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by Zankruti Dave, Per Sandgren and André Görgens; Joel Nordin

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Platelet-derived extracellular vesicles are relevant for clot stability in cryopreserved platelets

Zankruti Dave[1,#], Per Sandgren[2,#], André Görgens[1,3,4,§,*], Joel Nordin[1,4,5,§,**]

[1]Division of Biomolecular and Cellular Medicine, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

[2]Department of Center for Hematology and Regenerative Medicine (HERM), Karolinska Institutet, Stockholm, Sweden

[3]Department of Cellular Therapy and Allogeneic Stem Cell Transplantation (CAST), Karolinska University Hospital, Huddinge, Sweden

[4]Karolinska ATMP Center, Karolinska Institutet, ANA Futura, Huddinge, Sweden

[5]Department of Clinical Immunology and Transfusion Medicine (KITM), Karolinska University Hospital, Stockholm, Sweden

# These authors contributed equally

§ These authors contributed equally

* Corresponding author: Division of Biomolecular and Cellular Medicine, Karolinska Institutet, ANA Futura, Alfred Nobels Allé 8, 14152 Stockholm, Sweden; andre.gorgens@ki.se

** Corresponding author: Division of Biomolecular and Cellular Medicine, Karolinska Institutet, ANA Futura, Alfred Nobels Allé 8, 14152 Stockholm, Sweden; joel.nordin@ki.se

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Data sharing statement: Data supporting this study are available from the corresponding author upon reasonable request.

Author contributions

Z.D. performed experiments, analyzed and curated data, and wrote the manuscript. P.S. conceived the study, performed experiments, analyzed data, and drafted the manuscript. A.G. designed the methodology, performed experiments, analyzed and curated data, and contributed to manuscript writing. J.N. conceived the study and methodology, supervised the project, provided resources, and edited the manuscript.

Disclosures

AG and JN have been consultants for and have equity interest in Evox Therapeutics, Oxford, UK. All other authors have no conflicts of interest to disclose.

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Main Text

Platelets are indispensable components of the hematological system, serving as the frontline mediators of hemostasis and wound repair. Consequently, transfused platelets are a critical life-saving intervention for patients suffering from trauma or chemotherapy-induced thrombocytopenia. Transfused platelets are vital for treating life-threatening thrombocytopenia across numerous clinical settings, yet their therapeutic utility is severely restricted by storage conditions. Unlike other blood products, platelets are conventionally stored at ambient temperature to preserve their functional post-transfusion integrity, although cold storage at 4 °C has been associated with enhanced procoagulant and clot-forming activity[1] and thus has been proposed as a viable alternative in some instances. The requirement for storage at room temperature limits their shelf life to 5–7 days and adds significant complexity to supply chain management, particularly in remote or resource-constrained settings such as small hospitals, isolated healthcare facilities, and military operational environments. The cryopreservation of platelets using dimethyl sulfoxide (DMSO) as a cryoprotective agent (CPA) has several logistical advantages and has been considered as an established approach since the 1970s. Over the past few decades, cryopreserved platelets have been extensively evaluated after thawing and are considered functional and haemostatically effective both *in vitro*[2,3] and *in vivo*[4].

Our recent publications[5,6] present a novel approach by omitting DMSO and freezing platelets in isotonic saline (0.9% NaCl, also referred to as DMSO-free). Regardless of the cryopreservation method used, platelets are significantly affected by the freezing process itself, leading to cell lysis and activation. Interestingly, this also results in a high secretion of extracellular vesicles (EVs), which play significant roles both in hemostasis and in coagulation by providing catalytic surfaces for coagulation factors[7,8].

Platelet-derived extracellular vesicles (pEVs) represent one of the most abundant subpopulations found in circulation[9], and promote clot formation, angiogenesis, and wound healing[9,10]. Recent studies demonstrate that cryopreservation profoundly alters platelet integrity and drives extensive release of EVs. Raynel et al.[7] showed that freeze–thawing with DMSO triggers a dramatic increase in platelet-derived microparticles, while Tegegn et al.[11] further reported widespread membrane disintegration and a surge in exosome-sized EVs. Together, these findings indicate that EVs produced during cryopreservation are bioactive entities with

altered composition and functional properties, underscoring the need to understand their biological roles when using cryopreserved platelets as cellular or vesicular sources.

Given the increasing recognition of EVs in cryopreserved platelet concentrates[7,11], we here comprehensively characterized EV-enriched supernatants generated from cryopreserved platelets (CPPs) that were frozen using either conventional (DMSO-based)[3,6,12] and novel NaCl-based (DMSO-free)[6] platelet cryopreservation protocols.

For this study, CPPs were prepared from double-dose platelet concentrate (n=6) produced as previously described[3,6]. Platelet concentrates (~200 mL) were mixed with freezing medium, either 25% dimethyl sulfoxide (DMSO) in isotonic NaCl (50 mL total) or isotonic NaCl alone (100 mL), and centrifuged at 1,200 × g for 10 min. Most of the supernatant was removed, leaving ~10 mL of freezing medium with the platelet pellet, resulting in ~10 mL of 5% DMSO or isotonic NaCl associated with the original platelet concentrate solution. These CPPs were frozen at –80 °C by direct transfer without the use of a controlled-rate freezing device (uncontrolled-rate freezing) (**Fig. 1A**). All CPPs were stored at -80°C for >100 days and subsequently thawed by gentle reconstitution with 100% of compatible freshly thawed donor platelet-free fresh-frozen plasma (PFFP), to a final volume of 200 mL (**Fig. 1A**)[6]. Subsequently, the samples were immediately processed to obtain the CPP supernatant and residual platelet fraction (RPF) (**Fig. 1A**). The study was conducted in accordance with the Declaration of Helsinki with approved ethical permissions granted by the Swedish Regional Ethics Review Board under ethical approval 2024-00754-02.

For assessment of cryopreserved platelet hemostatic function, we performed platelet mapping assays using the TEG 6s automated analyzer (Haemonetics Corporation, Braintree) for both types of thawed CPPs (conditions 2 and 3 in **Fig. 1B**), comparing them against unmatched fresh platelets, PFFP, and RPF of DMSO-CPP resuspended in PFFP. The Activator F (ActF), Arachidonic Acid (AA), and Adenosine Diphosphate (ADP) channels were assessed using maximum amplitude (MA) values as the primary readout. ActF (MA) reflects fibrin contribution to clot strength, ADP (MA) assesses platelet responsiveness to ADP, and AA (MA) evaluates platelet function via thromboxane A_2 generation. This approach captures interactions between platelets, plasma components, and EVs.

Fresh platelets have preserved platelet quality and functional integrity, but the ActF channel actively suppresses the mechanical contribution of platelets to the clot strength, and hence this channel displayed low, but visible, MA values (**Fig. 1B**). However, for the ADP and AA

conditions, stable MA levels were achieved due to preserved platelet quality and functional integrity. Thawed CPP, despite containing platelets with reduced functional integrity, generated stable clot firmness comparable to fresh platelets, as well as a stable clot in the ActF condition. In contrast, PFFP and RPF+PFFP failed to induce clotting in the ActF assay and induced significantly lower clot strength in the ADP and AA conditions compared to the whole CPP, demonstrating that frozen platelets fail to induce a stable clot without external factors in the CPP supernatant (**Fig. 1B**). These results suggest that stable MA can be achieved via different pathways: intact fresh platelets rely primarily on intrinsic platelet functionality, whereas affected platelets in thawed CPP appear to depend on CPP supernatant-associated procoagulant activity to reach sufficient hemostatic efficiency. EVs may therefore play a compensatory role in sustaining clot formation when platelet function is compromised.

Therefore, next, nanoparticles in CPP supernatants derived from Step 4a in **Fig. 1A** were characterized by ZetaView® Twin platform (ParticleMetrix) which facilitates quantification of particle diameters and concentrations. Analysis of particle size profiles revealed that nanoparticles derived from cryopreserved units had significantly larger mean diameters than those from fresh platelets (**Fig. 1C, Supplemental Fig. 1**). The supernatant from DMSO-supplemented CPPs appeared to contain a higher concentration of particles compared to the DMSO-free group (**Fig. 1D**).

EV-specific analysis in respective samples by multiplex bead-based EV flow cytometry (MACSPlex EV Kit, human, Miltenyi Biotec) with previously optimized protocols[13] confirmed a high abundance of platelet markers (CD41, CD42b, CD61, CD62P) and tetraspanins (CD9, CD63, CD81) specifically in the CPP supernatants (**Fig. 2A, Supplemental Fig. 2**).

EVs were next analyzed at the single vesicle level by high resolution Imaging Flow Cytometry (IFCM) on a Cellstream instrument (Amnis/Cytek) following staining with FITC-conjugated antibodies (CD9, CD81, CD61, or PAN-FITC) and gated as SSC(low) based on reference material, as described previously[13,14]. IFCM analysis identified a novel population of larger EVs, SSC(int), positive for the EV marker CD9 and the platelet marker CD61, appearing specifically post-cryopreservation in the DMSO-free group (**Fig. 2B**).

To further substantiate the role of EVs particularly with regard to their contribution to clot stability, we next isolated 10k and 100k EV fractions using an established protocol (**Fig. 3A**). For preparation of EVs, DMSO and DMSO-Free CPPs were first centrifuged at 1450 × g for

10 minutes. EVs were then isolated via differential centrifugation (10,000 × g for 45 minutes; 100,000 × g for 2 hours) as detailed previously[14]. The final pellets were resuspended in PBS-HAT buffer[13] and stored at -80°C until usage.

Both 10k and 100k EV fractions were profiled by NTA and single EV IFCM, as before, and results confirmed previous findings on isolated EV preparations (**Fig. 3B/C**). These results were further supported by scanning electron microscopy data (analysis was performed as reported previously[15]) of the newly arising CD9+ EVs pulled down with CD9 capture beads from the 10k fraction of DMSO-free CPP supernatants, revealing a higher abundance of $CD9^+$ particles with EV-like characteristics in the CPP sample compared to the same fraction derived from fresh platelets (**Supplemental Fig. 3**).

TEG assays demonstrated that adding isolated EVs to the plasma "Base" enhanced clot firmness (ActF MA) in a dose-dependent manner (**Fig. 3D**). Notably, the dilution of EVs directly influenced clot firmness, with the highest concentrations yielding the strongest clots, thereby corroborating that pEVs released during the freeze-thaw process are crucial for the observed clot stability in cryopreserved units.

Our findings provide evidence for a haemostatic mechanism associated with cryopreserved platelet products and highlight the substantial contribution of pEVs to coagulation. Importantly, the conclusions of this study are intentionally limited to the observed EV-mediated effects and do not extend to comparative assessments between cryopreservation methods. Accordingly, baseline differences between conditions are presented as contextual observations and should not be interpreted as evidence for or against the haemostatic superiority of either approach. Notably, TEG parameters were more consistently aligned with EV abundance than with platelet count across experiments suggesting that vesicles released during cryopreservation may play a proportionally greater role in driving clot formation. Under physiologic activation, pEVs retain functional adhesion receptors (e.g., GPIIb–IIIa), promoting their retention within the developing clot. In contrast, under more drastic or pathologic activation, impaired adhesion receptor activation may allow such EVs to disseminate, spreading their procoagulant potential beyond the initial site of injury[16].

These insights prompt a reconsideration of current quality indicators for platelet products. Traditional platelet quality metrics may not fully reflect the additional contribution pEVs to coagulation. Future work should investigate whether specific subtypes of platelet-derived vesicles differ in procoagulant potency, how storage conditions modulate their biochemical

features, and whether engineering of vesicle membranes could enhance therapeutic outcomes. Our findings suggest that the therapeutic value of cryopreserved platelet transfusions may rest on the quality and quantity of pEVs released during storage and preparation, rather than on platelet count alone. Transfusion protocols may need to differentiate between indications where cellular content or EV release provides greater clinical benefit, such as trauma versus chronic thrombocytopenia. A deeper understanding of EV biology could inspire novel diagnostic markers based on vesicle abundance and composition rather than cell counts.

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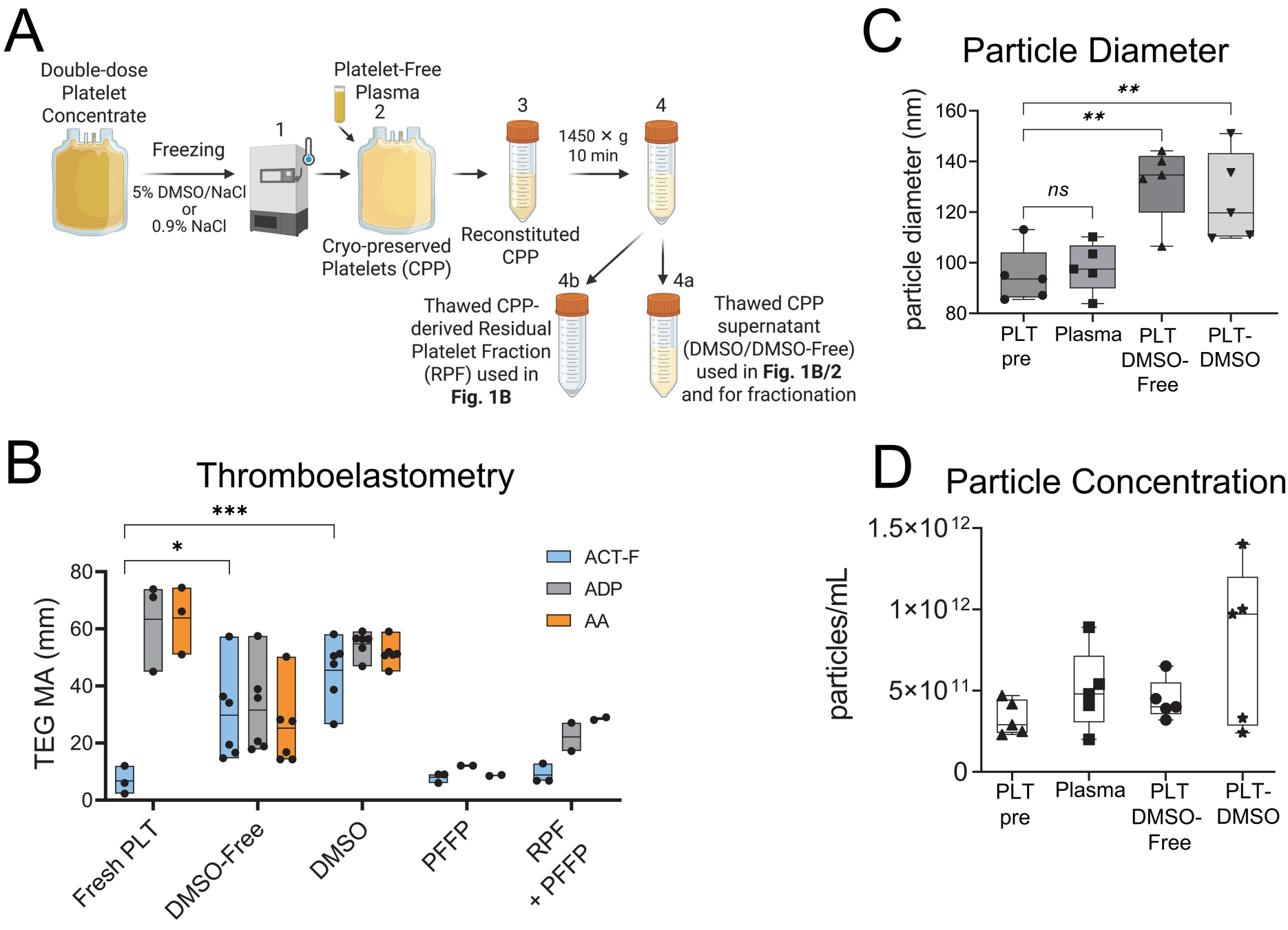
Figure Legends

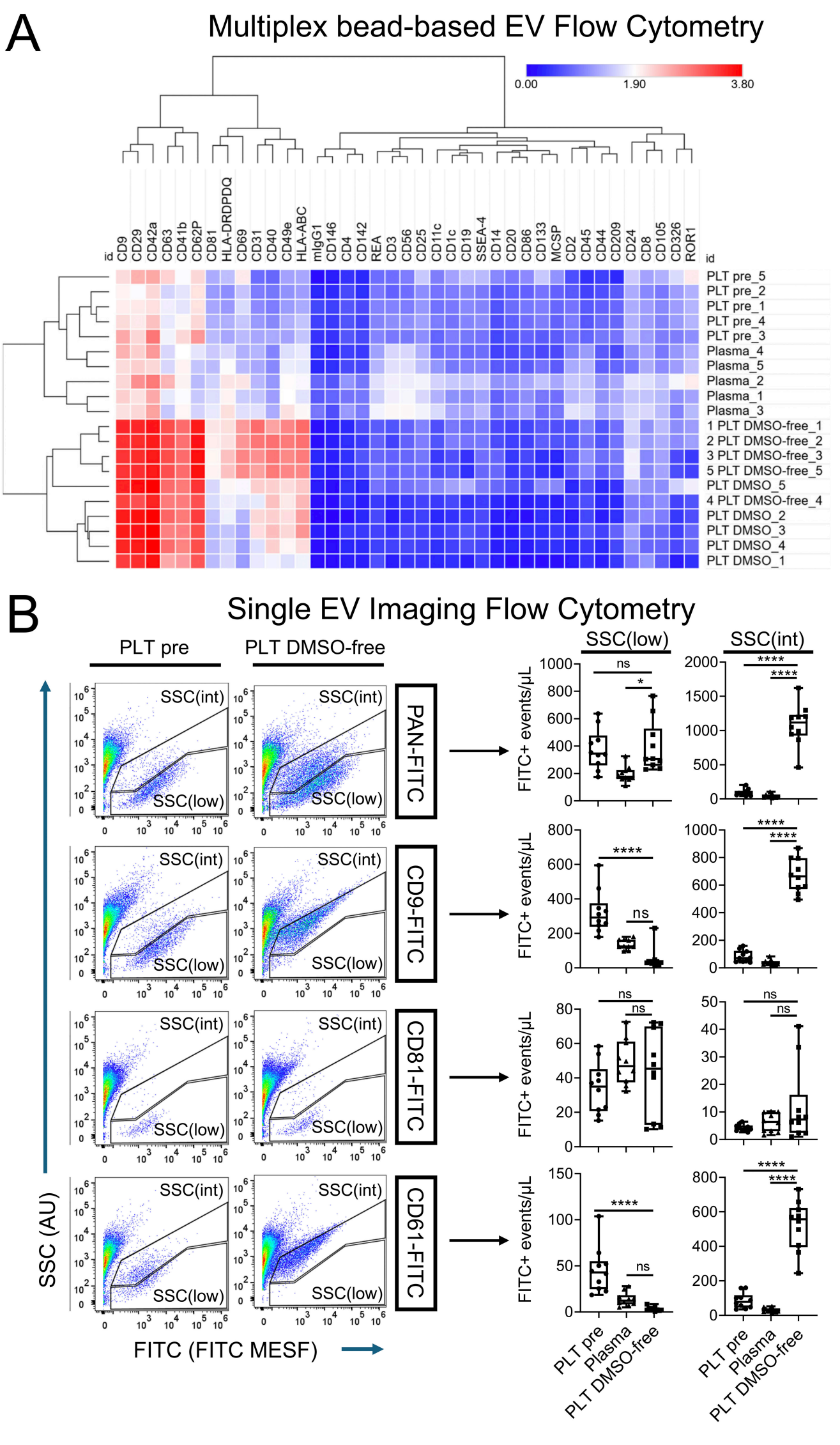
Fig. 1: Assessment of cryopreserved platelet hemostatic function and characterization of supernatant-derived nanoparticles. (A) Schematic overview of the preparation of the residual platelet fraction (RPF) and supernatant from platelets cryopreserved in DMSO-supplemented (DMSO) or DMSO-free media (DMSO-Free). The supernatants were used for TEG analysis and further EV isolation. *PFFP* refers to platelet free plasma. Created in BioRender. (B) TEG 6s (Thromboelastograph 6S) analysis of fresh platelet concentrates (Fresh PLT), the supernatant of thawed platelet concentrates cryopreserved in DMSO or DMSO-free media, platelet-free fresh plasma used for reconstitution (PFFP), and RPF diluted in PFFP (RPF+PFFP). The Act-F parameter assesses fibrin contribution in the absence of platelet function (via GPIIb/IIIa blockade), while ADP and AA assays measure clot formation stimulated through the $P2Y_{12}$ and Thromboxane A2 pathways, respectively. (C) Comparative analysis of mean particle diameters for nanoparticles in supernatants from fresh platelets (PLT pre), platelet-free plasma (PLASMA), DMSO-free cryopreserved platelets (PLT DMSO-free), and DMSO-supplemented cryopreserved platelets (PLT DMSO). See Supplemental Fig. 1 for additional details. (D) Quantification of the particle concentration across conditions. Statistical significance was assessed by one-way ANOVA using GraphPad Prism 8.

Fig. 2: Analysis of EV surface markers in CPP supernatants by EV flow cytometry. Clustering heatmap summarizing the multiplexed EV surface protein data. See also Supplemental Fig. 2. (B) Single EV IFCM-based quantification of EV concentrations by surface marker. DMSO-free CPP supernatants (PLT DMSO-free), supernatants from fresh platelet concentrates (PLT pre), and reconstitution plasma samples (Plasma) were stained with respective antibodies alongside non-EV containing buffer controls (PAN-FITC: CD9+CD63+CD81).

Fig. 3: Analysis of EVs isolated from DMSO-free and DMSO CPPs. (A) Illustration of the differential centrifugation/ultracentrifugation procedure applied to isolate EVs pelleting at 10,000 x g (10k) and 100,000 x g (100k). Created in BioRender. (B) NTA analysis for 10k and 100k EV samples prepared from fresh platelet concentrates (PLT pre) and DMSO-free and DMSO-based CPP supernatants (n=4 per group). (C) Quantification of the ratio of bigger SSC(int) to small SSC(low) EVs detected as CD9+ or PAN+ by IFCM, derived from respective preparations. (D) Comparison of the influence of DMSO-free vs DMSO storage medium derived EVs on clot firmness assessed through thromboelastography parameters Act-F, ADP and AA (n=1 per group). Cryo-DMSO Free or DMSO unit refers to the thawed, reconstituted CPPs in plasma which contain the EVs release during the freeze/thaw process. Base refers to the PFFP, which is used for the reconstitution post thawing of CPPs with the inclusion of 30% erythrocyte volume fraction (EVF) and RPF. For the remaining conditions, 10k and 100k EVs were used in combination or as standalone conditions mixed with the Base as per the indicated ratios, i.e. (10k+100k): Base 1:2 refers to 10k and 100k EVs taken in equal volumes and mixed

with twice the volume of the Base. Due to the lack of biological replicates statistical analysis was not possible; however, the dilution of EVs influences the clot firmness as evidenced by TEG ACT-F results of DMSO-Free and DMSO CPP samples.


A
Double-dose Platelet Concentrate
Freezing
5% DMSO/NaCl or 0.9% NaCl
1
Platelet-Free Plasma
2
Cryo-preserved Platelets (CPP)
3
Reconstituted CPP
1450 × g 10 min
4
4b
Thawed CPP-derived Residual Platelet Fraction (RPF) used in Fig. 1B
4a
Thawed CPP supernatant (DMSO/DMSO-Free) used in Fig. 1B/2 and for fractionation
B
Thromboelastometry
TEG MA (mm)
ACT-F
ADP
AA
***
*
Fresh PLT
DMSO-Free
DMSO
PFFP
RPF + PFFP
C
Particle Diameter
particle diameter (nm)
**
**
ns
PLT pre
Plasma
PLT DMSO-Free
PLT-DMSO
D
Particle Concentration
particles/mL
1.5×10^12
1×10^12
5×10^11
0
PLT pre
Plasma
PLT DMSO-Free
PLT-DMSO


A
Multiplex bead-based EV Flow Cytometry
0.00
1.90
3.80
id
CD9
CD29
CD42a
CD63
CD41b
CD62P
CD81
HLA-DRDPDQ
CD69
CD31
CD40
CD49e
HLA-ABC
mIgG1
CD146
CD4
CD142
REA
CD3
CD56
CD25
CD11c
CD1c
CD19
SSEA-4
CD14
CD20
CD86
CD133
MCSP
CD2
CD45
CD44
CD209
CD24
CD8
CD105
CD326
ROR1
id
PLT pre_5
PLT pre_2
PLT pre_1
PLT pre_4
PLT pre_3
Plasma_4
Plasma_5
Plasma_2
Plasma_1
Plasma_3
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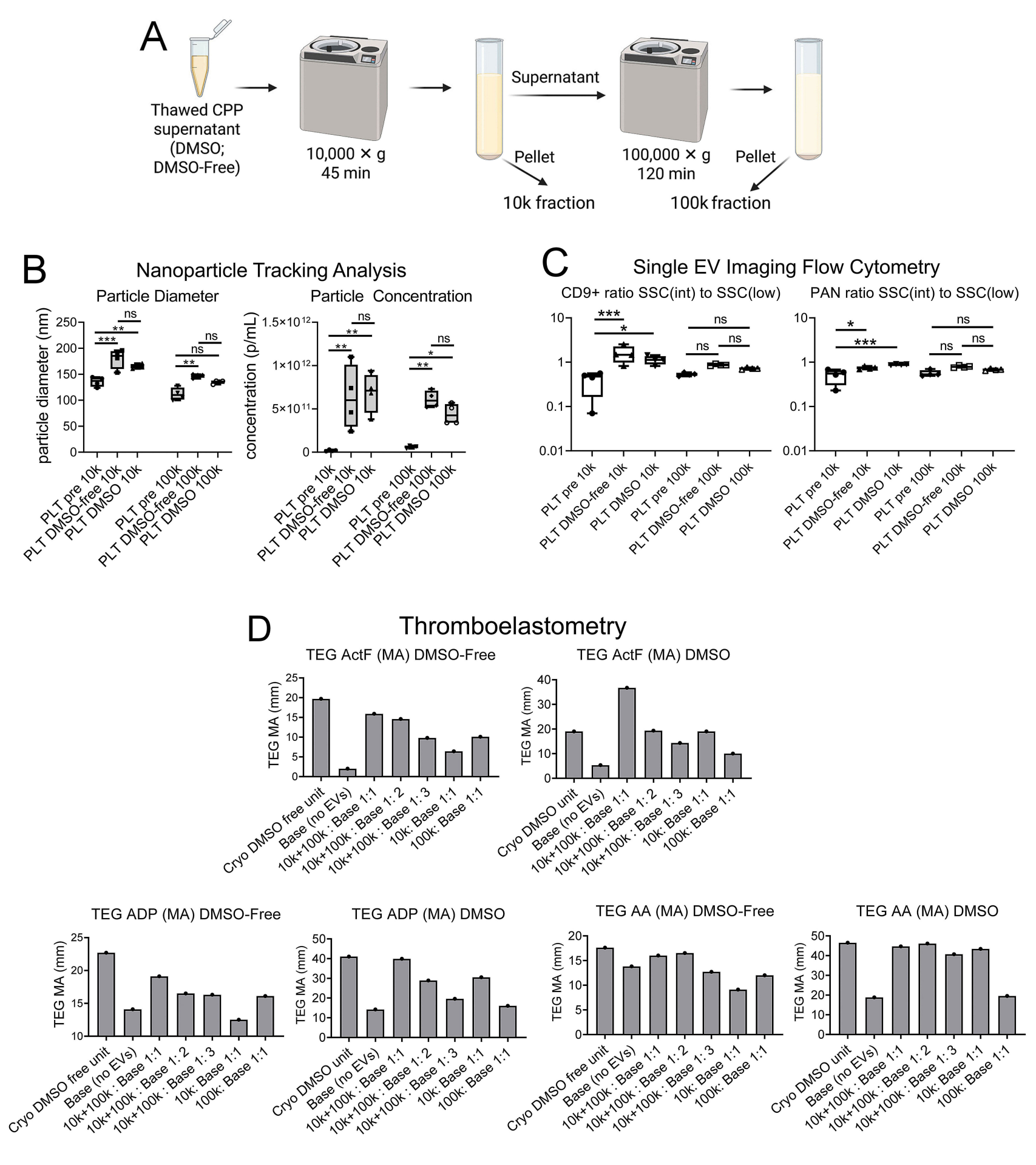
2 PLT DMSO-free_2
3 PLT DMSO-free_3
5 PLT DMSO-free_5
PLT DMSO_5
4 PLT DMSO-free_4
PLT DMSO_2
PLT DMSO_3
PLT DMSO_4
PLT DMSO_1
B
Single EV Imaging Flow Cytometry
PLT pre
PLT DMSO-free
SSC(int)
SSC(low)
PAN-FITC
CD9-FITC
CD81-FITC
CD61-FITC
SSC (AU)
FITC (FITC MESF)
SSC(low)
SSC(int)
FITC+ events/µL
ns
*
****
PLT pre
Plasma
PLT DMSO-free


A
Thawed CPP supernatant (DMSO; DMSO-Free)
10,000 × g 45 min
Supernatant
Pellet
10k fraction
100,000 × g 120 min
Pellet
100k fraction
B
Nanoparticle Tracking Analysis
Particle Diameter
particle diameter (nm)
Particle Concentration
concentration (p/mL)
PLT pre 10k
PLT DMSO-free 10k
PLT DMSO 10k
PLT pre 100k
PLT DMSO-free 100k
PLT DMSO 100k
C
Single EV Imaging Flow Cytometry
CD9+ ratio SSC(int) to SSC(low)
PAN ratio SSC(int) to SSC(low)
D
Thromboelastometry
TEG ActF (MA) DMSO-Free
TEG ActF (MA) DMSO
TEG ADP (MA) DMSO-Free
TEG ADP (MA) DMSO
TEG AA (MA) DMSO-Free
TEG AA (MA) DMSO
TEG MA (mm)
Cryo DMSO free unit
Base (no EVs)
10k+100k : Base 1:1
10k+100k : Base 1: 2
10k+100k : Base 1: 3
10k: Base 1:1
100k: Base 1:1

Platelet-derived extracellular vesicles are relevant for clot stability in cryopreserved platelets

Zankruti Dave[1,#], Per Sandgren[2,#], André Görgens[1,3,4,§,*], Joel Nordin[1,5,§,**]

[1]Division of Biomolecular and Cellular Medicine, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

[2]Department of Center for Hematology and Regenerative Medicine (HERM), Karolinska Institutet, Stockholm, Sweden

[3]Department of Cellular Therapy and Allogeneic Stem Cell Transplantation (CAST), Karolinska University Hospital, Huddinge, Sweden

[4]Karolinska ATMP Center, Karolinska Institutet, ANA Futura, Huddinge, Sweden

[5]Department of Clinical Immunology and Transfusion Medicine (KITM), Karolinska University Hospital, Stockholm, Sweden

# These authors contributed equally

§ These authors contributed equally

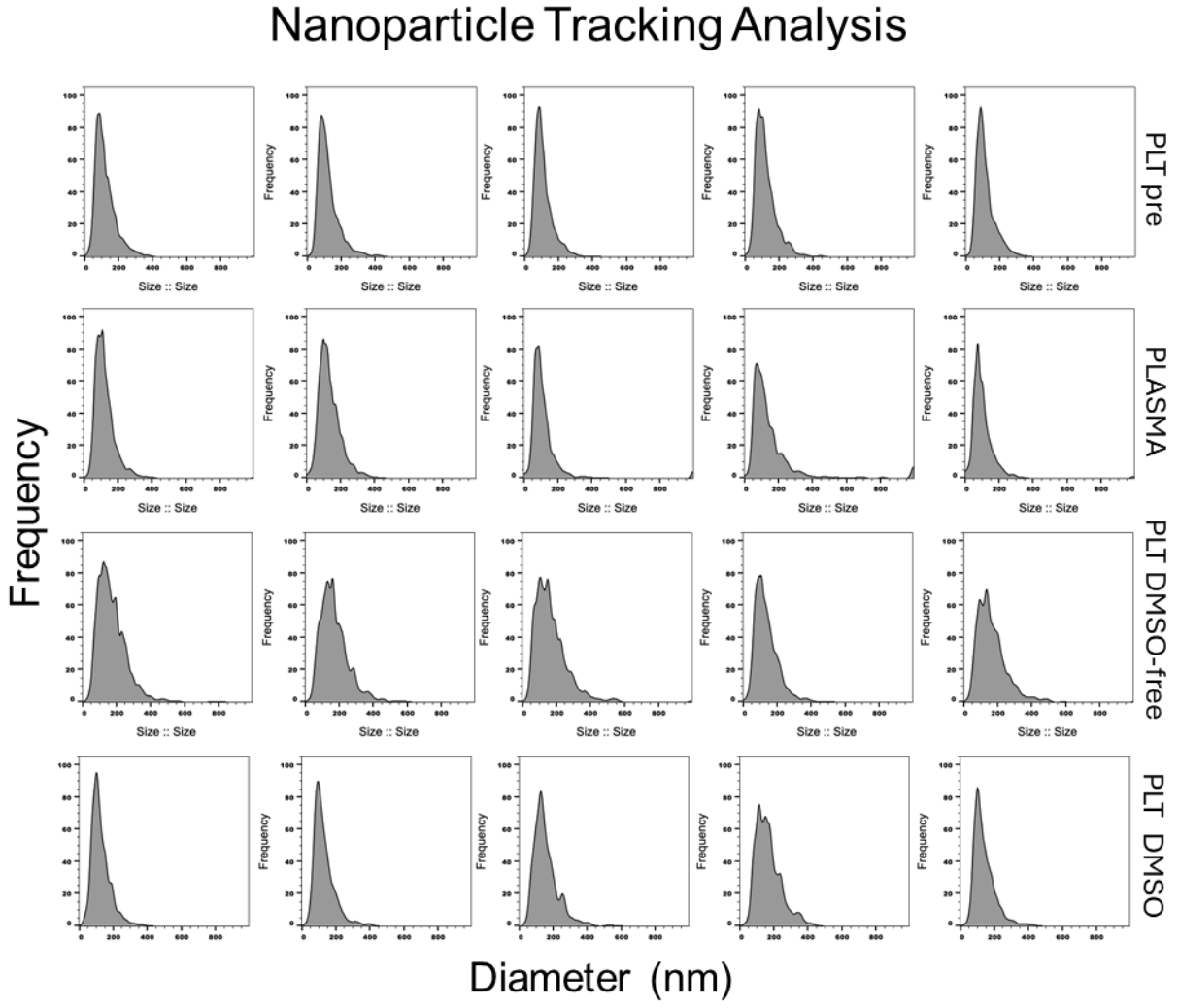
* Corresponding author: Division of Biomolecular and Cellular Medicine, Karolinska Institutet, ANA Futura, Alfred Nobels Allé 8, 14152 Stockholm, Sweden; andre.gorgens@ki.se

** Corresponding author: Division of Biomolecular and Cellular Medicine, Karolinska Institutet, ANA Futura, Alfred Nobels Allé 8, 14152 Stockholm, Sweden; joel.nordin@ki.se

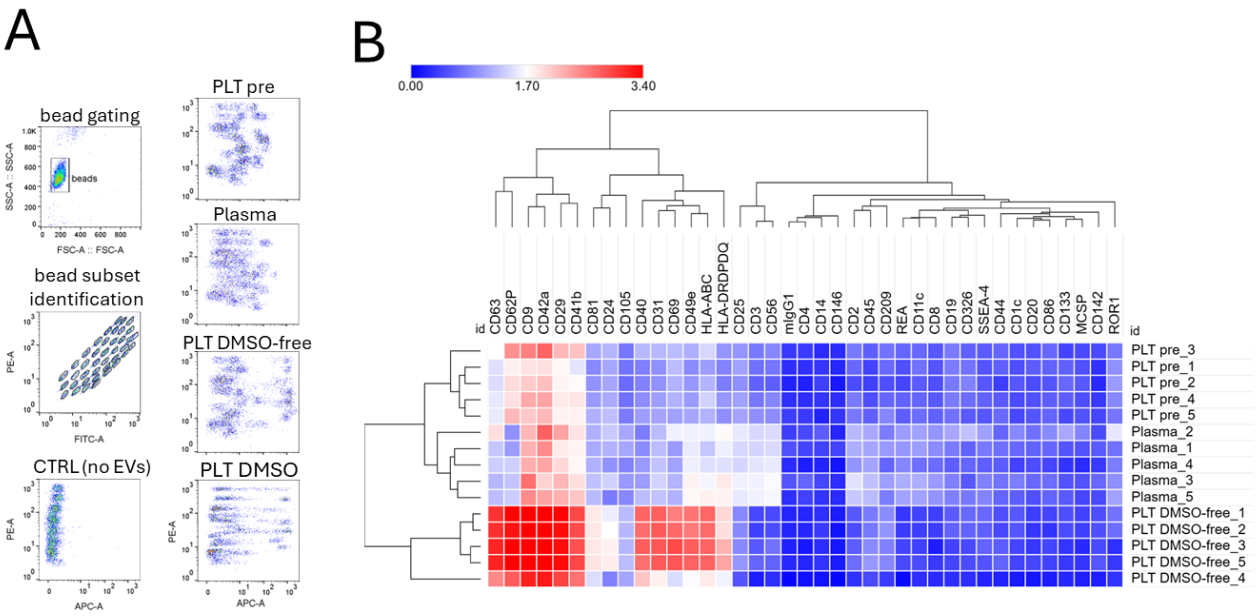
Keywords: Platelets, Platelet cryopreservation, Platelet transfusion, Extracellular Vesicles

Supplemental Figures

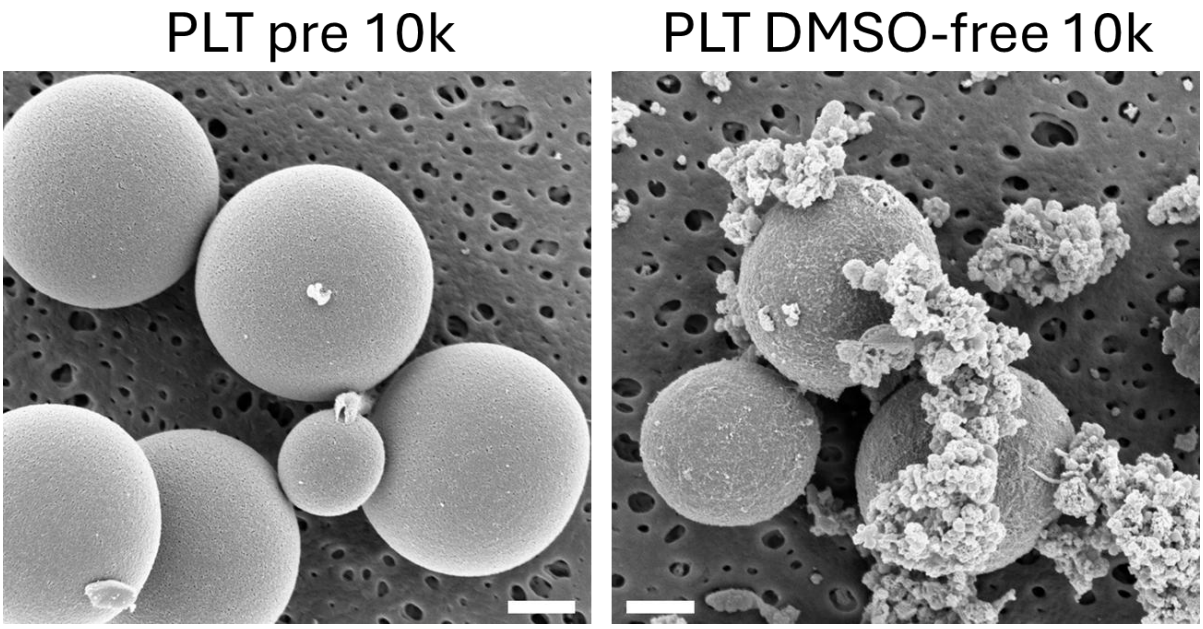
This supplement contains 3 supplemental figures.



Supplemental Figure 1: Representative size distribution histograms for nanoparticles in supernatants from fresh platelets (PLT pre), platelet-free plasma (PLASMA), DMSO-free cryopreserved platelets (PLT DMSO-free), and DMSO-supplemented cryopreserved platelets (PLT DMSO) (n=5 per condition) for data presented in Figure 1C/D.



Supplemental Figure 2: (A) Dotplots show the gating on the population of single EV capture beads (beads), the subsequent identification of capture bead subsets using the human MACSPlex EV Kit, IO panel, and examples of PAN-APC (CD9+CD63+CD81) stained control measurements (no EVs, beads + detection antibodies) and representative measurements for each sample group (beads+ sample + detection antibodies) for data presented in Figure 2A. (B) Analysis of the surface marker composition of EVs contained in CPP supernatants by multiplex bead-based EV flow cytometry using CD9 as detection antibody. Clustering heatmap summarizes the normalized protein surface marker data for all 39 different capture beads after staining with CD9-APC as detection antibody.



Supplemental Figure 3: Representative scanning electron microscopy images of CD9-coated capture beads incubated for 30 min with either PLT pre 10k or PLT DMSO-free 10k fractions (scale bar: 1 µm). Images show higher abundance of CD9+ particles captured from the PLT DMSO-free 10k fraction derived from CPP compared to the same fraction derived from fresh platelets (PLT pre). Respective 10k fractions of PLT pre and PLT DMSO-free samples were incubated with anti-CD9 capture beads for 30 min at 4 ºC and washed twice with PBS 1% BSA. Samples were immediately fixed, prepared for SEM and analyzed as described before.[15]