Received: 22 August 2023 | Accepted: 4 December 2023

DOI: 10.1002/jev2.12400

REVIEW ARTICLE

A beginner's guide to study extracellular vesicles in human blood plasma and serum

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Abstract

Blood is the most commonly used body fluid for obtaining and studying extracellular vesicles (EVs). While blood is a standard choice for clinical analysis, using blood as a source of EVs introduces multiple layers of complexity. At the Blood Extracellular Vesicle Workshop organized by the International Society for Extracellular Vesicles in Helsinki (2022), it became evident that beginner researchers lack trustworthy information on how to initiate their research and avoid common pitfalls. This educational guide explains the composition and frequently used terminology of blood, provides guidelines for blood collection, and the preparation of plasma and serum. It also introduces the basic principles of isolating and detecting blood EVs while considering blood-related factors. The goal of this guide is to assist beginners by offering a concise and evidence-based introduction to the current knowledge and available resources to study blood EVs.

KEYWORDS

blood, detection, exosomes, extracellular vesicles, guidelines, isolation, plasma, review, serum, vesicles

1 | COMPOSITION OF BLOOD

Blood and its derivatives plasma and serum are the most commonly studied body fluid for extracellular vesicle (EV) research (Royo et al., 2020). Blood is a complex body fluid, or rather a tissue matrix in a liquid form, which contains cells that are present within a fluid called plasma. The cell types present in blood are leukocytes (or white blood cells), erythrocytes (or red blood cells), and platelets (Figure 1, Table 1). Blood plasma contains mostly water and high concentrations of soluble proteins, lipoproteins, and importantly, relatively few EVs in proportion.

Blood EVs originate mostly from blood cells and to a lesser extent from endothelial cells, the cells lining the inside of the vessels and contacting the blood. The cellular origin of plasma EVs has been widely studied by detection methods such as (electron) microscopy (Arraud et al., 2014) or flow cytometry (Welsh et al., 2023). Additionally, low concentrations of EVs originating from tissues and organs may be present. The possibility of detecting these rare populations of EVs is often the basis of EV-based biomarker studies, where blood offers a semi-invasive opportunity for 'liquid biopsy'. Examples of such rare populations in blood are EVs originating from cancer cells (Nanou et al., 2023; Yang et al., 2018), cardiomyocytes (Hegyesi et al., 2022) or bacteria (Tulkens et al., 2020). Recently, the cellular origin of plasma EVs has also been deduced by measuring the presence of tissue-specific long RNA sequencing profiles (Li et al., 2020) or tissue-specific protein signatures (Abdelmohsen et al., 2023) in plasma or serum. Since EVs are only a small fraction of all particles present in blood, plasma and serum (Johnsen et al., 2019; Simonsen, 2017), detection of rare EV types is even more challenging. Often promising results in single studies have not been

TABLE 1 Physical properties of cells, extracellular vesicles and lipoproteins in human blood.

Cells, particles	Number concentration/mL[#]	Unique molecular identifier	Diameter (nm/μm)	Density (g/mL)	Shape
Erythrocytes	$4–6 \times 10^9$	CD235a	7–8 μm	1.098	Discoid
Leukocytes	$4–11 \times 10^6$	CD45	10–24 μm	1.067–1.1	Spherical
Platelets	$150–450 \times 10^6$	CD41	2–4 μm	1.061–1.091	Discoid *
Extracellular vesicles	1×10^{10}	–	30 nm–1 μm	1.02–1.19	Spherical
Chylomicrons		ApoB48	100 nm–1 μm	< 0.94	Spherical
VLDL	$0.7–8.6 \times 10^{13}$	ApoB100	30 nm–80 nm	0.94–1.006	Spherical
LDL	$0.6–1.2 \times 10^{15}$	ApoB100	20 nm–30 nm	1.006–1.063	Spherical
HDL	$1.4–2.3 \times 10^{16}$	ApoA1	7–12 nm	1.063–1.210	Spherical/discoid

The concentration of extracellular vesicles in human blood, plasma and serum is an estimation based on recent data; concentrations of lipoproteins (chylomicrons and lipoprotein particles) were calculated based on reported reference range values of unique molecular identifiers in fasting healthy human donors (Jeyarajah et al., 2006).
Abbreviations: CD41, Glycoprotein IIb; CD45, leukocyte common antigen; CD2351, glycophorin A; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

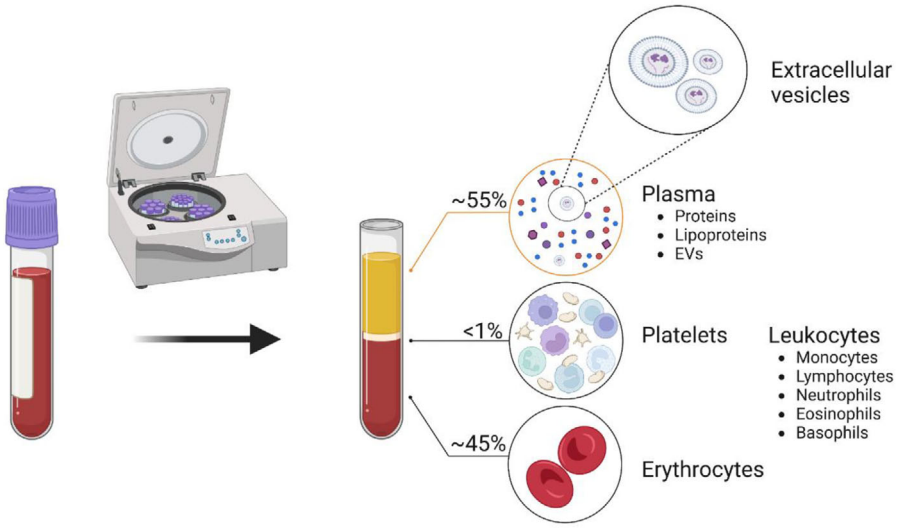

FIGURE 1 Fractionation of blood by centrifugation. The collected blood is centrifuged to separate cells from the blood fluid. Centrifugation separates blood into three distinct fractions. The red bottom fraction contains predominantly erythrocytes. The size of this fraction is approximately 40%–50%, but this depends on the donor. The intermediate thin white layer, also known as 'buffy coat', constitutes less than 1% of the total volume and contains leukocytes and most of the platelets. The leukocyte population comprises the peripheral blood mononuclear cells, such as monocytes, T- and B-lymphocytes, as well as polymorphonuclear cells like neutrophils, and the rarer cell types, basophils and eosinophils. The yellow top fraction is the blood plasma, which primarily consists of water (90%–92%), and the rest of this fraction is comprised of soluble proteins, lipoproteins, residual platelets, fragments of other cells such as 'ghosts' from red blood cells, and EVs. Image created by Biorender.com.

reported in sufficient methodological detail, or have not been reproducible, which makes it difficult to find reliable information on the tissue source of blood EVs.

The concentration of blood EVs is a balance between production and clearance. At present the mechanisms underlying the clearance of EVs as well as their half life in blood are virtually unexplored. Many older studies have indicated a half life of blood EVs in the order of minutes (van der Pol et al., 2012). In these studies methods such as ultracentrifugation were used to concentrate EVs *before* administration, which may affect the half life of EVs by inducing formation of EV aggregates and exposure of 'eat-me' signals such as phosphatidylserine (Dasgupta et al., 2009). EVs are likely to be cleared by phagocytosis in multiple organs including lungs, liver and spleen (Aimaletdinov & Gomzikova, 2022), and for example, the plasma concentration of EVs was reported to be elevated in splenectomised patients compared to controls (Rautou & Mackman, 2012). Thus, how long EVs remain in the circulation is a still unanswered question, but recent advantages in in vivo imaging of EVs (Verweij et al., 2021) may offer answers in the near future.

The physiological and pathological functions of blood EVs in vivo are also still largely unexplored (Yates et al., 2022). The early observations that blood cell-derived vesicles remove a redundant transmembrane receptor (Johnstone et al., 1987) and support coagulation (Chargaff & West, 1946) suggest that such EVs contribute to cellular homeostasis and haemostasis. In both cases the functional processes involve membrane surfaces, and the extent of the total surface area of EVs can be estimated from (i) the published particle size distributions of EVs in human body fluids (Arraud et al., 2014; van der Pol et al., 2014), and (ii) the estimated concentration of EVs (Welsh et al., 2023). The total surface area of EVs per mL of a body fluid such as plasma is estimated to be in the range of 1600 mm (Arraud et al., 2014), with a total volume of about 50 nL. Thus, EVs have a large surface to volume ratio, which supports the importance of the membrane surface in their functions. The recent observations that the surface of blood-derived EVs is covered by a protein corona (Palviainen et al., 2020; Tóth et al., 2021; Wolf et al., 2022) supports the relevance of the EV surface in functions such as cargo transport. Regarding the pathological functions of EVs, there are numerous papers summarizing the contribution of blood EVs to processes as coagulation, complement activation, immunity and inflammation, and to diseases as cardiovascular disease and cancer (Alberro et al., 2021; Pelissier Vatter et al., 2021; Shah et al., 2018; Yates et al., 2022). Therefore, we recommend the reader to look up their topic of interest for the most recent and comprehensive information.

Although the concentrations and cell sources of non-blood cell-derived EVs are not known, it is generally assumed that they change in pathological states. Since it is nearly impossible to study EVs directly in whole blood due to the presence of cells (approximately 50% of the blood volume, the first step involves separating cells from blood by centrifugation (Figure 1). In this way, (blood) EV-containing plasma is obtained (Lacroix et al., 2012), which is the most commonly used starting material to study blood EVs (Royo et al., 2020).

The composition of blood is donor-dependent, known as inter-individual or bio-specimen variation. For example, healthy male donors, on average, have a higher concentration of erythrocytes than females. Other factors that can affect the composition

of blood are age, circadian rhythm, life style, and medication. One well-known example of how life style directly impacts blood composition is eating. In the post-prandial state, which refers to the period after eating, blood (including plasma and serum) contains increased concentrations of lipoproteins (see Glossary) compared to the blood from the same donor when fasting (Nakajima et al., 2011). Since lipoproteins overlap in size and/or density with EVs (Simonsen, 2017), they hinder isolation and detection of EVs. The normal ranges of lipoprotein concentrations and densities are shown in Table 1. These lipoprotein concentrations were determined from the blood of fasting healthy individuals (Jeyarajah et al., 2006; Tikkanen et al., 2021). Throughout this paper, our focus will be on the blood of healthy humans.

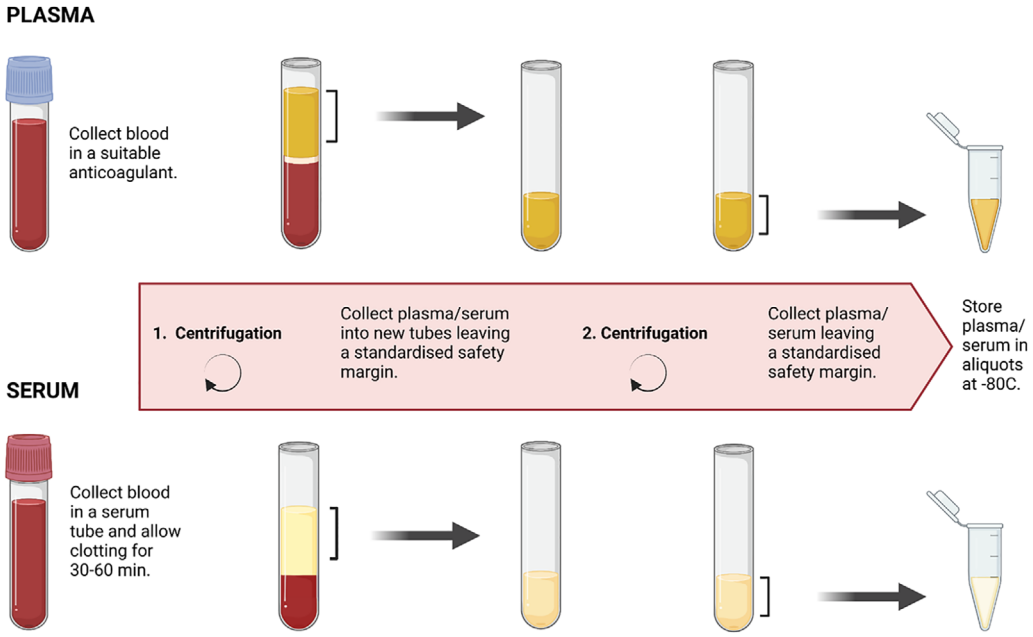

FIGURE 2 Preparation of plasma and serum. Plasma (top, from left to right) is prepared from anticoagulated blood by centrifugation. The protocol shown is an example, but this protocol is commonly used by dedicated blood EV laboratories. In this protocol, blood is first centrifuged at 2500×g for 15 min to separate the blood cells from the plasma. Centrifugation is performed at 20°C using a swing-out rotor and without applying a break (cold activates platelets and a fixed angle rotor introduces more platelets/material to the side of the tube, which may become loose when spinning is stopped). During centrifugation, the blood separates into three clearly distinct fractions, as explained in Figure 1. Next, the plasma is carefully collected by pipetting while leaving approximately 0.5–1 cm safety margin on top of the buffy coat. This safety margin aims to minimize the re-entry of platelets (predominantly present in the loose buffy-coat) into the collected plasma. Next, the plasma is then transferred to a new tube and subjected to another centrifugation step using the same conditions as before. The final plasma sample is collected without disturbing the cell pellet, which is best achieved by leaving another 0.5–1 cm from the bottom if possible. It is important to maintain a constant safety margin between samples and measure the platelet count in the final prepared plasma. Finally, plasma can be used directly, and/or stored at −80°C in single-use aliquots with suitable volumes for the planned downstream analyses to avoid multiple freeze-thaw cycles. Serum (bottom, from left to right). Similar to plasma, all serum samples should be treated identically, including standardizing the time the blood is allowed to clot on the bench, typically 30–60 min. There are various types of commercial serum tubes available, but glass tubes without any additives also suffice. At present, optimized serum preparation protocols for EV research have not yet been established. Therefore, we recommend using the same centrifugation protocol as for plasma preparation after the clotting step. Image created by Biorender.com.

1.1 | Plasma

Plasma is prepared from anticoagulated blood by centrifugation (Figure 2, Guidelines for the preparation of plasma and serum, and Glossary). Centrifugation is a key pre-analytical step, that generates an essentially (but not completely) cell-free plasma (Figure 2) (Rikkert et al., 2021). Centrifugation is a balancing act between removing cells while maintaining the EVs. Especially for blood, this balance is difficult to achieve for two reasons. Firstly, blood has high viscosity and density. Secondly, blood contains the smallest cells found in any human body fluid, namely, platelets (Glossary). Platelets (nearly) overlap in size and density with EVs in general, and their main biochemical marker composition is similar to platelet-derived EVs.

In comparison to serum, plasma is considered to be a more natural blood fluid. To which extent the EVs in plasma reflect the true circulating EVs in blood, however, is unclear for several reasons. Firstly, the concentration of EVs in blood is a balance between production and clearance. EVs or subpopulations of EVs may adhere to blood cells or endothelial cells, and become internalized. Secondly, removal of blood cells by centrifugation likely eliminates large or dense EVs, as well as EVs that adhere or have been taken-up by the blood cells. Thirdly, platelets are prone to activation during blood collection and plasma preparation, leading to the release of EVs that were not initially present in the blood or in the circulation. Platelet activation can be minimized

by following practical guidelines (Coumans et al., 2017), and by selecting appropriate anticoagulants (Guidelines). Fourthly, during storage and freeze-thaw cycles of plasma and serum, any residual cell that is present, including platelets, may fragment. These fragments are indistinguishable and inseparable from the EVs that were present in the blood at the time of blood collection.

Human plasma also contains non-spherical and low-density cell remnants originating from red blood cells and platelets (Arraud et al., 2014), and potentially from other cell sources. Some of these remnants manifest as elongated structures of several micrometres in length and are not removed by centrifugation, and therefore persist in plasma (Arraud et al., 2014). Plasma also contains extracellular particles that differ in morphology from EVs, particles that have multiple membranes (Broad et al., 2023; Buzas, 2023), exomeres and supermeres (Jeppesen et al., 2023; Zhang et al., 2021). At present, the biogenesis routes of such particles is currently unknown.

Plasma is a clear and transparent yellow fluid. However, when blood collection is imperfect, for example, due to poor venipuncture or when blood collection tubes have excessive vacuum, the plasma may be orange or red. This colour change is a result of haemolysis, which refers to the disruption of the red blood cell membrane, and the subsequent release of haemoglobin. Furthermore, in the postprandial state, when elevated numbers of lipoproteins are present, the plasma may lose its transparency and appear milky or opaque. This type of plasma is referred to as lipemic.

The presence of an anticoagulant prevents the blood or plasma from clotting. Coagulation (Glossary) is the underlying process that triggers clotting of blood and plasma (Figure 3). In the absence or insufficient anticoagulation, fibrinogen, a common soluble protein in blood and plasma, is converted into an insoluble mesh of fibrin polymers. During clotting, the clear yellow plasma transforms into a turbid and non-transparent gel.

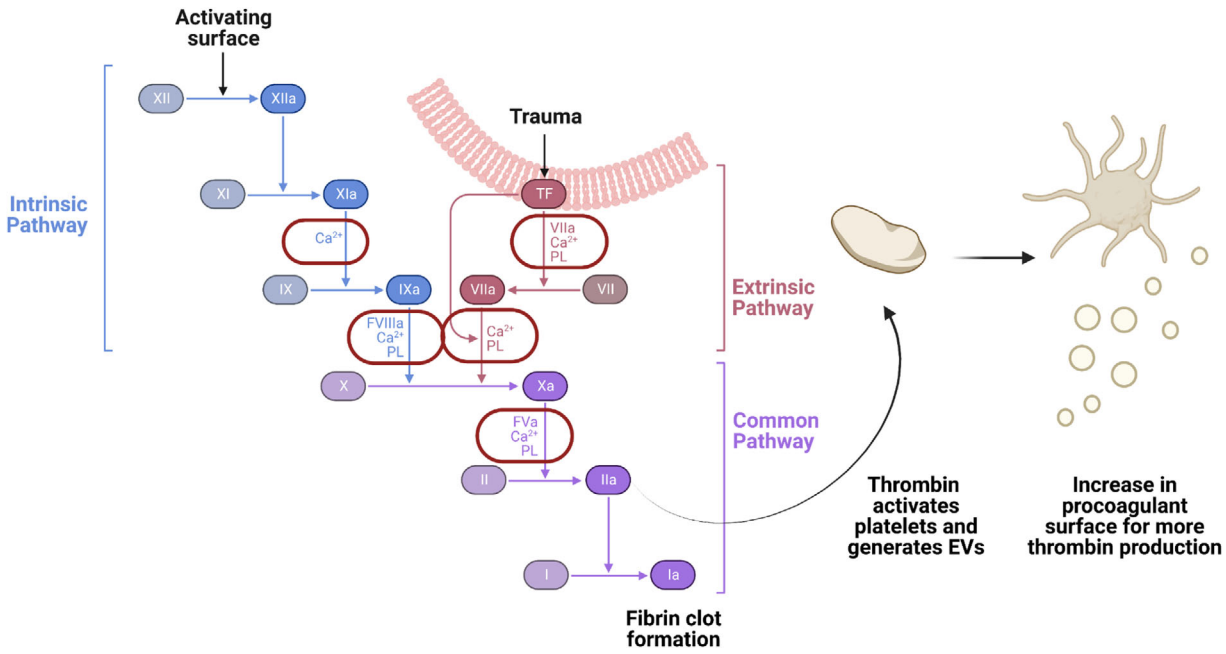

FIGURE 3 Coagulation pathways and platelet activation. Coagulation can be initiated through two different routes: the intrinsic and extrinsic pathways. The intrinsic pathway of coagulation (top left) is triggered by an activating surface. A well-known example of intrinsic coagulation pathway initiation is contact activation by glass, which activates factor XII to factor XIIa. Glass tubes were commonly used for blood collection in the past. In contrast, in a physiological setting, blood can contact extravascular tissue in a bleeding wound, which results in the activation of the extrinsic pathway of coagulation (top right). This pathway begins with the transmembrane protein tissue factor (TF), which acts as a receptor for coagulation factor VII (present in the blood), and this (extrinsic tenase) complex results in the formation of (activated) factor VIIa. Both the intrinsic and extrinsic pathways converge into a final common pathway. In the common pathway, factor X is activated into factor Xa, and factors Xa and factor Va together form the prothrombinase complex, which catalyses thrombin formation (IIa) from prothrombin (II). The formation of tenase and prothrombinase complexes require a surface, which is present as membranes (platelets, EVs) that expose negatively charged phospholipids (PL) as phosphatidylserine. Several coagulation factors (II, VII, IX, X) require Ca^{2+} ions for binding to the procoagulant membrane. Certain anticoagulants inhibit coagulation by chelating Ca^{2+} ions thereby impeding the formation of the tenase and prothrombinase complexes. Thrombin also activates platelets (right), leading to the secretion of granule contents, platelet aggregation, and the release of EVs. In turn, platelet-derived EVs promote coagulation by generating additional procoagulant (coagulation-promoting) PL membrane surface. It is important to note that this is a highly simplified overview, and the coagulation pathway is interconnected to the fibrinolytic pathway and complement activation. From the regulatory perspective, the coagulation process is actively counter-counterbalanced by natural anticoagulants in blood and on the vascular wall. When preparing serum, spontaneously generated thrombin triggers fibrin formation and activates platelets, which explains the higher concentration of platelet-derived EVs in serum compared to plasma prepared from the blood of the same donor (as shown in Figure 4). Image created by Biorender.com.

1.2 | Serum

Serum is another commonly used blood-derived fluid for studying EVs. If blood is *not* mixed with an anticoagulant, it will spontaneously clot, usually within 1 h (Figure 3). The final common step in coagulation is the activation of prothrombin (also known as coagulation factor II, FII) into active thrombin (FIIa). Thrombin not only triggers fibrin formation, but also strongly activates platelets, as does fibrin (Mammadova-Bach et al., 2015; Sang et al., 2021) (simplified in Figure 3, also see the KEGG pathway for molecular interactions https://www.genome.jp/pathway/hsa04610). Upon activation, platelets release the content of their storage granules, which among others contain growth factors, cytokines, and anti-microbial compounds (Pagel et al., 2017), and they also release platelet-derived EVs. As a result, the concentration of platelet-derived EVs is higher in serum compared to plasma from the same donor (shown in many studies and Figure 4) (Palviainen et al., 2020). When blood is centrifuged after clotting is completed (Figure 2), a pellet of cells and fibrin forms, and the serum can be collected as a clear yellow supernatant.

The preparation of serum and the differences between plasma and serum are shown in Figure 2 and Table 2, respectively. The main advantages of serum are the ease of preparation, and its 'stability' since serum does not clot. However, there are currently

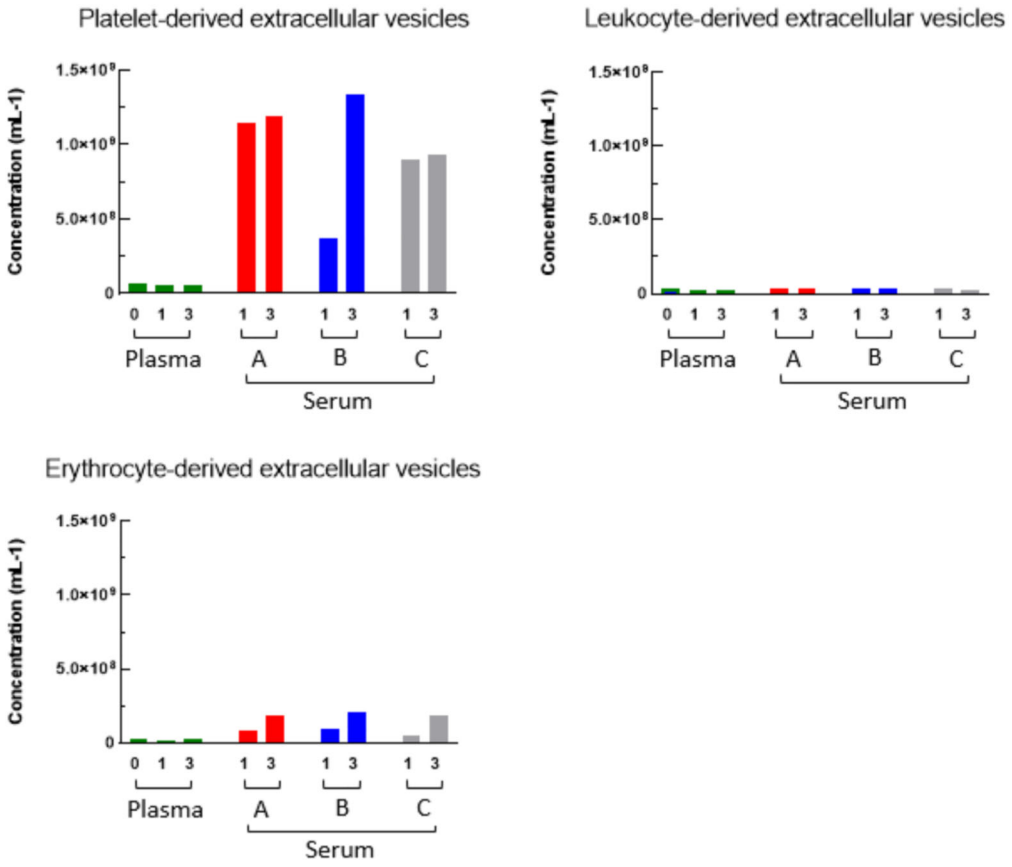

FIGURE 4 Concentrations of blood cell-derived extracellular vesicles in plasma and serum. Blood was collected with informed consent from a single healthy donor in accordance with the rules of the Medical Ethical Committee of the Amsterdam Medical Centre, University of Amsterdam (W19_271#19.421). Venous blood was collected using a 21-Gauge needle, and the first 2 mL of blood was discarded. To prepare plasma and serum, blood was centrifuged for 15 min at 2500 g and 20°C, without using a brake. After centrifugation, the tube was marked 1 cm above the cells using a Lego brick. The supernatant was carefully collected using a plastic Pasteur pipet to 1 cm above the cells, and pipetted into a new tube. This new tube containing either plasma or serum was centrifuged as described for the first centrifugation step, and then the (supernatant) plasma and serum were collected to determine the concentrations of EVs and platelets as described earlier (van der Pol et al., 2012). Blood was collected into four different blood collection tubes. One tube type contained the anticoagulant EDTA for plasma preparation (Becton & Dickenson ((BD), New Jersey, US; 368861), while the other three tubes were used for serum preparation: A (serum CAT, activator silica; BD; 368815), B (serum SSTII, activator silica; BD; 367955), and C (serum, no activator, Greiner Bio-one, Kremsmünster, Austria; 455001). Multiple tubes of EDTA plasma were collected and prepared either immediately after blood collection (arbitrarily denoted as $t = 0$) or after the tubes had been left standing on the lab bench for 1 h or 3 h ($t = 1$ or $t = 3$). Similarly, the different serum blood collection tubes (which require time to clot, hence no $t = 0$) were also left on the bench and centrifuged after 1 h or 3 h. The figure presents the concentrations of the following extracellular vesicles (EVs): (A) leukocyte-derived EVs; CD45+), (B) platelet-derived EVs (CD61+), and (C) erythrocyte-derived EVs (CD235a+), measured by calibrated Apogee A 60 enabling measurement of EVs within the size range of 160–1000 nm. Measuring EVs and platelets by flow cytometry was performed as described (van der Pol et al., 2012; Zhang et al., 2021). This figure demonstrates that (1) the concentrations, particularly of platelet-derived EVs, differ between plasma and serum, and (2) differences in pre-analytics of serum preparation (such as the choice of collection tube, the time between blood collection and centrifugation) also impact the measured concentrations of EVs.

no established protocols for serum preparation specifically for EV research, and there is limited research on the impact of using serum as an EV source. Figure 4 illustrates the differences, particularly for platelet-derived EVs, between plasma and serum from the same healthy donor. This figure also shows that the detection of EVs also in serum depends on preanalytical variables, such as the type of serum tube used and the time between blood collection and centrifugation to prepare serum. In essence, the concentration, composition, and function of EVs in plasma and serum cannot be directly compared, even when blood from the same donor is used.

TABLE 2 Comparison of plasma and serum for blood extracellular vesicle research.

	Plasma	Serum
Anticoagulation	Required	No
Generation of additional EVs during collection and handling	Anticoagulant-dependent	Release of platelet EVs during blood clotting
Removal of EVs by clot removal	Not applicable	Clot removal removes fibrin-binding EVs
Composition resembles blood fluid	Yes, except the presence of an anticoagulant	No. Centrifugation removes fibrin and other endogenous proteins. Thrombin and fibrin activate platelets, which secrete the contents of their granules and release additional EVs.

Abbreviation: EVs, extracellular vesicles.

2 | BASIC PRINCIPLES OF ISOLATION AND DETECTION

2.1 | Isolation

For EV research, most downstream analytical assays require the isolation of EVs from plasma or serum. The isolation of EVs is primarily based on four physical and biochemical principles: (i) size (diameter, assuming that all EVs are spherical), (ii) density, (iii) charge, and (iv) molecular composition/ immuno-phenotype (presence or absence of a particular biomolecule, often transmembrane or membrane-associated proteins or lipids, which can be specifically identified, e.g., using antibodies). Currently, the most commonly used isolation methods are differential ultracentrifugation (UC), density gradient centrifugation (DGC), and size-exclusion chromatography (SEC), or combinations thereof. These methods rely on separation based on the differences in EVs in size and density (UC), density (DGC), or size (SEC) (Coumans et al., 2017). Affinity-based chromatography methods that utilize molecular properties of the EVs, such as marker proteins or charge for capture, are also gaining popularity.

In addition to total yields, these isolation methods vary in terms of time, laboriousness, and the need for specialized equipment and experience (Coumans et al., 2017). It is important to note that none of the currently available methods can isolate all EVs or exclusively EVs from plasma or serum, as platelets and lipoproteins overlap with EVs in size and/or density (Table 1) (Johnsen et al., 2019; Rikkert et al., 2020). Since the used isolation methods can preferentially isolate certain EV subpopulations or remove confounders such as plasma proteins, the choice of the method or methods will impact downstream results. Therefore, direct comparisons of EVs that were isolated by different methods can be challenging.

Finally, commercial precipitation kits that employ reagents such as polyethylene glycol can quickly and efficiently precipitate EVs with a high yield, but since these methods concentrate all precipitable material without selectivity, the purity of the EVs is low. Higher purity of isolated EVs can be achieved by methods such as SEC or DGC, although this may come at the cost of lower recovery. Consequently, the choice of the isolation method should be carefully considered based on the study's objectives.

The main confounders in the isolation and detection of EVs are lipoproteins (Simonsen, 2017) and plasma proteins (György et al., 2011). Lipoproteins are particles composed of lipids and apolipoproteins, classified based on their density and biochemical composition (Glossary, Table 1). Co-isolation of lipoproteins with EVs is inevitable due to their overlapping size range and density, as well as their significantly higher number concentration compared to EVs, which can be up to a million-fold higher (Simonsen, 2017).+ Overcoming the latter problem is difficult, as illustrated by the example of high-density lipoprotein (HDL). HDL has a reported diameter of 7–12 nm (Table 1). When isolating EVs using a size-based separation method like SEC, with a resin cut-off of approximately 70 nm, one would expect efficient separation of HDL from EVs with a diameter of 70 nm and larger. Indeed, this is the case and about 99% of HDL is removed, resulting in a 100-fold enrichment of EVs compared to HDL (assuming no EV loss) (Böing et al., 2014). However, despite the removal of 99% of HDL, 1% of HDL remains, which still represent a 10,000-fold excess of HDL (particle) concentration compared to the estimated concentration of plasma EVs (10 (Johnsen et al., 2019); Table 1). This means that for every single EV, there are still about 10,000 HDL particles present in the sample, despite the efficient size-based separation resulting in a 100-fold enrichment of EVs compared to HDL.

Plasma and serum contain 60–80 mg of soluble proteins per mL (György et al., 2011), Among the total proteins, over 90% are comprised of ten major proteins such as albumin, immunoglobulins, and fibrinogen, while the remaining 10% consist of more

than 1500 of less abundant proteins. It is worth noting that the protein composition in plasma is dynamic and dependent on both individuals' genetic and environmental factors, as well as longitudinal changes. Given that EVs constitute a tiny fraction of plasma and serum, with an estimated volumetric presence approximately 50 nL per mL for plasma, (which accounts for 0.00005% of the plasma volume), EV proteins and protein cargo *within* EVs are present at extremely low concentrations, posing a challenge for their analysis. Moreover, recent findings have shown that EVs isolated from human plasma and serum carry a plasma protein (or molecular) corona (Palviainen et al., 2020; Tóth et al., 2021). Both soluble plasma proteins and lipoproteins interact with the membrane surface of EVs, and this corona is dynamic and influenced by factors such as anticoagulation (Palviainen et al., 2020). The corona is likely to be an essential part of EVs, carrying molecular information relevant for biomarker studies (Hoshino et al., 2020) and functional properties EVs, such as cellular/tissue uptake (Wolf et al., 2022). Thus, considering the optimal level of purity for the isolated EVs becomes relevant and depends on the purpose of the study.

Of note, for multiple reasons it is difficult to completely separate platelets from platelet-derived EVs. Firstly, plasma and serum as fluids have high viscosity and density which hinder platelet separation from platelet-derived EVs by centrifugation. Secondly, since the size distribution of platelets and platelet-derived EVs nearly approaches a continuum, also their separation by size-based separation methods such as size-exclusion chromatography is difficult. Thirdly, due to the overlap in density and biochemical phenotype of platelets and platelet-derived EVs (Rikkert et al., 2020), separation by density-gradient centrifugation or immunoaffinity is also not exclusive. Therefore, as suggested by the ISEV Blood EV task force, we recommend to measure the platelet concentration in prepared plasma and serum samples before freezing the samples (Clayton et al., 2019). Since some platelets fragment during a freeze-thaw cycle, the concentrations of both platelet-derived EVs and platelet fragments (which are indistinguishable from each other by methods such as flow cytometry) correlate with the remaining platelet concentration (Bettin et al., 2022).

In summary, co-isolation of confounders can introduce biases in EV analyses and hinder the comparability of results obtained through different preanalytical and isolation protocols. Optimizing conditions of blood collection, plasma and serum preparation can help to reduce the presence of confounders. The Guidelines for preparation of plasma and serum provide practical information to improve the quality and reproducibility of blood EV research by harmonizing the preanalytical steps. We also recommend measuring and quantitating confounding factors, such as lipoproteins, platelets and other residual blood cells in the prepared plasma and serum samples, especially during protocol development and optimization. Additionally, the relative enrichment of EVs compared to confounders serves as an important quality control parameter offering insights into the efficacy of the applied isolation protocol (Théry et al., 2018). Such values are, for example, particle/protein or protein/lipid ratios. Removal of abundant plasma proteins and lipoprotein can be monitored during protocol optimization, for example, by Western blot.

2.2 | Detection

The choice of analytical methods for characterizing EVs depends on whether the objective is to detect the intact EVs themselves or their cargo. There are three important considerations regarding the detection of plasma and serum EVs.

The first consideration is whether isolation of EVs is necessary. For example, flow cytometry is a commonly used method to detect single EVs within a certain size range, and flow cytometry does not require isolation of EVs before detection (Welsh et al., 2023). Similarly, functional coagulation assays using EV-containing plasma do not require the isolation of EVs (Berckmans et al., 2019). In the case of biomarker analysis, both quantitative and qualitative detection of single blood EVs can be used, whereas currently bulk analysis remains mandatory for omics analyses of EV molecular cargo before single EV omics evolve.

The second consideration is whether the chosen method detects all particles or exclusively EVs. For example, (nano) particle tracking analysis (NTA) is a frequently employed for sizing and determining EV concentration, but it is an optical method that detects all particles that are present in a sample. As mentioned earlier, EVs are outnumbered by lipoproteins in plasma and serum, and so methods like NTA measure mostly lipoproteins and not EVs in plasma or serum (Gardiner et al., 2014).

The third consideration is the limit of detection (LoD) (van der Pol et al., 2014). Methods and instruments have a dynamic range, meaning that they can detect EVs within specific diameter ranges, such as between 100 and 500 nm. Consequently, EVs with a diameter smaller than 100 nm or larger than 500 nm cannot be detected. It is particularly important to know the lower LoD, that is, what is the smallest particle or weakest signal that can be measured with the said method/instrument, for accurately measuring, reporting, and comparing EV concentrations (Welsh et al., 2023). Even slight differences in the LoD can significantly impact the measured concentration or signal intensity, since the concentration of small EVs, for example, with a diameter of approximately 30–50 nm, is much higher than the concentration of EVs with a larger diameter (Arraud et al., 2014). Consequently, even minor variations in the lower LoD will markedly affect the analysis of EV concentrations. At present, there are no methods that can measure the entire particle size distribution of EVs.

Finally, most, if not all methods used to detect single particles or EVs in suspension are optical methods. The light scattering of particles, including EVs, depends on their size, biochemical composition, and refractive index. However, the high refractive index of lipoproteins compared to EVs further complicates EV detection by optical methods such as NTA (Dragovic et al., 2011). The biophysical properties and biochemical composition of EVs necessitate the use of specialized reference materials

and test samples, which are currently being developed in the EV community. For instance, an infrastructure has recently been established, encompassing reference materials, test samples, software, and reporting standards, to calibrate flow cytometers used for EV detection, measurement, and reporting in a standardized and transparent manner (Welsh et al., 2023). In general, reference materials and test samples can be employed to determine the LoD of an instrument for EVs. It is crucial not to confuse appropriate reference materials for EV detection with the manufacturer's calibration or reference beads which are solely intended to test and calibrate the equipment.

3 | GUIDELINES FOR PREPARATION OF HUMAN BLOOD PLASMA AND SERUM

3.1 | Donors

- Use matched controls in terms of age, gender, medication, etc. for comparison.
- Collect blood at the same time of the day, if possible.
- If possible, exclude the first 2–3 mL of collected blood from EV studies. This tube can be, for example, an EDTA tube used for haematological analyses such as cell counts. Adhere to clinical venipuncture guidelines regarding needle size, use of stasis, and monitor the blood flow until the last tube, as blood may become activated and even begin to clot when collecting multiple tubes.
- Overnight fasting reduces the concentrations of lipoproteins.
- Standardize all possible steps of blood sample collection and handling. Systematic differences in collection and handling, for example, between patients and healthy controls, may affect blood EVs and the sample quality.

3.2 | Anticoagulation

- Plasma preparation requires anticoagulation. All anticoagulants inhibit coagulation but their efficacy differs due to variations in their mode of action (see Glossary for more information).
- Some anticoagulants also efficiently inhibit platelet activation (ACD, EDTA), thereby reducing the release of platelet-derived EVs during blood collection and handling.
- Some anticoagulants incompletely inhibit platelet activation (citrate), or may even activate platelets (heparin).
- Ensure that the chosen anticoagulant is compatible with downstream analysis. For example, heparin binds to DNA, thereby inhibiting the polymerase chain reaction.
- Serum samples differ from plasma samples. For EV studies, the most significant impact comes from the *ex vivo* platelet EVs and molecules secreted from activated platelets.
- Do not mix and match differently anticoagulated samples, or plasma and serum samples.

3.3 | Preparation of plasma and serum

- Currently one of the most common protocols for preparing plasma involves two centrifugation steps (Figure 2). We recommend using the same protocol for serum preparation.
- Use established and commonly used plasma preparation protocols to avoid discussions with reviewers and editors, or provide evidence that the modified protocol is effective in, for example, removal of platelets.
- Standardize processing time and anticoagulants for all donors, as they may affect the concentration of plasma EVs. Similarly, standardize the serum preparation procedures as the serum EV concentration depends on the time the blood is allowed to clot.
- Discard or report plasma or serum samples that appear opaque (fat), orange, or red (haemolysis), or contain visible clumps or insoluble strands (insufficient anticoagulation).
- Platelets are sensitive to cold activation. Never put tubes containing blood on ice or in the fridge, and perform all procedures at room temperature.
- Do not use a brake during the centrifugation of blood or plasma, as platelets (present in the buffy-coat) can easily re-enter the plasma.
- Note that centrifuges can vary, even with the same brand, type and rotor, leading to slightly different results. Use the same centrifuge or establish the protocol for each instrument used at the different sample collection sites. Alternatively, monitor the quality of the prepared plasma or serum samples by measuring, for example, the residual platelet concentration.
- Use polypropylene plastics for plasma handling (see Glossary, section on Coagualtion). For serum preparation, glass tubes or commercial serum tubes can be used. Different types of serum tubes are available, but their impact on the presence, composition, and function of EVs remains largely unexplored and requires further studies.

- The concentration of residual platelets can be reduced by staying well above the cells (buffy coat or clot) upon plasma collection (Figure 2). Standardizing this step improves the quality of prepared plasma samples, and reduces inter-individual variation between operators. For example, a (2 × 4) Lego® brick can be used to mark a tube one centimetre above the cells. The caveat is the loss of plasma.
- The concentration of remaining platelets in plasma and serum samples after centrifugation can be determined using clinical haematology analysers or flow cytometry. Even when using a double centrifugation protocol, for example, 2 × 2500 g (without brake and maintaining a one-centimetre safety distance during pipetting), the prepared plasma will still contains approximately 0.5%–1.0% of the original platelet concentration. This platelet concentration may be below the LoD of a haematology analyser, which is commonly around 10 (Hegyesi et al., 2022) platelets/mL.
- If necessary for biomarker studies, the remaining platelets can be removed by filtration. Keep in mind that filtration may also remove large EVs.
- Measure and report the residual concentration of platelets in the plasma and serum samples once your protocol has been established.
- Platelets and any other residual cells or remnants, including 'ery-ghosts', present in plasma and serum may fragment during freeze-thaw cycles.
- The effects of storage on the presence, composition, and functional activity of plasma and serum EVs are insufficiently known.

3.4 | Reporting

- Avoid using commonly used terms such as *platelet-free* plasma, *platelet-depleted* plasma, or *platelet-poor* plasma, since these terms are unclear and scientifically undefined. Instead, define the starting material obtained by the used protocol by reporting the concentration of remaining platelets. It is also recommended to evaluate the number of residual lipoproteins after setting up a method in the laboratory.
- Provide detailed information about the sample properties, preanalytical handling, and the level of detection of the instrumentation used in your manuscript. For help on what to report, use the newest checklists available such as those for flow cytometry or biobank samples.
- Stay updated with the newest educational material provided by ISEV to enhance your understanding and adherence to current guidelines and best practices.

4 | SUGGESTIONS FOR MORE INFORMATION

In 2017, the American Heart Association published the first guidelines to study EVs in human blood and its fractions (Coumans et al., 2017). This manuscript contains practical information on how to work with blood and blood-derived EVs, including blood collection, plasma and serum preparation, storage, EV isolation, and more. Most of these guidelines remain valid, and form the basis of the present manuscript.

In 2019, the Blood EV task force of the ISEV Rigor and Standardization Subcommittee published a manuscript titled '*Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles*' (Clayton et al., 2019). This publication presents the results of a survey conducted amongst ISEV members with at least 5 years of experience in working with blood and blood-derived EVs. In this manuscript, 'a roadmap' was proposed to overcome the identified sources of pre-analytical variation. Recently, as a follow-up, the task force is working on a minimal reporting checklist to enhance the quality of prepared plasma and serum samples used in EV research or obtained from biobanks as starting materials.

The complexity of the presence of lipoproteins and EVs in blood has been well documented emphasizing their biophysical similarity, which, combined with their abundances in plasma, causes challenges regarding the isolation and detection of EVs. For further reading, https://www.lipidmaps.org/ provides additional information on lipoproteins. For a comprehensive overview of coagulation, we recommend the paper of Smith et al. (2016) For plasma protein content, we recommend clinical haematology textbooks and literature on protein corona.

The ISEV website (www.isev.org) contains educational material including massive open online courses on coagulation, isolation, and detection. It also provides links to reports from recent workshops, ISEV Rigor and Standardization task forces, and intersocietal working groups.

It is important to note that this paper is not a literature review, and we acknowledge that many references of all the foundational work in the EV field, such as studies on preferential anticoagulants and optimal preanalytical considerations to enhance repeatability, have been omitted. We deliberately refrained from highlighting specific papers, as benchmarked protocols from the literature should be validated by measuring the resulting samples in the researcher's laboratory rather than just strictly following protocols. A recent study demonstrated that even when EV laboratories use the same protocol to collect and handle blood, the composition of the prepared plasma still differenced significantly regarding the concentration of residual platelets, which, as explained, is one of the confounders that is difficult to get rid off when studying EVs from blood (Bettin et al., 2022).

GLOSSARY

Anticoagulant. A reagent or a mixture of reagents that is added to whole blood during blood collection. Its purpose is to inhibit coagulation, preventing blood and plasma from clotting. Anticoagulants differ in their working mechanism and potential side effects. In EV research, commonly used anticoagulants include (tri-sodium) citrate, acid citrated dextrose (ACD), and ethylenediaminetetraacetic acid (EDTA). These anticoagulants chelate calcium ions albeit with different affinities. Calcium ions enable binding of coagulation factors to membranes of cells and EVs, which is a prerequisite for thrombin formation (see Figure 3). Some anticoagulants, such as hirudin derived from leeches, directly inhibit thrombin, while for example, heparin indirectly inhibits thrombin by binding to anti-thrombin. Anti-thrombin is a naturally occurring anticoagulant protein present in plasma. Binding heparin to anti-thrombin significantly enhances its affinity to inhibit thrombin function. In the absence of an anticoagulant during blood collection, the blood will spontaneously clot, and serum can be obtained by centrifugation. For more information on anticoagulants and their effects on blood EVs, the reader is referred to two recent publications as examples (Dhondt et al., 2023; Lima-Oliveira et al., 2021).

Coagulation. Coagulation, along with platelet activation and aggregation, plays an essential role in haemostasis, the process that stops bleeding. Platelet activation and aggregate formation are referred to as 'primary haemostasis', while coagulation is known as 'secondary haemostasis'. The term 'coagulation cascade' describes the sequential conversion of inactive enzymes (zymogens) into active enzymes (designated by Roman numbers and an 'a', such as 'factor X' for the inactive zymogen, and 'factor Xa' for the active enzyme). Thrombin (factor IIa) is the final active enzyme of the coagulation cascade. The cascade structure amplifies thrombin and fibrin formation unless inhibited by natural anticoagulants that are present in the blood and on the vessel wall. Coagulation can be initiated through extrinsic and intrinsic pathways, both of which lead to thrombin generation and the conversion of soluble fibrinogen (factor I) to insoluble fibrin, resulting in fibrin strand crosslinking and clot formation (Figure 3). Fibrinogen is the third most abundant soluble protein in human plasma and is also stored in platelet granules. Thrombin strongly activates platelets by, for example, cleavage of protease-activated receptors. Upon platelet activation, the molecular contents of the platelet granules, including fibrinogen, are released further promoting clot formation and platelet aggregation, but also wound healing. Additionally, thrombin activates fibrinolysis, the process responsible for clot dissolution. The enzyme responsible for clot dissolution is plasmin. Under normal conditions in a healthy individual, coagulation and fibrinolysis are carefully balanced and regulated by multiple inhibitors. EV-induced coagulation can be assessed in coagulation assays that measure, for example, the presence of phosphatidylserine or tissue factor, the transmembrane protein that activates factor VII into factor VIIa of the extrinsic pathway.

Lipoproteins. By far the most abundant colloid particles present in blood, plasma and serum. Chylomicrons transport dietary lipids from the intestine, while very low density lipoprotein (VLDL) is secreted by the liver and metabolized in the circulation into intermediate density lipoprotein (IDL) and low density lipoprotein (LDL). High density lipoprotein (HDL) is secreted both from the intestine and the liver. Lipoproteins are classified based on their density and size (Table 1), and they can be further classified, for example, based on the presence or absence of specific apolipoproteins (such as A, B, C or E). Chylomicrons have a single ApoB-48 protein molecule per particle, VLDL, IDL and LDL have a single ApoB-100 protein molecule per particle, whereas HDL has two or three ApoA-I protein molecules per particle, which affects their shape (Table 1). In contrast to the heterogeneity observed in EVs, lipoproteins exhibit specific size and density distributions and contain well-defined molecular identifiers such as ApoB-100 or ApoA-I. The known number of apolipoprotein molecules per lipoprotein allows for the calculation of absolute particle concentrations of each of the classes of lipoprotein particles based on the apolipoprotein concentration (Table 1).

Platelets. Platelets are the smallest type of cells found in any human body fluid. A nuclear platelets originate from bone marrow precursor cells known as megakaryocytes. Platelets play crucial roles in physiological processes such as haemostasis and vascular integrity, wound healing, inflammation, and immune responses. While the size of a resting, discoid platelet is approximately 2–4 μm, they are agile cells with large intracellular membrane reservoirs which will change their size, volume and shape upon activation (depicted in Figure 3). Platelets become easily activated during cold, and during blood collection and handling, which can result in release of EVs. Due to their discoid shape and size and density similarity to larger EVs (as shown in Table 1), it is impossible to completely remove platelets from plasma or serum by centrifugation *without* affecting the presence of EVs. Therefore, even after performing a double centrifugation protocol, some residual platelets will still be present in plasma, accounting for approximately 0.5%–1.0% of the original blood platelet concentration. Consequently, from an EV researcher's perspective, the commonly used terms such as '*platelet-free* plasma' and '*platelet-depleted* plasma' are misleading.

AUTHOR CONTRIBUTIONS

Rienk Nieuwland: Conceptualization; methodology; visualization; writing—original draft; writing—review and editing. **Pia R-M Siljander**: Conceptualization; methodology; visualization; writing—original draft; writing—review and editing.

ACKNOWLEDGEMENTS

This work was supported by the Academy of Finland [grant 330486 (PRMS); Business Finland [grant EVE (PRMS)]; Magnus Ehrnrooth Foundation (PRMS); Medicinska Understödsföreningen Liv och Hälsa rf (PRMS). We thank Chi M. Hau for performing the experiment shown in Figure 4, Mendel Engelaer for critical reading of the first draft, and Katariina Öörni for critical reading the provided information on lipoproteins.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

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How to cite this article: Nieuwland, R., & Siljander, P. R.-M. (2024). A beginner's guide to study extracellular vesicles in human blood plasma and serum. *Journal of Extracellular Vesicles*, *13,* e12400. https://doi.org/10.1002/jev2.12400