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RESEARCH ARTICLE

Small extracellular vesicles are released ex vivo from platelets into serum and from residual blood cells into stored plasma

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Abstract

Small extracellular vesicles (sEV) purified from blood have great potential clinically as biomarkers for systemic disease; however interpretation is complicated by release of sEV ex vivo after blood taking. To quantify the problem and devise ways to minimise it, we characterised sEV in paired serum, plasma and platelet poor plasma (PPP) samples from healthy donors. Immunoblotting showed twofold greater abundance of CD9 in sEV fractions from fresh serum than from fresh plasma or PPP. MACSPlex confirmed this, and showed that proteins expressed on platelet sEV, either exclusively (CD41b, CD42a and CD62P) or more widely (HLA-ABC, CD24, CD29 and CD31) were also twofold more abundant; by contrast non-platelet proteins (including CD81) were no different. Storage of plasma (but not serum) increased abundance of platelet and selected leukocyte sEV proteins to at least that of serum, and this could be recapitulated by activating cells in fresh plasma by Ca^{2+}, an effect abrogated in PPP. This suggests that a substantial proportion of sEV in serum and stored plasma were generated ex vivo, which is not the case for fresh plasma or PPP. Thus we provide strategies to minimise ex vivo sEV generation and criteria for identifying those that were present in vivo.

KEYWORDS
biobanking, exosomes, extracellular vesicles, ex vivo generation, MACSPlex, platelets, storage

1 | INTRODUCTION

Extracellular vesicles (EV) are a heterogeneous group of lipid-bound particles released by cells into the interstitium where they are important for cell-cell communication (Bebelman et al., 2018; Devhare & Ray et al., 2018; Doyle & Wang et al., 2019); typically they are classified on grounds of biogenesis and release pathway (Doyle & Wang et al., 2019; Yáñez-Mó et al., 2015). However different categories of EV overlap in size and density, and it is currently impossible to isolate pure populations of the smallest ones, namely microvesicles and exosomes (Doyle & Wang et al., 2019; Simons & Raposo et al., 2009; Théry et al., 2018; Willms et al., 2018). EV are of particular interest because the surface molecules they express indicate their cellular origin, and their cargo communicates information about cellular injury and activation state both locally, to nearby cells and more distantly, after transport via the circulation. Released EV achieve this by fusing with cells thus incorporating their surface proteins into the plasma membrane and discharging their lipid, protein and RNA cargo into the cytoplasm; alternatively the cells internalise intact EV, which then release their cargo (Gurung et al., 2021). It follows that EV not only transfer information about the donor cell but also signals

that alter the function of the recipient cell. The presence of EV in the circulation and the possibility of identifying their cellular origins emphasises their potential as diagnostic and disease activity biomarkers, and also as vehicles to deliver therapeutic drugs (Doyle & Wang et al., 2019; Vlassov et al., 2012; Zhou et al., 2006)

Robust biomarkers are typically directly involved in pathogenesis as would be the case for circulating EV that reflect pathological changes in situ. This requires confidence that EV purified from blood originated in vivo and were not generated ex vivo after blood withdrawal. Numerous techniques and sample types have been used to purify EV from human blood which introduces risks for down-stream analysis and demands rigorous standardisation of the pre-analytical handling to avoid errors when quantifying their protein, lipid or RNA contents (Brennan et al., 2020; Hong et al., 2016; Ibsen et al., 2017; Jamaly et al., 2018; Lacroix et al., 2012; Li et al., 2017; Ludwig et al., 2019; Muller et al., 2014; Serrano-Pertierra et al., 2019; Théry et al., 2006; Welton et al., 2015; Witwer et al., 2013). The problem of co-purifying plasma proteins and lipids is well documented (Brennan et al., 2020; Małys et al., 2021; Welton et al., 2015) but much less is known about the origin of the EV themselves. However recent reports have identified populations of EV in both human serum and plasma that were released from platelets ex vivo after blood withdrawal rather than directly into the circulation in vivo (Bæk et al., 2016; Karimi et al., 2022; Zhang et al., 2022). Currently neither of these distinct populations has been extensively characterised; it is not clear how they can be distinguished; whether they are influenced by blood sample type and storage, or by other aspects of the pre-analytical protocol. Such information is required for the design of robust studies searching for biomarkers that reflect disease activity, which often require the use of archived samples from well-characterised clinical cohorts.

Here we compared EV isolated from matched samples of human serum, plasma and platelet poor plasma (PPP) samples, the three common sample types archived in human biobanks. The EV were purified either by differential ultracentrifugation (DUC) or with Exo-spin columns both of which produce mixed EV population, relatively depleted in microvesicles and enriched in exosomes, which hereafter we designate as small EV (sEV). Extensive characterisation of these sEV using MACSPlex arrays together with experiments with Ca^{2+} pulsing to release EV acutely from residual cells, confirmed the abundance of ex vivo generated platelet-derived sEV in fresh human serum and stored plasma. They also facilitated the identification of circulating sEV of differing origins, and thus defined a novel population of leukocyte-derived ex vivo generated sEV in stored plasma that was not present in serum. The use of PPP minimised the complicating effect of ex vivo sEV generation. We then performed a proof-of-concept study in two chronic autoimmune inflammatory diseases, which supports the potential of EV as a disease biomarker and confirms PPP is the best sample type for such studies but does not exclude the use of stored plasma.

2 | MATERIALS AND METHODS

2.1 | Blood samples

Whole blood from healthy volunteers and patients with either ANCA-associated vasculitis or Rheumatoid Arthritis (sample size was experiment dependent—between 4 and 7) was drawn into VACUETTE TUBE Z No Additive (Greiner Bio-One, Austria) and VACUETTE TUBE K3EDTA (Greiner Bio-One, Austria) and centrifuged within maximally 15 min to obtain plasma (for EDTA) or left to clot for 20−30 min at room temperature before centrifugation for 15 min, 2500 × g at room temperature. To obtain PPP, according to the International Society on Thrombosis and Haemostasis (ISTH), we collected plasma and centrifuged it one more time at the same settings (Coumans et al., 2017). Plasma, PPP and serum were used immediately for purification of sEV and the paired aliquots of processed blood samples were stored in 4°C and −80°C for 14 days for the next sEV isolation. For the experiment with Ca^{2+} we incubated plasma, PPP and serum overnight at room temperature after addition of 100 μL $CaCl_2$ (1 M) into a tube including 4 mL of a prepared blood sample. On the next day samples were centrifuged for 10 min 2500 × g and the supernatant was used for sEV isolation.

2.2 | Isolation of sEV by differential ultracentrifugation

Plasma, PPP and serum samples (each 2 mL) were diluted in 30 mL of cold PBS before ultracentrifugation (Allegra X-15R, Beckman Coulter, Brea, CA, USA; Ultracentrifuge CP100NX, Hitachi, Tokyo, Japan). We removed large vesicles by ultracentrifugation at 30,000 × g for 2 h at 4°C. The supernatant was collected as a source of sEV and pelleted at 100,000 × g for 2 h, 4°C. Further, the obtained pellet was washed in 32 mL of cold PBS (100,000 g; 2 h; 4°C). Afterwards, the sEV pellet was collected into an Eppendorf tube, in which the volume was adjusted to a total of 200 μL by addition of cold PBS and down-stream analysis was performed immediately or within the next hours of 4°C storage. This method was previously validated by us showing purity and quality of the sEV preparations according to MISEV standards (Małys et al., 2021; Théry et al., 2018).

2.3 | Isolation of sEV by Exo-spin mini-HD column EX05

We used commercial columns—Exo-spin mini-HD column EX05 (Cell Guidance Systems, Cambridge, UK) to isolate sEV from 100 μL of paired plasma, PPP and serum samples. Briefly, 100 μL of serum/plasma/PPP were centrifuged for 30 min, 14,000 × g and layered onto the columns after equilibrating them with 3 × 2.5 mL PBS. The first fraction (900 μL) was discarded and the second sEV fraction (400 μL) was collected into an Eppendorf tube. The down-stream analysis was performed immediately or within the next hours of 4°C storage. This method was also validated by us according to MISEV standards (Małys et al., 2021; Théry et al., 2018).

2.4 | Nanoparticle tracking analysis (NTA)

We used a Zetaview Basic NTA PMX 120 (Particle Metrix, Inning am Ammersee, Germany) system to measure the size and the number of particles in sEV preparations. We kept the same standard settings throughout all experiments: sensitivity = 68; shutter = 65; temperature = 24°C. The sEV preparations were diluted in PBS before an analysis in order to fit the concentration range: 10^5–10^9 particles/mL in following dilution factors (DF): for DUC, DF = 25–150; for Exo-spin, DF = 150–1500. Each sample we run in three cycles and scanned 11 cell positions with capturing 30 frames per position. The data were analysed with FlowJo_v10.6.1 (FlowJo LLC, Ashland, OR) and R version 3.6.3 (R Core Team) for Windows.

2.5 | Primary and secondary antibodies

The primary antibody used in transmission electron microscopy and Western blotting was mouse anti-CD9 (AHS0902, Invitrogen, Waltham, Massachusetts, USA) and mouse anti-CD63 (10628D; Invitrogen, Waltham, MA, USA).

The secondary antibody used in transmission electron microscopy (TEM) was gold-labelled (EM:GAM15, goat anti-mouse 15 nm immunogold-conjugate; BBI Solution, Crumlin, UK). For Western blotting, we used fluorescently labelled secondary antibodies: goat anti mouse IRDye 800 or goat anti mouse IRDye 680 (LI-COR Biosciences, Lincoln, NE, USA).

2.6 | Transmission electron microscopy and immuno-electron microscopy

We performed transmission electron microscopy (TEM) according to the protocol described by Thery et al. (2006). Briefly, we fixed the sEV preparations in 2% PFA and placed 5 μL drop on formvar-coated 200 mesh Ni-grids (S162N; Agar Scientific Ltd, Standsted, UK). CD9 was detected on sEV by immuno–electron microscopy after incubating the grids overnight in a humid chamber at 4°C with the mouse anti-CD9 diluted at 1:40 in 1% Eggalbumin. The antibody was detected with a gold labelled secondary antibody (goat anti-mouse) in dilution 1:50. Between antibody incubation preparations were washed with PBS followed by post-fixation with 1% glutaraldehyde and incubation in a solution of neutral uranyl oxalate. The grids were covered with a polyvinylalcohol film. After air drying, the grids were examined in a TEM JEOL 1400 PLUS (JEOL, Tokyo, Japan) at 60 kV. Pictures were taken in Radius software with Quemesa_Camera (Fa. Olympus, Tokyo, Japan).

2.7 | SDS-PAGE and western blotting

Small extracellular vesicle preparations of volume 30 μL were mixed with 10 μL 4× Protein Loading Buffer (LI-COR, Biosciences, Lincoln, NE, USA) and incubated at 95°C for 5 min. The samples were separated by SDS-PAGE (10% SDS) in non-reducing conditions. Dilutions of human serum or lysates of THP-1 cells were added as protein loading controls. We lysed the cells in RIPA buffer (150 mM NaCl; 5 mM EDTA, pH 8.0; 50 mM TRIS, pH 8.0; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS) and with sonication.

2.8 | MACSPlex human exosome pan kit

We used MACSPlex Exosome Kit, human (Miltenyi Biotec, Bergisch-Gladbach, Germany) for surface protein profiling on sEV according to the manufacturer's instructions. Briefly, we incubated sEV preparations (120 μL) overnight with MACSPlex Exosome Capture Beads (15 μL; containing 39 different antibody coated bead subsets) on an orbital shaker (450 rpm) at room temperature without access to light. Next, we washed samples by addition of MACSPlex buffer (500 μL) into each tube and we pelleted the

beads by centrifugation at RT for 5 min 3000 × g. The supernatant was discarded, and the pelleted beads were incubated with MACSPlex Exosome Detection Reagent (APC-conjugated anti-CD63, anti-CD9, anti-CD81) for 1 h at room temperature on a shaker (450 rpm) without access to light. Further, we washed the beads twice with MACSPlex buffer and fluorescence intensities for FITC, PE and APC were acquired by the BD FACSCanto II Cell Analyzer (BD Biosciences, Franklin Lakes, NJ, USA), resulting in approximately 5000−20,000 single bead events being recorded per sample. Analysis was performed in FlowJo_v10.8.1. and we separated the bead populations according to their fluorescence properties in the PE and FITC channel. Median APC fluorescence intensity was used to quantify sEV bound to the beads.

2.9 | Counting of residual platelets in blood preparations

Complete blood count of leukocytes, erythrocytes and leukocytes in whole blood and blood preparations was performed with the use of XN-2000 Sysmex.

2.10 | Statistical analysis

Statistical analysis and graphs were performed with R version 3.6.3 for Windows (R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/; and Microsoft Excel (Redmond, WA, USA); and in Graphpad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Differences between sEV preparations from various sample types suggest that many of sEV were released ex vivo during coagulation

The sEV were purified immediately by DUC from samples of freshly prepared serum, plasma and PPP from individual blood samples taken from seven healthy donors. Western blotting for the commonly used exosome marker CD9 ($n = 3$) uniformly showed a threefold greater abundance in sEV purified from serum than from paired samples of the same donor's plasma or PPP (Figure 1a), suggesting that serum contains appreciably more sEV. This was confirmed by MACSPlex arrays, which quantify the abundance of 37 documented EV surface proteins, including CD9 and two other established exosome markers CD63 and CD81. The mean median fluorescence intensity (MFI) for CD9 was 2.5-fold higher in sEV purified from serum than from plasma ($p = 0.078$), and 2.3-fold higher than from PPP ($p = 0.031$) (Figure 1c). The difference for CD63 was even greater: 7.6-fold higher in serum than plasma ($p = 0.031$) and 6.4 than PPP ($p = 0.016$). By contrast, the CD81 abundance was similar in all three sample types. The sEV purified by Exo-spin gave qualitatively similar results, albeit with lower abundance, reflecting the paucity of sEV obtained by this method (Figure 1b, d) (Małys et al., 2021). Indeed, CD9 and CD63 were only detectable in serum-derived sEV and CD81 was uniformly low (Figure 1d). The consistently greater abundance of CD9 and CD63 provides compelling evidence that serum contains more sEV than plasma and PPP. NTA has commonly been used to enumerate sEV, but surprisingly the particle counts and diameters were similar in all three sample types (Figure 1b and Figure S1a); we attribute this documented unreliability of NTA due to its inability to distinguish between sEV, protein aggregates and lipids (Gardiner et al., 2013; Mørk et al., 2017; Witwer et al., 2013).

Previous reports have documented ex vivo release of EV from platelets during coagulation (Bæk et al., 2016; Zhang et al., 2022), which would explain the greater abundance of CD9 and CD63 in fresh serum compared to fresh plasma or PPP and why the CD81 abundance was similar because platelet sEV lack CD81 (Koliha et al., 2016). To confirm this, we compared the concentrations of other proteins expressed on platelet-derived sEV (Aatonen et al., 2014; Heijnen et al., 1999; Rui et al., 2021). MACSPlex arrays contain three proteins expressed uniquely on platelet-derived sEV: namely, CD41b, CD42a and CD62P. All three proteins gave strong MFI signals in sEV preparations from fresh serum, plasma and PPP, but with appreciably greater MFI in those from serum (Figure 2a–c): the increased fold differences for serum compared to plasma and PPP were respectively: CD41b – 2.1 and 2.5 fold; CD42a – 1.9 and 2.3 fold, which are similar to those of CD9; and for CD62P, which is uniquely expressed on activated platelets (Lu & Malinauskas et al., 2011), was even greater at 5.4 and 4.9 fold, respectively (Figure 2c) and similar that of CD63, which is also particularly expressed by EV from activated platelets (Heijnen et al., 2003). The little difference between results using fresh plasma and PPP shows that sEV isolation protocol does not affect platelet activation because with such large numbers of residual platelets that are found in plasma, we would expect substantial fold difference between those two sample types; probably due to the first centrifugation step (30,000 × g) that removes both large EV and residual platelets. The other four proteins whose abundance was greatly increased in the MACSPlex arrays can be expressed on platelet EV as well on those from other sources. These were: the adhesion molecule and CD62P-ligand CD24, which is also expressed on neutrophils and B cells

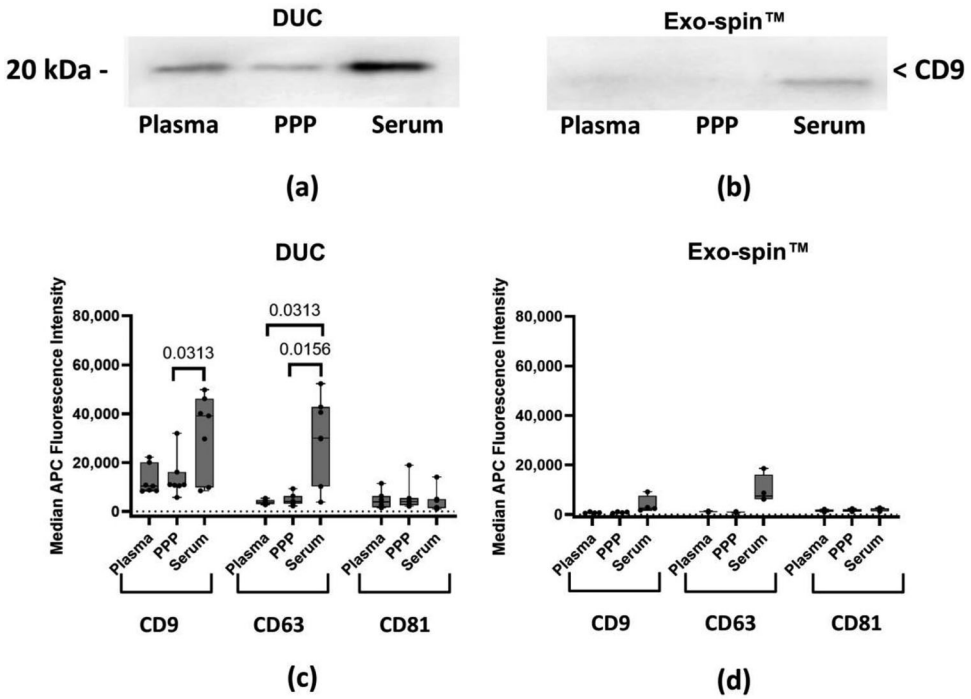

FIGURE 1 The figure shows the differences between small extracellular vesicles (sEV) that were isolated from following sample types (plasma, PPP—platelet poor plasma, serum). The representative Western blots for CD9 bands are shown on the picture (a) – for sEV purified by differential ultracentrifugation (DUC) and (b) – for sEV purified by Exo-spin, and they show observed differences for the expression of CD9 marker in sEV preparations purified from different sample types. The semi-quantitative analysis of exosomal markers (CD9, CD63 and CD81) measured by MACSPlex human exosome kit is shown on the graph (c) – for sEV purified by DUC and (d) – for sEV purified by Exo-spin. The values are shown as the Median Allophycocyanin (APC) Fluorescence Intensity (MFI) averaged from 7 (DUC) and 4 (Exo-spin) blood samples for each paired group. Analysis for differences between sample types was performed in GraphPad Prism 9 using Wilcoxon matched-pairs signed rank test. The numeric values on the graphs show p values that are below $p < 0.05$. The graphs (e) and (f) present the number of particles (a) and the mean diameter size (b) of sEV purified by DUC and measured by nanoparticle tracking analysis.

(increased in four of seven donors); the beta-1 integrin CD29, which is commonly expressed on leukocytes and activated platelets (Varga-Szabo et al., 2008) (increased in five of seven donors); and HLA-ABC, which is almost universally expressed including platelet microvesicles (Pannetier et al., 2021) (increased in five of seven donors). The data show that despite donor-to-donor variation there was a close correlation between all the platelet proteins in a given donor (Figure 2e). Thus all nine platelet sEV proteins in the MACSPlex arrays have at least a two-fold greater MFI signal in sEV from serum than from fresh plasma or PPP samples. By contrast only CD25 of 28 proteins not expressed on platelet EV had a twofold difference (Figure 3). CD44 that binds to fibrin(ogen) (Alves et al., 2009; Ponta et al., 2003) was the only protein that was less abundant in serum suggesting the sEV expressing it might be adsorbed onto the clotting mesh. Altogether results provide powerful evidence that fresh serum contains more sEV than fresh plasma and PPP, and that additional sEV are released ex vivo from platelets, especially when activated; there was little difference in results from fresh samples of plasma and PPP.

The MACSPlex arrays also contain proteins not expressed on platelets that were detected in sEV from almost all the healthy donors. The MFI for these proteins—CD81, CD45, HLA-DRDPDQ—were similar in preparations from fresh serum and plasma or PPP, thus providing no evidence for ex vivo generation and implying they were already present in the circulation (Figure 3). Notably the strong MFI signal for HLA-DRDPDQ which is expressed by antigen presenting cells, including monocytes, macrophages and dendritic cells was similar in all three sample types. The definite weaker MFI for the endothelial markers CD105 and CD146 were similar in sEV from fresh plasma, PPP, and serum; again this suggests their in vivo origin and suggests that the ex vivo generated sEV that expressed CD31 originated from platelets not endothelium. The data are less certain for the pan-lymphocyte marker CD3 and for CD56, which had a stronger expression in serum. MFI signals for seven proteins (CD19; CD20; CD1c; CD11c; CD209; MCSP; CD133/1) were uniformly negative, which implied that sEV that express them are absent or rare in blood, at least of healthy donors. The absence of detectable MFI for the B cell proteins CD19 and CD20 suggests that B cells were not the source of CD24 signals but that they originated from platelets or neutrophils (Fang et al., 2010; Koliha et al., 2016). Exo-spin purified sEV gave qualitatively similar results but with far lower MFI that were only detectable for platelet proteins demonstrating the ex vivo origin of Exo-spin-purified sEV. The data are summarised in (Figure 3). This contrasts with results recently reported by Karimi et al. (2022) who identified more sEV in human plasma than in serum and showed that they originated from platelets. When we applied similar protocols (iodixanol gradient isolation and magnetic bead separation), but

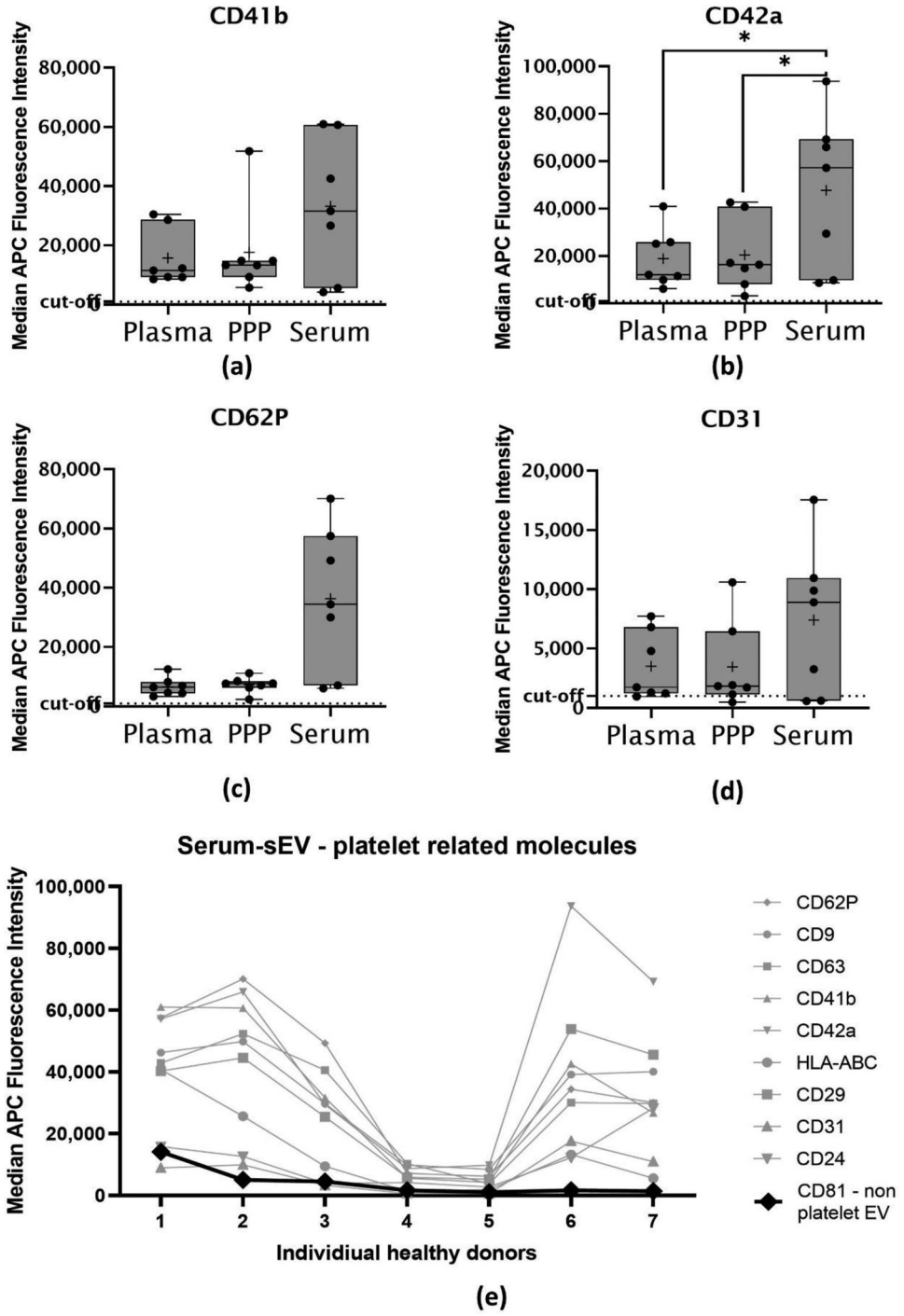

FIGURE 2 The figure shows the differences in the expression of platelet associated markers in small extracellular vesicles (sEV) preparations purified from differently processed blood samples: plasma, platelet poor plasma (PPP), serum by differential ultracentrifugation (DUC). The analysis was performed by MACSPlex human exosome kit that semi-quantifies the abundance of sEV surface proteins by measuring the median fluorescence intensity (MFI) of allophycocyanin (APC) on beads pre-coupled with antibodies. The graphs show the abundance of CD41b (a); CD42a (b); CD62P (c); CD31 (d) in sEV preparations and the statistical analysis was performed with Wilcoxon matched-pairs signed rank test ($n = 7$ for each group). The asterisk * corresponds to $p \leq 0.05$. The graph (e) shows the correlation between the platelet associated marker intensity in each different donor. Note that CD81 is not found on platelet-sEV and is the only marker that is not correlated.

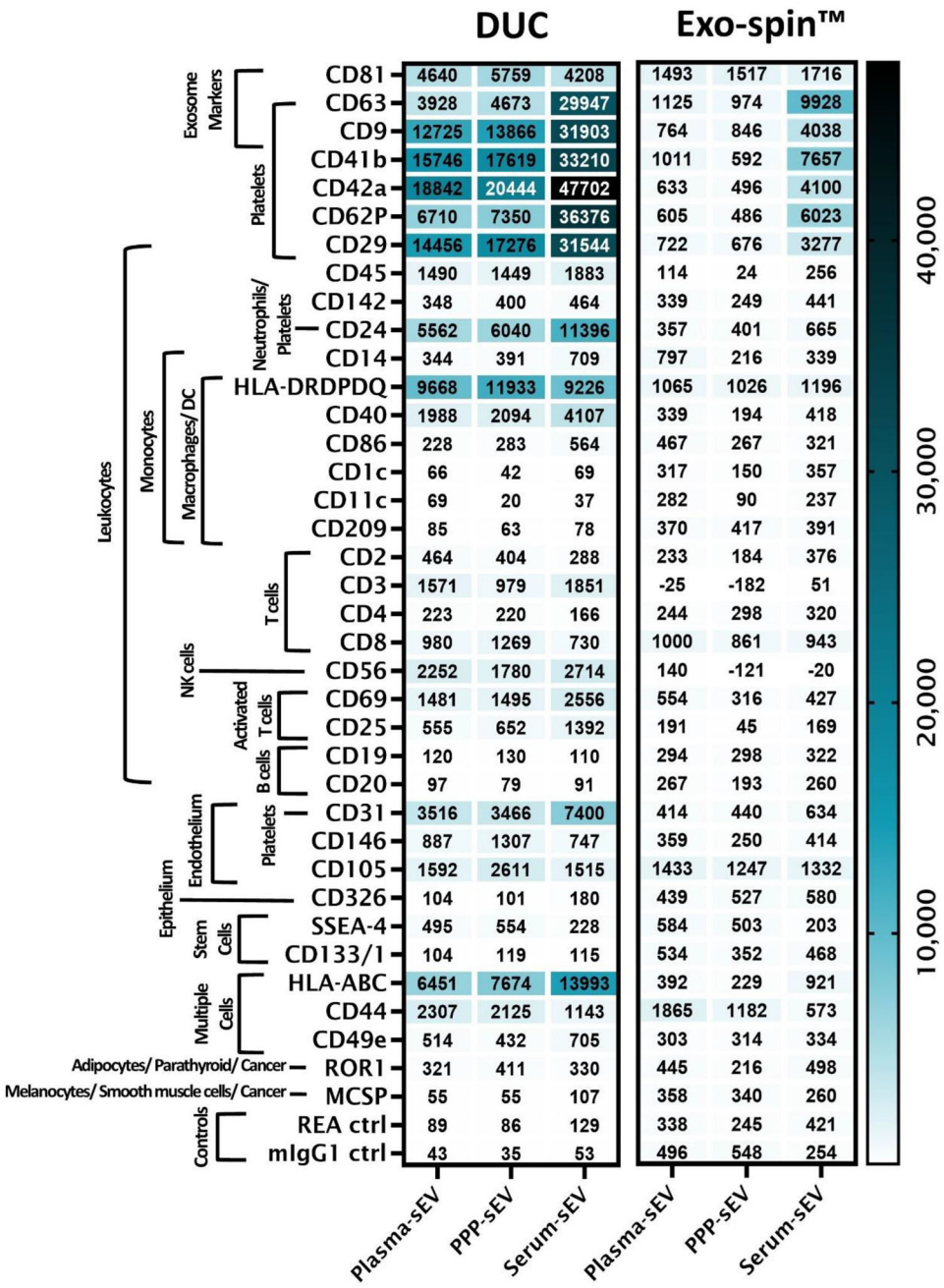

FIGURE 3 MACSPlex results of 37 potentially expressed markers on the surface of small extracellular vesicles (sEV) purified from fresh plasma, platelet poor plasma (PPP) and serum. The intensity of the square in the heatmap corresponds to the value of Median Allophycocyanin (APC) Fluorescence Intensity (MFI) measured on the beads pre-coupled with antibodies that bind to sEV from healthy donors and were purified by both methods: differential ultracentrifugation (DUC; $n = 7$) and Exo-spin ($n = 4$).

modified according to our technical possibilities, we observed that regardless whether we analyse EV (pre-clearance with 10,000 × g for 45 min) or sEV (pre-clearance with 30,000 × g for 2 h), CD9 and CD63 are consistently higher in sEV purified from serum (Figures S2 and S3).

3.2 | Residual platelets and leukocytes generate sEV ex vivo in stored plasma

We next quantified the effects of storage on ex vivo release of sEV into serum, plasma and PPP. Western blotting ($n = 3$) showed that CD9 was more abundant in sEV purified from stored than fresh plasma and PPP; indeed the CD9 concentration was similar to that in serum in samples stored at 4°C (Figure 4a and Table S1), but not after −80°C storage. These results were confirmed

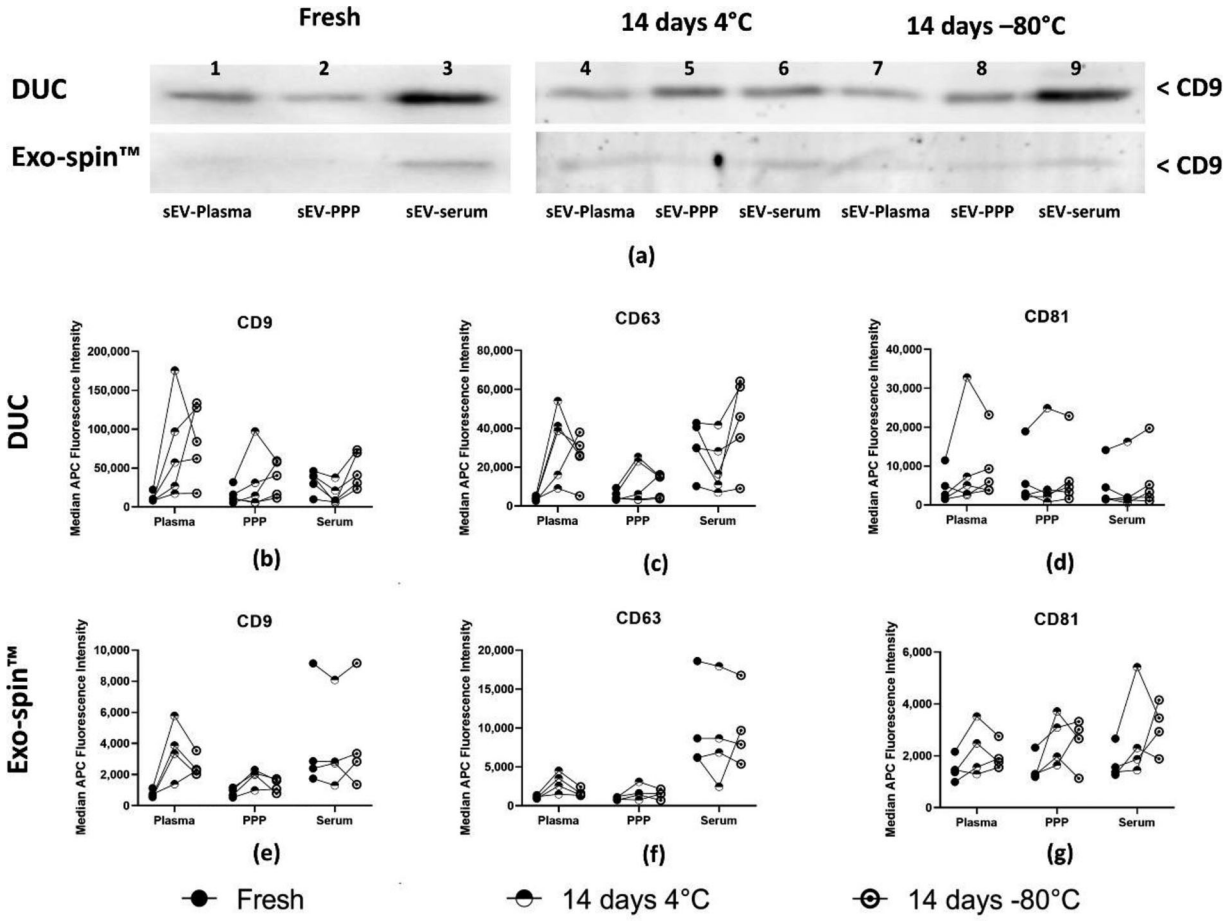

FIGURE 4 Storage of samples prior to isolation of small extracellular vesicles (sEV) influences the expression of exosomal markers in sEV preparations. Representative Western blots for CD9 bands of one donor are shown on the picture (a) – for sEV purified by differential ultracentrifugation (DUC) and Exo-spin from different sample types stored in 4°C and −80°C for 14 days. The numbered bands are as follows: 1 – sEV purified from fresh plasma; 2 – sEV purified from fresh platelet poor plasma (PPP); 3 – sEV purified from fresh serum; 4 – sEV purified from plasma stored for 14 days in 4°C; 5 – sEV purified from PPP stored for 14 days in 4°C; 6 – sEV purified from serum stored for 14 days in 4°C; 7 – sEV purified from plasma stored for 14 days in −80°C; 8 – sEV purified from PPP stored for 14 days in −80°C; 9 – sEV purified from serum stored for 14 days in −80°C. Notice that storage of plasma and PPP increased the intensity of CD9 expression in sEV preparations. The graphs (b–g) show the Median Allophycocyanin (APC) Fluorescence Intensity (MFI) of exosomal markers (CD9; CD63; CD81) evaluated by flow cytometry with the MACSPlex exosome human kit ($n = 5$ for DUC; and $n = 4$ for Exo-spin for each paired group). The analysis confirmed that expression of exosomal markers increases due to storage of blood samples. Notice that positive MFI signals were detected in the sEV preparations purified by Exo-spin from stored plasma, stored PPP and serum, suggesting that purified sEV are released ex vivo from residual cells.

with MACSPlex arrays ($n = 5$): the CD9 MFI were respectively 5.9 and 8.5-fold higher in sEV from plasma stored at 4°C and −80°C than in fresh plasma (Figure 4b); and there were similar changes in CD63 abundance with 7.7 and 6.9 fold increases in 4°C and −80°C samples (Figure 4c); and also a small but consistent increase in CD81 abundance of 2.2 fold after storage at 4°C storage, and 2.5 at −80°C (Figure 4d). The effect was greatly attenuated with PPP with MFI-fold increases of: CD9 – 1.7 and 3.4 at 4°C and −80°C respectively; CD63 – 2.0 and 2.2 at 4°C and −80°C respectively; and CD81 – 0.9 and 1.3 at 4°C and −80°C respectively. By contrast the MACSPlex arrays showed little change in serum stored at −80°C, and the MFI decreased to below that of plasma after storage at 4°C. Collectively the results demonstrate the release of platelet-derived sEV into plasma and PPP during freezing or storage, but not into serum. NTA showed that sEV preparations from plasma, PPP and serum ($n = 6$ for each group) consistently had increased particle counts when stored for 14 days at either 4°C or −80°C before sEV isolation, again not correlating with measured protein signals in sEV preparations (Figure S4a). Whereas the mean particle diameter was unchanged (Figure S4b). Nevertheless, since we did not find the correlation between sEV proteins signal and particle number, we translate this increase in number due to the analysis of artefacts, such as protein aggregates. Importantly, CD9 immunogold labelled electron microscopy images of the isolated sEV uniformly showed intact cup-shape vesicles with similar morphology and density of immunogold particles regardless of sample types and whether stored at 4°C or −80°C (Figure S4c–e). Again, the MACSPlex results with Exo-spin purified sEV were qualitatively similar, except samples from fresh plasma and PPP were typically below the limits of detection (Figure 4e–g).

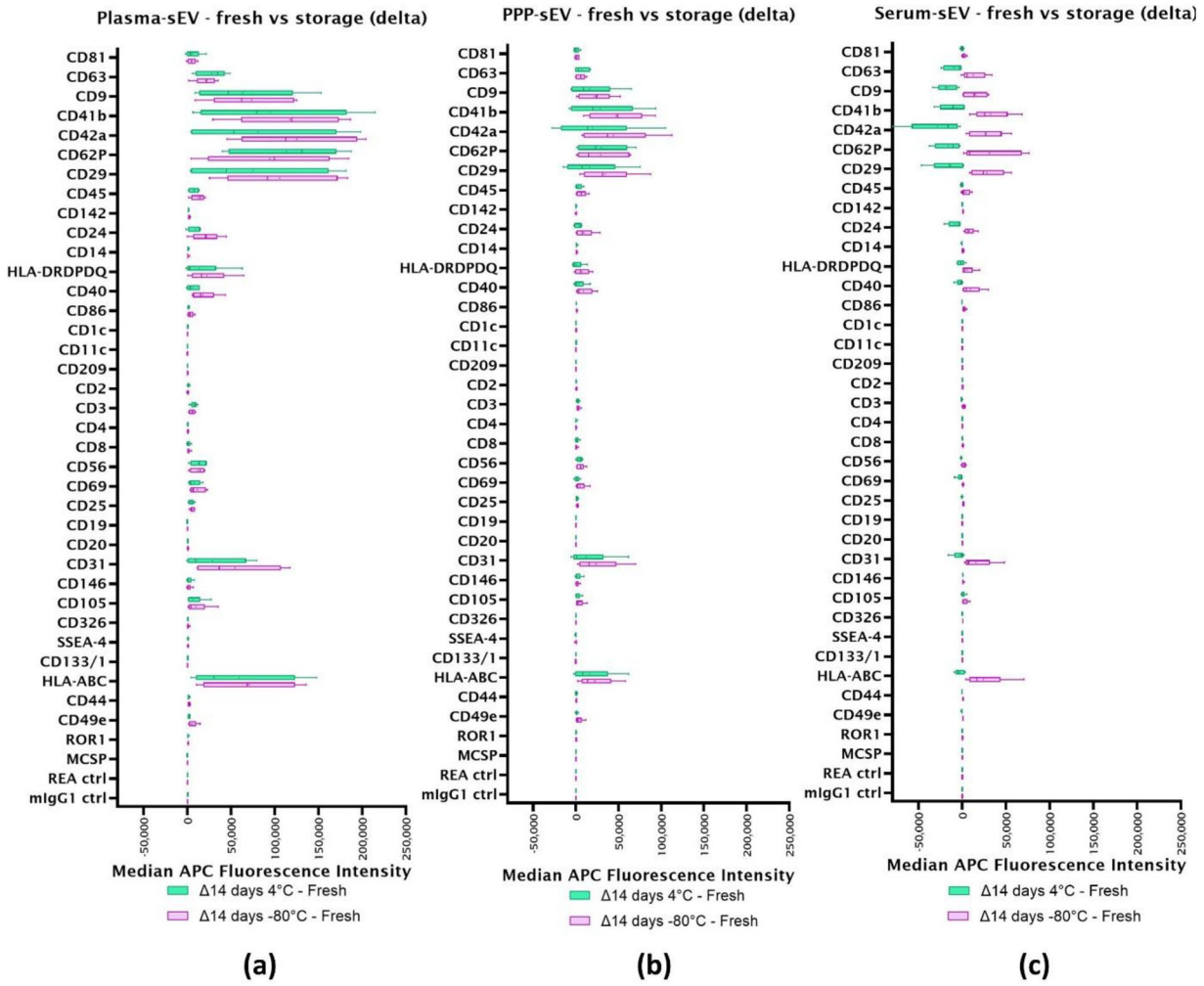

FIGURE 5 MACSPlex results shown as delta values between Median Allophycocyanin (APC) Fluorescence Intensity (MFI) measured on small extracellular vesicles (sEV) purified by differential ultracentrifugation from fresh plasma, platelet poor plasma (PPP) and serum of paired samples stored for 14 days in either 4°C or −80°C. MFI signals are measured on the bead populations pre-coated with antibodies that captured sEV from preparations ($n = 5$ for each group). The data are visualised as box and whisker plots: the box—extends from the 25th to 75th percentiles with a line inside corresponding to the median and a dot showing the mean; the whiskers show minimum and the maximum value. Notice that the major differences in expression after storage are associated with platelet and leukocyte markers.

The complete MACSPlex data (summarised in Figure 5) show that, in addition to the uniformly increased signal for platelet proteins, stored plasma had increased MFI of subset of leukocyte-expressed EV molecules. These included: pan-leukocyte marker CD45; myeloid antigen presenting cell proteins HLA-DRDPDQ and CD40; the natural killer (NK) cell protein CD56, and the activated T cell markers CD69 and CD25. PPP-derived sEV show an identical pattern but with much reduced delta values, and in both cases storage temperature made little difference. This was in marked contrast to the results from stored serum in which the mean delta values for sEV purified from −80°C stored samples generally had positive delta values whereas those from the 4°C samples were consistently negative, indicating loss of sEV during storage. The results for individual donors confirmed the effect on the MFI for platelet-expressed molecules was consistent, albeit with donor-to-donor variation (Figure 6); and the same was true for the leukocyte molecules but with greater individual variation (Figure 7). Consequently, the magnitude of the mean MFI was sometimes dominated by the results from a single or few individuals, for example: HLA-DRDPDQ and CD105 and to a lesser extent for CD40 (Figure 7f–h), which are all expressed on activated monocytes, macrophages and dendritic cells (Rohde et al., 2006; Schmidt-Weber et al., 2005; Zhang et al., 1996). Importantly donors with the highest MFI for one leukocyte molecule also had the highest values for the others. This strongly supports quantitative differences in the number of residual leukocytes (or their subtypes) between donor samples although it does not exclude dominating differences in the number of circulating leukocyte-derived sEV. Notably the effect of storage was abrogated (HLA-DRDPDQ and CD105) or markedly attenuated in PPP-derived sEV, again providing further evidence that sEV originated from residual leukocytes. The MFI for sEV purified from fresh serum, which were higher than those from fresh plasma, were less elevated than the paired plasma sample after storage for 14 days at

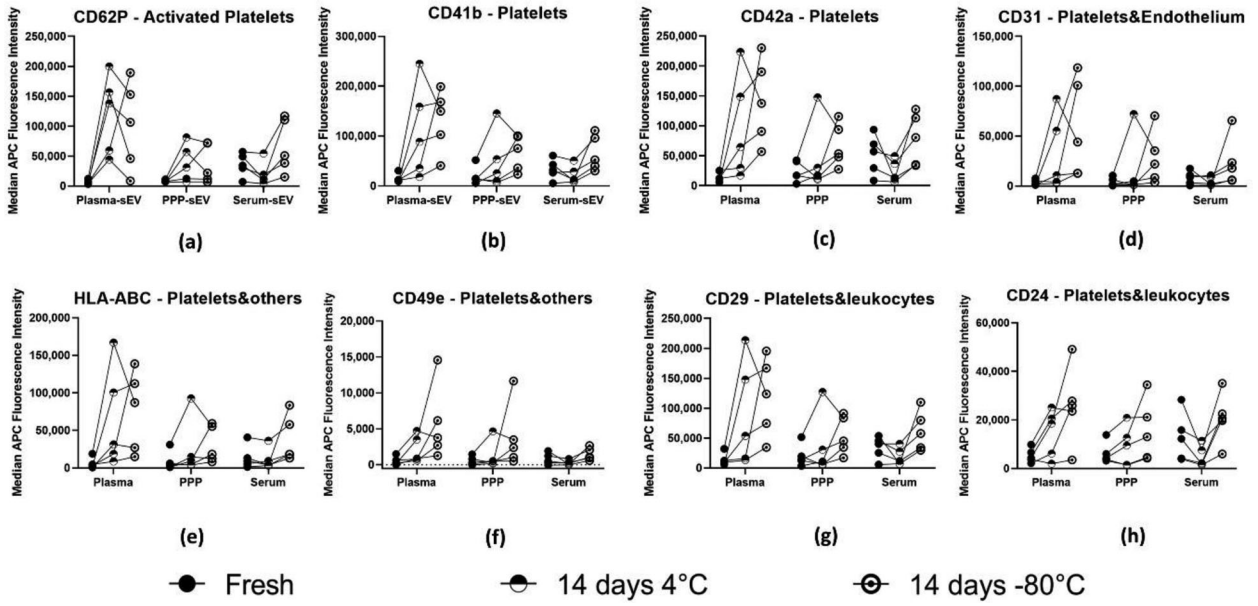

FIGURE 6 Storage of blood preparations prior to isolation of small extracellular vesicles (sEV) has an influence on the expression of surface proteins in sEV preparations that are linked to platelets. The figure shows the expression of proteins: CD62P, CD42a, CD41b, CD31, HLA-ABC, CD49e, CD29, CD24 in sEV preparations that were isolated by differential ultracentrifugation (DUC) from the following sample types: plasma, platelet poor plasma (PPP) and serum ($n = 5$ for each group). Notice that sample storage strongly influences profiles of sEV purified from plasma and PPP, but not serum.

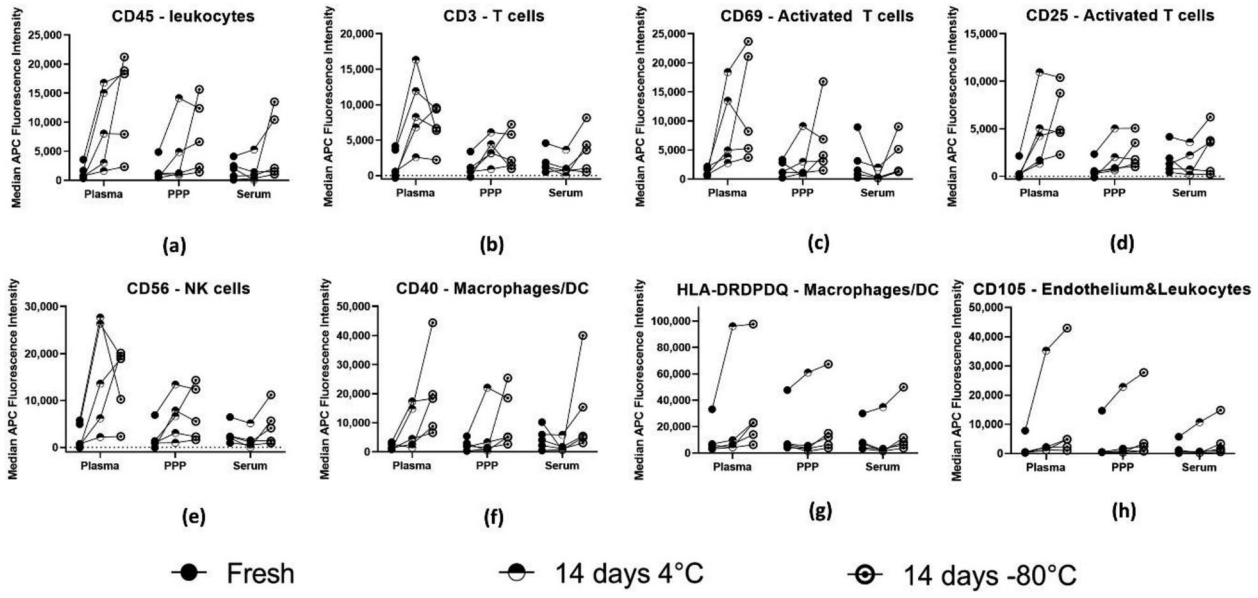

FIGURE 7 Storage of samples prior to isolation of small extracellular vesicles (sEV) has an influence on the expression of surface proteins in sEV preparations that are linked to leukocytes. The figure shows the expression of surface proteins: CD45, CD3, CD69, CD25, CD56, CD40, HLA-DRDPDQ, CD105 in sEV preparations that were isolated freshly or after 14 days of storage in 4°C and −80°C by differential ultracentrifugation from various sample types: plasma, platelet poor plasma (PPP) and serum ($n = 5$ for each group).

4°C. Again, sEV purified by Exo-spin gave similar results, albeit with the expected much lower signals, which were often only detectable in serum and stored plasma samples (Figure S5).

These results document the importance of ex vivo generation of sEV not only from platelets, but also from leukocytes in blood samples prior to isolation, and emphasise the complications this poses for interpreting expression levels of molecules expressed on them. This is exemplified by the canonical "endothelial markers," CD31, CD105 and CD146: the MFI for CD31, which is also expressed on platelets was markedly greater in serum and stored plasma compared to fresh plasma and stored PPP; whereas the MFI for CD105, which is expressed on endothelium, activated macrophages and activated CD4+ T cells but not

platelets (O'Connell et al., 1992; Piao & Tokunaga et al., 2006; Schmidt-Weber et al., 2005), was higher in stored compared to fresh plasma. There was also a smaller increase of MFI for CD146, which is not only expressed by endothelium but also by subsets of activated T and B cells (Elshal et al., 2005). These data caution against the assumption that sEV expressing CD31 and possibly CD105 or CD146 are necessarily generated in vivo by endothelial cells. The pattern of MFI signals generated by sEV prepared from fresh PPP is most likely to reflect the situation in vivo, but even here we cannot be certain whether the weak but definite MFI signals originate from sEV that were present in the circulation, or alternatively were released ex vivo during the earliest phase of centrifugation whilst preparing PPP.

3.3 | Pulsing with Ca2+ induces release of sEV from residual cells in plasma

Pulsing with Ca^{2+} activates platelets and leukocytes and promotes the rapid release of EV, thus it could be used as a surrogate measure for the number of residual platelets and leukocytes in the blood samples, or alternatively show that the changing MFI were caused by storage induced artifacts, such as aggregation or clumping. First we counted the number of residuals platelets in blood preparations by haemocytometer and showed that plasma contains 19.2% ± 8.7% of initial platelets found in whole blood, while PPP contains around 1% ± 0.4%, and serum none (Figure S6a). Interestingly, the platelet counts were unchanged after storage of plasma samples in −80°C, but their size did increase, indicating their activation and therefore potential to release more sEV because of storage (Figure S6b–e) (Heijnen & Korporaal et al., 2017).

Next we pulsed fresh plasma, PPP and serum with Ca^{2+} and used MACSPlex arrays to measure ex vivo sEV generation. The sEV purified from Ca^{2+}-pulsed plasma uniformly showed significantly increased abundance from the full range of molecules expressed on platelet sEV (Figure 8a–f), together with some of those expressed on T cells (CD3 and CD25) and myeloid cells (CD40 and HLA-DRDPDQ) (Figure 8g–i); Figure 8j shows the delta values for a wider range of platelet and leukocyte proteins. By contrast pulsing PPP with Ca^{2+} to PPP had no or little effect on the MFI of any of the proteins in the array, proving that the increased expression is due to sEV released from residual blood cells rather than simply a technical artefact. Similarly, pulsing serum with Ca^{2+} had no effect on the MFI of either platelet or leukocyte molecules. The abundance of detected proteins after pulsing was similar to the ones for stored samples and evident by the change of CD29, CD142, CD24, HLA-DRDPDQ, CD40, CD56, CD31, HLA-ABC and shown as delta values between Ca^{2+}-treated blood samples and control samples (Figure 8a–i). This is supported by immuno-electron microscopy, which showed there was no difference in the mean size of CD9 stained vesicles in the different sample types either with or without Ca^{2+} treatment (Figure 9a–f). Thus standard haemocytometry confirms the persistence of platelets even in PPP and their ability to release sEV is demonstrated by the Ca^{2+} pulsing experiments. The number of residual leukocytes in plasma and PPP were both below the level that could be detected by hemocytometry but, despite this Ca^{2+} pulsing showed that enough were present to release detectable number of sEV into both plasma and PPP. This confirms that standard plasma samples contain sufficient numbers of residual platelets and leukocytes to release a new population of sEV ex vivo and provides a method for quantifying them. The data also demonstrate the importance of using immediately prepared PPP when attempting to purify circulating EV from human blood. This has important implications for the use of EV for disease biomarker studies, especially for systemic inflammatory diseases and infections in which leukocytes and platelets are central to pathogenesis.

3.4 | Effect of sample storage on indication of biomarkers in rheumatoid arthritis and ANCA-associated vasculitis

Circulating EV are most faithfully represented by populations purified from fresh PPP, which would be the ideal resource for biomarker discovery, if the consequences of ex vivo EV generation are to be avoided. This would preclude the use of archived samples from well-characterised clinical cohorts, which are the foundation of such studies, especially of chronic autoimmune inflammatory disease. Consequently, we performed a small proof-of-concept study from newly recruited nine individuals with rheumatoid arthritis (four active (RA-A); five in remission (RA-R)) and five with ANCA-associated vasculitis (AAV) (Table 1 and Tables S2-S4). It was designed simply to determine whether there were clear differences between MACSPlex MFI profiles of sEV from fresh PPP of healthy subjects and those of patients; and if so whether the differences were maintained in profiles of sEV from stored samples (Figure S7a–d). The PPP-derived sEV from the healthy control and patients´ groups had broadly similar levels of expression of the expected clusters of molecules but with a few obvious exceptions (Figure 10). Three molecules were more abundant on sEV from the patient groups than controls: the mesenchymal stem cell protein SSEA-4, associated tissue regeneration, was uniformly present in sEV from patients but absent from controls (Figure 11a); expression of CD44, which is also involved in tissue regeneration, was low in controls but much higher in patients (Figure 11b); and the exosome marker CD81, which is absent on platelets, was similarly higher in patients (Figure 11c) (Gang et al., 2007; Koliha et al., 2016; McDonald & Kubes et al., 2015; Zöller et al., 2011). Notably sEV expression of common leukocyte (CD45), lymphocyte (CD3, CD8) and the myeloid cell (HLA-DRDPDQ, CD40 and CD86) markers were similar in controls and patients. Nevertheless, sEV from two of

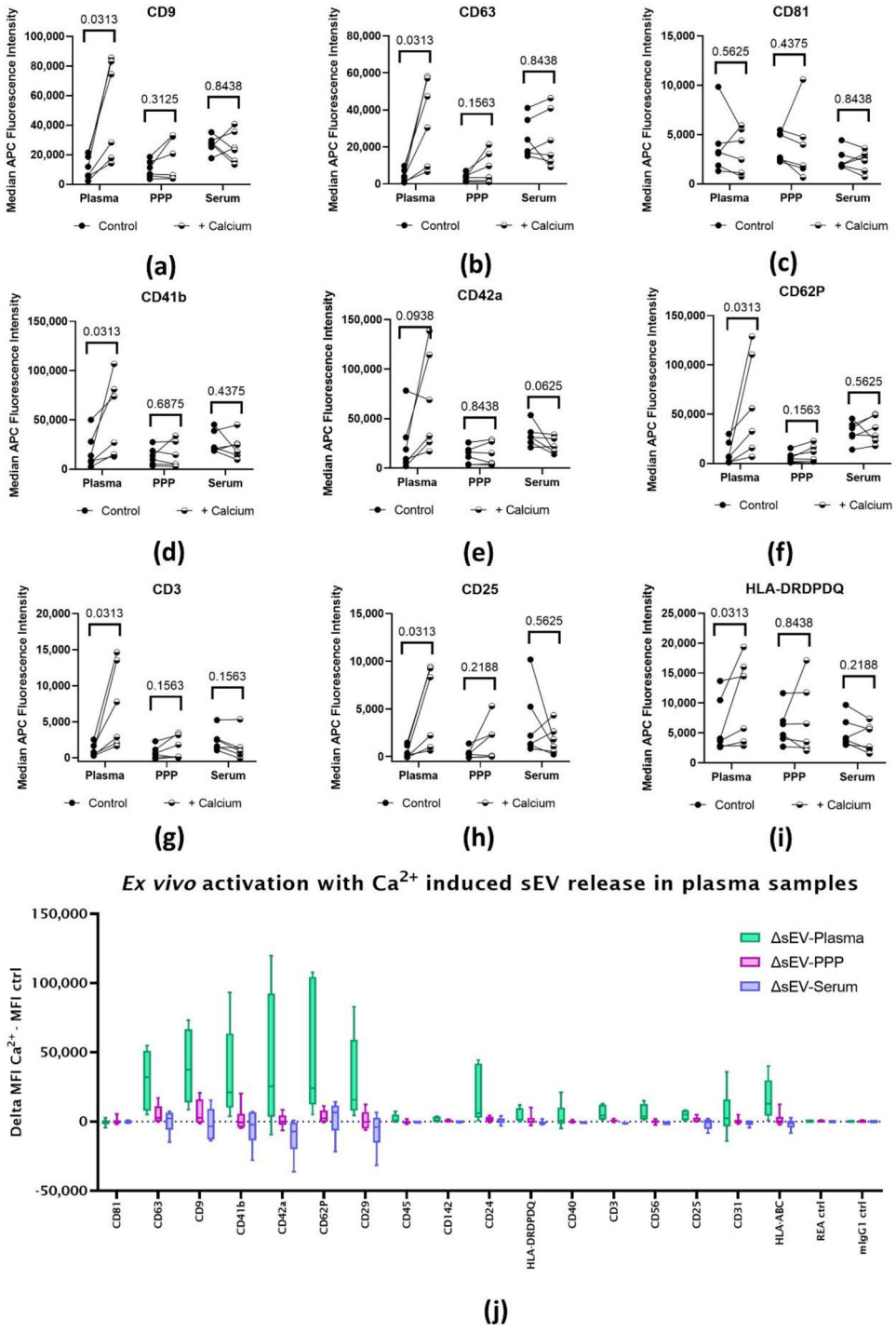

FIGURE 8 Activation of plasma, platelet poor plasma (PPP) and serum by Ca^{2+} addition. Graphs (a–e, g–i) show the Median Allophycocyanin (APC) Fluorescence Intensity (MFI) for the common exosome markers: CD9 (a), CD63 (b), CD81 (c); and platelet markers: CD41b (d), CD42a (e), CD62P (f); and leukocyte markers: CD3 (g), CD25 (h), HLA-DRDPDQ (i) from sEV (small extracellular vesicles) preparations purified from normal plasma (control); normal PPP (control); normal serum (control); calcium activated plasma (+ calcium); calcium activated PPP (+ calcium); calcium activated serum (+ calcium). The MFI was measured by MACSPlex human exosome kit ($n = 6$). Graph (g) shows a delta value between the Ca^{2+}-activated and control plasma, PPP and serum samples. Statistical analysis was performed with Wilcoxon matched-pairs signed rank test.

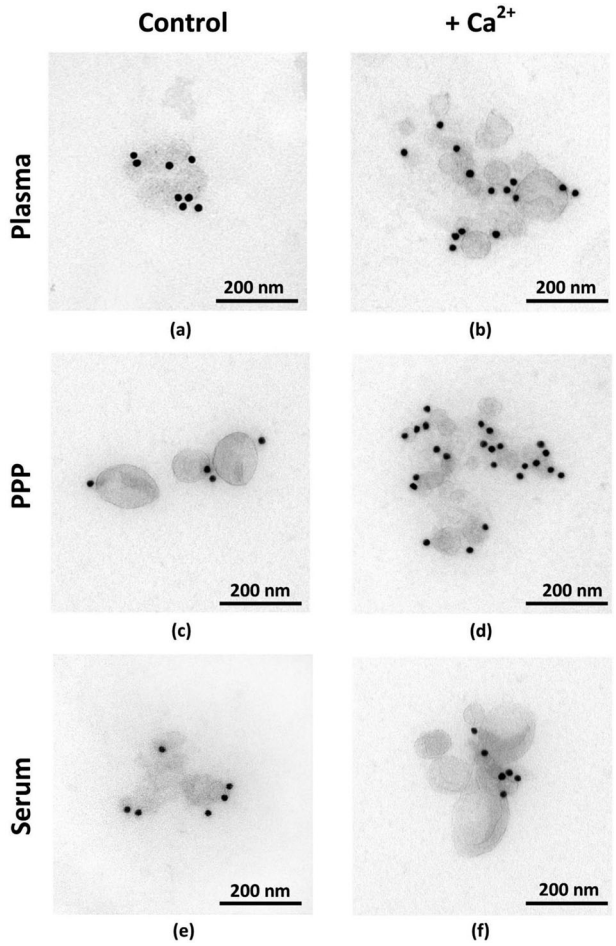

FIGURE 9 Transmission electron microscopy images presenting the typical morphology of small extracellular vesicles (sEV) purified by differential ultracentrifugation from different sample types: plasma (a and b); PPP—platelet poor plasma (c and d) and serum (d and f) that were either non-treated before isolation (a, c, e) or treated with Ca^{2+} (b, d, f). sEV preparations were identified using an antibody specific to CD9 and antibody binding was visualised by a secondary antibody conjugated to 15 nm gold particles. We have applied the image enhancement methods to reduce noise and perform shading correction.

TABLE 1 Clinical parameters of patients diagnosed with Rheumatoid Arthritis (RA) in active state (RA-A) and remission (RA-R) and of ANCA-associated vasculitis patients (AAV)

Patient group	AAV Mean (SD)	RA-R mean (SD)	RA-A mean (SD)
Gender F/M	3/2	4/1	4/0
Age	61.6 (14.6)	54.6 (11.7)	59.5 (18.1)
CRP	0.61 (0.76)	0.08 (0.07)	0.11 (0.06)
Leukocyte G/L	6.85 (2.45)	6.96 (1.33)	7.7 (2.9)
Platelets G/L	300.6 (80.7)	254.8 (66.6)	260.5 (40.9)
CDAI	N/A	0.6 (0.8)	29.0 (12.0)
RF	N/A	31.2 (46.9)	6.8 (9.6)
CCP-antibody	N/A	45.7 (50)	3.0 (3.5)
Creatinine	0.98 (0.34)	N/A	N/A
a-MPO ELISA	96.06 (191.97)	N/A	N/A
a-PR3 ELISA	4.18 (7.92)	N/A	N/A

Abbreviations: CCP, Cyclic Citrullinated Peptide; CDAI, Clinical Disease Activity Index; CRP, C-reactive Protein; ELISA, Enzyme-linked immunosorbent assay; MPO, Myeloperoxidase; PR3, Proteinase 3; RF, Rheumatoid Factor.

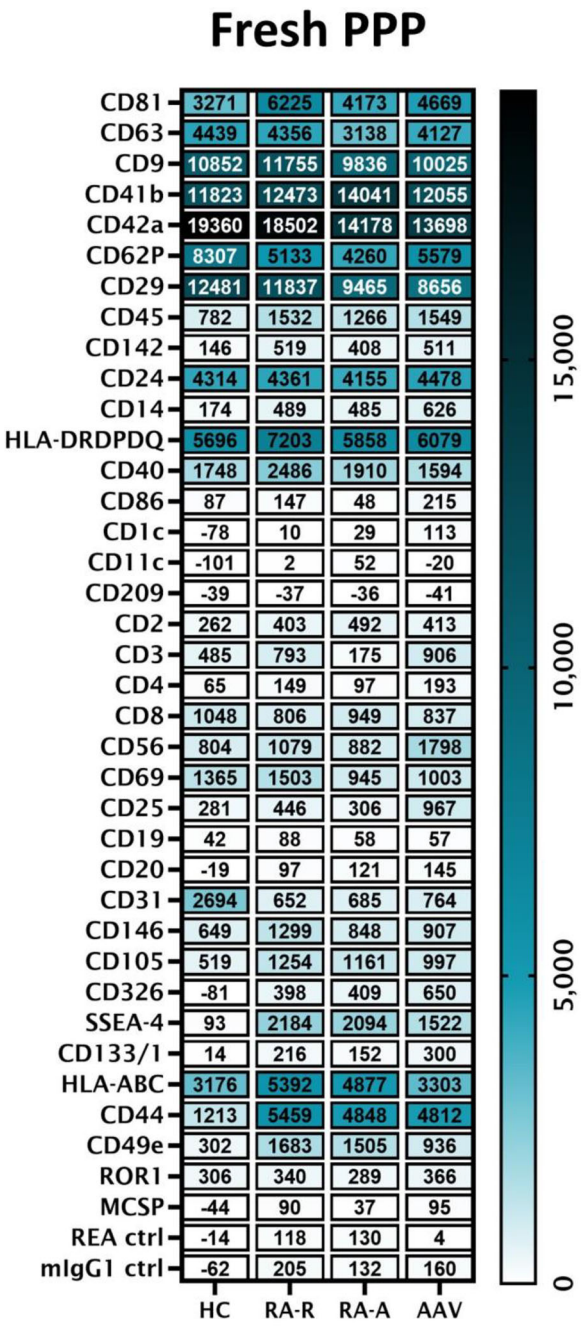

FIGURE 10 MACSPlex analysis of surface protein expression on small extracellular vesicles. Heatmap of MFI (Median Fluorescence Intensity) mean signals of sEV purified from fresh platelet poor plasma (PPP) from HC—healthy controls ($n = 4$) and patients: RA-R, Rheumatoid Arthritis in remission ($n = 5$); RA-A, Rheumatoid Arthritis active ($n = 4$); AAV, ANCA associated vasculitis ($n = 5$).

the five AAV patients had increased expression of a set of leukocyte proteins typical of activated T cells (CD25 but not CD69) and NK cells (CD56) and CD24, which is found on a variety of leukocytes, platelets and also some epithelial cells (Figure 11d–f). In general, sEV from patients and controls expressed similarly the molecules found on platelets with the possible exception for CD63 and CD62P, which are characteristic of activated platelet sEV and were reduced in active RA (Figure 11g, h) (Heijnen et al., 1999). Finally, the classical endothelial cell marker, CD31, which is also expressed on leukocytes and platelets and that is critical for adhesion and transmigration, was less abundant in all the patient groups, which is interesting because decreased CD31 is associated with excessive immunoreactivity (Figure 11j) (Marelli-Berg et al., 2013). Collectively the results identify consistent differences in protein expression patterns in sEV purified from fresh PPP between the patients and healthy controls in our cohorts, which are likely to reflect real differences in circulating sEV.

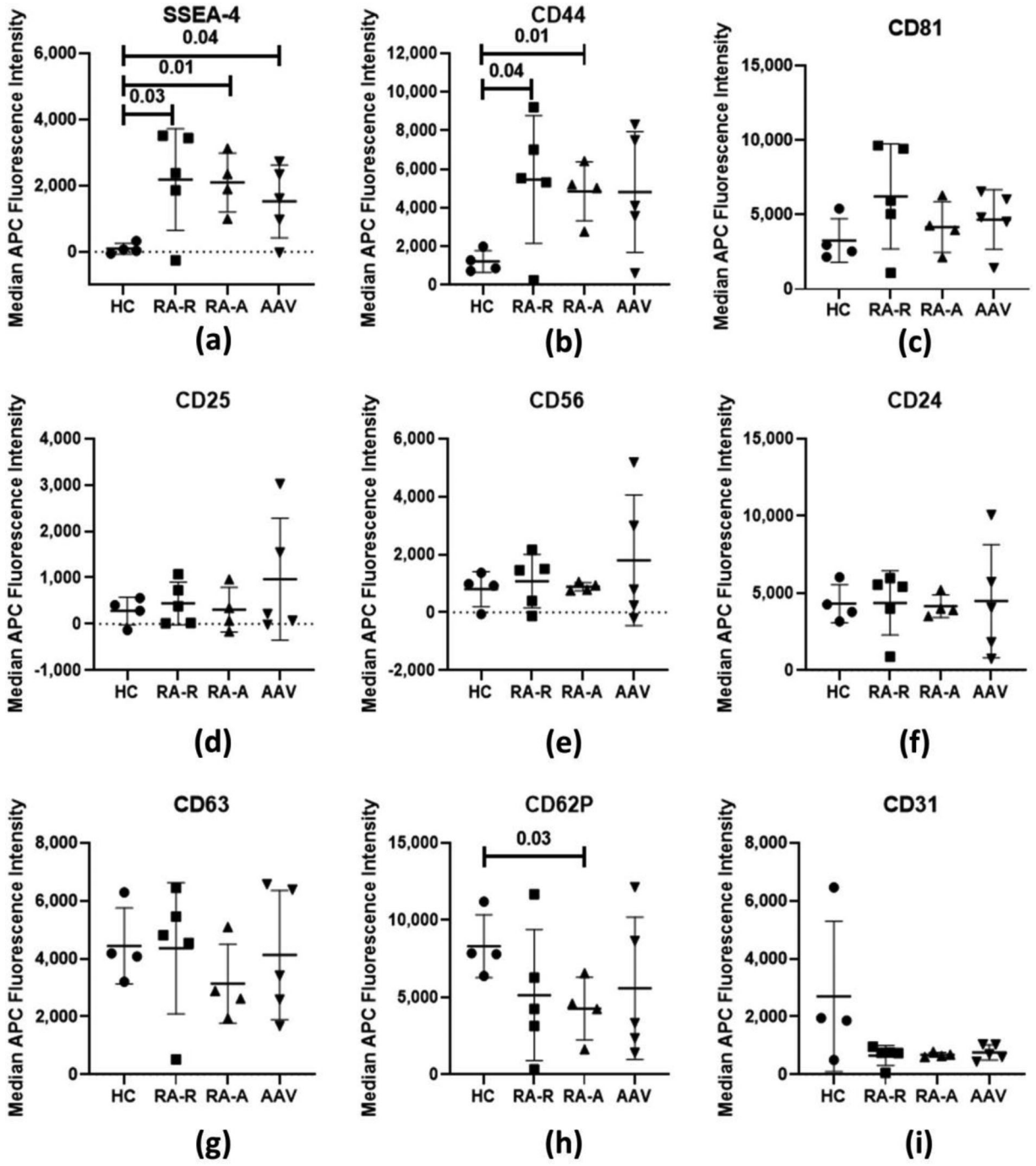

FIGURE 11 Plots with individual points representing differences that were found across patients´ groups and healthy control in fresh platelet poor plasma (PPP). Statistical analysis was performed in GraphPad with unpaired *t* test. HC, Healthy Control; AAV, ANCA associated vasculitis; RA-R, Rheumatoid Arthritis in remission; RA-A, Rheumatoid Arthritis active.

We next examined the effect of storage on the disease-associated protein expression patterns using sEV purified from PPP, plasma and serum stored at −80°C (Figure S7). The high expression of SSEA-4 (Figure 12a–d), CD44 (Figure 12e–h) in patients' sEV was barely changed in stored PPP and plasma, but the pattern in stored serum was decreased (as was the case with fresh serum). This was expected since few if any sEV expressing them were generated ex vivo, which is not the case for platelet expressed molecules whose abundance increased two to three folds in stored PPP and five to ten folds in stored plasma; or for some leukocyte sEV which increased roughly three folds and ten folds, respectively, in stored PPP and plasma. The expression of CD81 after storage was still visibly increased in patients, but with more variations (Figure 12i–l). Storage-associated MACSPlex MFI signal amplification obscured the difference in CD56, CD25, CD24, CD63 and CD62P expression by sEV purified from plasma between

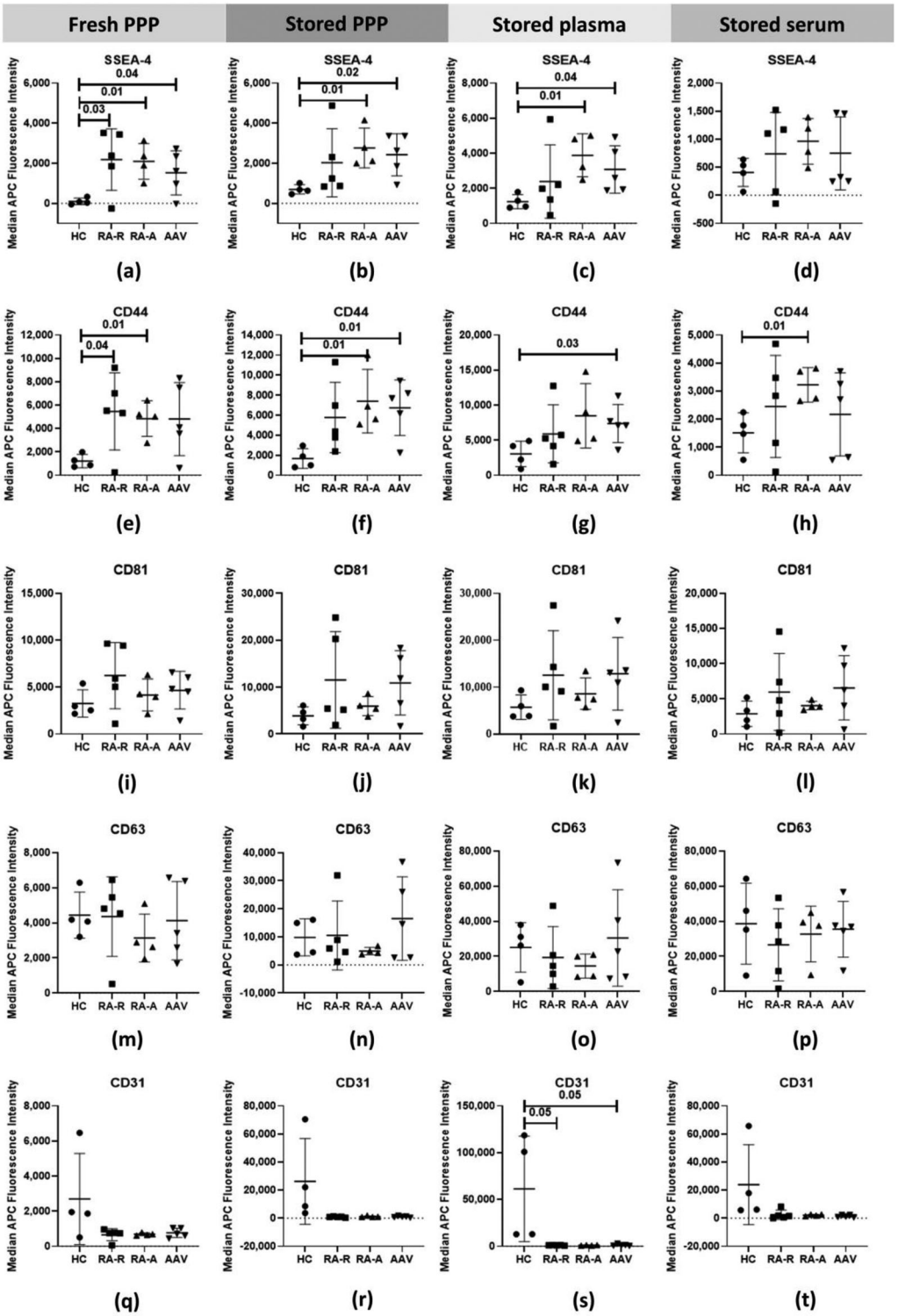

FIGURE 12 Plots with individual values showing MFI (Median Fluorescence Intensity) values of MACSPlex analysis of sEV proteins from healthy control and patients´ groups. sEV were purified from differently processed sample types (plasma, platelet poor plasma, serum) freshly or after −80°C storage. Selected markers were determined to be different across patients groups and those differences were preserved (to different degree) across samples. HC, Healthy Control; AAV, ANCA associated vasculitis; RA-R, Rheumatoid Arthritis in remission; RA-A, Rheumatoid Arthritis active; PPP, platelet poor plasma. Statistical analysis was performed with unpaired *t* test.

patients and controls, although it was still apparent in PPP-derived sEV (Figure 12m–p and Figure S8a–p). By contrast storage enhanced the difference in CD31 by increasing its abundance on sEV from healthy controls but not on those from patients, which resulted in all patient groups having lower CD31 expression (Figure 12q–t). There were few apparent differences of expression patterns using sEV purified from serum. Thus stored PPP samples are a reasonable substitute for fresh samples in biomarker discovery studies. Stored plasma samples are appreciably less good and probably inadequate for studies directed at potential biomarkers leukocyte (and platelet)-derived sEV biomarkers.

4 | DISCUSSION

Realising the potential of circulating sEV to be non-invasive biomarkers of systemic disease demands clear understanding of the influence of the pre-analytical techniques used during their purification. The issue of co-purified plasma proteins and lipids is well known (Ludwig et al., 2019; Małys et al., 2021) but there are few data examining the origins of purified sEV, and specifically whether a significant proportion were generated ex vivo after blood sampling (Karimi et al., 2022; Kim et al., 2022; Zhang et al., 2022). Here we have used two different purification methods and three discrete experimental strategies to resolve this issue. The results confirm a substantial proportion of sEV in human serum samples that were generated ex vivo and show that their populations are determined by the sample type and how it was stored. The MACSPlex arrays showed that platelets are the sole source of these sEV in serum, whereas in stored plasma it is augmented by contribution from leukocytes that is greatly diminished in PPP. Our results provide a template for identifying sEV that were unequivocally generated in vivo, and for characterising their cellular origins. We demonstrate the importance of this for biomarker discovery studies in a proof-of-concept study that identifies disease associated signals in PPP-derived sEV from individuals with rheumatoid arthritis and AAV that are preserved in sEV purified from stored PPP but attenuated or lost when stored plasma or serum is used as the source. This contributes as an essential prerequisite for the introduction of sEV as clinical biomarkers of disease activity as well as carries obvious implications for exploring disease pathogenesis.

The consistently greater abundance of the canonical exosome proteins CD9 and CD63 in sEV fractions purified from serum to plasma from the same freshly taken human blood samples showed ex vivo sEV generation and the differences were not restricted to these proteins. They also affected a large subset of sEV proteins represented on the MACSPlex arrays, which notably did not include CD81, the third canonical exosome protein on the arrays. These data are in apparent contradiction to the recent study of Karimi et al. (2022) which showed that fresh EDTA-plasma-derived EV contain more CD9 positive vesicles than sEV preparations from paired sera and that the CD9-EV were most probably released from platelets during blood withdrawal or sample handling. Our results strongly support the case for the release of platelet sEV into plasma ex vivo but show that our protocols for plasma preparation and sEV purification, unlike those used by Karimi et al., reduce ex vivo release of sEV to a level far below that occurring in serum. The explanation probably lies in methodological differences between the studies, and specifically in: (i) our more rigorous centrifugation protocols during plasma preparation based on ISTH recommendations; (ii) our use of DUC and Exo-spin to purify sEV which rapidly separates platelets; and (iii) the centrifugation step to remove platelet remnants and membranes from sEV preparations before isolation. Critically, both Karimi's study and ours demonstrate the potential confusion caused by ex vivo sEV in human plasma samples, and the necessity for removal of residual platelets and leukocytes as rapidly and completely as possible. Our Ca^{2+}-pulsing experiments show that immediate preparation of PPP from blood is the most effective way to achieve this. The results also demonstrate that PPP is essential when using sEV isolation method that retains platelets and sEV in the same compartment for relatively prolonged periods of time, such as the density gradient ultracentrifugation and magnetic bead isolation methods used by Karimi et al. (2022).

The issue of ex vivo sEV release into plasma is shown explicitly by our observations on the effects of storage. Specifically, after 14 days at either 4°C or at −80°C, the abundance of platelet sEV markers in plasma had increased to at least the level found in fresh serum, whereas there was little increase in serum; indeed, their abundance decreased after 14 days at 4°C. As expected, the effect of storage was strongly attenuated when PPP rather than plasma was used, given the haemocytometer counts of residual platelets (Figure S6). More importantly, sensitivity of haemocytometer was too low to quantify the small number of residual leukocytes in plasma needed for ex vivo EV generation, as confirmed by our Ca^{2+} pulsing experiments, which provoked release of platelet and leukocyte sEV into fresh plasma and but very few into PPP; they were absent in serum, presumptively because platelets and leukocytes had been consumed during coagulation. This is consistent with a previous report by Bæk et al. (2016) who used an in-house microarray to show that the signal intensities for CD9, CD63 and CD81 increased in sEV purified from EDTA blood samples after long term storage; and of Kim et al. (2022) who demonstrated that freezing plasma provoked ex vivo generation of EV from residual platelets (Palviainen et al., 2020). Platelet activation by agonists, such as Ca^{2+} and thrombin, also stimulates granules and sEV release (Aatonen et al., 2014; Blair & Flaumenhaft et al., 2009; van der Meijden & Heemskerk et al., 2019). This provided the foundation for our Ca^{2+} pulsing experiments which confirmed that residual platelets and leukocytes in fresh plasma release sEV acutely when activated and that the effect is abrogated by prior depletion of cells as in PPP. Addition of Ca^{2+} to serum had no effect on the abundance of sEV proteins, presumably because the platelets had already been activated by coagulation. Collectively our data exclude the possibility that the change in abundance of the sEV proteins could be explained

by alterations in the concentration on individual sEV. Instead, they prove that sEV purified from human sera and stored plasma contain a large sEV-populations that were not present in the circulation but were generated ex vivo.

The MACSPlex analysis of the Ca^{2+} pulsing experiments allowed closer scrutiny of the nature and origins of sEV. All platelet-expressed proteins represented in the arrays were at least twofold more abundant in sEV from Ca^{2+} treated plasma than those from control plasma, confirming platelets as the predominant source. These included the six proteins that were not restricted to platelets, but also expressed on sEV from other single (CD24 and CD29 – leukocytes, and CD31 - endothelium) or multiple other sources (HLA-ABC and the canonical exosome markers CD9 and CD63). The data also provide clear evidence for ex vivo release of sEV from residual leukocytes albeit in smaller numbers. The observed increase was prominent for sEV expressing HLA-DRDPDQ, which is expressed on monocytes, macrophages and dendritic cells, but also present for sEV expressing a second myeloid cell marker (CD40) and to a lesser degree as those expressing the pan leukocyte marker CD45, the pan-T cell marker (CD3) and markers for activated T cells (CD25 and CD69).

The large population of sEV released ex vivo into human sera and plasma samples during preparation and storage complicates the use of sEV as disease biomarkers and carries particular implications for the interpretation of the individual sEV proteins commonly used to determine their origin. The issue is exemplified by the contrasting behaviour of the canonical exosome proteins CD9, CD63 and CD81. All three proteins gave clear positive MACSPlex MFI with sEV fractions from fresh plasma. However, the abundance of CD9 and CD63 was appreciably greater in fractions from the paired serum and stored plasma samples, whereas there was little difference in the abundance of CD81. This implies that most CD81 expressing sEV were already present in the circulation, compared to perhaps less than half (Figure 5a–c) of those expressing CD9 and CD63. Similar caution applies to other molecules expressed on platelet or leukocyte sEV and other cells. These include the classical endothelial markers CD31 and CD105, and HLA class II molecules DRDPDQ. The latter are characteristically expressed on macrophages and dendritic cell-derived sEV and have powerful effects in antigen presentation to T cells (Leone et al., 2017). As well as those expressing T cell markers including CD3 and CD8, for activated T cells (CD25 and CD69), and NK cells (CD56) can be affected. By contrast the presence of sEV proteins, like CD81, were similarly abundant in serum and fresh plasma can be assumed with greater confidence to have originated in vivo.

Biomarker discovery studies are performed most efficiently using archived blood samples from well-characterised longitudinally followed clinical cohorts. Our proof-of-concept study demonstrates the importance of sample type for the success of such studies, at least for chronic autoimmune inflammatory diseases such as RA and AAV. The results showed that there were differences between patients and healthy controls in patterns of expression of proteins represented on the MACSPlex arrays when sEV purified from fresh plasma were used. The patterns were preserved in sEV from frozen PPP but altered in sEV from frozen plasma or serum. Notably signals from molecules that were not generated ex vivo were preserved across all three stored sample types, whereas those derived from leukocytes, CD69, CD56 and CD24, or platelets, such as the activated platelet markers CD62P and CD63, were lost or attenuated because of the signal generated by sEV released ex vivo. CD31 was the exception since the ex vivo generation of sEV that occurs in healthy individuals was supressed in the patients; this provides an interesting insight into the pathogenesis of RA and AAV, or more likely the treatment. The study also emphasised the need for more tailored arrays for characterising EV in biomarker studies. Currently at least 9 of the 37 proteins included in the MACSPlex arrays bind proteins expressed on platelet-derived sEV, including some such as CD31, CD24 and CD29 commonly thought to also indicate other cellular sources. This clearly complicates their use for biomarker discovery.

Our study provides strong support for the potential of blood-derived sEV as surrogates to monitor human health and disease, but emphasises the need to take stringent precautions to minimize, or at least take account of ex vivo release by residual platelets and leukocytes. We show that the magnitude of their effect is critically affected by pre-analytical protocols and sEV purification methods used. Indeed, the signals from Exo-spin purified sEV comes almost exclusively from platelet-derived ex vivo generated sEV. Finally, our results provide a template for identifying sEV in human blood samples that originated in the circulation and so provide a firm foundation for the use of sEV as clinical biomarkers. In conclusion, our data show that archived PPP is a suitable sample type however the use of PFP remains desirable for biomarker research when available.

AUTHOR CONTRIBUTIONS

Małgorzata S. Małys: Conceptualization Lead, Data curation Lead, Formal analysis Lead, Investigation Equal, Methodology Lead, Visualization Lead, Writing-original draft Lead. **Maximilian C. Köller**: Data curation Supporting, Formal analysis Supporting. **Kristin Papp**: Methodology Supporting. **Christof Aigner**: Methodology Supporting. **Daffodil Dioso**: Resources Supporting. **Patrick Mucher**: Resources Supporting. **Helga Schachner**: Methodology Supporting. **Michael Bonelli**: Resources Supporting. **Helmuth Haslacher**: Resources Supporting. **Andrew J. Rees**: Conceptualization Equal, Data curation Equal, Investigation Equal, Methodology Equal, Supervision Equal, Writing-original draft Supporting, Writing-review & editing Lead. **Renate Kain**: Conceptualization Equal, Funding acquisition Lead, Investigation Equal, Methodology Equal, Resources Equal, Supervision Lead, Writing-review & editing Equal

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV220319) (van Deun et al., 2017).

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Medical University Vienna Ethics Committee (ECS 1089/2012 and IRB 1945/2018).

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

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