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RESEARCH ARTICLE

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Complement C3 inhibition reduces complement activation in clinical platelet concentrates but does not counteract platelet storage lesions

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Abstract

Platelet storage is associated with storage lesions, including platelet morphological changes and a gradual functional loss. We investigated the impact of complement C3 inhibition on complement activation and platelet storage lesions in clinical platelet concentrates. Platelet concentrates ($n = 8$) were prepared in PAS-E and stored for seven days at 22°C. Each concentrate was split in two, with the C3 inhibitor compstatin Cp40 added to one part, and the other serving as the control. Complement and platelet activation markers, platelet function, and metabolic measures were analyzed every second day. Cp40 significantly reduced C3bc and sC5b-9 levels, but not C4c, indicating inhibition of complement activation at the level of C3. However, Cp40 did not affect platelet-specific or metabolic measures. Surface expression of CD62P and NAP-2 release increased significantly over the storage time, whereas CD63 expression and PF4 and TSP-1 release remained stable. Platelet responses to TRAP-6 mediated PAR-1 activation and U46619 mediated TXA_2R stimulation decreased over time, recorded as CD62P and CD63 expression and release of soluble factors. No drop in platelet count was observed, and metabolic markers remained within their critical limits. While C3 inhibition effectively reduced complement activation in stored platelet concentrates, it did not mitigate platelet storage lesions.

Plain Language Summary

What is the context?

- Storage of clinical platelet concentrates in blood banks is associated with storage lesions, defined as a gradual platelet functional loss due to changes in platelet morphology and altered platelet metabolism.
- The complement system, part of the innate immune response, is gradually activated in the plasma of stored platelet concentrates.
- Platelets and the complement system are mutually interconnected and complement activation fragments could potentially exacerbate platelet storage lesions.

What is new?

- Here, we investigated whether inhibiting complement activation at the C3 level could reduce platelet storage lesions and improve the quality of stored platelet concentrates containing 35% plasma.

Keywords
Complement, complement inhibition, platelet concentrates, platelet storage lesions, platelet transfusion, platelets

History
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- The C3-inhibitor was found to significantly reduce the level of complement activation in the stored concentrates. The C3-inhibitor did not affect any platelet-specific or metabolic measures.

What is the impact?
Thisstudy provides evidence that the degree of complement activation fragments atand downstream of complement C3, generated in stored platelets containing 35%plasma, does not impact platelet activation or function.

Introduction

The storage of platelets in concentrates requires an environment that supports their metabolic needs and maintains their function without activating the plasma components stored alongside the platelets. However, platelet storage lesions, defined as a gradual functional loss in stored platelets due to changes in platelet morphology, modification of expressed surface molecules, and altered platelet metabolism, as well as the extracellular release of proteins, RNA, and other biological modifiers, increase over time.[1,2] Various platelet additive solutions (PAS) have been developed to replace part of the plasma in stored concentrates, thereby reducing platelet storage lesions and transfusion-associated adverse reactions.[3,4] Storage in PAS-E, which contains potassium, magnesium, phosphate, and citrate, with approximately 35% plasma, has shown good outcomes compared to other PAS or those stored with plasma only, including reduction in allergic reactions, decreased platelet activation and reduced complement activation.[4–6] According to national guidelines, platelet concentrates with PAS-E in Swedish blood banks can be stored for up to seven days at 22°C before transfusion.[7,8]

The complement system, a protein-based cascade featuring plasma and membrane-bound components (Figure 1), is an essential part of the innate immune system that protects the host from invading pathogens, malignant cells, and scavenges cell debris.[9] However, unwanted complement activation can be initiated by contact with artificial surfaces, such as the inside of a platelet concentrate storage bag. Soluble complement pattern recognition molecules of the classical and lectin pathways can interact with the bag's surface, initiating complement activation and the generation of C4bC2b C3-convertases.[10–12] Activation can also occur via fluid-phase generation of C3 (H_2O) through C3 hydrolysis in plasma, possibly accelerated by contact with the storage bag surface.[13,14] C3(H_2O) exhibits a C3b-like function and forms the C3(H_2O)Bb alternative pathway C3-convertase.[15,16]

Once C3 is cleaved, effector molecules such as C3a and C5a anaphylatoxins, C3b/iC3b opsonins, and the C5b-9 membrane

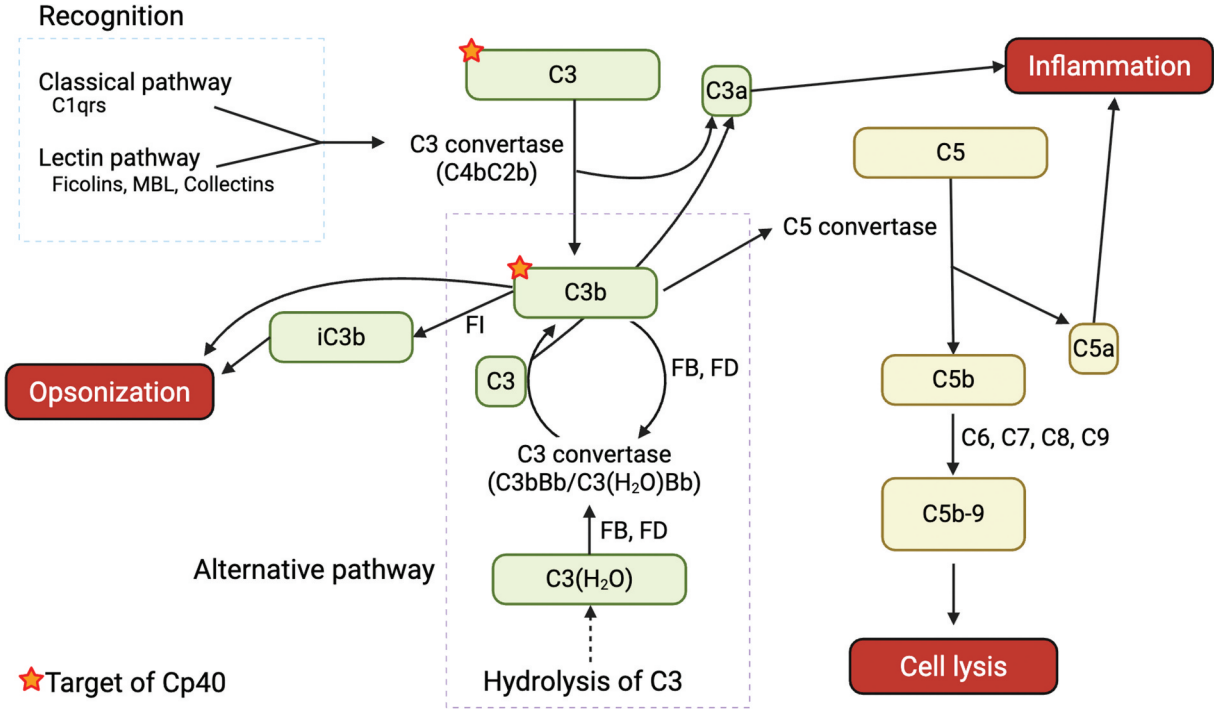

Figure 1. The complement system can be activated via three pathways[1]: by binding of recognition molecule C1q in the classical pathway (CP),[2] by binding of mannose-binding lectin (MBL), ficolins, or collectins in the lectin pathway (LP), or[3] by hydrolysis of the internal thioester in C3 ("tick-over"), forming C3(H_2O), in the alternative pathway (AP). Activation proceeds through the formation of C3 convertases: C4bC2b in the CP and LP, and C3(H_2O)Bb in the AP. Deposition of C3b on a surface can further amplify AP activation via formation of the C3bBb C3 convertase. As C3b accumulates, the substrate specificity of the convertase shifts to C5, leading to the generation of C5a and C5b. C5b associates with C6, C7, C8, and multiple C9 proteins to form the C5b-9 membrane attack complex (MAC). Effector functions generated through the cascade include the C3a and C5a anaphylatoxins, which, through activation of their receptors, can trigger inflammation. The degradation fragments C3b and iC3b opsonize substrates for phagocytosis, while the MAC is capable of lysing sensitive cells or activating nucleated cells. Compstatin Cp40 targets C3 and C3b, as indicated by a star. The specific markers used to assess complement activation in this context include the CP/LP-specific activation fragment C4c, the C3 activation fragments C3b and C3c (collectively referred to as C3bc), the hydrolyzed form of C3 [C3(H_2O)], and the soluble terminal complement complex (sC5b-9). Abbreviations: factor B (FB), factor D (FD), factor I (FI).

attack complex are generated. There is an intricate and mutual dependence between complement and platelets. Complement anaphylatoxins, C3a and C5a, have been demonstrated to stimulate platelet activation,[17,18] although the context for the expression of the anaphylatoxin receptors on platelets is not entirely clear.[19] Older studies have also shown platelet activation and release of platelet microvesicles after insertion the C5b-9 membrane attack complex.[20,21] Inhibition of C3 has been shown to reduce *E. coli*-induced platelet aggregation, an effect mediated via C3b.[22] The platelet release reaction has the potential to initiate complement activation in the fluid phase through the extracellular release of intracellularly stored chondroitin sulfate-A, which can activate the classical pathway and, feed forward on platelet activation.[23]

Several reports have shown increased complement activation in platelet concentrates stored over time in either plasma or PAS.[5,11,24–26] We recently showed that complement activation increased over time in stored platelet concentrates with PAS-E and that complement activation was inversely correlated with platelet function.[11] By inhibiting complement at the level of C3, we here evaluated whether there is a causative relationship between complement activation and platelet function in stored clinical platelet concentrates.

Methods

Preparation of random donor platelet concentrates

Blood was collected from healthy human volunteers within the Region Kalmar Län, Sweden, as part of the routine clinical blood donation program. Using the Reveos® Automated Blood Processing System from Terumo BCT (Larne, UK), the blood was processed into plasma, erythrocytes, and an interim platelet unit (IPU) containing platelets and plasma, either the day of donation or after overnight storage. To fulfill a therapeutic dose of the Random Donor Platelets (RDPs), IPUs from four to five donors were pooled with PAS-E (Macopharma, Mouvaux, France) to approximately 35% plasma content, using a platelet pooling set with a leukocyte filter (Terumo BCT). In total, eight concentrates were produced as follows: Two concentrates contained only IPUs from blood processed on the day of donation, four concentrates were a mixture of donation-day processed and overnight-stored blood, and two contained only IPUs from donations stored as whole blood overnight before processing. The concentrates were analyzed for bacterial contamination using the BacT/Alert® Virtuo® blood culture detection system (BioMérieux SA RCS, Lyon, France). Platelet count was assessed using an automated hematology system (XN-9100, Sysmex, Kobe, Japan), and ensuring leukocyte numbers below hospital guidelines was conducted using flow cytometry (BD FACSLyric, Franklin Lakes, NJ). Subsequently, the RDPs were exposed to a 25 Gy gamma radiation dose using the TrueBeam system (Varian, Palo Alto, CA), to inactivate lymphocytes, thereby preventing transfusion-associated graft-versus-host disease. All platforms mentioned above were in accordance with the manufacturer's protocols and employed to treat the RDPs as if they were to be transfused to patients. All platelet concentrates were de-identified after RDP processing.

Platelet concentrate preparation and inhibitor addition

The platelet concentrates ($n = 8$) were split into two equal parts, each weighing 143 g ±1 g (~100 mL), using an accessory platelet storage set (Terumo BCT, Inc., Lakewood, CO), and a sterile welder TSCD-II (Terumo BCT). To maintain a constant volume-to-area ratio from the original concentrate, the new storage bags were sterile welded to half the original volume before filling. The C3-inhibitor compstatin Cp40[27] ($n = 8$, final concentration 20 μM) or control, consisting of either a scrambled peptide ($n = 2$, 20 μM) or PBS ($n = 6$), was injected into the tubing connected to the storage bag using BD Micro-Fine™ (BD, Franklin Lakes, NJ). Cp40 fully blocks C3-cleavage at 40 μM in 100% serum and plasma.[28,29] Here, 20 μM was used to overcome C3-clevage in the RDPs containing 35% plasma. Cp40 and the scrambled peptide was initially dissolved in ultrapure water to 20 mm, and subsequently dissolved to a 2 mm stock solution in PBS. These steps were performed in a laminar air flow bench under controlled aseptic conditions to minimize the risk of bacterial contamination. The eight concentrates were run in a paired setup, and every part of the inhibitor application and sample analysis was blinded.

Platelet storage, sampling and metabolic measurements

The concentrates were incubated for seven days in a 22°C incubator (Forma platelet incubator, Thermo Electron Corporation, Waltham, MA) with agitation (Meloc Engineering Corp., Glendale, CA). On Days 1, 3, 5, and 7, a sampling bag (Macopharma) was sterile welded (TSCD-II, Terumo BCT) to the concentrate, and a 6–8 mL sample was isolated. The sample was transferred into a 15 mL Falcon tube (Fisherbrand, Pittsburgh, PA). Samples were isolated for platelet count and metabolic measurements. Platelet count, expressed as the mean of two technical replicates, was performed using a Swelab Alfa (Boule Diagnostics, Spånga, Sweden). Metabolic analysis of glucose and lactate was performed using an ABL 800 (Radiometer Medical ApS, Copenhagen, Denmark).

Platelet activation

A $CaCl_2$ solution was added to a final concentration of 25 mm to counteract the 10.8 mm citrate in the PAS-E solution. To prevent coagulation from the recalcification, the thrombin inhibitor lepirudin (Refludan®, Celgene, Uxbridge, UK) was added at a final concentration of 50 μg/mL before the calcium solution. All platelet concentrate samples were split into three aliquots. Dulbecco's PBS (Thermo Fisher, Waltham, MA) was added to a baseline sample followed by the immediate addition of a 10× stop solution containing CTAD (0.11 M buffered trisodium citrate solution, 15 M theophylline, 3.7 M adenosine, and 0.198 M dipyridamole), and EDTA at a final concentration of 10 mm. The two remaining aliquots were incubated for 20 minutes in 1.8 mL cryotubes (CryoPure, Sarstedt, Nümbrecht, Germany) at 37°C in a water bath with either the PAR-1 agonist thrombin activating peptide-6 (TRAP-6) (25 μg/mL, Batch No: 8A Tocris, Bristol, UK) or the thromboxane A_2 receptor (TXA_2R) agonist U46619 (3 μM, Tocris, No: 7E/249404). The reaction was stopped by adding the CTAD/EDTA stop solution. All three aliquots were centrifuged for 20 minutes at 3000 × g at 22°C. The supernatants were isolated into CryoPure tubes (Sarstedt). The samples were subjected to snap-freezing in liquid nitrogen and stored at −80°C until subsequent analyses.

Complement activation and soluble platelet markers analysis

Markers of complement activation were analyzed in the baseline samples using ELISAs. The common classical and lectin pathway marker, C4c (Hycult® Biotech, Uden, The Netherlands), was analyzed according to the manufacturer's instructions. Markers for C3-cleavage and terminal complement pathway activation, i.e., C3bc and sC5b-9 markers respectively, were analyzed using methods described by Bergseth *et al.*.[30] In brief, the Bh6 mAb, which recognizes a neoepitope in the C3 activation fragments C3b, iC3b, and C3c, was used as the capture antibody for the

quantification of C3bc. A polyclonal rabbit anti-human C3c (Siemens, Erlangen, Germany), followed by a peroxidase-labeled anti-rabbit Ig (GE Healthcare, Uppsala, Sweden), was used to detect C3bc. The aE11 mAb, which recognizes a neoepitope in C9 when in C5b-9, was used as a capture antibody in the sC5b-9 ELISA. A biotinylated mAb (9C4-RQ-B) recognizing C6, followed by streptavidin-HRP (GE Healthcare), was used for detection. C3(H_2O) was analyzed according to the method described by Fromell *et al*. using a neoepitope-specific monoclonal anti-C3a (clone 4SD17.3) antibody for capturing and a biotinylated polyclonal anti-C3d antibody (Dako, Glostrup, Denmark) and streptavidin-HRP for detection.[31] Absorbance was measured using a Tecan plate reader Sunrise (Tecan Nordic, Stockholm, Sweden), and the data analysis was performed using Magellan software, version 7.1 (Tecan Nordic AB). Platelet activation markers thrombospondin-1 (TSP-1), neutrophil activating peptide-2 (NAP-2), and platelet factor 4 (PF4) were analyzed in the baseline, as well as in the TRAP-6 and U46619 activated samples using R&D DuoSets® ELISAs (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Platelet surface activation markers

Platelets in the baseline samples and those incubated with either the PAR-1 agonist TRAP-6 or the TXA_2R agonist U46619 were analyzed for platelet surface activation markers CD62P and CD63 expression. The platelets were stained using anti-CD62P-PE (BD, clone: AK-4) and anti-CD63-FITC (BD, clone: H5C6). Each antibody (5 µL) was mixed with 10 µL of platelet sample and incubated for 20 minutes at 4°C in the dark, followed by fixation with 0.2% formalin (Histolab, Gothenburg, Sweden) in PBS with 1% bovine serum albumin (BSA) (Thermo Scientific). Flow cytometry was performed using CytoFLEX (Beckman Coulter, Inc., Brea, CA), gating platelets using forward and side scatter. Data were obtained using CytoExpert V. 2.5.0.77 (Beckman Coulter) and analyzed using FlowJo, version 10.10.0 (BD).

Data presentation and statistical analyses

Data visualization and statistical analysis were performed using GraphPad Prism 10 for Mac (San Diego, CA, USA). Due to limited availability of the scrambled peptide, only two concentrates were treated with this peptide, and the remaining control concentrates were given PBS. The PBS-treated concentrates showed the same trend as the scrambled-peptide treated controls (Supplementary Figure S1-S4) and these were therefore merged into a single control group. Statistical differences between treatments for each sampling day were analyzed using two-way ANOVA with Šídák's multiple comparisons test. Statistical differences over time within each treatment were analyzed for all sampling days relative to Day 1 using two-way ANOVA with Dunnett's multiple comparisons test.

Results

No effects from complement inhibition on platelet decay, lactate or glucose

Platelet concentrates were released by the blood bank, all fulfilling the requirements for platelet and leukocyte concentrations, as well as absence of bacterial contamination. No changes in platelet counts were observed during the storage period in any of the groups. Platelet counts in the Cp40-treated samples on days 1, 3, 5, and 7 were, respectively, mean ± SD: 800 ± 77, 785 ± 72, 778 ± 88, and 790 ± 92 × 10^9/L. In the control group, counts on days 1, 3, 5, and 7 were 790 ± 97, 809 ± 61, 767 ± 97, and 795 ± 89 × 10^9/L, respectively. Glucose levels dropped significantly in both groups from day 3, but glucose was not fully depleted by the end of the storage period, which is crucial for maintaining a balanced metabolism ($p < .01$) (Figure 2A). Lactate increased linearly and was significantly elevated from day 3 ($p < .001$), although it did not reach the critical threshold of 20 mm (Figure 2B). No significant differences were observed between the Cp40-treated concentrates and the controls for any of the markers.

Reduced activation of C3 and the terminal pathway in concentrates with Cp40

Complement activation markers were analyzed in all samples (Figure 3). C4bc, a marker upstream of C3, increased 1.52-fold in concentrates containing Cp40 and 1.63-fold in the control concentrates throughout the storage period (Figure 3A). This increase was statistically significant for both conditions ($p < .001$).

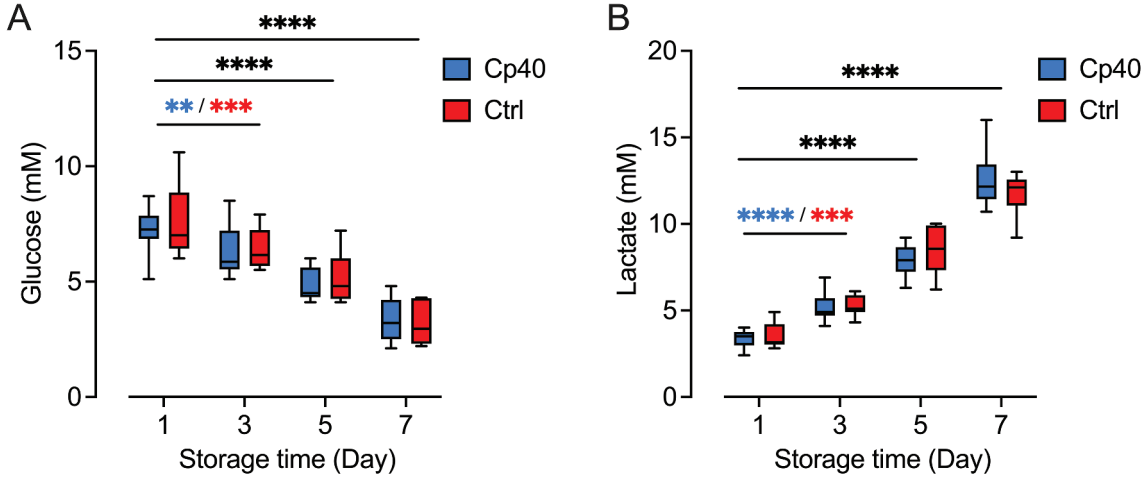

Figure 2. Metabolic markers. metabolic markers analyzed as glucose (A) and lactate (B) in aliquots isolated from the stored concentrates at Days 1, 3, 5, and 7 with (blue) and without (red) Cp40 (n = 8). Data are shown in box and whisker plots with range showing 5–95 percentile. Statistical differences between treatments for each day were analyzed using two-way ANOVA with šídák's multiple comparisons test. Statistical differences over time within each treatment were analyzed for all sampling days relative to Day 1 using two-way ANOVA with Dunnett's multiple comparisons test, **p <.01, ***p <.001, ****p <.0001, black asterisk = significance level for both Cp40 and control, blue asterisk = significance level for Cp40, red asterisk = significance level for control.

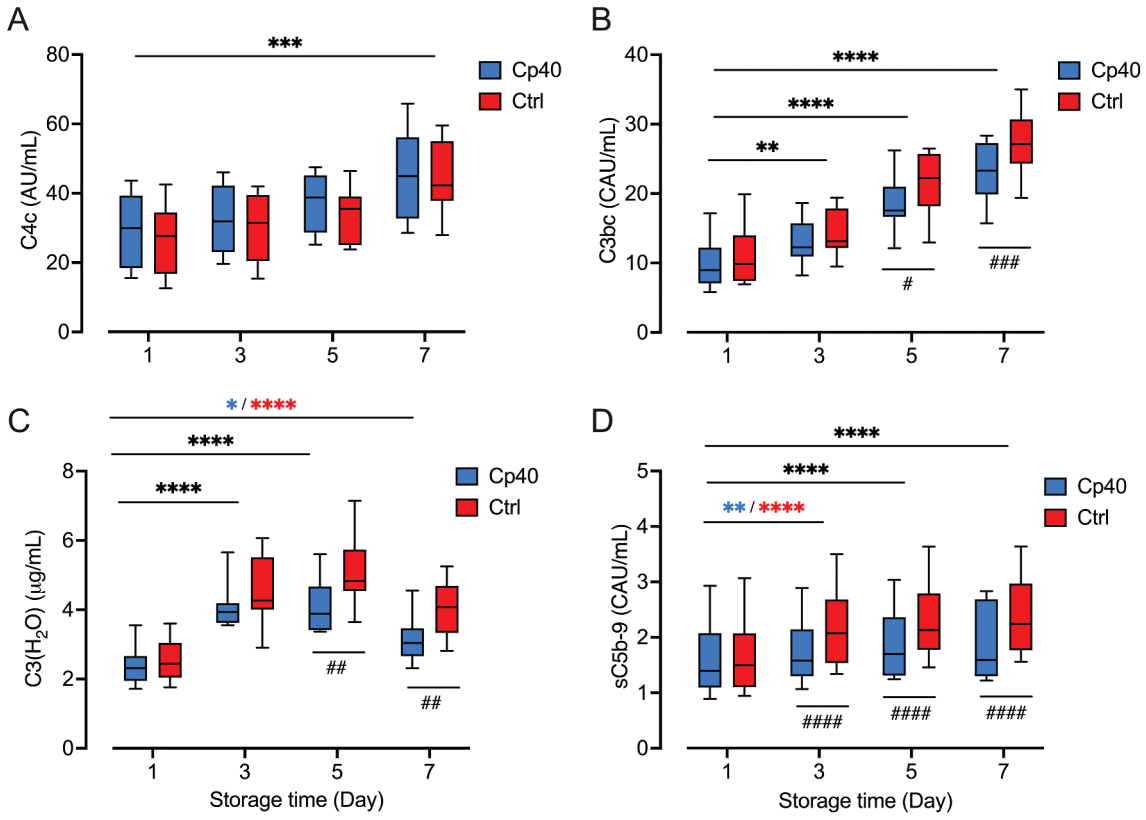

Figure 3. Complement activation in stored platelet concentrates. Complement activation analyzed as C4c (A), C3bc (B), C3(H_2O) (C), and sC5b-9 (D) in CTAD/EDTA-aliquots from stored concentrates at days 1, 3, 5, and 7 with (blue) and without (red) Cp40 (n = 8). Data are shown in box and whisker plots with range showing 5–95 percentile. Statistical differences between treatments for each day were analyzed using two-way ANOVA with šídák's multiple comparisons test, $^{\#}p$ <.05, $^{\#\#}p$ <.01, $^{\#\#\#}p$ <.001, $^{\#\#\#\#}p$ <.0001. Statistical differences over time within each treatment were analyzed for all sampling days relative to Day 1 using two-way ANOVA with Dunnett's multiple comparison test, *p <.05, **p <.01, ***p <.001, ****p <.0001, black asterisk = significance level for both Cp40 and control, blue asterisk = significance level for Cp40, red asterisk = significance level for control.

Cp40 had no significant effect on the level of C4bc. C3bc increased significantly at all sampling days compared to Day 1, both with and without Cp40 ($p < .01$) (Figure 3B). The increase was 2.35-fold for Cp40 and 2.46-fold for the control at Day 7. Cp40 significantly attenuated the level of C3bc on Day 5 ($p < .05$) and Day 7 ($p < .001$) compared to the control. C3(H_2O), a marker of C3-hydrolysis, formed independently of convertase-mediated cleavage of C3, significantly increased from Day 3 until the end of storage on Day 7 ($p < .05$), both with and without Cp40 (Figure 3C). The peak C3(H_2O) was reached on Day 5 with a 1.72-fold increase in Cp40 and a 1.99-fold increase in the control. The levels of C3(H_2O) were significantly lower in the Cp40-treated concentrates on Day 5 and Day 7 ($p < .01$). Cp40 significantly inhibited the generation of sC5b-9 in the concentrates at Day 3–7 ($p < .0001$) (Figure 3D). The level of sC5b-9 increased by 1.18-fold in the Cp40-samples at Day 7, compared to 1.45-fold in the control at Day 7. Although Cp40 had an inhibitory effect on sC5b-9, the levels increased significantly from Day 3 as an effect of storage time under both conditions ($p < .01$).

No effects from complement inhibition on the release of soluble platelet activation markers

The soluble platelet activation markers TSP-1, PF4, NAP-2, and TGF-β1 were measured in baseline PBS samples and in samples treated with the TXA_2R agonist U46619 and the PAR-1 agonist TRAP-6. Treatment with Cp40 did not significantly affect the levels of any of these markers (Figure 4). TSP-1 levels stayed stable during storage in untreated samples (Figure 4A). The strongest release of TSP-1 after stimulation with U46619 and TRAP-6 occurred on Day 1 and dropped significantly from Day 5 ($p < .001$) (Figure 4B-C). PF4 levels remained stable in non-activated samples during storage (Figure 4D). Its release after U46619 stimulation was highest on Day 1 and significantly lower from Day 5 ($p < .001$) (Figure 4E). TRAP-6 caused a smaller drop in PF4 release, but levels were still significantly lower on Day 7 compared to Day 1 ($p < .05$) (Figure 4F). NAP-2 levels increased spontaneously over storage time (Days 5–7, $p < .01$) (Figure 4G). Its release after U46619 stimulation was significantly reduced on Days 5 and 7 ($p < .001$). In response to TRAP-6, no change was seen in control samples, but Cp40-treated samples showed a significant reduction on Day 7. TGF-β1 levels significantly increased from Day 3 ($p < .01$) (Figure 4J). TGF-β1 release in response to U46619 was significantly reduced in control samples ($p < .01$), with no change seen in any samples after TRAP-6 stimulation.

Reduced responsiveness toward TXA_2R stimulation but no effect from complement inhibition

The expression of the platelet activation markers CD62P (from alpha granules) and CD63 (from dense granules) was analyzed in PBS (non-activated) samples and in samples treated with U46619 and TRAP-6 (Figure 5). Platelets showed a significant increase in CD62P expression over the storage period in both Cp40-treated and control samples starting from Day 3 ($p < .05$) (Figure 5A). On Day 7, the Cp40 group had a slightly but significantly higher level of CD62P expression than the control

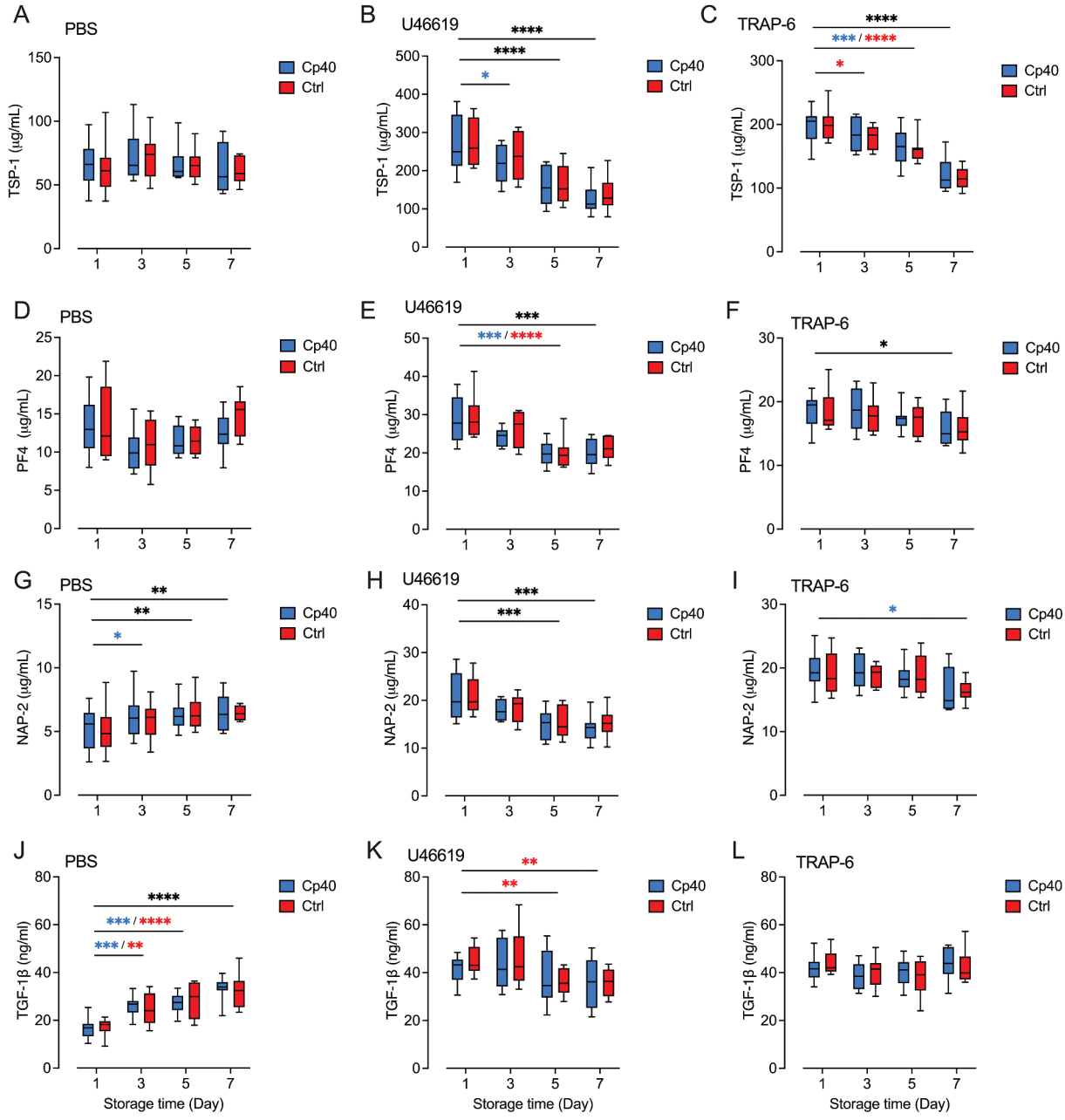

Figure 4. Release of soluble platelet activation markers. the release of thrombospondin-1 (TSP-1) (A-C), platelet factor 4 (PF4) (D-F), neutrophil-activating peptide-2 (NAP-2) (G-I), and transforming growth factor-beta 1 (TGF-β1) (J-L) in aliquots from stored concentrate samples, with (blue) and without (red) Cp40, and immediately treated with CTAD and EDTA (PBS) (A, D, G, J), or incubated for 20 minutes with the TXA_2R agonist U46619 (B, E, H, K), or the PAR-1 agonist TRAP-6 (C, F, I, L) before adding CTAD and EDTA (n = 8). Data are shown in box and whisker plots with range showing 5–95 percentile. Statistical differences between treatments for each day were analyzed using two-way ANOVA with šídák's multiple comparisons test. Statistical differences over time within each treatment were analyzed for all sampling days relative to Day 1 using two-way ANOVA with Dunnett's multiple comparisons test. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$, black asterisk = significance level for both Cp40 and control, blue asterisk = significance level for Cp40, red asterisk = significance level for control.

group ($p < .05$); otherwise, no significant differences were observed. Storage did not trigger CD63 expression (Figure 5B). There were no significant differences between Cp40 and control samples for either U46619 or TRAP-6 treatments on any day (Figure 5C-F). All samples became less responsive to the TXA_2R agonist U46619 over time, with a significant drop in CD62P and CD63 expression ($p < .0001$) (Figure 5C-D). PAR-1 responsiveness to TRAP-6 was better preserved, but from Day 5, both markers showed a significant reduction in expression in both treatments ($p < .0001$) (Figure 5E-F).

Discussion

Our objective here was to study if the limited but statistically significant complement activation in RDPs containing 35% plasma in PAS-E could propel platelet activation, and thus, complement inhibition could improve platelet in vitro quality. Indeed, we found significantly increased complement activation within the platelet concentrates over time, which was in line with our recent study.[11] Most pronounced was the activation of C3, which aligns with the propensity of artificial surfaces to activate complement via the alternative pathway.[32] The C3-inhibitor Cp40

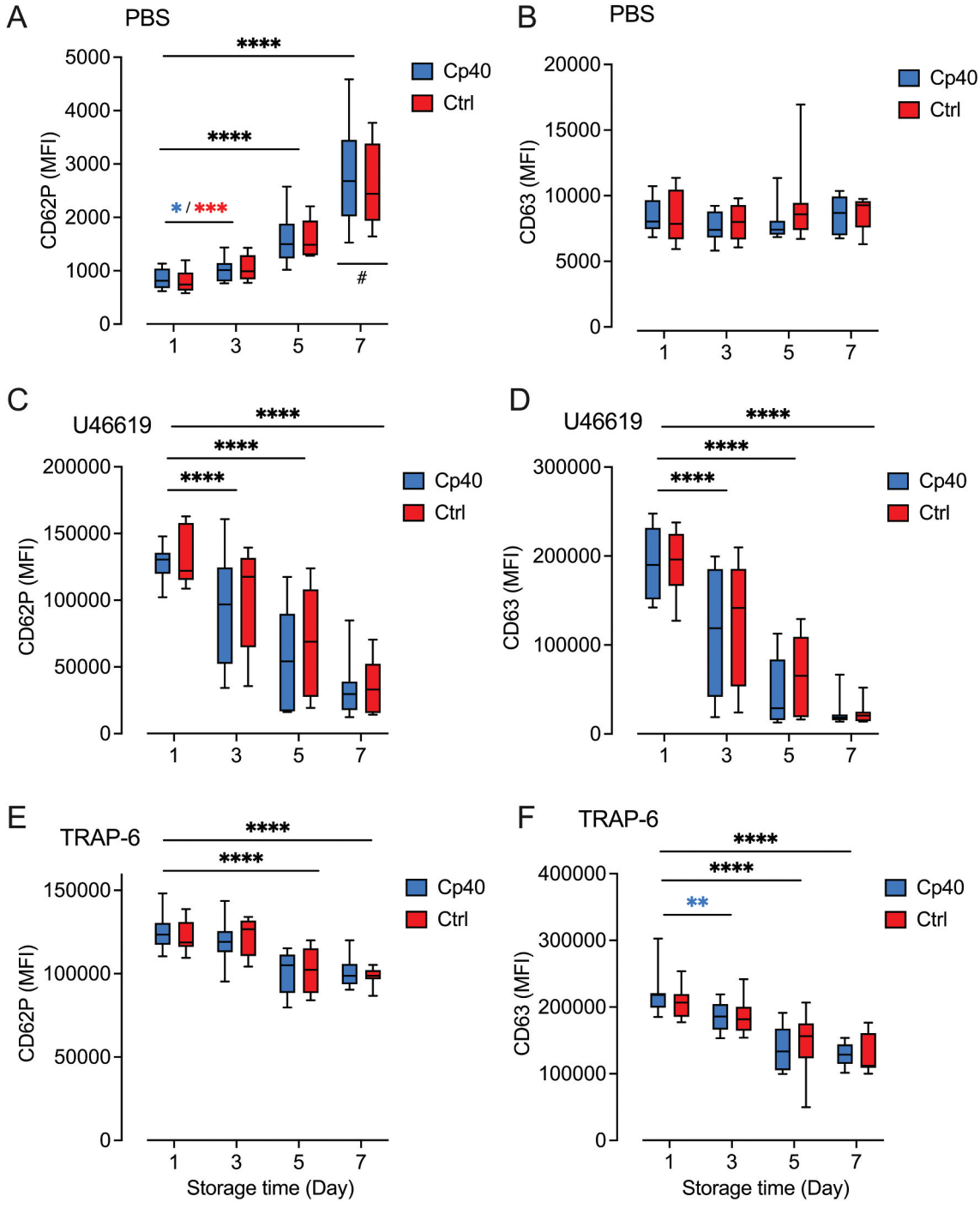

Figure 5. Expression of platelet surface activation markers. expression of CD62P (A, C, E) and CD63 (B, D, F) in aliquots from stored concentrate samples immediately treated with CTAD and EDTA (PBS) (A-B) or incubated for 20 minutes with the TXA_2R agonist U46619 (C-D) or the PAR-1 agonist TRAP-6 (E-F) before adding CTAD and EDTA (n = 8). Data are shown in box and whisker plots with range showing 5–95 percentile. Statistical differences between treatments for each day were analyzed using two-way ANOVA with šídák's multiple comparisons test. $^{\#}p$ <.05. Statistical differences over time within each treatment were analyzed for all sampling days relative to Day 1 using two-way ANOVA with Dunnett's multiple comparisons test, $^{*}p$ <.05, $^{**}p$ <.01, $^{***}p$ <.001, $^{****}p$ <.0001, black asterisk = significance level for both Cp40 and control, blue asterisk = significance level for Cp40, red asterisk = significance level for control.

effectively reduced the levels of C3bc, and sC5b-9, indicating successful inhibition of the complement cascade at the C3 level but did not impact C4-activation, as expected. Despite that Cp40 limited complement activation, complement inhibition had no effect on any of the markers for platelet activation, platelet function or metabolic markers. This indicates that while complement activation occurred, it was not to the extent to impact platelet functional or metabolic parameters in RDPs containing PAS-E and 35% plasma.

Cp40 binds C3 and C3b and efficiently blocks the cleavage of native C3 by the C3-convertases.[33,34] However, we observed elevated levels of the marker C3bc in concentrates containing Cp40. The increased C3bc was possibly due to increased levels of $C3(H_2O)$. $C3(H_2O)$ shares functional and conformational similarities with C3b[35] and is recognized by several of the C3b/iC3b/C3c neoepitope-specific antibodies.[36,37] We showed that $C3(H_2O)$ was elevated on Days 3 and 5 in the stored concentrates but decreased by Day 7. Although we do not have a definitive

explanation for the drop observed on Day 7, it is possible that the increased degree of platelet activation at this time point led to partial depletion of C3(H_2O) from the fluid phase due to surface binding, as C3(H_2O) binds to the surface of activated platelets.[38] Cp40 does not prevent C3 hydrolysis into C3(H_2O),[39] but somewhat surprisingly, Cp40 reduced the levels of C3(H_2O). This non-proteolytic activation of C3 is catalyzed by various surfaces, including activated platelets[38] and artificial surfaces,[13] which can explain the elevated C3(H_2O). C3(H_2O) can form the C3(H_2O)Bb C3-convertase, which is a rather inefficient convertase but can contribute to C3 cleavage. Along with complement activation, complement effector molecules are generated in the concentrates, including the anaphylatoxins C3a and C5a, the C3b-opsonin, and the terminal sC5b-9 complex. Collectively, these have been implicated in complement-mediated platelet activation,[17,19,22] but the inhibition of C3 did not translate to improved platelet storage outcomes, nor did it worsen them. One explanation could be that the level of complement activation was only minor. C3 activation, including C3(H_2O), increased two-fold and sC5b-9 increased 1.5 times in the concentrates without inhibitor. Even if our data suggests that complement inhibition does not limit platelet activation or rescues platelet function, it might be beneficial to limit transfusion of complement activation fragments to patients along with the platelets. This is particularly important for the C5a molecule, the inflammatory most potent of the complement activation products.[40] Although it is rapidly converted to C5a-desArg, this des-arginated form still has biological activity.[41] de Wit et al recently showed an association between increased complement C4- and C3-cleavage fragments and adverse transfusion reactions apheresis platelet concentrates.[42] This would be even more important in storage in 100 % plasma, where complement activation is more pronounced.[26] The activity of the alternative pathway is highest in undiluted serum and is gradually attenuated by plasma dilution.[43] Since the classical and lectin pathways are also attenuated by the presence of citrate in PAS-E, plasma dilution may be a significant determinant in the degree of complement activation. Future studies should examine the role of complement inhibition in platelet concentrates containing 100% plasma.

Here, we reduced the volume in the platelet storage bags by half but kept the volume-to-surface ratio intact. This did not affect the metabolic markers, indicating that the storage conditions were adequate to maintain platelets over the storage period. The present study shows a strong coherence with our previous study[11] regarding platelet activation and function. Some observations we made, independent of complement inhibition, were that the alpha-granule derived CD62P and NAP-2 increased as a function of storage, and the platelets attenuated ability to respond to TXA_2R stimulation was striking. Loss of TXA_2-TXA_2R function is associated with increased bleeding times and decreased platelet aggregation.[44] Further, we found that platelet sensitivity to U46619-induced activation of the TXA_2 receptor declined more rapidly than TRAP-6-induced activation of PAR-1. Additionally, the expression of surface receptors CD62P and CD63 in response to U46619 declined more than the release of soluble mediators (e.g., NAP-2 and TGF-β1). As platelets age, natural alterations in protein content can affect their functional responses.[45] Moreover, low-grade platelet activation in stored concentrates may induce receptor desensitization.[46–48] Whether these observations were due to natural platelet aging, effects from storage lesions or a combination thereof were beyond the scope of this study but would be interesting to investigate further.

In conclusion, complement activation occurs also in stored platelet concentrates in PAS-E/plasma, but our results from inhibiting C3 complement activation with Cp40 does not show a causal relationship between complement activation and platelet activation in this set-up. While C3 inhibition lowers complement C3 cleavage and terminal pathway activation, it does not impact platelet function or metabolic markers.

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Authors' contributions

LIA, PS, DJS, TEM, and PHN designed the study. LIA, PS, DJS, CM, KH, IT acquired the data. LIA, PS, DJS, CM, TEM, and PHN analyzed the data. LIA and PHN wrote the original draft of the manuscript. All authors participated in the interpretation of the data. All reviewed and revised the manuscript, and approved the final manuscript as submitted.

Ethical statement

This study was designed and conducted following the ethical guidelines outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects involved in the study, approved by Ethical Review Board in Linköping, Sweden. All platelet concentrates were de-identified after RDP procesing at Region Kalmar Län.

Supplementary material

Supplemental data for this article can be accessed online at https://doi.org/10.1080/09537104.2025.2513298.

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