Analysis of platelet-derived extracellular vesicles in plateletpheresis concentrates: a multicenter study

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BACKGROUND: Routine quantification of platelet-derived extracellular vesicles (PL-EVs) may be useful in the quality control (QC) of platelet concentrates (PCs). The aim of this multicenter study was to establish and validate a consensus protocol for the standardized PL-EV quantification using conventional flow cytometers.

STUDY DESIGN AMD METHODS: Eighty-six PCs were investigated in five blood transfusion centers (A-E) on Days 0 and 5. The centers used different apheresis instruments: Trima Accel (n = 56) and/or Amicus (n = 30). PCs were prepared using standard methods (sd-PCs; n = 73; A-D) or with pathogen inactivation (PI [PI-PCs]; n = 13; E). Platelet (PLT) count was determined using conventional hematology analyzers. PLT degranulation (P-selectin expression in response to thrombin receptor PAR1 activation) and PL-EVs were analyzed by flow cytometry.

RESULTS: During storage, PLT count remained stable in 58 PCs (A, C, E), whereas a decrease was observed in 12 PCs (B). PLT degranulation declined in all PCs ($p < 0.001$) and PL-EVs increased in 74 PCs (A, C-E; $p < 0.001$). Certain donor variables (e.g., plasma cholesterol, immature PLT fraction) were associated with lower PL-EVs. In Trima-produced PCs, PL-EVs were significantly lower (D) and PLT degranulation was superior compared to PCs prepared with the Amicus (A, D). PL-EVs were 10-fold lower in PI-PCs, compared to sd-PCs. However, similar QC trends were demonstrated for both PC groups during storage.

CONCLUSION: PL-EV analysis in a QC program of PCs was successfully performed with results comparable among the different centers. PLT degranulation and vesiculation were primarily affected by preparation techniques.

Platelet concentrates (PCs) stored at room temperature under gentle agitation for transfusion purposes undergo alterations of platelet (PLT) structure and function, which are known as the PLT storage lesion (PSL).[1] PSL is characterized by reduced PLT recovery and survival in vivo.[2] The relationship between PSL and PLT apoptosis, similar to eryptosis during storage of red blood cells, is under discussion.

ABBREVIATIONS: CAD = compound adsorption device; cc = correlation coefficient; CD = cluster of differentiation; CHOL = cholesterol; DPBS = Dulbecco's modified phosphate-buffered saline; EV(s) = extracellular vesicle(s); FCM = flow cytometry; IPF = immature platelet fraction; LDL-CHOL = low-density lipoprotein cholesterol; PC(s) = platelet concentrate(s); PI = pathogen inactivation; PI-PCs = PCs prepared with pathogen inactivation; PL-EV(s) = platelet-derived extracellular vesicle(s); PSL = platelet storage lesion; sd-PCs = standard PCs prepared without pathogen inactivation; TRAP-6 = thrombin receptor–activating peptide 6.

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A potential relevance for PLT vesiculation during storage of PCs has been suggested.[3]

Several processing and storage conditions of PCs, such as PLTs obtained by apheresis or from whole blood and the use of different additive solutions (ASs), were found to be important for PSL.[4,5] Established variables to detect PSL of PCs include monitoring of cell counts (e.g., PLTs, residual white blood cells [WBCs]), pH values, hypotonic shock response, or P-selectin (CD62P) surface exposure on PLTs by flow cytometry (FCM) as a surrogate marker for their activation and alpha degranulation.[6-9] Preparation and storage of PCs (e.g., different cell separators, cold storage, several ASs, or leukoreduction filters) were also found to alter the amount of PLT-derived extracellular vesicles (PL-EVs).[3,10-15] Maurer-Spurej and colleagues[16,17] established a quality scoring system for PCs (ThromboLUX DLS score) that includes number and shape of PLTs and microparticles and found a strong correlation between PLT quality and transfusion outcome in patients with hematologic malignancies.[18] Pathogen inactivation (PI) of PCs, which prevents replication of contaminating pathogens and intends to reduce the risk of transfusion-transmitted infections, was found to enhance PSL as a consequence of the preparation procedure.[19] Therefore, vesiculation is altered by certain preparation techniques, which should be known when evaluating PL-EVs in PCs as a variable for PSL.

The potential therapeutic application of EVs was proposed by the International Society for Extracellular Vesicles for infectious diseases, systemic inflammatory response syndrome or sepsis, and for immune modulation in antitumor or regenerative therapies.[20] EVs may become novel biologic medicinal products, either alone or as part of blood components, or alternatively as targets for elimination from the circulation (e.g., apheresis, immune adsorption) in patients with pathologically elevated EV levels. The prognostic value of in vivo–formed EVs has been demonstrated in transfused critically ill patients, with an association to the required blood components and mortality rate.[21] The overall benefit of vesicle measurement in blood concentrates has not yet been fully evaluated, especially for PL-EVs as by-products in PCs. Therefore, a standardized quantification of (PL-)EVs originating from blood components is required and may provide a new surrogate marker for product quality.

There are known limitations of the FCM method in assessing PL-EVs due to preanalytical variables, such as blood collection handling and centrifugation conditions, and analytical requirements, such as calibration beads and instrument settings. However, FCM is a widely used technique in approximately 75% of studies to enumerate EVs.[22] The first step toward this was achieved by a collaborative workshop from the International Society on Thrombosis and Haemostasis,[22] which concluded that PL-EV analysis by FCM depends on calibration materials and flow cytometer settings that are applicable to "wide forward scatter platforms." Preanalytical variations that have an impact on EV analysis were identified and their validation was recommended before being applied to multicenter studies.[23,24] Further steps toward standardization of vesicle analysis were summarized in a recent position paper of the International Society for Extracellular Vesicles.[25] In addition, several other working groups defined criteria necessary for a standardized EV analysis by FCM,[26-28] which also may serve as recommendations for current best practices to EV analysis.[29] More recent studies utilize the new generation of high-resolution flow cytometers (e.g., Apogee A50-Micro, Apogee Flow Systems Ltd; CytoFLEX, Beckman Coulter) to obtain more sensitive and accurate data on EVs through optimized scatter performance, resolution, and sensitivity.[30-33] Unfortunately, these techniques are not yet available for most routine laboratories. Based on recommendations for standardization and on the availability of conventional flow cytometers in routine laboratories, we recently published data demonstrating improvements of PC quality control (QC) using EV analysis as a plausibility check for PLT degranulation.[34] Here we provide data from five independent transfusion centers demonstrating the successful evaluation of the QC consensus protocol and its applicability for routine use in transfusion medicine.

MATERIALS AND METHODS

Protocols for multicenter studies

Standardized protocols for PC processing, sampling, and analysis were designed and validated separately in each center according to previously published protocols.[34] The study design is depicted in Fig. S1 (available as supporting information in the online version of this paper). Analysis of PC samples was performed before (Day 0) and after storage (Day 5). Five blood donation centers participated in this study: Center A, Institute for Clinical Chemistry and Laboratory Medicine, University Hospital of Regensburg; Center B, Institute of Transfusion Medicine and Transplantation Immunology, University Hospital Muenster; Center C, Institute of Transfusion Medicine, Charité University Medical Centre, Berlin; Center D, Department for Health Sciences and Biomedicine, Danube University Krems, Austria; and Center E, Regional Blood Transfusion Service Zurich SRK, Zurich, Switzerland.

Data were collected and interpreted for PCs regarding identical instrument settings (Trima Accel vs. Amicus) and the preparation technique (standard PCs [sd-PCs] vs. pathogen-inactivated PCs (PI-PCs)]. For the period of QC analysis as part of the multicenter study, identical consumables and reagents (manufacturer, lot number) were purchased. Each center confirmed gating strategies for

vesicle analysis and use of PLT-specific antibodies under saturating conditions by FCM.

Characteristics of PLT donors, apheresis, and PC storage

All 86 healthy donors volunteered in five different blood donation centers and were free from medication. Informed consent and approval from the ethics committee for each center was obtained. Single-donor PLT units (PCs) were obtained by hemapheresis according to the German regulations for blood donation.[6]

PCs were collected using either the Trima Accel (Terumo BCT; n = 56; all centers) or Amicus collection system (Fenwal, Inc.; n = 30; Centers A, D, and E; see listed instruments in Table S1, available as supporting information in the online version of this paper). Two PC preparation techniques were used. First, 73 PCs (Centers A-D) were collected in autologous plasma and ACD was added (sd-PCs). Second, in 13 PCs (Center E), the AS (InterSol, Fenwal, Fresenius Kabi) was replaced to a final amount of approximately 53% to 68% per unit (32%-47% residual plasma) and PI was performed using photochemical treatment with amotosalen (150 μmol/L) and UVA light (3 J/cm^2, Intercept Blood System, Cerus Europe BV).[35]

All PCs were stored for up to 5 days under constant agitation at 60 cycles/min and incubated at 22 ± 2°C (e.g., PLT agitator PF96i, Helmer Laboratories). For analysis, PC samples were drawn after 2 hours of resting storage (Day 0) and after agitated storage (Day 5).

Total blood count, PLT count, and pH analysis

Blood counts of donors were measured on hematology analyzers (e.g., XE-5000 hematology analyzer, Sysmex) according to standard procedures using EDTA blood samples obtained on Day 0. PC samples (50 μL) were diluted in capillary tubes (e.g., Sysmex) with 200 μL of buffer (e.g., Cellpack, Sysmex) in a mixed ratio of 1:5. PLT counts were subsequently analyzed using hematology analyzers as described. Measurement of pH values of PC samples (e.g., Blood Gas Monovette, Sarstedt) on Day 0 and Day 5 at 22 ± 2°C was performed using a potentiometric method on a blood gas analyzer (ABL 90 FLEX, e.g., Radiometer GmbH).

Serum variables of donors

Serum variables of glucose and lipid metabolism were determined on a clinical chemistry analyzer (e.g., Dimension Vista 1500, Siemens Healthcare Diagnostics) according to standard manufacturer procedures. Cholesterol (CHOL), low-density lipoprotein CHOL (LDL-CHOL), high-density lipoprotein CHOL, triglycerides, and nonfasting serum glucose were analyzed from donor blood samples with clot activator (Day 0).

Detection of PL-EVs by FCM

The scatter size of the flow cytometers (two BD FACSCanto II, BD Biosciences; two Navios and one Gallios, Beckman Coulter) was calibrated by fluorescent polystyrene beads with a refractive index of 1.61 (Megamix beads; BioCytex) to discriminate PLTs (approx. 2-3.0 μm) from PL-EVs (≤0.5 μm), as described by Lacroix and colleagues[22] and applied in an earlier study[34] (Fig. S2, available as supporting information in the online version of this paper). Staining of 100 μL of PC sample (diluted 1:500 in Dulbecco's modified phosphate-buffered saline [DPBS] without Ca^{2+}/Mg^{2+}; Biochrom AG) was performed by incubation with saturating amounts (2.5 μL) of R-phycoerythrin-cyanine 7–labeled mouse IgG_1 against CD61 (IOTest, Beckman Coulter) at room temperature for 10 minutes in the dark. After addition of 500 μL of DPBS (final dilution, 1:2500), samples were analyzed immediately. The relative PL-EV and PLT counts were used to calculate the ratio of PL-EVs to PLT (in percentages).

PLT degranulation test by FCM

Basal externalization of P-selectin (CD62P) on the PLT surface and in response to thrombin receptor–activating peptide-6 (TRAP-6) was assessed on Days 0 and 5 as previously described.[34] Evaluation was performed using FCM (triplicate with BD FACSCanto II, BD Biosciences; once with Navios, once with Gallios, Beckman Coulter) according to standard protocols for evaluation of the expression of PLT membrane glycoproteins. The fluorescence of CD62P-positive labeled PLTs was indicated in mean fluorescent intensity (MFI) and a ratio was calculated ($MFI_{TRAP\text{-}6} \times 100\%/MFI_{basal}$; Fig. 3). PC samples were diluted with DPBS (Biochrom AG) to a final PLT concentration of not more than 2×10^4/μL. Diluted PC samples (20 μL) were incubated either with 10 μmol/L TRAP-6 (10 μmol/L, peptide SFLLRN, Roche Diagnostics GmbH) for 10 minutes at room temperature or with 10 μL of DPBS. Immunostaining was performed with the monoclonal antibodies CD62P-FITC (IOT Beckman-Coulter; clone CLB-Thromb/6, IgG_1), CD41-PE (IOT Beckman-Coulter; clone P2, IgG_1), and CD61-PerCP (BD-Biosciences; clone RUU-PL 7F12, IgG_1).

Statistical analysis and data presentation

Statistical analysis was performed with computer software (SPSS 19.0, IBM SPSS Statistics for Windows, Version 19.0, IBM Corp.). Raw data from 86 PCs produced by the five centers were collected and analyzed after testing for normal distribution using the Kolmogorov-Smirnov test and expressed as mean ± SD. Wilcoxon signed-rank test was used for all paired groups and Kruskal-Wallis' one-way analysis of variance on ranks was used for independent groups. The two-tailed Pearson test was used for sample correlation (correlation coefficient [cc]). A significant

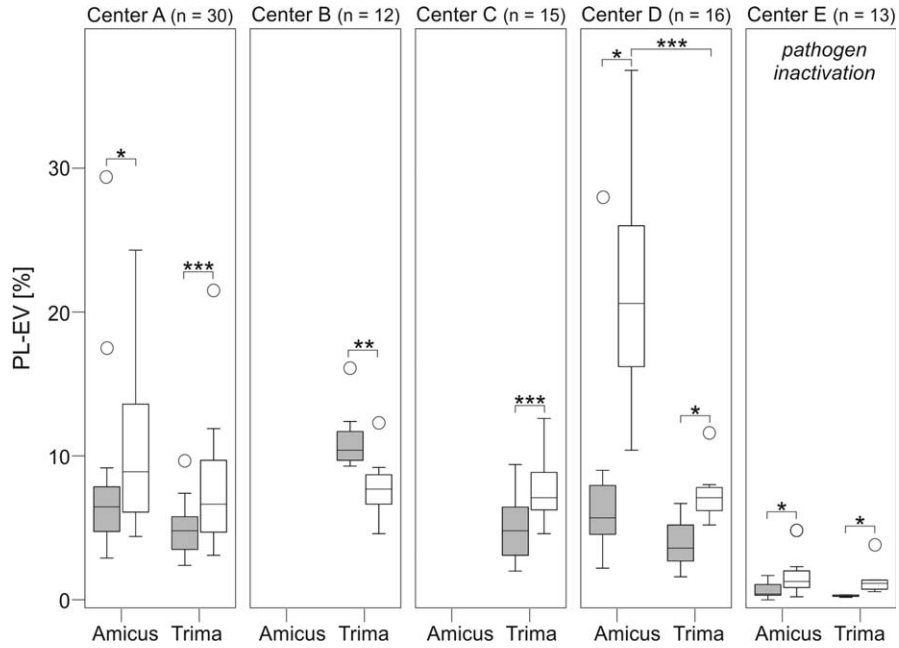


Fig. 1. PL-EVs are influenced by the type of apheresis instrument. CD61-positive labeled PL-EVs were analyzed by FCM (values correspond to the ratio of PL-EVs to PLT in percentages). Apheresis instruments (Amicus vs. Trima Accel) differentially influenced PL-EVs in expired PCs (Center D, $p < 0.001$; not significant for Center A), but not on Day 0, nor in PCs with PI (Center E). During storage, a significant increase of PL-EVs was found for 74 PCs (Centers A and C–E) and a decrease was observed in PCs from Center B (n = 12). Centers B and C used Trima instruments. (■) Day 0; (▢) Day 5.

difference or correlation was considered when p values were less than 0.05 (*), 0.01 (**), or 0.001 (***). Graphical representations were performed using computer software (SPSS 19.0 and CorelDRAW X7). For box plots, dark horizontal lines of boxes represent the mean, within boxes corresponding to the 25th and 75th percentiles, whiskers depict the 5th and 95th percentiles, and outliers are represented by circles.

RESULTS

Donor-specific variables

Donor characteristics were comparable between the five donation centers; however, not all variables were evaluated for the 86 donors from all centers. Twenty-eight females and 58 males were of normal weight and clinically healthy, with no pathologic laboratory values for blood counts, plasma lipids, proteins, and glucose profile. Further descriptive information on donors is provided in Table S2 (available as supporting information in the online version of this paper). The ABO blood group was observed to have a normal distribution for Caucasians (O, 41.9%; A, 40.7%; B, 16.3%, AB, 1.2%) with no relevant correlation between ABO blood groups and PL-EVs or PLT degranulation.

In fresh PCs (on Day 0), there was a significant inverse correlation between PL-EVs and donor plasma lipid levels for CHOL and LDL-CHOL (n = 57, cc = −0.37**, cc = −0.34*; Fig. 4A). After 5 days of storage of PCs, there was a significant correlation between PL-EVs and donor age (n = 87, cc = −0.24*), LDL-CHOL (n = 57, cc = −0.28*), PLT count before apheresis (n = 87, cc = 0.30**), immature PLT fraction (IPF; n = 42, cc = −0.59**), PLT distribution width (PDW; n = 57, cc = −0.38**), and mean PLT volume (MPV; n = 87, cc = −0.24*; Fig. 4B, including 13 samples from donors in Center E). In Center E, no other relevant correlation was found for PI-PCs (n = 13) between all donor-specific variables and PL-EVs (Days 0 and 5) or CD62P (Days 0 and 5).

Apheresis settings

The time of plateletpheresis varied between 37 and 110 minutes, with a processed blood volume between 1789 and 5426 mL. A longer apheresis time resulted in higher PL-EVs and lower PLT degranulation on Day 5 (cc = 0.272*, cc = −0.285*). A higher volume of processed blood, as a direct consequence of longer apheresis time, resulted in higher PL-EV levels on Day 5 (cc = 0.31**), but correlated with a slight increase in PLT degranulation (CD62P expression after stimulation) on Day 0 (cc = 0.237*).

All blood donation centers processed PCs from a single PLT donation. In two centers (B and C), only one apheresis instrument type (Trima Accel) was used. In three centers (A, D, and E), PC production was additionally performed using a second instrument type (Amicus), corresponding to

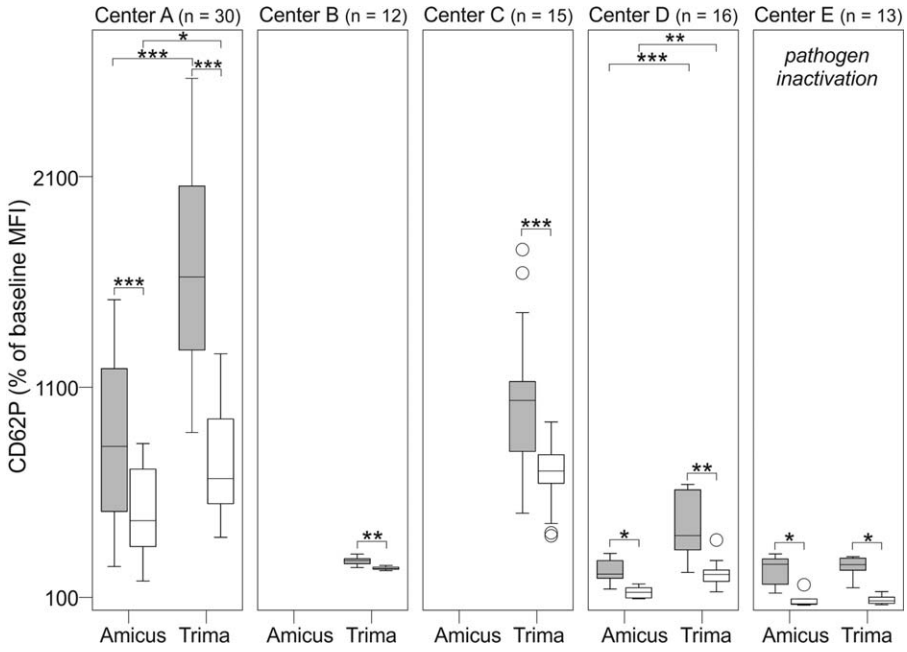


Fig. 2. PLT degranulation is influenced by the type of apheresis instrument. PLT degranulation (P-selectin [CD62P] expression in response to thrombin receptor PAR1 activation; values correspond to MFI × 100%/MFI_{basal}) was analyzed by FCM. Degranulation was affected by the type of apheresis instrument (Amicus vs. Trima Accel) on Day 0 (■, Center A, $p < 0.001$; Center D, $p < 0.001$) and on Day 5 (□, Center A, $p = 0.038$; Center D, $p = 0.01$), but not in PCs with PI (Center E). During storage, loss of PLT degranulation after stimulation was observed in all PCs (n = 86). Centers B and C used Trima instruments.

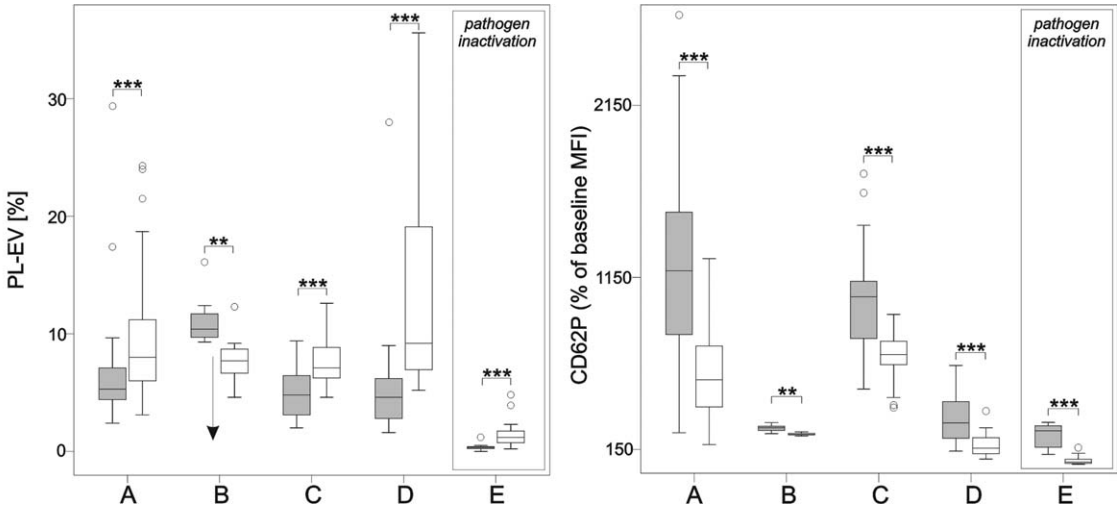


Fig. 3. Characteristics of 86 PCs during storage. PL-EVs and CD62P expression on PLTs on Day 0 (■) and Day 5 (□) in order of the five participating apheresis centers (Centers A-E). PL-EV levels increased in 74 PCs (Centers A and C-E) and decreased in all PCs from one center (B; n = 12, $p = 0.002$). CD62P expression after stimulation of PLTs with TRAP-6 (SFLLRN) decreased in all PCs. Framed box plots depict the Intercept-treated PCs in Center E.

34.9% of 86 PCs. No relevant differences between both instrument types were found for all centers for PL-EVs (n = 86, Mann-Whitney U test; Day 0, $p = 0.89$; Day 5, $p = 0.08$). However, center-specific data revealed significantly lower PL-EV levels in PCs prepared with Trima Accel (Center D, n = 16; Day 5, $p < 0.001$; Fig. 1).

PLT function capacity was significantly influenced by the type of instrument used for all 86 PCs (Day 0, $p = 0.031$; Day 5, $p = 0.011$) and for center-specific data (n = 49) from Center A (Day 0, $p < 0.001$; Day 5, $p = 0.038$) and Center D (Day 0, $p = 0.002$; Day 5, $p = 0.005$; Fig. 2). A comparison of apheresis-specific data between

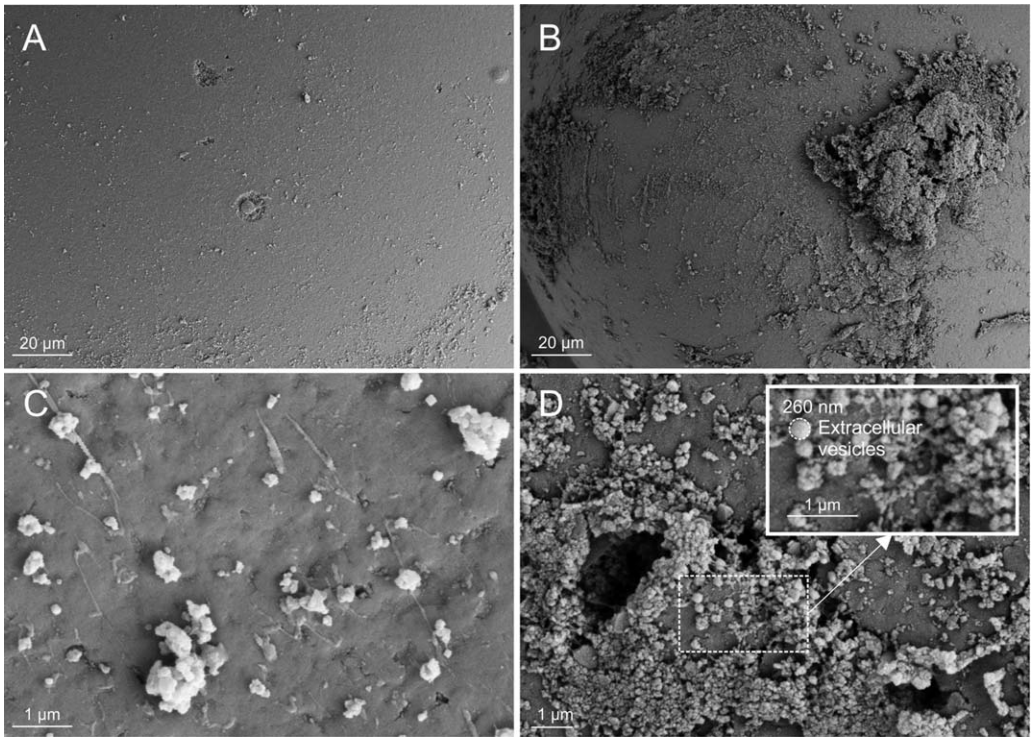


Fig. 4. Scanning electron micrographs of (A, C) unused and (B, D) used CAD. The CAD filter system was implemented in the PI process of PCs to remove the remaining psoralen and its photodegraded by-products. PLTs and PL-EVs adhered also on the CAD surface. The scale bars present 20 µm (A, B) or 1 µm (C, D). Images are used with the permission of B. Frey, Regional Blood Transfusion Service Zurich SRK, Switzerland, and A. Kaech from the Center for Microscopy and Image Analysis, University of Zurich.

sd-PCs (Centers A-D) and PI-PCs (Center E) revealed a higher blood volume processed for sd-PCs (3598 mL vs. 2549 mL, $p < 0.001$).

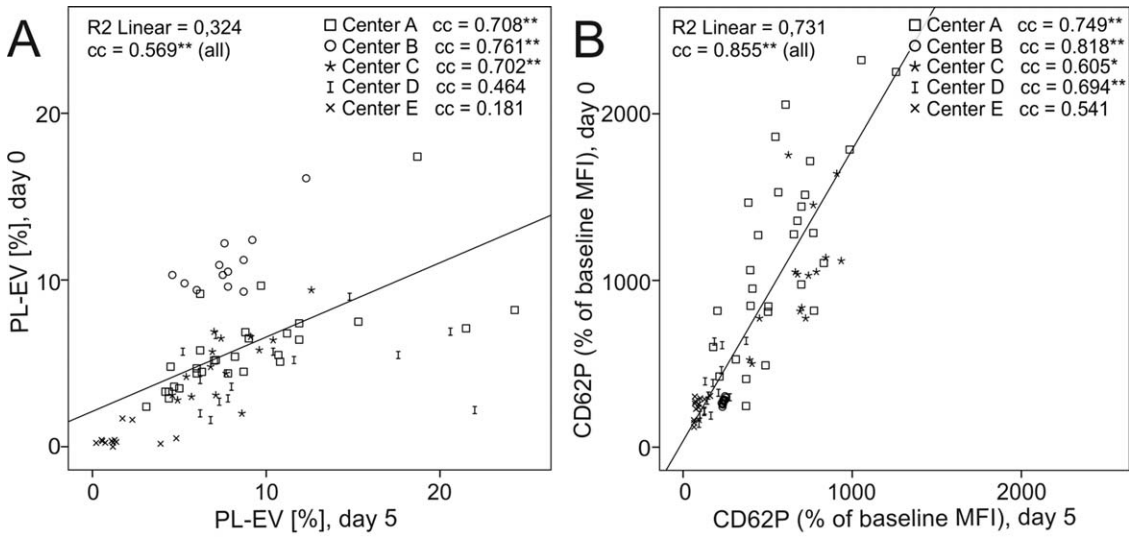
PC variables during storage

The volume of the concentrates was reduced by up to 28% ($n = 57$, $p = 0.001$) corresponding to the sampling volume on Days 0 and 5. PCs were observed to have a relative constant PLT count over 5 days of storage in 58 PCs. However, in 12 PCs (Center B), PLT count significantly decreased but remained in the range of approval regulations. PL-EVs were also observed to decrease in these 12 PCs compared to the other PCs ($n = 74$), which showed an increase in PL-EVs over time (Fig. 3). In contrast to relative PL-EV analysis (PL-EV in percentage to PLT count), the absolute quantification of PL-EV count (PL-EV/µL), which was only determined in Center B, also showed a slight increase in PL-EVs during storage (Day 0, PL-EVs = 659/µL; Day 5, PL-EVs = 710/µL); however, this was not significant. PLT degranulation significantly declined in all PCs ($n = 86$) after storage time ($p < 0.001$, Fig. 3).

No acidic pH pattern was observed during storage. The mean pH value on Day 0 was 7.26 (7.06-7.48; $n = 72$) and 7.36 (7.04-7.6) on Day 5 ($n = 57$). Visual evaluation of PCs revealed intact PC containers at all times and visible swirling on Day 0 (mean, 1.74; $n = 58$) and Day 5 (mean, 2.66; $n = 15$). PC variables are specified in detail in Table S3 (available as supporting information in the online version of this paper).

In Center E, PI-PCs on Day 0 were analyzed before and after PI (Table S4, available as supporting information in the online version of this paper). After PI, no relevant differences of PLT count ($p = 0.116$) and PL-EVs ($p = 0.221$) were found. In contrast, PI-PC volume ($p = 0.001$) and PLT degranulation significantly decreased after PI ($p = 0.003$, Wilcoxon test, two-tailed).

After storage (Fig. 3), similar changes of quality variables were observed in sd-PCs and PI-PCs (e.g., lower PLT degranulation and higher PL-EV levels). When comparing data of both PC groups (sd-PCs vs. PI-PCs), there was a significantly higher PC volume on Day 0 in PI-PCs ($n = 13$, $p = 0.012$) compared to sd-PCs ($n = 57$; Centers A-C). PLT count was lower in PI-PCs compared to 42 sd-PCs (Centers A and B, Day 0, $p = 0.005$; Day 5, $p = 0.012$). Most discrepancies regarding the two different preparation techniques were associated with PL-EVs and PLT degranulation. Significantly lower PL-EVs (10-fold lower, $p < 0.001$, on Days 0 and 5) and lower PLT degranulation ($p < 0.001$, on Days 0 and 5) were detected in 13 PI-PCs (vs. 73 sd-PCs). Reasons for the "loss of PL-EVs" in PI-PCs may be the two additional preparation steps. First, the composition of PLT



C

		PL-EV [%]			
		day 0		day 5	
CD62P (% of baseline MFI)	day 0	A	-0.430*	A	-0.492**
		B	-0.135	B	-0.086
		C	0.204	C	0.374
		D	-0.492	D	-0.512*
		E	-0.624*	E	-0.737**
		All	-0.121	All	-0.093
	day 5	A	-0.268	A	-0.535**
		B	-0.512	B	-0.382
		C	-0.372	C	-0.329
		D	-0.400	D	-0.512*
		E	-0.213	E	-0.561*
		All	-0.021	All	-0.111

Fig. 5. Correlation between PL-EVs and CD62P. Scatter plots figure the correlation between variables of fresh and expired PCs. Both variables, (A) PL-EVs and (B) CD62P, correlated positively between Day 0 and Day 5 in Centers A through E (in Center E without significance). (C) The table presents cc's between PL-EVs and CD62P, in each case on Days 0 and 5. CD62P = PLT surface expression of P-selectin in response to TRAP-6 (values correspond to $MFI_{TRAP\text{-}6} \times 100\%/MFI_{basal}$). Data are shown for the PC subgroups (A-E) in regard to the apheresis Centers A (n = 30), B (n = 12), C (n = 15), D (n = 16), and E (n = 13) and for all PCs together (n = 86).

surrounding solution of PI-PCs differs to that of sd-PCs. The AS InterSol results in the dilution of PL-EVs, which are present in residual donor plasma. Second, PL-EVs of PI-PCs adsorb to nonphysiologic surfaces (e.g., compound adsorption device [CAD]; Fig. 4).

Vesiculation indicative of PC quality

As depicted in Fig. 5, high levels of PL-EVs in fresh PCs reveal even higher PL-EV levels on Day 5 (n = 86, cc = 0.569**). Higher PLT degranulation (CD62P) on Day 0 resulted in higher PLT degranulation on Day 5 (n = 86, cc = 0.855**).

No significant correlation between PL-EVs and CD62P was found, indicating that interlaboratory variability was a storage determinant. When evaluating center-specific data, significant inverse correlations were found between PL-EVs and CD62P on Day 0 for 43 PCs (Center A, −0.43*; Center E, −0.624*) and on Day 5 for 59 PCs (Center A, −0.535**; Center D, −0.512*; Center E, −0.561*). For these 59 PCs, a correlation was found between PL-EVs on Day 5 and CD62P on Day 0 (Center A, −0.492**; Center D, −0.512*; Center E, −0.737**). These associations were not specific for a particular preparation technique (sd-PCs vs. PI-PCs).

DISCUSSION

PL-EVs may play a role in coagulation, immune function, and cell-to-cell communication.[36-40] Circulating in patients with cardiovascular diseases, they become more important

as players in pathogenesis and as biomarkers.[41-43] In trauma patients, endogenous PL-EVs negatively correlated with mortality and transfusion requirements.[21,44] PL-EVs have been suggested to be responsible for numerous side effects after PC transfusion.[45,46] However, the role and function of PL-EVs during apheresis, PLT storage, and after transfusion remains unclear and is in the focus of current research.

This study aimed to evaluate the potential use of PL-EV analysis in the QC of PCs. To achieve this, we successfully applied a previously established QC panel[34] in a multicenter study. Each blood donation center validated the protocol in their instrument settings and procedures. Using a bead solution for calibration of the different conventional flow cytometers, the dependence of flow cytometer settings and resolution was minimized as described by Robert and colleagues and the International Society on Thrombosis and Haemostasis workshop.[22,47] The use of calibration beads, as published by several groups,[27,29,30,32,47-49] provides an appropriate strategy for standardizing the determination of FCM-based PL-EV levels.

Two main factors influencing PL-EVs in PCs have become apparent: donor-related and apheresis-related variables. PL-EV analysis in PCs revealed a possible relationship between metabolomic variables from healthy donors and PLT degranulation. Blood CHOL levels may be important for short-time vesiculation; however, PLT-specific variables of the donors (PLT count, IPF, MPV) appear to be more useful in assessing PLT degranulation during storage under in vitro conditions. CHOL is an important determinant for the membrane fluidity of blood cells and an increased amount of free CHOL relative to phospholipids in PLTs has been previously reported.[50] The characteristic lipid composition of PLTs reflects nonfusogenic membrane features compared to WBCs and may be required for protection against early PLT activation. Incorporation of CHOL into the PLT membrane after the addition of lipid solutions has been observed to limit PLT responsiveness to shear stress,[51] which may be indicative of fewer membrane vesicle formation processes. In our study, this hypothesis was supported by the negative correlation between PL-EVs and CHOL and LDL-CHOL in donor blood samples.

The IPF of donor blood samples is a marker of PLT turnover and megakaryopoietic activity in the marrow. The negative correlation between PL-EVs and PLT variables (IPF, PLT distribution width, and MPV) of donors shown in this study may demonstrate that the age of PLTs in peripheral blood before apheresis influences their vesiculation in concentrates, although IPF contributes only to approximately 1% in blood.

Furthermore, apheresis settings influenced PL-EVs, such as the type of instrument and additional preparation techniques (e.g., PI). PLT activation during PC preparation is an unavoidable consequence and results in PLT fragmentation, vesiculation, and degradation. PL-EVs as a QC variable for PCs can be used to estimate the mechanical stress of preparation techniques and storage (agitation). Our data demonstrate that PLTs in PCs from Amicus instruments were more significantly activated and shed more PL-EVs than those collected with the Trima Accel. Similar findings with Amicus instruments were previously demonstrated by Hagberg and coworkers,[12] who found an increased size of PLT aggregates, microparticle fraction, and degranulation and a reduced response to agonist stimulation. Analysis of PL-EVs influenced by apheresis instruments could be used for optimization of the apheresis process to prevent avoidable PLT activation.

PL-EVs increased during storage in 86% of investigated PCs. The decrease in PL-EV levels in PCs from Center B only may be explained by different instrument settings of their conventional flow cytometer, causing a relevant variation in size resolution. This variation affected relative PL-EV analysis (percent to PLT count), but not the absolute quantification of PL-EV count (/μL), which showed a slight but nonsignificant increase. PL-EVs should also be subsequently quantitatively (/μL) counted when using conventional FCM. The decrease in PC volume after storage was due to PC sampling for laboratory analysis. We assumed that this did not have an important influence on the PLT to PL-EVs ratio, because both were equally distributed within the samples.

In this study, PI was used by one center and autologous plasma of PCs was replaced with InterSol AS before photochemical treatment with the Intercept technology.[35] This procedure consists of two major steps. First, the blood component is mixed with amotosalen and the suspension is illuminated with UVA light. Second, residual amotosalen and photoproducts are adsorbed in a CAD enclosed in a 35 μm/11% polyester mesh pouch. Finally, the PI-PC is filtered to eliminate microaggregates.

As previously described, this procedure influences PLT activation and fragmentation, thus altering vesicle concentration.[52-55] Compared with other ASs (Composol, SSP+, M-sol), InterSol induced high baseline activation and metabolism of PLTs (lower TRAP-6–induced PLT aggregation or decreased hypertonic shock response).[4,53] The proposed in vitro rating system for stored PCs by van der Meer and coworkers[55] (including lactate concentration, CD62P expression, and annexin A5 binding) showed a higher rating for PLTs in plasma, SSP+, and Composol compared to PLTs in other ASs (e.g., InterSol, T-sol). There are also reports showing that functional and proteomic properties of PLTs treated with the Intercept System (using ASs such as InterSol, SSP+) are well preserved and do not differ from the controls.[52,54] The low content of PL-EVs in Intercept- and Intersol-treated PCs from Center E is attributable to the replacement of donor plasma including PL-EVs by AS and most likely to the absorption processes (CAD, filtration) as shown by electron microscopy.

Therefore, PI procedures require separate PL-EV quantification strategies, reference values, and additional validation studies, because vesicle concentration is significantly different in comparison to sd-PCs.

In summary, evaluation of agonist-induced PLT degranulation and vesicle analysis in stored PCs was successfully performed in a multicenter study. The analysis protocol included the measurement of CD62P expression on PLTs after stimulation (e.g., TRAP-6) and PL-EVs by FCM. This protocol for PC QC offers additional information on the quality of the blood component. Furthermore, it may contribute to refining the production process and provide increased knowledge regarding factors important for an effective PLT transfusion therapy.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

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Fig. S1. Protocol for quality control (QC) program of the multicenter study. Five blood donation centers participated in the study (A–E). Analysis of platelet concentrates (PC) was performed before (day 0) and after storage (day 5). The QC program included determination of platelet (PLT) count in hematology analyzers, PLT degranulation (P-selectin expression in response to thrombin receptor PAR1 activation), and platelet-derived extracellular vesicles (PL-EVs) by flow cytometry.

Fig. S2. PL-EV analysis of platelet concentrates using size-calibrated fluorescent beads and PC7-labeled mouse IgG_1 against CD61. **A**: For calibration of the flow cytometer, polystyrene bead subsets (e.g., 0.5 μm, 0.9 μm, and 3.0 μm) were selected and instruments settings (PMT voltage) were adjusted on the basis of side scatter (SSC) and fluorescence (fluorescein isothiocyanate = FITC). **B**: Gating of platelets (PLTs) was based on selection of events greater than 0.9 μm in a FS log x SSC log cytogram (not shown) that were CD61-PC7 positive. **C**: PL-EV region was constructed including CD61-PC7 positive events in maximal size of 0.5 μm (FS log x SSC log cytogram). Ratio of PL-EV counts to PLT counts was calculated in % (PL-EVs = 7%; representative for platelet concentrates on day 0).

Fig. S3. PLT degranulation test using CD62P externalization on PLTs surface measured by flow cytometry. Immunostaining of platelets (PLTs) was performed with fluorescent labeled IgG1 against CD62P (FITC) and CD61 (PerCP). Gating strategies based on (**A**, **D**) forward scatter (FSC) and side scatter (SSC) of all cells, and fluorescence of (**B**, **E**) CD61 positively labeled PLTs, and (**C**, **F**) CD62P/CD61 positively labeled PLTs. The fluorescence of basal CD62P (without agonist) and agonist-induced CD62P (10 μM TRAP-6 = thrombin receptor activating peptide 6) was indicated in the mean fluorescent intensity (MFI) and a ratio was calculated (CD62P: MFITRAP-6 *100%/MFIbasal).

Fig. S4. Correlation between donor-specific parameters and PL-EVs. Scatter plots show a negative correlation between PL-EVs and donors parameters for **A**: cholesterol and LDL-cholesterol (day 0, sd-PCs in centers A–C), and **B**: on day 5 for immature platelet fraction (IPF; sd-PCs in centers A–B); platelet distribution width (PDW; sd-PCs in centers A–C); mean platelet volume (MPV; sd- and PI-PCs in centers A–E) (cc, correlation coefficient).

Table S1. Listed instrument settings used in the multicenter study. In five blood donation centers (A–E), platelet concentrates (PCs) were collected using either the collection system Trima Accel or Amicus. In center E, pathogen inactivation of PCs was performed (*). For detection of platelet-derived extracellular vesicles (PL-EVs) in PCs and platelet degranulation (P-selectin [CD62P] expression in response to thrombin receptor activating peptide 6 [TRAP-6]), three flow cytometers were used: FACSCanto II, Navios, or Gallios.

Table S2. Donor characteristics evaluated in five blood donation centers A–E on day 0. n.s., not specified; BMI, body mass index; HBG, hemoglobin; HCT, hematocrit; IPF, immature platelet factor; PDW, platelet distribution width; MPV, mean platelet volume; CHOL, cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TRIG, triglycerides; GLC, glucose; CRP, C-reactive protein.

Table S3. PC parameters evaluated in five blood donation centers A–E. Values describe range (min, max), mean, and standard deviation (SD) for the number of cases (n; except for parameters marked with *, n = 15).

Table S4. PC-specific parameters on day 0 of PCs produced in center E using pathogen inactivation (PI). Differences were found in 13 PI-PCs before vs. after PI by Wilcoxon test, two-tailed. In centers A–D, PI was not used.