Short Communication

Platelet Extracellular Vesicles in Cord Blood of Term and Preterm Newborns Assayed by Flow Cytometry: the Effect of Delay in Sample Preparation and of Sample Freezing

(platelets / cord blood / newborns / extracellular vesicles / flow cytometry)

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Abstract. Plasma levels of circulating platelet extracellular vesicles (PEVs) are an emerging marker of platelet activation, thrombosis, inflammation, and endothelial dysfunction. Analysis of PEVs in cord blood of preterm newborns may reflect the underlying pathology and possibly serve as a new diagnostic and prognostic tool. However, collection, preparation and analysis of cord blood samples in clinical settings is a logistically complex process. We have studied the effect of delay in sample preparation and sample freezing on the PEV analysis by flow cytometry. PEVs in the cord blood plasma were identified after double labelling with monoclonal antibodies CD36+CD41 or CD41+CD62. Both, the delay and the freezing significantly affected the count and often also fluorescence of the detected PEVs. Additionally, our pilot study utilizing fresh cord blood samples of term and preterm newborns demonstrated significantly decreased CD36 and CD62 PEV fluorescence in preterm newborns. Our data highlight the importance of pre-analytical steps in the analysis of cord blood PEVs and suggest that not only the count, but also the level of PEV fluorescence may have possible diagnostic potential.

Received September 26, 2020. Accepted January 4, 2021.

The study was supported by grant NV17-31403A from the Czech Health Research Council.

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Abbreviations: EVs – extracellular vesicles, FIRS – foetal inflammatory response syndrom, FSC – forward scatter, MAb – monoclonal antibody, PBS-BSA – phosphate-buffered saline with bovine serum albumin, PEVs – platelet extracellular vesicles, PFP – platelet-free plasma, PPP – platelet-poor plasma, SSC – side scatter.

Introduction

Blood platelets are well recognized for their role in haemostasis, but they also participate in many other physiological and pathological processes. The importance of platelet involvement in the immune response and inflammation is increasingly recognized (Kerris et al., 2020). Platelets express complement and Toll-like receptors, which allow them to recognize invading microbes. Platelets can directly participate in the clearance of bacterial infection by sequestering and killing pathogens or by limiting their growth by secretion of antimicrobial peptides. They also play a part in the presentation of the trapped bacteria to neutrophils and other immune cells. Upon activation, platelets release a plethora of active substances, many of them with a direct effect on inflammation processes. Among them, soluble CD40L modulates differentiation of dendritic cells and B lymphocytes and triggers expression of adhesion receptors by endothelial cells, facilitating leukocyte homing. Cytokines and chemokines released from platelet storage granules exert both pro-inflammatory and anti-inflammatory properties (Kapur et al., 2015). In addition, platelets also release different populations of extracellular membrane vesicles, which carry biologically active molecules and participate in modulation of coagulation and inflammatory response (De Paoli et al., 2018). Plasma levels of circulating platelet extracellular vesicles (PEVs) are an emerging marker of platelet activation, thrombosis, inflammation and endothelial dysfunction, with a possible prognostic value (Kerris et al., 2020).

One area where the involvement of PEVs remains insufficiently studied are newborn complications. Preterm birth contributes to over 70 % of perinatal mortality in developed countries. The most common cause of preterm birth is inflammation of the foetal membranes. The infection is usually caused by microorganisms ascending from the vagina, but can include haematogenous

spread, invasion from peritoneal cavity or iatrogenic infection after invasive procedures (Galinsky et al., 2013). Microbial colonization initiates the maternal and foetal inflammatory response (FIRS), which is accompanied by recruitment and activation of immune cells (Gotsch et al., 2007). The involvement of platelets and PEVs in the process and the possible diagnostic potential of PEVs in FIRS remain to be elucidated. Sampling venous cord blood represents an attractive alternative to venous blood collection, especially in extremely preterm birth infants where the availability of venous blood is limited. A number of studies utilized flow cytometry for quantitative analysis of PEVs in the blood plasma, but studies of PEVs in the cord blood are extremely rare (Schweintzger et al., 2011; Campello et al., 2015). Collection and analysis of cord blood samples in clinical settings is complicated by numerous logistic issues. Unpredictable time of delivery may lead to delays in sample preparation, creating space for possible confounding factors of PEV analysis. Our study was focused on evaluating the effect of delay in the sample preparation on the results of PEV analysis by flow cytometry. We suggest an alternative procedure for analysis of fresh cord blood samples that we utilized in a pilot comparative study of PEVs in term and preterm newborns. Additionally, we describe substantial changes in the number and fluorescence of PEVs after cord blood plasma freezing.

Material and Methods

Monoclonal antibodies and calibrating beads

Mouse monoclonal antibodies (MAb) against platelet markers: CD36 FITC (IgG1, clone TR9); CD41 FITC and CD41 PE (IgG1, clone MEM-06) and mouse IgG1 isotype FITC and PE controls (clones MOPC-21 and PPV-06) were from Exbio (Vestec, Czech Republic). MAb CD62 PE (IgG1, clone CRC81) was from Thermo Fisher Scientific (Waltham, MA). ApogeeMix beads were obtained from Apogee Flow Systems (cat. no. 1493, Hemel Hempstead, UK). All MAbs were titrated to estimate their saturating concentration using EDTA-anticoagulated blood of a healthy donor (Vostal et al., 2001).

Patients and cord blood collection

Only patients undergoing caesarean section were included into the study. The study was approved by the ethical committee of ICMC (no. 2015/06-02-4) and signed informed consents were obtained from all participants. Placenta manually removed at caesarean section was immediately transported at room temperature to an adjacent collection room, and 7.2 ml of umbilical venous blood was collected into a syringe containing 0.8 ml of 3.8% sodium citrate. The blood was transferred into a 12 ml polypropylene tube and gently mixed by the tube inversion. Further processing of the samples proceeded after their transport to the laboratory located 15 min away by public transport. The samples were transported at ambient temperature in a polystyrene transport box with care to prevent shaking of the samples. In indicated instances, automated blood cell count was done using cell counter Bayer Advia 60 (Siemens, Germany).

Preparation of cord blood plasma

The blood was centrifuged in a swing-out rotor (3000 *g*, 15 min, RT) to prepare platelet-poor plasma (PPP) devoid of blood cells and the majority of platelets. Upper 2.5 ml of PPP was transferred into a fresh 12 ml polypropylene tube and centrifuged again (3000 *g*, 15 min, 4°C) to sediment the remaining platelets. The platelet-free plasma (PFP) was kept on ice and 1 ml aliquot was used for isolation of EVs. In the study of the effect of plasma freezing, PPP was prepared in the hospital and transported on ice to the laboratory. Afterward, one 1.2 ml aliquot of PPP was processed immediately, and the second 1.2 ml aliquot was frozen on dry ice and stored at −80 °C for 1–2 weeks. After thawing, preparation continued with the second centrifugation to prepare PFP as with the fresh PPP aliquot. The samples in the freezing study were labelled and analysed in duplicates and the presented data are mean values of the duplicates.

Study of the effect of delay in the sample preparation

Two approaches to sample preparation were compared. In the first one, aliquots of anticoagulated cord blood transported to the laboratory were either processed immediately or left on table for 6 h at 24 °C before the processing. In the second one, preparation of the plasma in the laboratory started within 30 min after cord blood collection, and one PPP aliquot was processed directly and the second aliquot after 6 h refrigeration at 4 °C.

Comparative study of PEVs in the cord blood of term and preterm newborns

The cord blood of term (gestational age 39 ± 0.6 weeks, birth weight 3233 ± 271 g, N = 7) and preterm (gestational age 31.6 ± 2.7 weeks, birth weight 1568 ± 220 g, N = 5) newborns were processed by the approach incorporating immediate preparation of PPP in the hospital and its storage in a fridge before the transport on ice to the laboratory for direct analysis.

Isolation and labelling of EVs

The aliquot of PFP (1 ml) was centrifuged in a fixed-angle rotor (15,000 *g*, 20 min, 4 °C) to sediment EVs. The supernatant was carefully removed and the EVs resuspended in 0.5 ml of 0.1 μm filtered phosphate-buffered saline with 0.1 % bovine serum albumin, pH 7.4 (PBS-BSA). The labelling was carried out in 1.5 ml Eppendorf tubes containing 40 μl aliquots of EV suspension and 10 μl of a mix of monoclonal antibodies (CD36 FITC + CD41 PE, CD41 FITC + CD62 PE or

isotype controls IgG1 FITC + IgG1 PE). After 30 min incubation on ice, the samples were washed with 1 ml of ice-cold PBS-BSA (15,000 *g*, 20 min, 4 °C) and the pelleted EVs resuspended in 300 µl of ice-cold PBS-BSA.

Flow cytometry analysis of EVs

The analysis was carried out in a FACSCanto™ II cytometer (Becton Dickinson) in original configuration. The performance of the cytometer was checked daily by running CST calibration beads (Becton Dickinson), as suggested by the manufacturer. The flow rate was set on low (10 µl/min) and the threshold was lowered to the minimum (200 for both FSC and SSC with parameter "AND"). Diluted whole blood was used to create the platelet gate in the upper right corner of the FSC-A/SSC-A logarithmic plot and the rectangular EV gate was created bellow the platelet gate. ApogeeMix beads were used to estimate the size of particles detected within the EV gate and the resolution limit of the cytometer. Each sample was collected for 2 min (equivalent of 20 µl). PBS-BSA buffer used for EV dilution was checked for the background noise level. The compensation of the samples and the data analysis were carried out using FlowJo software ver. 8.8.6. (Tree Star, Inc, Pasadena, CA). The non-labelled sample of EVs was used to set the position of the negative population. Only double-labelled platelet EVs (CD36+CD41+ and CD41+CD62+) were included into the analysis, and their number described as count/µl of plasma was corrected by subtraction of the number of EVs double-labelled using the isotype control MAbs. The median fluorescence intensity of PEVs was utilized to compare the level of binding of individual MAbs to PEVs.

Statistical analysis

Statistical analysis was performed using SigmaStat (version 3.5, Systat Software). The data were analysed by paired *t*-test or Wilcoxon signed rank test for data with or without normal distribution, respectively. Unpaired *t*-test was used to analyse the data comparing term and preterm cord blood. Data in the text are presented as mean ± s.d. and the differences were considered as significant for the P value < 0.05.

Results and Discussion

Flow cytometry is a well-established technique of PEV analysis allowing their straightforward quantification (Nolan and Jones, 2017), but it has some inherent limitations. The resolution of standard flow cytometers permits analysis of large EVs with a diameter > 0.3 µm (De Rond et al., 2020). More numerous smaller EVs are not discriminated and their signal may overlap with the background noise. To establish the EV gate on the scattergram plot, we defined its upper limit to leave the signal of platelets in the diluted blood sample out (Fig. 1A). Analysis of standard silica beads with a refractive index (RI =1.43) similar to EVs demonstrated that 1300 nm beads were detected outside and 880 nm beads inside the EV gate. While 880 nm and 590 nm beads were detected as separate populations, smaller beads (300 + 240 + 180 nm) formed one population, suggesting that the resolution of our cytometer is about 500 nm for the SSC channel, which is in agreement with the information provided by the manufacturer (Fig. 1B). PEVs were double labelled using MAbs directed against well-established platelet markers, and only double-positive events were counted as PEVs. The reason for utilization of double labelling was to limit the chance of inadvertent

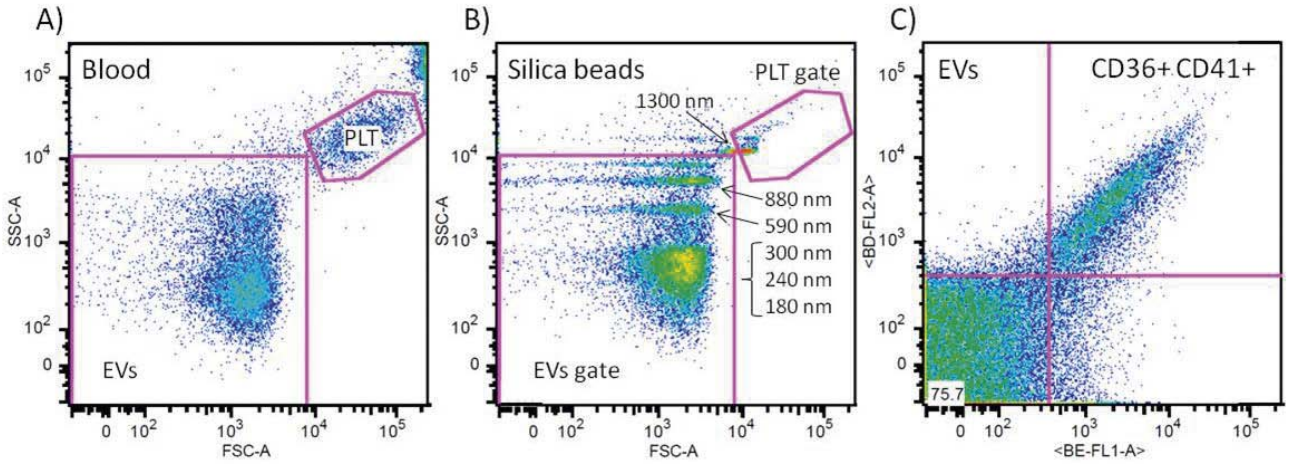

Fig. 1. Gating strategy for detection of platelet extracellular vesicles by flow cytometry. The EV gate was set up on the scattergram to exclude platelets (PLT) and other blood cells (**A**). The size of EVs detected in the gate was estimated by standard silica beads (**B**). 880 nm and 1300 nm beads were detected inside and outside the gate, respectively. Typical fluorescent signal of PEVs in the EV gate after labelling with CD36 FITC (FL1) and CD41 PE (FL2) MAbs (**C**). Platelet CD36+CD41+ double-positive EVs are present in the upper right quadrant of the dot plot

detection of non-specifically labelled particles as PEVs. The signal of double-labelled PEVs was well separated from the negative particle population (Fig. 1C) and their count varied from several hundreds to thousands/µl of cord blood plasma. In contrast, the count of double-positive particles after labelling with isotype control MAbs was very low, reaching < 12 events/µl of cord blood plasma (data not shown).

The calculation of PEV counts was based on the assumption that the low flow rate of sample analysis was 10 µl/min. The control experiment with manually counted fluorescent beads carried out after the completion of our studies suggested the actual flow rate 9.4 µl/min (data not shown), which is reasonably close to the value provided by the manufacturer. As platelet markers we utilized thrombospondin receptor (CD36), integrin subunit α IIb (CD41) and platelet activation marker P-selectin (CD62). While CD36 and CD41 are expressed on the surface of both resting and activated platelets, the expression of CD62 on resting platelets is low and increases after platelet activation (Simak et al., 1999). In our hands, the count of CD36+CD41+ PEVs in the cord blood was always higher than the count of CD41+CD62+ PEVs, suggesting that just a portion of detected PEVs originate from activated platelets. Development of the PEV analytical protocol was carried out with fresh cord blood processed within 1 h after its collection. Immediate analysis of fresh blood samples is recommended to limit possible pre-analytical artefacts of PEV analysis (Dey-Hazra et al., 2010).

However, as our flow cytometry laboratory is not located in the hospital and the time of patient cord blood sampling is unpredictable, we have evaluated the effect of the delay in sample preparation on the results of PEV analysis. The comparison of blood aliquots analysed within 1–2 h after the cord blood sampling with the aliquots that were left 6 h standing upright on the table at room temperature demonstrated significant changes in the counts of both platelets and detected PEVs. While the count of platelets in anticoagulated blood measured by the cell counter decreased from $212 \pm 35 \times 10^3$/µl to $168 \pm 43 \times 10^3$ / µl (Fig. 2A), the count of CD36+CD41+ PEVs tripled from 1830 ± 1010/µl to 5540 ± 1660 / µl (Fig. 2B). Similarly, the count of CD41+CD62+ PEVs increased from 890 ± 450/µl to 2530 ± 630/µl (Fig. 2C). The changes in the median fluorescence intensity of the detected PEVs were modest, reaching significance only for CD41 MAbs (Fig. 2D-F).

Our data demonstrate that the delay in the processing of citrate-anticoagulated cord blood may grossly affect the count of detected PEVs. The increase of the PEV count in venous citrated blood during storage was reported previously (Ayers et al., 2011) and together with our results suggests that the delay in the cord blood processing should be avoided. Next, we tested a method of sample preparation aimed at limiting storage-related artificial generation of PEVs. The method consisted of a single centrifugation step aimed to remove blood cells, including the majority of platelets, and of plasma withdrawal. The PPP was stored in a fridge for 6 h. The com-

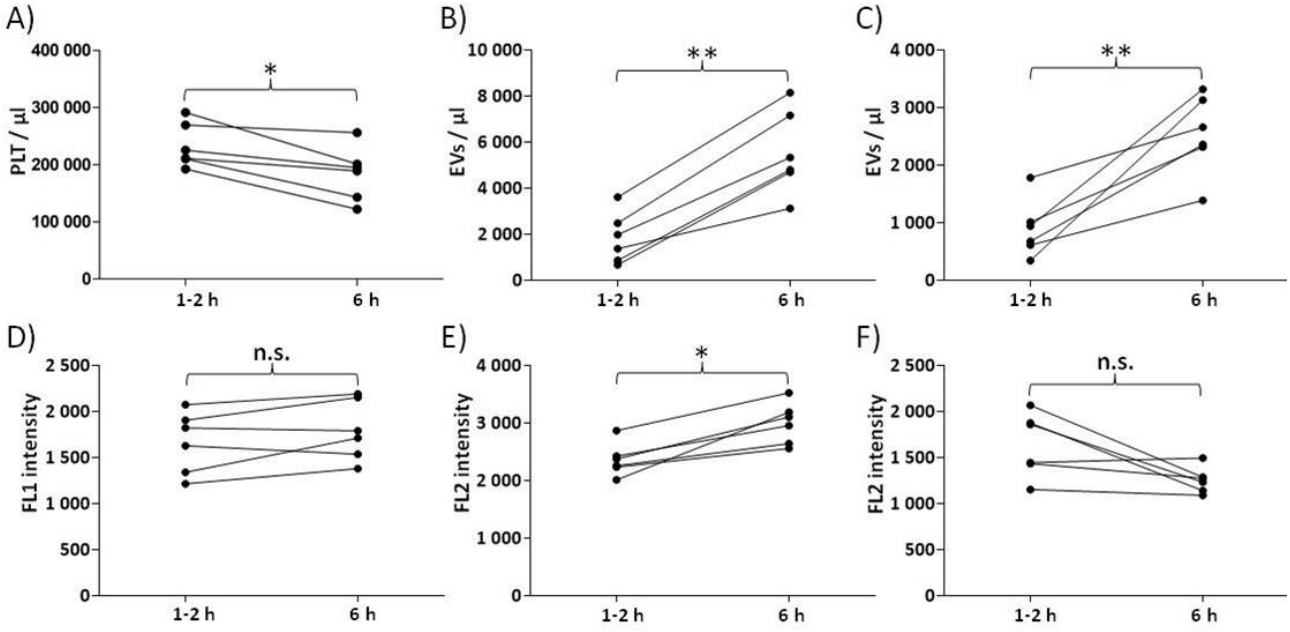

Fig. 2. The effect of the delay in preparation of cord blood plasma on the detection of platelet EVs by flow cytometry. The anticoagulated cord blood was processed either within 1–2 h after its collection or after 6 h (N = 6). The delay in the preparation of blood plasma was associated with a decrease in platelet count in the blood (**A**) and increased presence of CD36+CD41+ (**B**) and CD41+CD62+ (**C**) PEVs in the prepared plasma. The changes in the median fluorescence intensity of PEVs labelled by CD36 FITC (D), CD41 PE (E) and CD62 PE (F) were less dramatic (*P < 0.05, **P < 0.01).

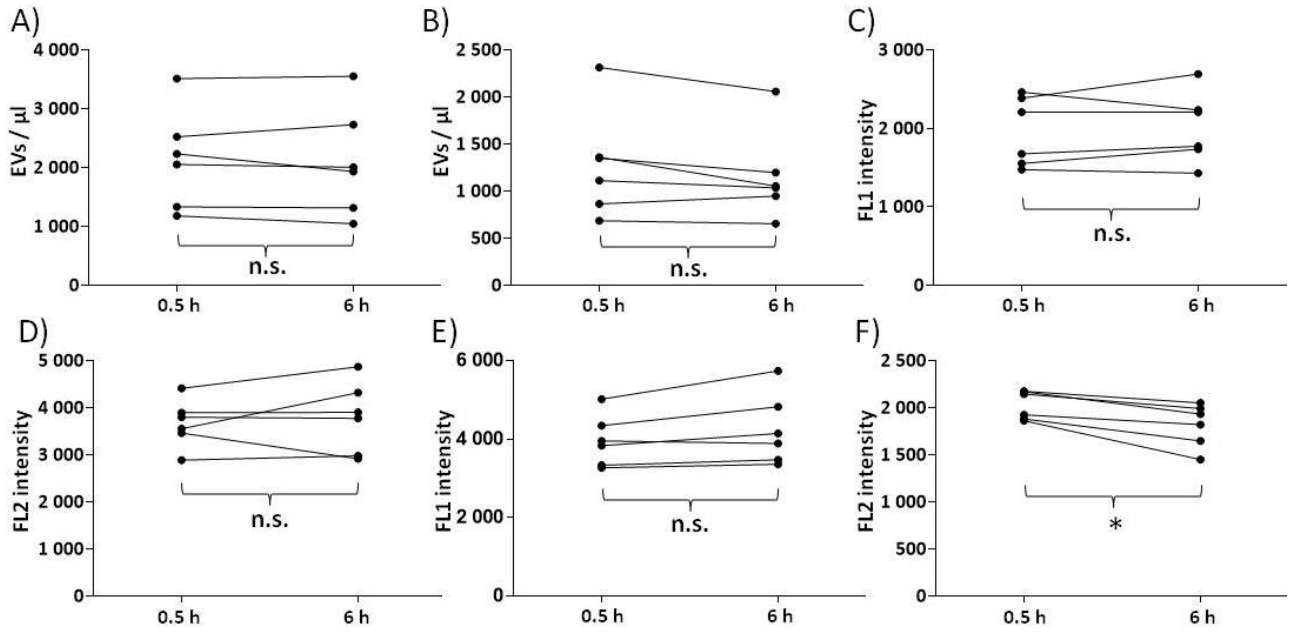

Fig. 3. The effect of storage of cord blood plasma on the detection of platelet EVs by flow cytometry. The plasma (N = 6) was prepared immediately after collection of the cord blood and analysed within 0.5 h or stored for 6 h in a fridge before analysis. No differences in the counts of detected platelet EVs double CD36+CD41+ (**A**) or CD41+CD62+ (**B**) positive were observed. Similarly, changes in the median fluorescence intensity of PEVs labelled with CD36 FITC (**C**), CD41 PE (**D**), CD41 FITC (**E**) were not significant. The only exception was slightly decreased fluorescence after labelling with CD62 PE (**F**) (*P < 0.05).

parison with fresh, immediately processed blood aliquots did not show any differences in the count of CD36+CD41+ or CD41+CD62+ PEVs (Fig. 3 A,B). Similarly, no difference in the median fluorescence intensity of the detected PEVs was detected, except for slightly decreased signal of CD62 PE MAb (Fig. 3 D-F).

Our data are in agreement with a previous study of Jayachandran et al. (2012) on venous blood and demonstrate the stability of PEVs in the plasma stored at 4 °C. To test the method in clinical settings, we carried out a small comparative study of PEVs in the cord blood of term and preterm newborns. The PPP samples were prepared directly in the hospital and stored in a fridge before the transport on ice to the laboratory.

We found no significant differences in the count of CD36+CD41+ and CD41+CD62+ PEVs between term and preterm newborns (Fig. 4 A,B). While this finding must be treated with caution as the number of patients included in the study was small, our data suggest that the counts of PEVs in the cord blood of preterm newborns do not deviate grossly from the counts recorded in the term newborns. On the other hand, PEVs of preterm newborns had significantly lower median intensity of fluorescence after labelling with CD36 and CD62 (Fig. 4 C,D), but not with CD41 (data not shown). The correlation of platelet CD62 expression with gestational age was reported previously (Wasiluk et al., 2008), suggesting that lower fluorescence of PEVs after CD62 labelling may reflect its lower expression in preterm newborns. Recently, lower formation of platelet-neutrophil aggregates in the cord blood of preterm neonates was reported (Esiaba et al., 2019), implying that the CD62 deficit may also have functional consequences. Also, the expression of CD41a, CD42b and CD61 was reported lower on platelets of immature preterm newborns (Sitaru et al., 2005), suggesting that quantitative analysis of PEVs in the cord blood may be complicated by their gestational age-related differences in the expression of platelet markers. In our hands, the intensity of PEV labelling with CD41 FITC or CD41 PE MAbs was gestational age insensitive, but this result must be treated with caution due to a small size of experimental cohorts. Even though our study demonstrated that analysis of PEVs in fresh cord blood samples is possible, most published studies of PEVs have utilized frozen samples of blood plasma.

The sample freezing in PEV analysis represents a controversial issue, but many times it is the only option to overcome the logistic hurdles of clinical sample analysis. To better understand the effect of plasma freezing on the analysis of PEVs, we compared aliquots of fresh and on dry ice frozen PPP samples. In our hands, single freezing of PPP led to a significant decrease in detected PEVs: 990 ± 480 vs 1650 ± 650 µl for CD36+CD41+ PEVs and 240 ± 160 vs 440 ± 240 µl for CD41+CD62+ PEVs (Fig. 2 A,B). The decrease in the PEV count was accompanied by a significant decrease in PEV median fluorescence intensity detected by all utilized MAbs

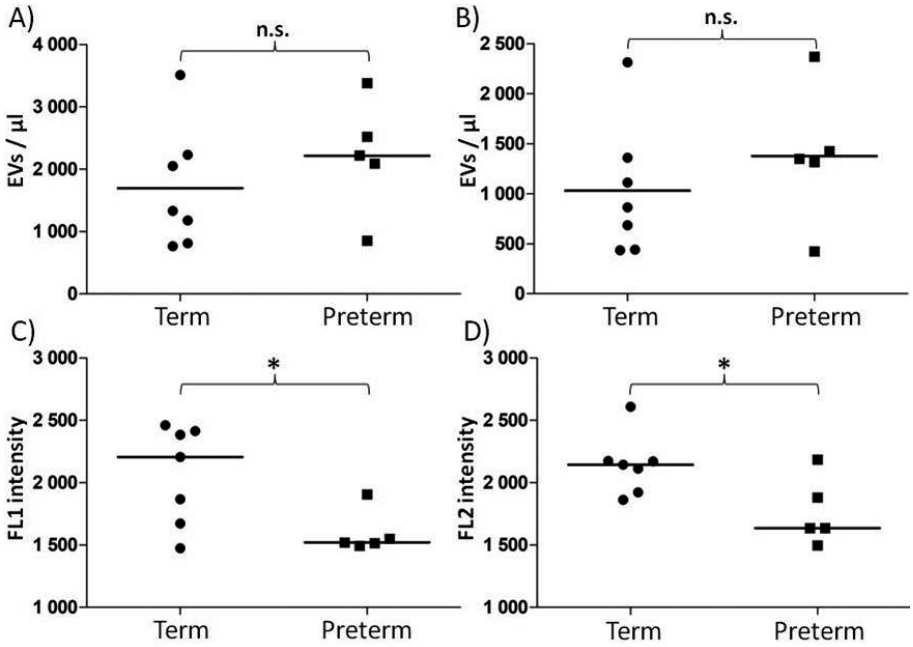

Fig. 4. Analysis of platelet EVs in the cord blood of term and preterm newborns by flow cytometry. The plasma from the cord blood of term (N = 7) and preterm (N = 5) newborns was prepared immediately after blood collection and stored in a fridge before the analysis. Differences in the numbers of double CD36+CD41+ (**A**) or CD41+CD62+ (**B**) positive PEVs were not detected. In contrast, preterm PEVs displayed lower median fluorescence intensity after labelling with CD36 FITC (**C**) and CD62 PE (**D**) (*P < 0.05).

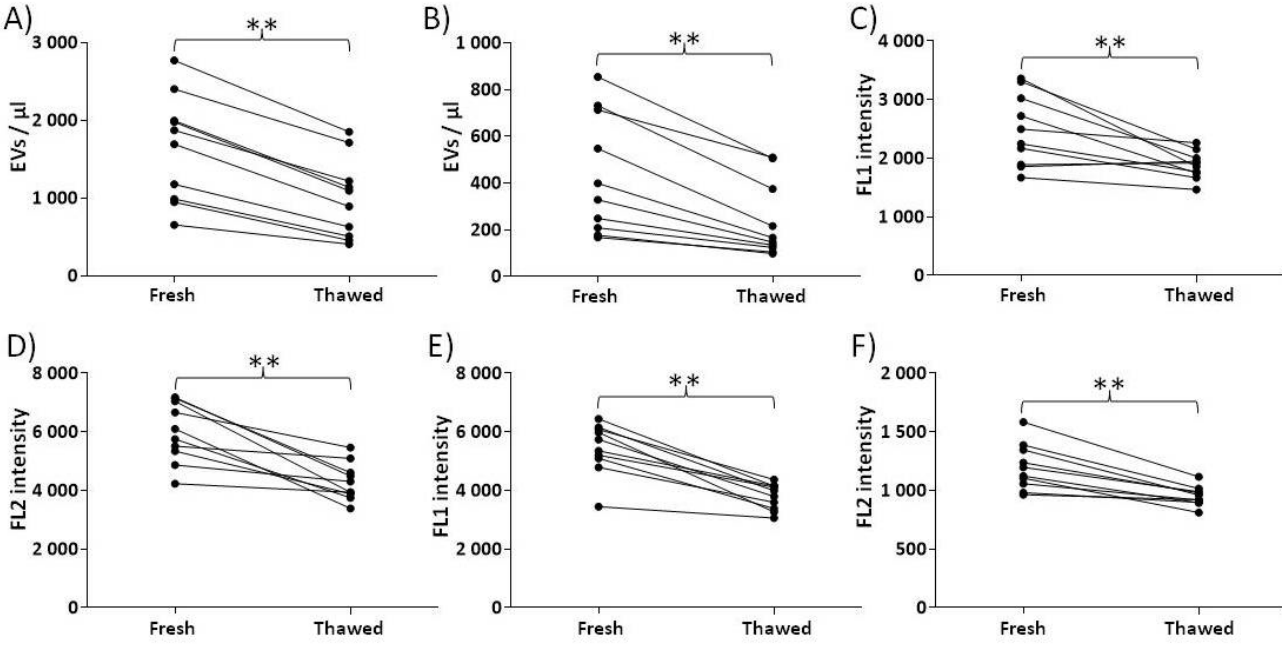

Fig. 5. The effect of cord blood plasma freezing on the detection of platelet EVs by flow cytometry. The presence of PEVs was measured in isolated plasma immediately after its preparation (Fresh) or after its freezing (Thawed) (N = 10). A significant decrease in the number of CD36+CD41+ (**A**) and CD41+CD62+ (**B**) PEVs was detected (**P < 0.01). Similarly, the freezing led to a decrease in the median fluorescence intensity of the detected PEVs for all MAbs used for their labelling: CD36 FITC (**C**), CD41 PE (**D**), CD41 FITC (**E**), and CD62 PE (**F**). Data represent the mean values of separately labelled duplicates (**P < 0.01).

(Fig. 2 C-E). The differences between fresh and thawed samples were 2480 ± 580 vs 1880 ± 220 for CD36 FITC; 5980 ± 970 vs 4290 ± 610 for CD41 PE; 5430 ± 830 vs 3790 ± 420 for CD41 FITC; and 1200 ± 190 vs 950 ± 80 for CD62 PE. The mechanism behind the concomitant decrease in the PEV count and fluorescence in our study is not known. It seems improbable that the simple loss of a portion of PEVs after freezing (e.g., by their adhesion to tube walls) would also cause the decrease in the PEV fluorescence. We can speculate that formation of ice crystals and changes in the plasma osmolality during freezing (Elliott et al., 2017) led to damage to the PEV membranes resulting in PEV fragmentation and/or partial inside-out membrane inversion.

The fragmentation would lead to formation of smaller, less fluorescent PEV populations that might not be detected by standard flow cytometry. Similarly, the inside-out inversion would cause decreased availability of marker antigens on the PEV surface, leading to their lower labelling and decreased detection. The fragmentation hypothesis seems to be supported by significantly lower side scatter (SSC-A) of thawed CD36+CD41+ PEVs (566 ± 99 vs 814 ± 194; $P < 0.001$; not shown). Our data contrast with studies reporting no significant effect or increase in the EV count detected by flow cytometry after the plasma freezing (Ayers et al., 2011; Yuana et al. 2015). While the discrepancy likely originates in the differences among the freezing and thawing protocols, it also revitalizes discussion about the possible effect of freezing on the PEV analysis.

In conclusion, the diagnostic potential of cord blood PEVs in preterm newborns represents an interesting area of biomedical research and may contribute to the ongoing search for new prognostic tools for management of newborn complications. Our study suggests that analysis of fresh cord blood plasma samples may be preferable to the analysis of frozen samples. Lower labelling of PEVs in frozen samples described in our study could complicate flow cytometry analysis, especially in immature preterm newborns expressing lower levels of platelet markers utilized for the PEV detection. It is possible that the full diagnostic potential of PEVs in the cord blood could be unlocked only after technological improvements leading to significant improvements of flow cytometry resolution (Tertel et al., 2020) and standardization of all aspects of PEV analysis (Welsh et al., 2020). Our study described significant differences in the fluorescence intensity of PEVs between term and preterm newborns. Further studies utilizing well-defined patient cohorts will clarify whether the PEV count and the level of platelet marker expression on PEVs have a diagnostic and prognostic value in preterm newborns with a foetal inflammatory response.

Acknowledgement

We thank Dr. Jan Simak (FDA, Maryland) for helpful comments and suggestions.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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