Assessment of time-dependent platelet activation using extracellular vesicles, CD62P exposure, and soluble glycoprotein V content of platelet concentrates with two different platelet additive solutions

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Running Title

EVs as a marker of platelet activation

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Summary

Novel analytical measures are needed to accurately monitor the properties of platelet concentrates (PC). Since activated platelets produce platelet-derived extracellular vesicles (EVs), analysing EVs of PCs may provide additional information about the condition of platelets. The prospect of using EVs as auxiliary measure of platelet activation state was investigated by examining the effect of platelet additive solutions (PAS) on EV formation and platelet activation during PC storage.

The time-dependent activation of platelets in PCs with PAS-B or with the further developed PAS-E was compared by measuring the exposure of CD62P by flow cytometry and the content of soluble glycoprotein V (sGPV) of PCs by an immunoassay. Changes in the concentration and size distribution of EVs were determined using nanoparticle tracking analysis.

A time-dependent increase of platelet activation in PCs was demonstrated by the increased CD62P exposure, sGPV content, and EV concentration. Using these strongly correlating parameters, PAS-B platelets were shown to be more activated compared to PAS-E platelets.

Since the pEV concentration correlated well with the established platelet activation markers CD62P and sGPV, it could potentially be used as a complementary parameter for platelet activation for PCs. More detailed characterization of the resulting EVs could help to understand how the PC components contribute the functional effects of transfused PCs.

Keywords

Platelet concentrate; extracellular vesicle; platelet activation; platelet additive solution; CD62P; soluble glycoprotein V

1 Introduction

Platelet concentrates (PC) are manufactured for patients with haemostatic problems, e.g., excessive bleeding or thrombocytopenia in cancer. The average lifespan of a platelet in blood circulation is 10 days [1], and the storage time of PC is typically 5 to 7 days [2] mainly due to an increased risk of bacterial contamination during extended storage at room temperature [3]. Efforts are made to increase the storage time of PCs by developing the platelet additive solutions (PAS), improving protocols in the PC preparation, pathogen inactivation and by more detailed quality control (QC) [4–6].

A crucial aspect of QC of PCs is the determination of platelet activation state. One widely used marker of platelet activation is P-selectin (CD62P) exposure of platelets. CD62P is transferred to the platelet plasma membrane through the fusion of α-granules upon activation [7]. Another platelet-specific marker is the soluble form of glycoprotein V (sGPV), which is released from activated platelets through proteolytic cleavage [8]. The transmembrane form of glycoprotein V is located on the platelet surface as a part of a complex with glycoproteins Ib and IX, which is the major receptor for von Willebrand factor [9] and it also participates in thrombin [10] and collagen [11] binding. Other current QC assays of PCs include the quantification of platelet metabolites (glucose, lactate, pH) and dissolved gases (pO_2, pCO_2), and the determination of platelet function using the extent of shape change and hypotonic shock response. Also, different platelet parameters, such as the mean platelet volume and platelet count are commonly monitored to examine the quality of platelets [6].

Besides the platelets' main role in regulation of haemostasis, they have been shown to influence immune responses [7], which is an aspect to consider when platelet concentrates are administered to patients. During storage of PCs, platelets liberate a large variety of bioactive components that have been proposed to relate to adverse pro-inflammatory effects observed in storage lesion [12,13]. Although several different markers for measuring storage lesion have been suggested, a gold standard to evaluate the usability of PCs for transfusion has not yet been established [6]. Therefore, novel markers are needed to assess the condition of platelets in more detail.

Besides platelets, the PCs contains extracellular vesicles (EVs), which in majority are derived from platelets, but which also originate from red blood cells and leukocytes residually present in the plasma fraction of PCs [14]. One of the first functions in which

platelet-derived EVs were shown to participate in was haemostasis [15,16], implemented by the interaction of coagulation factors on the phosphatidylserine surface of the EVs [17]. Additionally EVs are considered to be biomarkers of thrombotic and inflammatory diseases, as well as cancer [18,19], and in general EVs have already been shown to mediate several (patho)physiological processes [20,21]. Furthermore, EVs have been suggested to contribute to the adverse transfusion-related reactions [22], underscoring the need to understand the possible effector functions of EVs in transfusion. In addition to the possible effects of the transfused EVs in patients, EV generation during the storage of PCs could be considered as auxiliary parameter to monitor the activation state of platelets, since the generation of platelet-derived EVs is dependent on the aging and activation status of platelets [23,24].

In the current study, EVs of PC were quantified to see whether the EV content could be utilized as a parameter of the activation state of platelets in PCs together with the recognized platelet activation markers, CD62P exposure of platelets and the sGPV content. This was investigated by examining the platelet activation state in aging PCs with different PAS.

2 Materials and Methods

2.1 Sample Collection

Standard leukocyte-reduced PCs each derived from buffy coats of four ABO RhD - matched whole blood donations with platelet additive solution B (PAS-B) or platelet additive solution E (PAS-E) were obtained from the Finnish Red Cross Blood Service (Helsinki, Finland) and were handled anonymously, as accepted by Finnish Supervisory Authority for Welfare and Health (Valvira, Helsinki, Finland). The exact composition of PAS-B and PAS-E, also known as PAS-2 and modified PAS-3 [25], respectively, have been reported elsewhere [26].

Sterile sampling was done using 50 mL syringes (Henke-Sass, Wolf GmbH, Tuttlingen, Germany) and 18-gauge needles (Terumo, Tokyo, Japan). Before sampling, the contents of the storage bag's tube were emptied into the storage bag and the PC was mixed by gently turning it from side to side 5 times. This procedure was repeated 3 times to obtain a representative sample. After extracting 20 mL of sample via the storage bag's tube, the tube was resealed. The sampling days (d) were d1, d2, d5, and d8 counting from the blood donation (d0), where d1 was the production day of PC. The d1 sampling was performed

within 2 hours after the PCs were available from the production line, approximately at 3 p.m., whereas the d2 - d8 samplings were performed at 9 a.m. PCs were stored at 22 °C under constant horizontal agitation.

2.2 Determination of CD62P Exposure of Platelets

The CD62P expression on the platelet surface was determined by flow cytometry using 1 mL of PC sample. PC samples were diluted 1:100 (to approx. 1×10^7 per mL) using a diluent consisting of the same PAS used for PC production (either PAS-B or PAS-E; SSP or SSP+, (Macopharma, Tourcoing, France)) with 0.5% w/v Bovine Serum Albumin (Sigma-Aldrich, St. Louis, MO, USA). 50 µL of diluted PC sample was labelled using 2 µL of fluorescein isothiocyanate (FITC)-coupled anti-CD41 (FITC Mouse Anti-Human CD41, clone HIP8 (Becton Dickinson, Franklin Lakes, NJ, USA)) and 5 µL of phycoerythrin-cyanine 5 (PE-Cy5)-coupled anti-CD62P (PE-Cy5 Mouse Anti-Human CD62P, clone AK-4 (Becton Dickinson)). For each sample an isotype control sample (50 µL of diluted PC sample labelled with 5 µL of PE-Cy5 Mouse IgG1 κ isotype control, clone MOPC-21 (Becton Dickinson)) and a thrombin activated positive control sample with maximum CD62P expression (50 µL of diluted PC sample labelled with 2 µL of CD41-FITC and 5 µL of CD62P-PE-Cy5 and activated with 1 IU/mL thrombin (Roche, Basel, Switzerland)) were prepared. Samples and controls were labelled and analysed in BD TruCount-tubes (Becton Dickinson) containing a known number of fluorescent beads. Samples were analysed in singlicates, since previously the CD62P measurements had been found well repeatable [27]. After labelling samples were mixed, incubated for 20 minutes at room temperature (RT) in the dark, further diluted with 500 µL of diluent, and stored in the dark until analysis.

The samples and controls were analysed with Navios flow cytometer (Beckman Coulter, Brea, CA, USA) at “high-flow” speed. The forward (FS) and side scatter detectors’ (SS) volt and gain settings had been adjusted such that the platelet population was centred in the FS –SS dot plot (at around 10^1) and the fluorescence detectors’ FL1 (FITC) and FL4 (PE-Cy5) settings such that the detected fluorescence signals were well within the displayed ranges for all samples. The TruCount beads were gated based on their fluorescence in FL1, FL2 and FL3 channels and a platelet gate had been defined in the FL1 – SS dot plot. For each sample 5000 bead events were acquired, corresponding to

about 60000 platelet events. The gated platelet population was used to calculate the percentage of CD62P positive platelets, defined as:

- Based on the isotype control, a threshold was set to include 1% of all events with the highest fluorescence in the FL4-channel. All events with FL4-fluorescence above this threshold were defined as CD62P positive in comparison to the isotype control.
- Based on the positive control, a threshold was defined to include 95% of the thrombin activated platelet population with the highest fluorescence. All platelets with FL4-fluorescence above this threshold were considered CD62P positive in comparison to the positive control.

Platelet activation state was expressed in relation to both isotype and positive control as percentage of gated platelet population above the respective CD62P positivity threshold.

2.3 Quantification of sGPV

The quantification of soluble glycoprotein V (sGPV) was performed as reported previously [6]. Briefly, 1 mL of the PC was centrifuged (Biofuge 13 (Heraeus Sepatech, Hanau, Germany)) first at 3600 × g in RT for 15 minutes and the supernatant again at 11000 × g in RT for 5 minutes (Biofuge 13). The supernatant was transferred to new tubes in 500 µL aliquots and stored at -70 °C until sGPV quantification with a commercial kit (Asserachrom, Diagnostica Stago, France). For the measurement, samples were diluted 1:80 - 1:640 using phosphate buffer provided with the kit, and the amount of sGPV was expressed as pmol/10^9 platelets.

2.4 Isolation of EVs

A total of 17 mL of PC was used for EV isolation. To prevent platelet activation, Anticoagulant Citrate Dextrose Solution pH Eur Solution A (Terumo BCT, Lakewood, CO, USA) and Apyrase (Sigma-Aldrich) were added to the final concentrations of 4.25% v/v and 2 U/mL, respectively, and the PC was diluted 1:4 with phosphate buffered saline (PBS (Thermo Fisher, Waltham, MA, USA)). The diluted PC was centrifuged at 650 × g at RT for 7 minutes (Eppendorf centrifuge 5810R, (Eppendorf, Hamburg, Germany)) without brake, and the supernatant was centrifuged 1560 × g in RT for 20 minutes (Eppendorf centrifuge 5810R). The residual platelet content of the supernatant was reduced to 1 × 10^6 platelets/mL, as confirmed with Coulter Cell counter T-540 (Beckman

Coulter). To extract the whole EV population from the PC, the supernatant was ultracentrifuged at 100000 × g at 4 °C for 1 hour (MLA-50 rotor, k-factor 92 (Beckman Coulter)). The supernatant was carefully decanted and remaining supernatant was removed with a pipette, after which the EV sample was resuspended into 200 µL of PBS and stored in Protein LoBind tubes (Eppendorf) in -70 °C until analysis.

2.5 Quantification and Size Determination of Particles in EV samples

The concentration and size distribution of particles in EV samples was determined using Nanoparticle Tracking Analysis. The used LM14C model was equipped with 70 mW violet (405 nm) laser (Malvern Instruments Ltd., Malvern, UK) and sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan). Data were captured using camera level 14, and 3 videos of 90 seconds were recorded, manually mixing the sample between measurements. EV samples from PAS-B PCs were diluted 1:1000, 1:2000, 1:5000 and 1:10000 with filtered (0.2 µm) PBS on d1, d2, d5 and d8 samples, respectively, and EV samples from PAS-E PCs were diluted 1:1000, 1:2000, 1:5000 and 1:5000 - 1:10000 on d1, d2, d5 and d8 samples, respectively. Data analysis was performed with threshold 5 and gain 10. Data were recorded and analyzed with NanoSight software version 3.0 (Malvern Instruments Ltd.). The data were reported as EV concentration of the PC on the sampling day by calculating the particle content of EV sample using the determined particle concentration and taking into account that the particles were isolated from 17 mL sample, considered as a representative sample of PC.

2.6 Staining and Characterization of EV Samples on the ImageStreamX MkII

EV samples from PAS-E were labelled with Alexa Fluor 488C_5maleimide (Invitrogen, Carlsbad, CA, USA) for 60 minutes at RT as described previously [28]. Excess maleimide was removed by using exosome resin spin columns (Invitrogen) which were prepared according to manufacturer´s instructions. Maleimide labelling without EVs was performed in a parallel fashion to confirm the dye retention by columns and to get “mock” controls for the experiments.

Different fluorescent stains for further characterization of EVs were used according to manufacturer´s instructions. Antibodies were: CD41a AF647 (clone HIP8 (BioLegend, San Diego, CA, USA)), CD45 PerCP-Cy5.5 (clone 2DI (BioLegend)), CD63 BV510 (clone H5C6 (BD BioScience, San Jose, CA, USA)), and CD235a Pacific Blue (clone HI264 (BioLegend)). Apolipoprotein A contamination was surveyed with ApoA1 PerCP

(BioSite, Täby, Sweden). Antibodies were incubated for 30 minutes in RT in the dark in PBS.

Maleimide488 and fluorescent positive EVs were detected using a 12 channel Amnis® ImageStream®X Mark II (EMD Millipore, Burlington, MA, USA) imaging flow cytometer. Samples were acquired at 60 × magnification with low flow rate/high sensitivity. The integrated software INSPIRE® (EMD Millipore) was used for data collection. The instrument and INSPIRE software were set up as follows: Excitation lasers 488, 642 and 785 and channels (Ch)01 and Ch09 (bright field), Ch06 (scattering channel), plus fluorescence channels Ch02, Ch05, Ch07, Ch08 and Ch011 were activated for signal detections.

At least 10000 events for each sample were acquired. Positive events for maleimide488 were gated based on the intensity values and used for further analysis. Single colour controls were used for compensation and unlabelled EVs were used to determine the auto fluorescence. Buffer with and without antibody/maleimide488 molecules were used to determine the background noise. Compensated data files were analysed using image-based algorithms available in the IDEAS® statistical analysis software package (version 6.2.188.0).

2.7 Statistical Analysis

Kruskal-Wallis test together with Dunn's multiple comparison test to take into account the effect of multiple testing was used to determine the significance of the results within PASs, and p-values of < 0.05 (*), < 0.01 (**),< 0.001 (***), and < 0.0001 (****) were considered significant. To determine the significance between PASs on d5 sample Mann-Whitney test together with Bonferroni correction was used, * = Bonferroni adjusted $p < 0.05$. Spearman correlation coefficient and related p-value together with R^2 value of the standard curve was used to determine the correlation between the different platelet activation parameters. All statistical analysis was performed using GraphPad Prism v. 6.07 (GraphPad Software, Inc. La Jolla, CA, USA).

3 Results

3.1 CD62P Exposure of Platelets

A statistically significant, time-dependent increase in the CD62P exposure was observed during the eight day storage period. The exposure of CD62P in the PAS-B stored platelets

increased from 8.5% to 78% ($p = 0.0226$ and 0.0002 on d5 and d8, respectively) when compared to the positive, thrombin activated control (Fig. 1A). In PAS-E platelets, the average CD62P exposure increased from 3.6% to 71% ($p = 0.0028$ on d8) compared to the positive control (Fig. 1A).

When CD62P exposure was determined by comparison to the isotype control, the CD62P exposure of PAS-B platelets increased from 36% to 70% ($p = 0.0177$ and 0.0005 on d5 and d8, respectively) and from 24% to 53% ($p = 0.0117$ on d8) in the PAS-E platelets (Fig. 1B).

Regardless of the CD62P exposure determination method, on d5, the last day when the PC can be transfused to patients, platelets in PAS-B PCs exposed more CD62P than platelets in PAS-E PCs (Bonferroni adjusted $p = 0.0016$ for both, Fig. 1A and B)

3.2 Content of sGPV in PCs

The sGPV content of PAS-B PCs increased from the average of 2.1 to 24.5 pmol/10^9 platelets during the storage ($p = 0.0225$ and 0.0002 on d5 and d8, respectively (Fig. 1C)). Also in the PAS-E PCs, the increase in sGPV was statistically significant, but more subtle, as the sGPV concentration increased from 1.8 to 12.1 pmol/10^9 platelets ($p = 0.0224$ and 0.0002 on d5 and d8, respectively) during the 8 day storage (Fig. 1C). The sGPV content of PCs was significantly higher in PAS-B PCs than PAS-E PCs at d5 (Bonferroni adjusted $p = 0.0158$, Fig. 1C).

3.3 Concentration and Size Distribution of Particles in EV Samples

The particle concentration in EV samples of PAS-B PCs significantly increased during the 8 day storage period from 1.1×10^{10} particles/mL on d1 to 1.3×10^{11} particles/mL on d8 ($p = 0.0292$ and 0.0021 on d5 and d8, respectively) and in the PAS-E PCs from 7.9×10^9 particles/mL on d1 to 3.7×10^{10} particles/mL on d8 ($p = 0.0019$ and $p < 0.0001$ on d5 and d8, respectively (Fig. 1D)). Both the PAS-B and PAS-E PCs initially had similar particle concentration in EV samples, but from d2 time point onwards the particle concentration in EV samples of PAS-B PCs was higher compared to PAS-E PCs (Bonferroni adjusted $p = 0.016$ on d5, Fig. 1D).

The size distribution of particles in EV samples changed significantly during the aging only in the PAS-B PCs (Fig 1E). Initially, 61% of the particles were < 100 nm in diameter, but after 8 days of storage, the percentage of particles < 100 nm had decreased to 27% (p

= 0.0070). Consequently, the percentage of particles with a diameter of 101 – 200 nm was initially 31%, which increased to 56% at d8 ($p = 0.0484$). In contrast to PAS-B, no significant alteration in the size distribution of particles in EV samples was observed in PAS-E PCs (Fig. 1E).

3.4. Characteristics of EV Isolated from PCs

From all the maleimide-positive particles in the EV samples, the majority expressed CD41, indicating that the EVs isolated from the PCs are mainly derived from platelets. Also, EV-marker CD63 was abundantly present and the number of CD41 and CD63 positive particles increased during the 7 day storage. Besides the platelet-derived particles, minute amounts of leukocyte- and erythrocyte-derived particles and ApoA1 were detectable in the EV samples (Table 1).

3.5 Correlation of EV Sample Particle Concentration with CD62P Exposure and sGPV Content

A strong positive correlation was observed between the particle concentration of EV samples and the sGPV content of PCs ($R^2 = 0.7639$, Spearman $r = 0.7906$ with $p < 0.0001$, Fig. 2A), and very strong positive correlation was observed between the particle concentration of EV samples and the CD62P exposure of platelets ($R^2 = 0.6626$, Spearman $r = 0.8269$ with $p < 0.0001$, Fig. 2B), and between the sGPV content of PCs and the CD62P exposure of platelets ($R^2 = 0.8816$, Spearman $r = 0.9253$ with $p < 0.0001$, Fig. 2C).

4 Discussion

For a few decades PCs have been prepared with PAS together with some plasma. Initially only plasma-containing PCs were favoured due to better functionality (estimated by the corrected count increments and bleeding) compared to only PAS-containing PCs [29]. The disadvantages of the plasma-containing PCs include increased incidences of adverse transfusion-related reactions, mainly allergic reactions, but possibly also transfusion-related acute lung injury and ABO mismatched haemolysis [26]. Currently approximately 30% of the volume of PAS-containing PCs still contain plasma to maintain platelet functionality [30], but the development of PAS has resulted in a notable improvement of platelet quality and functionality, leading to experimentations with decreased content of plasma in PCs with PAS [31]. At the moment PAS-E is considered to be the best PAS

developed, having similar platelet functionality to the PCs with plasma in terms of corrected count increment [29], and it has been even hypothesized whether with the addition of further components such as glucose [30], the advantage of plasma could be surpassed in favour of the PAS-only PCs. The driving force behind reducing the plasma content in PCs is the potential decline in the incidence of adverse transfusion-related reactions. Additionally, the leftover plasma could be utilized for fractionation to produce other tranfusable products [26].

All common PASs contain NaCl and acetate [26]. NaCl is added in varying amounts to adjust the PC osmolarity, and acetate is added for two reasons: firstly, to provide an alternative energy source in addition to glucose, as it reduces lactate production and consequently influences the pH of the PC. Secondly, during the enzymatic processing of acetate carbon dioxide is formed, which further reacts with water to form bicarbonate providing increased buffer capacity to PCs [32]. Most PASs also contain citrate as anticoagulant, which provides yet another energy source, and added buffer capacity [26,33]. Furthermore, PAS-E contains phosphate, potassium, and magnesium whereas PAS-B does not [26]. Phosphate is added to PCs for improved buffer capacity and to stimulate platelet glycolysis [34]. PASs with different compositions have been extensively tested, and the addition of potassium and magnesium have been connected to decreased cytokine [35] and lactate [36,37] production, as well as decreased CD62P [37,38] and phosphatidylserine [38] exposure of platelets. Our results on time-dependent platelet activation are in line with these previous findings, as based on the three assessed platelet activation markers (CD62P exposure of platelets together with sGPV and EV content of PCs), platelets in PAS-B PCs were more activated than the PAS-E PC platelets on d5, the last day when PC could be transfused to patients. A significant increase in the activation state of the PAS-B platelets was detected with all three activation markers at d5 sampling. For PAS-E PCs a significant increase in the sGPV content and EV particle concentration was observed at d5 as well, whereas a significant increase in the CD62P exposure was observed only at d8. Although it is unclear how the altered PAS composition affects platelet activation, the mechanism might involve membrane potassium movement and permeability [39]. Similarly to CD62P and sGPV, a time-dependent increase of EV concentration in PCs was observed, in line with previously published results [40,41]. Based on the current data EV concentration correlated well with

the sGPV content and the CD62P exposure of platelets, indicating that the EV concentration could be used to indicate platelet activation in PCs.

As shown previously, determination of both the CD62P exposure of platelets and the sGPV content in PCs were sensitive and reproducible methods to detect platelet activation [6,27]. As an additional advantage of these methods, the maximum extent for both parameters can be determined, which helps to estimate the platelet activation state by giving either a relative or an absolute [42] boundary value (for CD62P and sGPV, respectively). Contrary to CD62P and sGPV measurements, it is not possible to generate an accurate control for a maximum EV production as different agonists produce varying amounts of platelet-derived EVs [23]. Although current PC manufacturing processes ensure minimal cell contamination, EVs from erythrocytes, platelets and leukocytes are present in PC already due to the plasma component of the PCs, as shown in this study and by others [14]. It is difficult to determine, whether the platelet-derived EVs are produced due to aging-related platelet activation, as a result of interaction of buffy coat components during the storage, or even apoptosis-like process [43]. The interaction of buffy coat EVs and platelets might explain the platelet activation to some degree [44] and consequently the high variation in the particle concentration of EV samples from PCs especially seen in the d8 samples. Additionally considering the variance in donors [45,46], current EV sample preparation methods [47] and the lack of standardized and accurate EV quantification methods [48–50], it must be stressed that although EV concentration seems as a potential marker of platelet activation, significant development and standardization will needed before the current methods could be replaced to determine platelet activation state in PCs. The authors would like to underline that EVs could still be used as a complementary platelet activation marker to CD62P and sGPV.

In addition to EV concentration being a marker for platelet activation similarly to CD62P or sGPV, EVs could also provide qualitative information of PC aging and possibly even functionality. Besides influencing the size [23,51] and the molecular cargo [23,24,52] of produced EVs, platelet activating conditions have been shown to affect the subsequent function of produced EVs [53], and future studies could concentrate on the qualitative information provided by PC-derived EVs. In the current study, we observed a time-dependent increase in the size distribution of particles in the EV samples from platelets stored in PAS-B. Since platelet activation was influenced by the PAS composition, it may also have an influence to the produced EVs. Another possible explanation to the altered

size could be an artefactual clumping of EVs, which has been shown previously [54]. However, the effect was only subtle compared to the size of e.g., EV doublets and without a corresponding decline in the particle concentration, so formation of stickier EVs is unlikely to explain the current results. A change in the EV population in the PC, reflected by the size change, could also have functional effects upon transfusion [55]. To understand the potential effects of EVs in storage lesion, adverse transfusion-related reactions, or immunomodulatory functions in general, it will be necessary to carefully examine the molecular composition of EV populations by lipidomics, proteomics, or metabolomics [56–58]. Moreover, characterizing the PC-derived EVs could be also a step towards personalized transfusion treatments, where patients could be targeted to receive PCs that would suit their needs the best [45,46], e.g., PCs that have more procoagulant potency in the case of severe bleeding. By doing this, the utilization of PCs could be optimized, leading to less wasted PCs and hopefully to transfusions with less adverse transfusion-related reactions.

In conclusion, EVs may be useful tools in QC of PCs in the future, and the molecular characterization of EVs could provide more information about the state and usability of the PCs, ultimately benefitting the patients receiving transfusions.

5 Statements

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5.2 Statement of Ethics

The authors have no ethical conflicts to disclose.

5.3 Disclosure Statement

The authors have no conflicts of interest to declare.

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5.5 Author Contributions

SV, PS, and SL designed the study that was critically reviewed by AV, JE, and KJ. SV completed the NTA data collection and data analysis. BM completed the CD62P data collection and data analysis. AV completed the sGPV data collection and data analysis. UI completed the Amnis sample preparation, data collection and analysis. SV, PS, and SL interpreted the results. SV, BM, PS, and SL wrote the manuscript, which was critically revised by AV, JE, and KJ. All authors approved the final manuscript.

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7 Figure Legends

Figure 1: Quality control markers used for the evaluation of platelet activation during storage of platelet concentrates with platelet additive solution (PAS-)B and PAS-E. Time-dependent changes in the CD62P exposure of platelets, when compared to a positive (A) or an isotype control (B); soluble glycoprotein V (sGPV) production of platelets (C); concentration (D) and size distribution (E) of particles in the extracellular vesicle samples isolated from platelet concentrates. Statistical difference within a given PAS,is indicated with black and grey stars for PAS-B and PAS-E platelet concentrates, respectively, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ compared to day (d)1 using Kruskal-Wallis test with Dunn's multiple comparison. Statistical difference between PAS-B and PAS-E platelet concentrates on d5, * = Bonferroni adjusted $p < 0.05$ using Mann-Whitney test with Bonferroni correction is indicated with a red star. Bars represent mean with standard deviation in A-D, columns present mean and bars standard deviation in E. Data were acquired in 3 independent experimental settings, n = 4-5 (PAS-B in all figures, PAS-E in A and B) or 10 (PAS-E in C, D, and E).

Figure 2: Correlation analysis of the three different markers for platelet activation: particle concentration in the extracellular vesicle samples and sGPV production of platelets (A); particle concentration in the extracellular vesicle samples and CD62P exposure of platelets, when compared to positive control (B); sGPV production and CD62P exposure of platelets (comparison to a positive control) (C). Figure was compiled using data from both platelet additive solution B and E platelet concentrates acquired from 3 independent experimental settings.

Table 1: Frequency (%) of surface markers identified from maleimide-positive particles of EV samples isolated from platelet concentrate on day 1 and day 8.