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VIEWPOINT

Extracellular vesicles in the transfusion medicine field: The potential of proteomics

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Abstract

In transfusion centres, blood components are divided and stored following specific guidelines. The storage temperature and time vary among the blood cells but all of them release extracellular vesicles (EVs) under blood bank conditions. The clinical impact of such vesicles in blood components for transfusion is an object of debate, but should be considered and is being investigated. In this context, proteomics is an excellent tool to study the cargo and composition of EVs derived from red blood cells and platelets, since such vesicles are enriched in lipids and proteins. The development of quantitative mass spectrometry techniques and the evolution of bioinformatics have allowed the identification of novel EVs biomarkers for different diseases. In this context, the application of high coverage proteomic tools to the analysis of EVs in the transfusion medicine field would provide information about storage lesions and possible transfusion adverse reactions. This viewpoint article approaches the potential of proteomics to investigate the impact of EVs in blood bank transfusion components, especially red blood cells and platelets.

KEYWORDS
extracellular vesicles, proteomics, transfusion medicine

1 | EXTRACELLULAR VESICLES IN THE TRANSFUSION MEDICINE FIELD

Extracellular vesicles (EVs) were depicted for the first time in 1967 when Peter Wolf described them as "platelet dust" [1]. Over the years, different studies confirmed that these small lipid particles come from different cellular types and reflect the processes that are taking part inside the cells. EVs are more complex than seemed at the beginning and have been classified according to their size, biochemical composition, and cell origin [2]. The traditional nomenclature divided EVs in three groups: exosomes, microvesicles (MV) or microparticles (MP), and apoptotic bodies. Since consensus has not yet emerged on specific markers of EV subtypes, currently The International Society of Extracellular Vesicles (ISEV) supports the use of the terms "small EVs" (<100 nm or <200 nm) and "medium/large EVs" (>200 nm) when the origin of EVs is uncertain [2]. Nevertheless, ISEV recommends using "extracellular vesicle" as the "generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate" [2].

The most interesting aspect of EVs, independently of their origin, is their cargo. EVs cargo includes proteins, lipids, nucleic acids such as mRNA, and other bioactive molecules. EVs participate in cell-to-cell communication triggering an effect on the acceptor cell by the delivery of their cargo or stimulating them via surface expression ligands. For instance, red blood cells-derived EVs regulate nitric oxide production under hypoxic conditions increasing vasodilation and smooth muscle relaxation [3,4]. In addition, they also take part in redox balance [5,6] and immunomodulation [7,8]. On the other hand, platelet-derived EVs

participate in haemostasis and thrombosis [9], inflammation [10], and immunity [11]. EVs are released by different cell types and are present in many body fluids. Their analysis would permit to know about the physiological state of the cells that release them and possible effects on other cells. Blood is one of the richest sources of EVs originated from different cell types but the majority of those present in plasma have a platelet origin [12].

Blood transfusion is performed nowadays as prophylaxis or treatment of different clinical scenarios such as surgery, trauma, and cancers. Over the last century, many discoveries in this field have increased the use and safety of blood products. Nowadays, transfusion centres keep blood components separately and with additive solutions in order to allow extending the storage time. Each blood component has different storage conditions. Thus, red blood cells (RBC) can be stored at 4°C for over 42 days. On the other hand, platelets are cold-sensitive leading to activation and, therefore, need to be stored at room temperature. The high risk of the presence of growing pathogens in platelet units led to the development of systems to inactivate them, the first one being approved by the FDA in 2014. Currently, there are various pathogen reduction technologies (PRT) based on the combination of different additive solutions and wavelength light able to inactivate a wide spectrum of viruses and bacteria. PRT consist of a compound that intercalates between DNA strands breaking them after a radiation pulse with a specific wavelength. These PRT are generally applied to platelets units due to their absence of nucleus and their storage at room temperature, which is not the case for instance for plasma, which is frozen and normally stored below −20°C (ideally at −80°C). Different equipment, steps to separate blood components and treatments to avoid pathogens, have an impact on blood units leading to storage lesions. In order to guarantee the safety of blood products, different parameters and markers of storage lesion are measured in RBC, platelets, and plasma units. In this context, EVs have started to gain attention in the transfusion medicine field due to their role in cell-to-cell communication and possible source of transfusion adverse reactions. In fact, the measure of EVs has been recently proposed as an internal parameter to check the quality of red cell concentrates (RCC) and platelet concentrates (PC) during storage in blood banks [13–16]. An extended storage time of RCC for transfusion and the introduction of PRT to improve the safety and storage time of PC have led to an increased number of papers quantifying EVs in blood concentrates. A selection of the most relevant and recent ones is summarised in Table 1.

2 | EVs ANALYSIS AS A SOURCE OF BIOMARKERS OF STORAGE LESIONS

Flow cytometry (FC) is the technique most frequently used to measure EVs present in different blood concentrates. The reason for that is the availability of flow cytometers in blood transfusion centres; however, it is important to consider many aspects related to this technique, which may have an impact on EVs quantification: the model of flow cytometer, including the instrument settings (calibration and data acquisition); the sample processing protocol, including washing and staining steps; and how EVs are measured, using fluorescence markers or only light scatter. Recently, the ISEV has provided specific guidelines to reduce this variability [35]. Other techniques based on particle size, such as dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA), are simple and do not require an expert technician. However, these techniques are unsuitable for EVs quantification from biological samples due to their poor specificity, sensitivity and dynamic range [36]. Besides, the application of these methodologies is difficult because it involves EVs isolation (to avoid interference with other particles present in the sample). Given the complexity of the analysis of EVs mentioned above, the ISEV advises to combine different techniques to characterise efficiently the population of EVs present in the sample.

Several papers have quantified the number of EVs present in different blood storage units during recent years, as indicated in Table 1. Most of the studies evaluated how different conditions (storage (cold, frozen, RT, DMSO), additive solutions (PAS-B/A) or blood extraction methodologies (apheresis, whole blood)) affected the number of released EVs. Nevertheless, none of studies present in Table 1 analysed the EVs cargo, which includes proteins, DNA, RNA, and lipids. Since the composition of EVs is related to the cell of origin, and RBC and platelets do not have nucleus and genomic DNA, proteomics is an ideal tool to study the EVs cargo and composition. That would allow obtaining information on the state of RCC and PC under storage conditions, which could help to predict potential transfusion adverse reactions (storage disease). Nevertheless, as indicated above, EVs contain not only proteins so the combination of proteomics with the analysis of lipids and microRNA could provide a broader overview on the state of the cell of origin upon storage, and possible adverse effects on the recipient patient.

3 | PROTEOMIC ANALYSIS OF BLOOD COMPONENTS IN THE TRANSFUSION MEDICINE FIELD

In recent years, several proteomics studies of blood components, especially platelets, have increased the knowledge of storage lesions in the transfusion medicine field. Many of these studies focused on the impact of PRT on platelets and led to the conclusion that PRT have a slighter impact on the platelet proteome than the PC storage time [37–39]. The first proteomics analyses performed in the platelet transfusion field were based on gel-based methodologies (1DE, 2DE and 2D-DIGE). As examples of the above, Prudent et al. [40] identified by 2DE modifications in DJ-1 after Intercept treatment. Marrocco et al. [41] also identified changes in this protein, which plays a major role in antioxidant stress responses in platelets following gamma irradiation. In addition, cytoskeleton proteins such as actin or gelsolin were also found impaired with storage time and PRT by different groups [42, 43].

Recent advances in bioinformatics tools combined with MS-based quantitative proteomics helped to identify new proteins related to PC storage lesions. In addition to the altered proteins mentioned above, the introduction of label free quantification (LFQ) methodologies allowed the identification of some new markers related to platelet

TABLE 1 Selection of recent EVs quantitative studies published in the transfusion medicine field

Article	Methods used to prepare EVs samples	Types of EVs measured	Technique	Molecular marker	Goal	Major outcomes
Veale et al., 2011 [17]	WB was leukoreduced prior to centrifugation at 5000 × *g* for 10 min at RT. RBC were resuspended in different additive solutions. Supernatant from RBC was collected for MP measurement	RMP	FC	GPA Annexin V	Compare blood storage additive solutions	GPA MP increased over storage time for all additive solutions.
Sparrow et al., 2012 [18]	WB was centrifuged at 5000 × *g* for 10 min at 22°C. Plasma was collected and frozen at -80°C. For analysis, plasma samples were thawed at 37°C	MP	FC	CD41 CD45 CD144 CD235a PS Annexin V	Analyse the effect of WB hold conditions before FFP preparation on the MP profile	FFP-derived MP number increased with longer WB hold time, especially PMP and PS MP.
Almizraq et al., 2013 [19]	Samples were collected directly from RCC, no additional steps were applied	RMP	FC	CD235a CD47 PS	Evaluate the effect of storage on RBC	MP levels increased over storage time.
Grisendi et al., 2015 [20]	Samples were collected directly from RCC, no additional steps were applied	RMP	FC	CFSE Annexin V GPA	Compare multiparametric stainings to detect and quantify MP over storage time	CFSE detected successfully MP with an intact membrane. Combination of CFSE and GPA increased MP detection versus the use of GPA/anexin V alone.
Bakkour et al., 2016 [21]	RCC were centrifuged at 2500 × *g* for 20 min at 20°C. Supernatant was collected to evaluate EVs	EVs	FC	CD235 CD41 CD62 CD66 CD14	Evaluate different manufacturing methods	EVs levels in RCC supernatant varied based on manufacturing method and increased over storage time.
Cho et al.; 2016 [22]	RBC were centrifuged at 1500 × *g* for 15 min to remove cells. Secondly, supernant was ultracentrifuged at 13,000 × *g* for 2 min. Pellet was resuspended in PBS	RMP	FC	CD235 CD41	Compare blood storage treatments	Irradiation increased significantly the number of CD-41 positive MP but not CD235a positive MP.
Serrano et al.; 2016 [23]	RCC supernatant was obtained centrifuging RCC at 2060 × *g* for 10 min at 4°C	RMP	FC	CD235	Verify plastic storage bags	Some polyvinyl plastics showed more MP formation than others.
Almizraq et al.; 2017 [24]	RCC were centrifuged at 2200 × *g* for 10 min at 4°C	RMP	FC	CD235	Test different methods of detection and storage time	Quantitative analysis using FC and a TRPS device showed an increase in the number of EVs during hypothermic storage. However, FC was unable to identify and quantify EVs ≥200 nm in size. A comparison of the TRPS and DLS techniques demonstrate considerable differences in the EVs size profile during storage. TRPS was able to differentiate among EVs subpopulations.

(Continues)

TABLE 1 (Continued)

Article	Methods used to prepare EVs samples	Types of EVs measured	Technique	Molecular marker	Goal	Major outcomes
			DLS	Size		
			TRPS	Size		
Bardyn et al., 2017 [25]	Samples were collected directly from RCC, no additional steps were applied	MV	FC	CD47	Analyse storage time	The number of MV increased linearly since day 1. However, the microvesiculation rose exponentially after day 43 of storage.
Black et al., 2017 [15]	Samples were collected directly from PC and diluted 1:500 in Dulbecco's modified phosphate-buffered saline	EVs	FC	CD61	Multicentre study: Standardise a protocol of EVs quantification	PMP increased during storage in 86% of investigated PC. Variability among centres could be explained by different instrument settings.
Chen et al., 2017 [26]	PPP was collected by two centrifugation steps of PC at 1000 × *g* at RT and then diluted 10-fold with PBS buffer containing 0.25% BSA and 200 mmol/L NaCl	MV	FC	CD41	Compare platelet storage treatment	MV release increased upon the Riboflavin/UV treatment during storage. However, microvesiculation decreased with p38 inhibitor.
Eker et al., 2017 [27]	Samples were collected directly from PC	PMP	FC	CD41 CD62	Evaluate platelet storage conditions	PMP levels of freeze-thawed PC were statistically significantly higher than fresh PC.
Marcoux et al., 2017 [28]	PC were centrifuged at 1430 × *g* for 25 min and then twice at 3200 × *g* for 5 min to obtain PFP.	MP	FC	CD41	Analyse blood preparation procedure and storage time	The mode of PC preparation had a bigger impact on MP release rather than storage time.
Noulsri et al., 2017 [29]	Platelet suspensions were collected directly from PC and diluted in HEPES buffer	PMP	FC	CD41 CD62 Annexin V	Compare blood preparation procedures	High variability of PMP in PC prepared by different procedures.
Waters et al., 2017 [30]	PC were centrifuged at 1600 × *g* for 20 min. followed by other centrifugation at 12,000 × *g* for 5 min at RT in order to obtain platelet supernatant.	PMP	FC	CD61	Evaluate storage conditions	Cryopreserved PC contained significantly more PMP than RT–stored PC.
Bouchard et al., 2018 [31]	RBC were diluted 1:2 with RBC wash buffer and centrifuged five times at 200 × *g* for 5 min. After each centrifugation step, the supernatants were collected and pooled. The pool supernatant was centrifuged at 800 × *g* during 10 min at RT following by other centrifugation at 1600 × *g* over 10 min at RT. Finally the last centrifugation was performed at 40,000 × *g* during 60 min at 4°C. MP were resuspended in 1 mL RBC wash buffer.	RMP	FC	GPA Lactadherin	Determine the thrombin generation via prothrombinase complex by RBC and RMP over storage time	MP increased over storage time and represent around 70% of the prothrombinase activity after 35 days. PS positive RMP were identified, but their amount over time did not correlate with thrombin formation

(Continues)

TABLE 1 (Continued)

Article	Methods used to prepare EVs samples	Types of EVs measured	Technique	Molecular marker	Goal	Major outcomes
Gao et al., 2018 [32]	PC samples were centrifuged at 1000 × *g* for 5 min to obtain PRP. Afterwards, samples were centrifuged at 2500 × *g* for 15 min to obtain PPP.	PMP RMP LMP MMP EMP	FC	CD41 CD235a CD45 CD14 CD144	Examine platelet preparation procedures and storage time	Apheresis procedure increased microvesiculation. Levels of EVs also increased during storage.
Ghasemzadeh et al., 2018 [33]	PPP was obtained from PRP with the platelet count of 5×10^8/mL.	PMP	FC	CD61 Annexin V	Evaluate storage time	MP formation increased during storage.
Oikawa et al., 2019 [34]	PC samples were centrifuged at 10,000 × *g* for 5 min at 22°C.	PMP	ELISA	Anti-GPIX Anti-GPIb	Compareplatelet preparation procedures	Levels of PMP were higher using an automated system than 100% plasma or a manual method.
Valkonen et al., 2019 [16]	PC were centrifuged at 650 × *g* for 7 min at RT after ACD and apyrase addition. PRP was centrifuged at 1560 × *g* for 20 min. also at RT. PPP was ultracentrifuged at 1,00,000 × *g* for 1 h at 4°C. Pellet was resuspended in PBS.	PMP	NTA	Size	Analyse platelet additive storage solution and storage time	PMP concentration increased over time and was higher in PAS-B. Moreover, EVs concentration correlated with the established platelet activation markers CD62P and soluble GPV.
			FC	CD41 CD45		

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; DLS, dynamic light scattering; EMP, endothelial derived micorparticles; EVs, extracellular vesicles; FC, flow cytometry; FFP, fresh frozen plasma; GPA, glycophorin A; LMP, lymphocyte derived microparticles; MMP, monocyte derived microparticles; MP, microparticles; MV, microvesicles; NTA, nanoparticle track analysis; PC, platelet concentrates; PMP, platelet microparticles; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PS, phospatidylserine; RCC, red cell concentrates; RMP, red blood cells-derived microparticles; RMV, red blood cell microvesicles; RT, room temperature; TRPS, tunable resistive pulse sensing; WB, whole blood.

activation, such as emilin and nidogen, among others [37, 38]. One of the last published papers that analysed PC storage lesions by LFQ found that many of the proteins that decrease over storage time are localised in alpha granules and contribute to coagulation, inflammation, and angiogenesis [37, 44]. In addition, other studies found decreasing levels of these proteins in platelets over storage time but increasing levels in the supernatant [45]. The latter highlights the need to analyse the proteome of EVs to obtain new information about the impact of the storage time in PC. Thus, the proteome analysis of PC-derived EVs could help to identify biomarkers of possible transfusion adverse reactions.

To date, there are two main studies analysing the proteome of PC-derived EVs. On one hand, Pienimaeki-Roemer et al. [46] performed a LFQ proteome analysis of EVs present in PC after 5 days of storage. EVs were divided into MV and exosomes during isolation. MVs were isolated by centrifugation applying a density gradient and exosomes were isolated only by ultracentrifugation. It is important to point out that this fractionation procedure is not accurate and leads to an overlap of different EVs subpopulations. The results showed a protein overlap between MV and exosomes; the protein composition was similar in both EVs populations although with differential enrichment in each of them. Thus, there was an enrichment of proteins such as ApoE and Rab11 in different fractions of MV whereas exosomes showed a higher expression of CD9 (typical exosomes marker), lipid raft markers (stomatin, flotillin) and complement factors compared to MV.

On the other hand, our group has recently analysed qualitatively and quantitatively the proteome of PC-derived EVs after a PRT treatment at different days of storage [47]. In this case, EVs populations were isolated together by ultracentrifugation, and many proteins were found in common among conditions. However, a suggested marker of platelet storage lesion, the platelet collagen receptor Glycoprotein VI (GPVI) [48], was only found in EVs at day 7. The quantitative analysis, based on SWATH MS, showed an enrichment of many proteins at day 7, such as Fructose-bisphosphate aldolase A and Triosephosphate isomerase, enzymes related to the glycolysis pathway; and cytoskeleton proteins related to exosome and MV shedding (CD9, actin and integrin alpha IIb, among others). The higher expression of these proteins at day 7 of storage indicates higher platelet activation levels so these proteins were suggested as EVs-derived biomarkers of platelet storage lesion. Moreover, proteins such as C-C motif chemokine 5 (CCL5) and Platelet Factor 4 (PF4), found with higher levels at day 7 of storage, have chemoattractant properties and could have an impact on the recipient after transfusion.

Regarding the red blood cells transfusion field, it is known that storage lesions are characterised by depletion of ATP and 2,3-diphosphoglycerate, and a loss of red cell membrane flexibility accompanied by the release of EVs [49]. The study of the RBC-derived EVs to date primarily focussed on their quantification over storage time and the analysis of different manufacturing methods, as it is summarised in Table 1.

In order to analyse the proteome of RBC-derived EVs, most studies were based on mass spectrometry and immunoblotting assays. In this way, several proteins including band 3 anion transport protein, stomatin and haemoglobin were found to be enriched in MV compared to the RBC membrane [50–52]. There is a hypothesis that RBC release EVs in storage conditions to prevent their removal from circulation after transfusion when they are still functional [53]. According to this, EVs are a protective mechanism to allow erythrocytes to eliminate altered proteins, lipids and other molecules that could trigger apoptosis [51]. This theory was reinforced by the presence of such proteins in RBC-derived EVs [54] (e.g., membrane-bound haemoglobin, band 3, flotillin, CD47 and inmunoglobin G), and by the identification of specific haemoglobin oxidised residues in vesicles over storage time [55].

In addition to the above, in recent years many publications have analysed and confirmed the immunomodulatory and procoagulant activity of RBC-derived EVs [8, 31, 56]. Moreover, Rhesus proteins [50, 57] and other antigens, Kell or Lutheran [57] were also identified by FC in MV derived from RBC. Although the release of EVs from RBC has been proposed as a protective mechanism, the discovery of their immunomodulatory activity and the presence of antigens in their membrane could lead to transfusion adverse reactions triggered by the development of alloantibodies in the recipient patient. In order to elucidate which explanation is the most suitable for the role of these EVs, further comprehensive analyses of their protein cargo are needed. The recent development of new strategies for haemoglobin depletion has allowed to decode the proteome of RBC in detail [58]. For this reason, it is a good moment to analyse the proteome of RBC-derived EVs because even today this is almost a mystery.

4 | FUTURE PERSPECTIVES AND CHALLENGES AHEAD

Research carried out in recent years illustrates how developments in mass spectrometry-based quantitative proteomics should be combined with more precise and efficient methods to isolate pure populations of EVs to achieve a thorough analysis of EVs derived from blood cells. In the context of the transfusion medicine field, the impact of EVs in blood transfusion components should be explored in detail. That should include quantifying the number of vesicles, analysing their size, and a quantitative analysis of the EVs protein cargo (Figure 1). Detailed functional studies should be carried out in parallel to the above.

The first proteomic studies of EVs present in blood transfusion components, especially PC, have been already carried out, providing clues on altered proteins related to storage lesions. Although these initial results are promising, we should not forget the limitations we face for this kind of studies, for example, the size of the smaller particles that makes difficult their analysis by FC; and the low protein yield, which requires high amounts of EVs to achieve an efficient proteomic analysis. The latter is an important issue that sometimes requires pooling biological replicates to have enough material for a complete analysis. And let's not forget than in addition to the initial proteomic analysis, further protein is needed for validations assays (e.g., immunoblotting). Another limitation is the variety of existing protocols for the isolation of EVs and the difficulty to obtain pure populations of EVs subtypes.

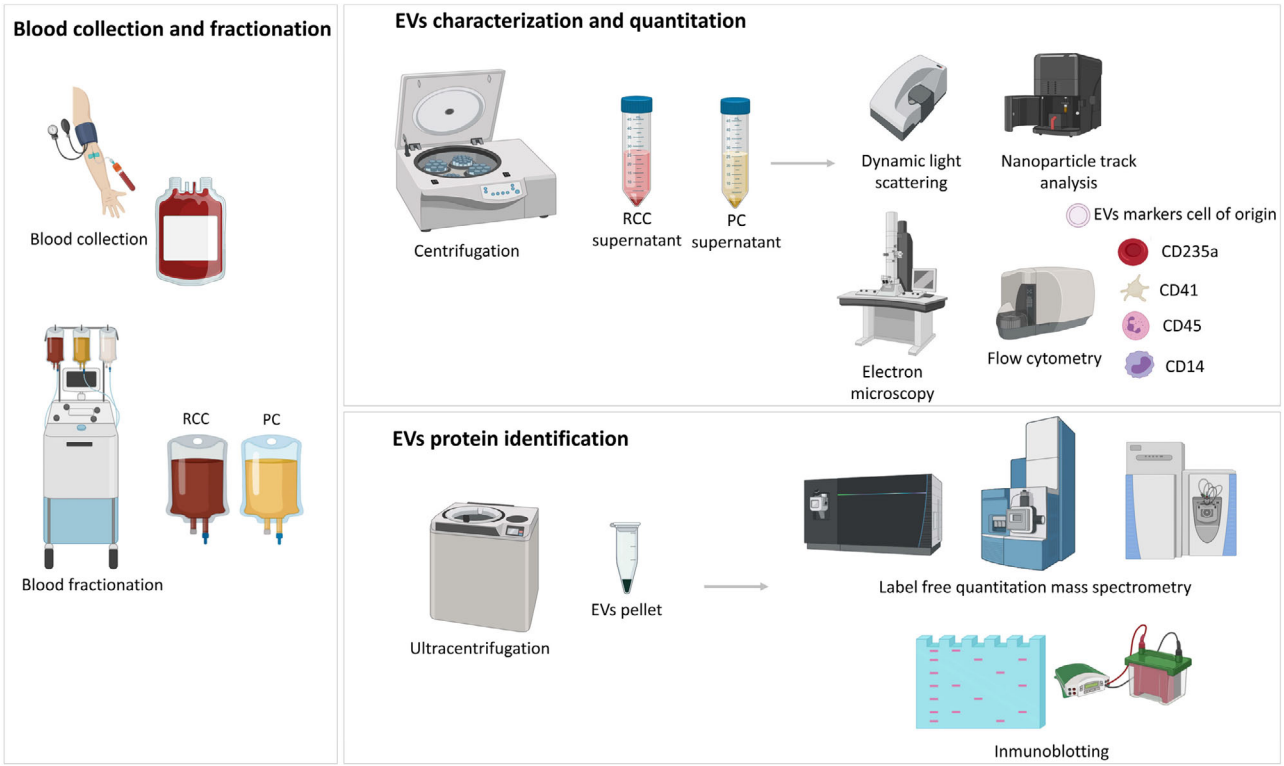

FIGURE 1 Schematic workflow for characterisation, quantitation, and protein identification of EVs in transfusion medicine

In this context, and thinking in an efficient proteomic analysis, it seems more plausible to initially analyse whole populations of EVs and focus on subpopulations in the validation phase, when lower amounts of protein are needed. The latter is of course assuming a EVs fractionation methodology is in place for efficient separation of subpopulations. In line with the above, the ISEV is providing guidelines and recommendations to improve the analysis of EVs with the aim of obtaining them with a high yield and purity [2]. This is key to obtain enough material to allow accurate proteomic analyses that can lead to the identification of specific EVs-related biomarkers in blood components that can be indicative of potential adverse transfusion events. In resume, we are still in early days for this young field and, as indicated above, several challenges lie ahead; however, the roadmap is ready and future studies should provide comprehensive data about EVs composition generated in blood banks storage conditions and their possible implications in transfusion adverse reactions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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