on a density gradient. Furthermore, NTA is not capable of detecting these fairly small 10 nm sized HDLs [61,64], and hence, a much lower EV concentration is likely obtained compared to the values derived from the PEG precipitation or SEC purification of EV samples, where larger lipoproteins may contaminate and become detected. The EV concentration deduced from the density gradient ultracentrifugation may therefore provide a good estimate of the EV concentration (~ 5 * 10^9 EVs per mL, Figure 2C) when determined using NTA, although the amount of data available on this method is scarce. Also, the impact of high sugar concentration and daylong centrifugation cycles at > 100,000 g has not been studied in detail, and thus, we know very little about the quality of the isolated sample.

Numbers matter – EVs as a minority population in human blood

No currently known isolation technique is able to provide pure EV samples due to the significant potential of co-isolating different classes of lipoproteins. This became especially evident with the estimation that EV samples contained at least one order of magnitude higher numbers of LDL particles compared to the EVs [37,65]. Furthermore, when platelet-free plasma was subjected to antibody-based pulldown of apoB-48 and apoB-100, the total number of particles measured on the NTA system decreased significantly [66]. Thus, there is little doubt that the number and physicochemical characteristics of lipoproteins are to be considered when wanting to isolate EVs from human blood for studies of their biological function or their potential as disease biomarkers. The different lipoprotein classes are defined according to their density as indicated by their names (Figure 4A). In addition, the apoB-48 protein is uniquely associated with chylomicrons in a 1 to 1 ratio, VLDL, IDL and LDL all contain one apoB-100 protein per particle, while the HDL on average contains three apoA-I protein molecules [9,67]. Taking these stoichiometries into account, the particle concentration of chylomicrons, the other low-density lipoprotein classes (VLDL, IDL and LDL) and HDL can be derived directly from the apoB-48, apoB-100 and apoA-I concentrations in plasma, respectively [9,67]. This is in direct opposition to the concentration of EVs, which can only be estimated indirectly due to its more complex and heterogenous structure and composition [55]. The variability of the total apoB and apoA-I concentrations is rather small in healthy individuals compared to EVs - about 30 % and 20 % in terms of relative standard deviations, respectively [68], whereas our data indicate that measurements of the plasma concentration of EVs span 6 – 7 orders of magnitude (Figure 4B).

Determining the concentration of EVs in human blood plasma is difficult, but our data indicates that a value of ~ 10^{10} EVs per mL is a relevant estimate (Figure 4B). This value is based on EV concentrations quantified using the NTA technique only and lying between the values obtained for all isolation methods combined (~ 2 * 10^{10} EVs per mL – considering the pitfalls of the NTA technique, this may actually illustrate an upper limit of EV concentration in human plasma) and for ultracentrifugation techniques (~ 5 * 10^9 EVs per mL, least varying data in the dataset). That being said, it does not consider the potential contamination from the lipoproteins as described above (Figure 4A), leading us to the relevant question: Is the EV concentration in plasma high enough compared to the lipoproteins to provide clear cut answers about their roles as biomarkers and the underlying importance this may have on disease biology? If not, how does this affect the interpretations we make on disease biology, when using EVs as biomarkers?

The total amount of lipoproteins in human plasma ranges around 10^{16} particles per mL (Figure 4C), making the EV the one in a million (information from Simonsen (2017)[9], example of calculation presented in the Supplementary Information). However, when looking at the different types of lipoproteins, their individual concentrations vary significantly (Figure 4C), making some of them less prone to contaminate EV samples using specific isolation methods. For example, chylomicrons are much lower in numbers compared to LDL and HDL particles, whereby they outnumber the EVs by a thousand-fold only. On the other hand, while this is indeed a low concentration compared to HDL particles, the chylomicron concentration will likely still become a severe problem when isolating EVs using SEC. This is further complicated by the indirect methodologies available for EV quantification, making it impossible to decipher how many of the particles measured on the NTA system that could be ascribed to chylomicron contamination, unless the molar concentration of apoB-48 (equal to the chylomicron concentration) was determined in the same samples. One could then go for another isolation method, since EVs and chylomicrons do not have overlapping densities (Figure 4A). The large lipoproteins (chylomicrons and VLDL) can therefore easily be removed from the EV samples by density gradient ultracentrifugation, although this would depict another potential contaminant, namely the HDL particles, which share densities with EVs (Figure 4A)[9]. In the current protocols for density gradient ultracentrifugation, this is rarely considered a problem, since HDL particles are thought to be removed in a prior standard ultracentrifugation step before loading of the density gradient (Figure 3C+D). This is true – in theory – but if we again consider the numeric differences in total HDL versus EV concentration, the problem persists (Figure 4C). Let us do a simple thought experiment: The sedimentation rate (V_{sed}) of spherical objects at constant acceleration is proportional to the difference between the density of the particle (ρ_{particle}) and the density of the medium (ρ_{medium}) and to the square of the particle radius ($r^2_{particle}$): $V_{sed} \propto (\rho_{particle} - \rho_{medium}) r^2_{particle}$. Thus, if we assume that the density of HDL and EVs is similar (Figure 4A), the sedimentation rate of 100 nm EVs is 100 times faster than 10 nm sized HDL particles. If all EVs are sedimented including the ones at the top of a cylindrical vial after a standard 100,000 g centrifugation step, 1/100 of the HDL particles will co-sediment due to their small but still significant sedimentation rate. Although the HDL contamination has then been reduced about 100 times, the HDL particles will still outnumber the EVs substantially (4 – 5 orders of magnitude) after the ultracentrifugation step (Figure 4C), especially when also considering that all of the HDL-containing supernatant is rarely removed. Since the HDL particles cannot be detected using the NTA technique, the input from them in EV enumerations may not be counted [61]. This is in principle very positive, because it could yield a more realistic value of the EV concentration. However, for subsequent interpretations made regarding the potential impact the investigated 'EV' biomarker may have on the underlying pathophysiology of the disease studied, unknown HDL contamination could have large impact, since these particles are also able to carry specific proteins and RNAs [69-75]. In the case of LDL that is about two times larger than HDL and has a density that is about 10 % less than HDL, the LDL fraction that co-sediment with EVs will be almost similar to that of HDL (~ 1/100 of the total LDL fraction contained in the samples). A significant LDL contamination was indeed shown when using both ultracentrifugation and density gradient ultracentrifugation [37,65]. Conversely, the lower density of chylomicrons relative to the density of plasma or serum should prevent the chylomicrons to co-sediment with EVs during ultracentrifugation due to their relatively fast buoyancy, meaning that they will flow towards the top of the liquid that is ultracentrifuged. This latter effect may explain why the vast majority of EV concentrations (all except for one) measured after ultracentrifugation or density gradient ultracentrifugation remain below 10^{11} EVs per mL (Figure 2C).

To handle these contamination issues, a two-step isolation protocol based on multiple physical parameters (size and density) was proposed to increase the purity of EV samples [9]. Some groups are now applying this two-step strategy to obtain purer EVs samples [10,76]. This combination is important if the

ambition is to increase the overall purity that can be obtained, and the current efforts have shown a significant reduction in the lipoprotein contamination, although the contamination is still fairly high [76]. Surprisingly, a substantial HDL contamination persists after SEC-based isolation (in combination with density gradient ultracentrifugation), which is peculiar given the very large size difference between HDL and EVs [76,77]. Such large differences should be possible to resolve using optimized SEC procedures, which points to the fact that the setup of currently used SEC systems (in-house made or commercially purchased) may be inadequate for true separation to happen [77]. Further optimizing the SEC setup for EV isolation should therefore be possible by introducing an HPLC-like format that would have little difficulties in resolving the different size ranges contained within a plasma sample, e.g. by considering the column type, packing material, flow rate, etc. Also, in relation to handling problems with EV purity, urine-derived EVs have shown great potential to predict prostate cancer in men with a prior negative biopsy [78]. Interestingly, while blood, lymph and cerebrospinal fluids contain significant amounts of lipoproteins, urine contains much less lipoprotein, and could therefore become an alternative way of obtaining the EV biomarkers.

EVs and lipoproteins as biomarkers

From these examples it seems clear that our current best practice for obtaining EV samples from human blood plasma is challenged by inevitable issues with co-isolating lipoproteins with concentrations that are orders of magnitude higher than what we expect of the EVs. This is the unpleasant truth based on which we have to make our interpretations on studies of EVs isolated from blood plasma, e.g. when evaluated as disease biomarkers. The biomarkers are of increasing interest since they possess the potential of diagnosing devastating cancer diseases at an early timepoint at which curable therapies might still be possible [3,79]. The highlighting of EV isolation methods for obtaining the EV biomarkers may seem irrelevant in the sense that whatever was analyzed in a given biomarker study (lipids, proteins, RNA, or DNA) seems to have diagnostic potential [45,79-82]. Can we be sure that these EV biomarkers are in fact EVs? No, we cannot at this moment. This may not necessarily be a problem, if the validity of the biomarker can be ensured by other means. However, in some future uses of EVs in the clinic, specific enumeration may be required.

If diagnostic or therapeutic tools are to be based on a minority population like the EVs, we must be sure that we know what we are analyzing. When using isolated EVs for drug delivery (obtained from blood or serum-containing cell cultures), we must be able to enumerate in great detail to provide a clear value of the therapeutic compound that was dosed to the patient [2,63,83,84]. When using the concentration of EVs in the plasma of a patient as a biomarker in itself, the fact that we may be counting mostly lipoproteins could make us blind to what the disease mechanism behind this effect is. When using the cargo contained in the EVs, it is also not possible to completely rule out that lipoproteins may be the particles carrying it [3,9]. In the recent years, several reports have described the potential of different lipoproteins to carry proteins and RNAs (small RNAs in particular)[85]. For example, advances in proteomics have extended the list of HDL-associated proteins, beyond the apolipoproteins associated to HDL, to over 85 suggesting that the composition of HDL is more complex than previously anticipated [69]. Studies have also shown that the levels of specific HDL-associated proteins are altered in disease states [86,87] and in polluted environments [88], and could therefore potentially also be used as disease biomarkers [89]. LDL and VLDL are also associated with a growing number of associated proteins that are known to have important functions in dyslipidemia, atherosclerosis, and coagulation disorders [90]. This has important implications on how we interpret EV biomarker data to obtain knowledge about the ongoing disease process, if the protein or RNA message could actually be released and transported by other means than EVs. Moreover, the use of lipid-based disease biomarkers contained in EVs is also highly challenged due to the difficulties in isolating EVs with high purity [3,91,92], because the lipid composition in lipoproteins is very heterogenous and could possibly yield a signal in ‘EV’ samples [91]. Again, the number of EVs versus lipoproteins could play a big role, since a very small fraction of lipoproteins carrying a lipid, protein, or RNA marker would easily

outnumber the total number of EVs carrying the same molecule. Our current knowledge on the stoichiometry of RNA-loading into EVs underscores this point, showing that only a very small fraction of EVs are expected to carry a specific type of RNA molecule with a very low copy number [92,93]. This is evident even if specific RNAs are overexpressed in cells to become loaded into EVs [93,94]. On the other hand, there may also be different kinds of molecules that are difficult to imagine in a lipoprotein context, e.g. transmembrane proteins like the tetraspanins, although these proteins may be associated with specific subtypes of EVs only [95]. It could then be possible to avoid overlapping cargoes between EVs and lipoproteins, whereby the robustness of the resulting EV biomarker may increase substantially. This highlights the potential of combining forces between the EV and lipoprotein fields to coordinate efforts and share knowledge to the benefit of the patient.

A continuum of lipid-containing particles

We are positive that the future is bright for EV biomarkers because their potential is being underscored in the literature with high frequency, and an introduction of these diagnostic tools based on detection of EVs to the clinicians may be coming soon. We provide here an estimate of the plasma EV concentration in healthy subjects of ~ 10^{10} EVs per mL that may serve as a reference point for future studies characterizing EV biomarker systems using EV quantification (Figure 4B). The future research into EV-based biomarkers must, however, have a stronger focus on optimizing the isolation protocols to obtain purer samples of EVs, although depending on the type of biomarker, it may be of less importance if it was expressed on EVs or lipoproteins. The higher purity may even yield more robust correlations to any ongoing disease, thereby increasing the clinical potential of the EVs as biomarkers especially in cancer diseases [3,79]. We suggest that new protocols that utilize the multifaceted physicochemical characteristics of EVs could improve the purity of the isolated sample, and therefore also the quality of the resulting EV biomarker, and we believe that the first attempts at using these protocols are encouraging. In this context, the numbers matter a lot. While diagnoses based on EV biomarkers should reach a high throughput stage for proper clinical relevance, there are some methodological issues that cannot be neglected. Thus, the more contamination we accept, the more the EVs will be outnumbered by the lipoproteins. We do not argue against the potential clinical value of the identified EV biomarkers, and it is our belief that EVs are nexuses that mediate a tremendous amount of interesting known and unknown biology that could and should be exploited in a clinical setting. That said, for some biomarker purposes, it may be just as useful to regard the EVs and lipoproteins as a continuum of lipid-containing particles to which biomarker molecules can be associated. Using such a holistic approach, increased strength of plasma-derived cancer biomarkers may soon be revealed.

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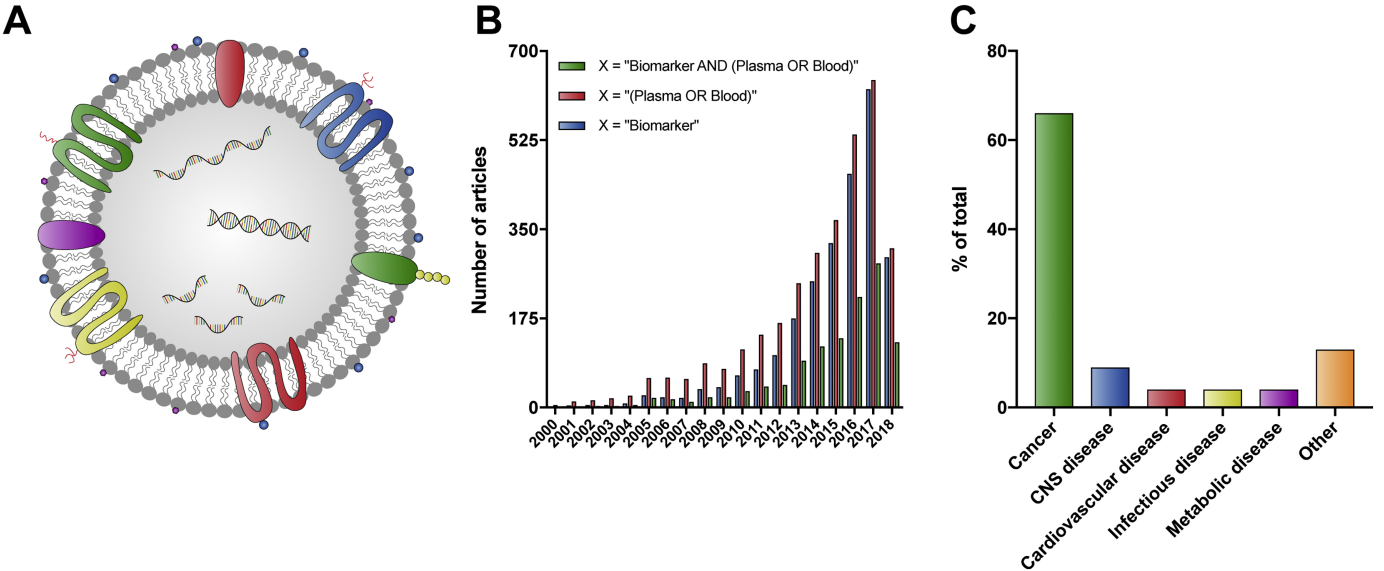
Figure 1. Extracellular vesicles as biomarkers of disease. (A) Extracellular vesicles (EVs) are a diverse family of lipid bilayer particles that are secreted by all cells of the body. They are composed of many different lipids (especially phospholipids and cholesterol), proteins (especially tetraspanin proteins), and nucleic acids. EVs are important for the intercellular communication, in which cells share different kinds of information with each other, either in a paracrine fashion to its immediate neighbors, or over large distances, where the EVs transport via the systemic circulation. (B) Publication frequency of studies investigating EVs in human blood. Data was derived from three different PubMed searches; *(exosome OR "extracellular vesicle") AND X AND year*, where *X* is explained in the figure. (C) Thematic analysis of the all EV biomarker studies from the search "(exosome OR "extracellular vesicle") AND (plasma OR blood) AND year" between 2013 – 2018.

Figure 2. Extracellular vesicle concentration in human blood. Estimates of the extracellular vesicle (EV) concentration in human blood was derived from the PubMed search "(exosome OR "extracellular vesicle") AND (plasma OR blood) AND human AND year" between 2013 – 2018, i.e. the period with the sharpest increase in publication frequency. Detailed analysis of the search outcome yielded 37 studies that provided 58 estimates of the EV concentration in blood from healthy human subjects. Estimates from subjects with disease or from other species were not included. (A) The plasma EV concentration measured in humans span more than 7 orders of magnitude with a geometric mean of approximately ~ 10^{10} EV per mL. Subanalysis revealed a substantial difference between the estimates derived from different isolation techniques, although with some intergroup variation as well. (B) When the quantification methodology was considered, flow cytometry was found to severely underestimate the EV concentration when compared to other techniques including the gold standard method, NTA. (C) Outlier estimates could largely be removed by considering estimates derived from nanoparticle tracking analysis only, after which the geometric mean of the EV concentration in human blood was found to be ~ 2 * 10^{10} EVs per mL. EV: Extracellular vesicle. SEC: Size exclusion chromatography. PEG: Polyethylene glycol-based precipitation. UC: Ultracentrifugation. DG: Density gradient ultracentrifugation. NTA: Nanoparticle tracking analysis. TRPS: Tunable resistive pulse sensing. FC: Flow cytometry. AEA: Acetylcholine esterase activity.

Figure 3. Methods for isolating extracellular vesicles. Extracellular vesicles (EVs) can be isolated using a variety of techniques depending on their specific physicochemical properties, most prominently the size and density. All currently used isolation methods are prone to lipoprotein contamination, although with differences as to which lipoproteins that will co-isolate and with varying impact. (A) Volume-excluding techniques such as polyethylene glycol (PEG)-based precipitation is a fast method for obtaining EV samples. The principle behind the technique is to mix a biological sample, e.g. plasma, with a specific amount of PEG-containing solution (black strands), followed by centrifugation at approximately 10,000 g. The method is a classical way of purifying HDL particles in the supernatant, and therefore, LDL, VLDL, and chylomicrons will likely co-isolate in the EV-containing pellet after centrifugation. (B) Size-exclusion

chromatography purifies particles in a solution as a function of their hydrodynamic diameter. The biological sample is passed through a column, wherein smaller particles (e.g. HDL) are slowed and larger particles (e.g. EVs) move freely through the column material. Due to the similarity in size distribution between EVs and chylomicrons, these two types of particles will likely co-isolate in the void fraction. (C) Ultracentrifugation is the most widely used isolation method, and its isolation capacity is based on pelleting the EVs at centrifugation speeds > 100,000 g. Due to the low number of EVs in a plasma sample compared to the different lipoproteins, a significant number of lipoproteins may be contained in the resulting pellet. This is depicted by the sedimentation rate and concentration of each type of lipoprotein and EVs. Given the low density of chylomicrons, these particles will likely move upwards instead of downwards to the pellet during ultracentrifugation. (D) In density gradient ultracentrifugation, the difference particles contained in the plasma sample are isolated based on their density. This means that HDL (and LDL) particles likely co-isolate with EVs, if these contaminants are not removed by other means. PEG: Polyethylene glycol. VLDL: Very low-density lipoprotein. LDL: Low-density lipoprotein. HDL: High-density lipoprotein.

Figure 4. Extracellular vesicles as a minority population in human plasma. (A) Extracellular vesicles (EVs) share several physicochemical properties with the different lipoprotein particles contained in the plasma. For example, the size distribution is very similar to chylomicrons, whereas the density distribution is similar to that of HDL. The figure was modified from *Simonsen* (2017) [9]. (B) Estimation of the EV concentration in human plasma based on data obtained from studies using nanoparticles tracking analysis as a quantification method. The geometric mean of the dataset suggests that the EV concentration in human plasma is ~ $2 * 10^{10}$ EVs per mL. (C) Particle concentration of lipoproteins and EVs in human plasma. EVs are outnumbered in human plasma by 6 orders of magnitude higher total concentration of the different lipoproteins. The VLDL concentration is not depicted since the majority of apoB-100 expression (present on VLDL) in human plasma can be ascribed to LDL particles. Lipoprotein particle concentrations were obtained from *Simonsen* (2017) [9].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2015
2016
2017
2018
% of total
80
60
40
20
0
Cancer
CNS disease
Cardiovascular disease
Infectious disease
Metabolic disease
Other

Figure 1

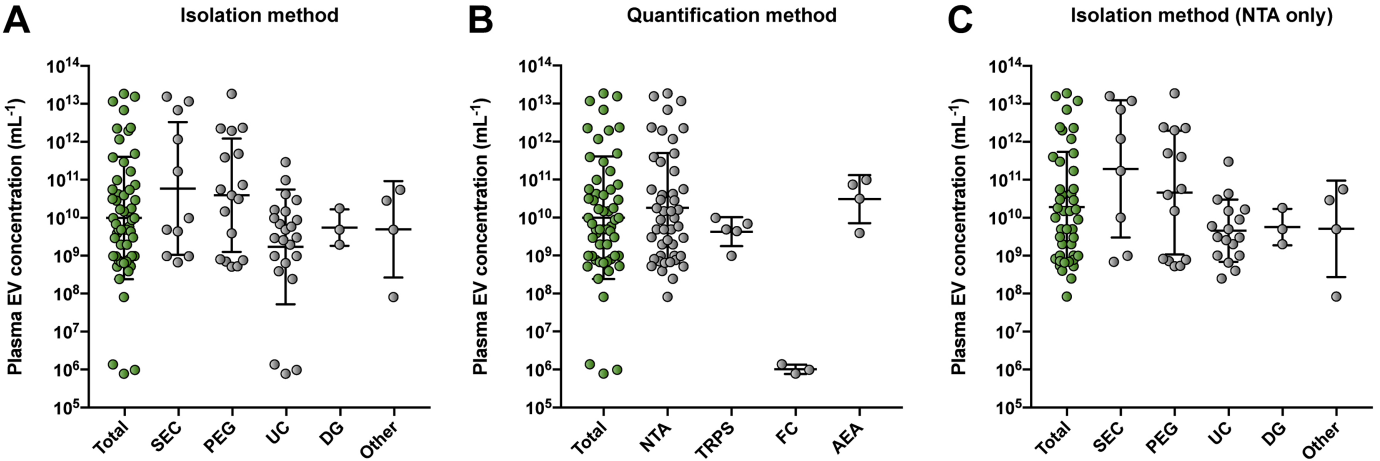
A
Isolation method
Plasma EV concentration (mL-1)
Total
SEC
PEG
UC
DG
Other
B
Quantification method
Plasma EV concentration (mL-1)
Total
NTA
TRPS
FC
AEA
C
Isolation method (NTA only)
Plasma EV concentration (mL-1)
Total
SEC
PEG
UC
DG
Other

Figure 2

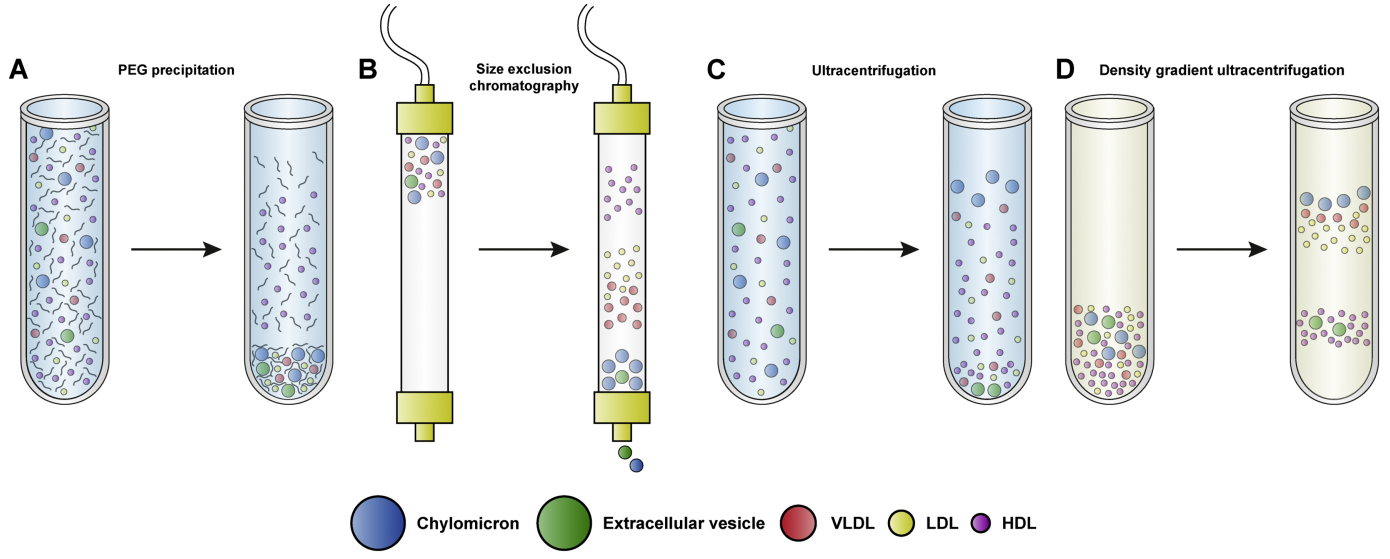
A
PEG precipitation
B
Size exclusion chromatography
C
Ultracentrifugation
D
Density gradient ultracentrifugation
Chylomicron
Extracellular vesicle
VLDL
LDL
HDL

Figure 3

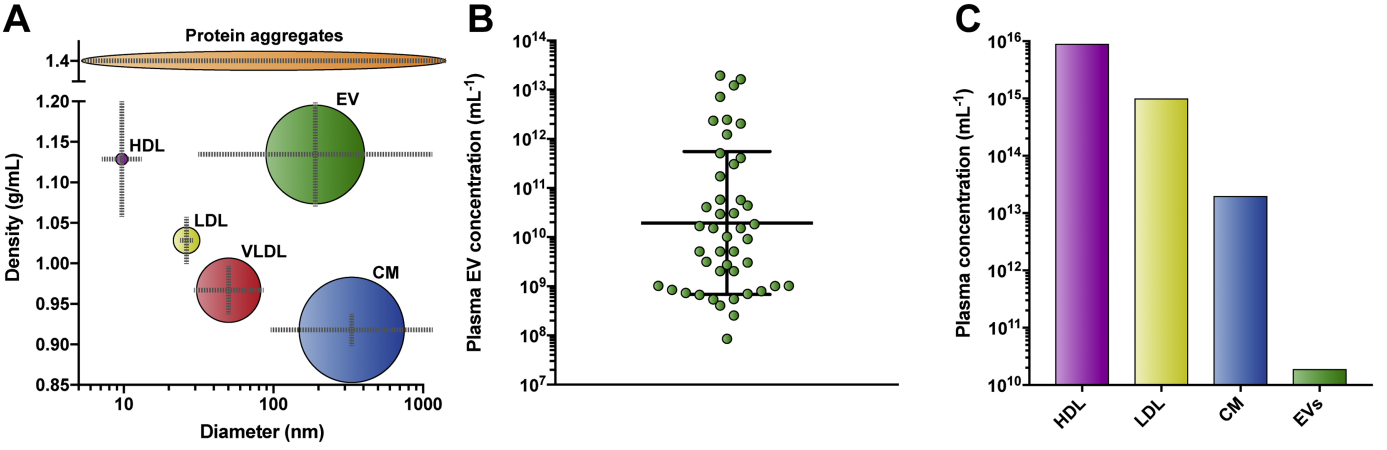
A
Protein aggregates
1.4
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
Density (g/mL)
HDL
EV
LDL
VLDL
CM
10
100
1000
Diameter (nm)
B
Plasma EV concentration (mL-1)
10^14
10^13
10^12
10^11
10^10
10^9
10^8
10^7
C
Plasma concentration (mL-1)
10^16
10^15
10^14
10^13
10^12
10^11
10^10
HDL
LDL
CM
EVs

Figure 4