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The contribution of von Willebrand factor–GPIbα interactions to persistent aggregate formation in apheresis platelet concentrates

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Background and Objectives Apheresis platelet concentrates sometimes contain persistent aggregates (PA). Because apheresis involves extracorporeal circulation, we hypothesized that interactions between GPIbα and von Willebrand factor (VWF) underlie their origin.

Materials and Methods Platelets in donations with PA were compared to aggregate-free (AF) controls. Flow cytometry was used to determine platelet bound VWF. Degranulation was measured using P-selectin expression in flow cytometry and cytokine release using immunosorbent assays. Platelet adhesion to VWF was assessed in hydrodynamic flow and real-time video microscopy.

Results Platelets in PA concentrates had significantly more ($P = 0{\cdot}009$, $n \geq 8$) bound VWF compared to AF platelets, but differences in VWF concentration, VWF collagen binding, activated VWF or GPIbα expression were not found. Degranulation was higher ($P = 0{\cdot}030$, $n = 7$) in PA than AF concentrates on day 1 of storage, but adhesion to immobilized VWF under hydrodynamic flow conditions was normal at that moment. On day 6, however, significantly less VWF adhesion ($P = 0{\cdot}009$, $n \geq 6$) was found for PA platelets compared to AF, indicating accelerated storage lesion in PA products. In a model that mimicks PA formation by chemically induced binding of VWF to platelets, we found that degranulation, phosphatidylserine expression and metabolism did not differ with paired controls at any time during subsequent storage.

Conclusion Accelerated storage lesion is found in concentrates with PA, but this cannot be explained solely by increased platelet bound VWF following apheresis. Therefore, additional stressors are probably responsible for the increases observed in platelet degranulation and storage lesion in products with PA.

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Introduction

Apheresis has specific advantages and disadvantages compared to other modalities of platelet concentrate production [1]. One practical problem in apheresis is the presence of platelet aggregates in the final product [2, 3]. These structures are poorly defined but manifest as macroscopic hyaline structures that are clearly visible to the naked eye, with or without backlighting. Most aggregates that reside in the collected product immediately after donation, spontaneously dissipate during a resting phase or following storage under standard blood banking conditions. However, in certain cases a significant number of aggregates will persist throughout storage and are

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designated 'persistent aggregates' (PA) (Fig. S1). Platelet concentrates prepared by the buffy coat method do not contain PA in the final storage bag in our experience. It is however not clear whether the leukofiltration step during component processing is successfully filtering out aggregates or whether these are simply absent to begin with. Therefore, this study focuses on PA in apheresis concentrates.

The advent of PA is unpredictable and yet a relatively common phenomenon, with roughly 4% of apheresis products being positive according to the internal scoring guideline used in our Blood Service [2, 4]. Depending on the institution's directives [2], products with PA may be distributed for transfusion, quarantined until free of aggregates or immediately destroyed. There are no clinical studies investigating these products, and the aetiology of PA in apheresis concentrates is not known [3].

During extracorporeal circulation in the apheresis machine, live endothelium is absent. Endothelial cells nonetheless provide crucial negative feedback signals to prevent spontaneous platelet activation *in vivo* [5]. In addition, the extracorporeal circulation involves centrifuging and pumping around blood (components) which cause hydrodynamic shear forces. Here, we hypothesize that PA formation can be explained by extracorporeal shear force and the loss of endothelial pacification which, respectively, favour primary VWF–platelet interactions and cellular activation. To address this, VWF and GPIbα molecular interactions were investigated in apheresis platelet concentrates with PA compared to aggregate-free (AF) controls. Effects on platelet quality during storage were studied in a model of increased VWF–GPIbα binding.

Materials and methods

Study design

Apheresis donations were by voluntary non-remunerated donors having given informed consent for the use of their donation for scientific purposes. Approval of an external ethics review commission was obtained under registration number B30020142040. Platelet collection from single donors was on a TRIMA ACCEL® (Terumo BCT, Lakewood, CO, USA) apheresis machine (software version 6.0.6). Standard operating procedures established at the Belgian Red Cross-Flanders Blood Service were followed. Collection targets are depending on prespecified donor parameters including haematocrit, whole blood platelet count, sex, body weight and length. Based on these parameters, an optimal target is proposed by the apheresis machine software. In all cases, there was dual component donation of plasma and platelets. The inlet to anticoagulant (acid citric dextrose) ratio was 11:1 in all cases. Following donation, the platelet bag was 'rested' label down, without flatbed agitation for at least 1 h at room temperature. Platelet concentrates were always supplemented with platelet additive solution (SSP+, Macopharma, Tourcoing, France).

Persistent aggregates are defined as free-floating precipitating hyaline particles, noticeable to the naked eye either during product processing or storage. The Belgian Red Cross-Flanders Blood Service routinely assesses platelet apheresis concentrates for the presence of aggregates using an in-house arbitrary scoring system reported previously [2]. In this study, platelets or supernatants of concentrates with PA were compared to unpaired AF products, that is the control group based on the scoring system ($n \geq 6$). Biochemical assays described below were performed on day one or six postdonation as indicated. All platelet assays were designed for the study of single platelets, the visible aggregates were not included.

Flow cytometry

Expression of glycoprotein Ibα (GPIbα) (anti-CD42b~fluorescein; Life technologies, Carlsbad, CA, USA) and P-selectin (anti-CD62P~phycoerythrin; BD Biosciences, Erembodegem, Belgium) was determined using an acoustic focusing Attune® flow cytometer (Life Technologies). Instrument stability and performance was controlled prior to every experiment using calibrated fluorescently labelled beads of different scatter properties. Binding of VWF to platelets was determined using in-house Alexa Fluor 488 labelled (A20181 from Life Technologies) polyclonal anti-VWF antibody (Dako, Glostrup, Denmark). Platelets were incubated with labelled antibodies for 10 min at room temperature in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7·4 with 0·9% NaCl (HBS), supplemented with 1 mM $MgSO_4$ according to Goodall *et al.* [6]. Phosphatidylserine exposure was measured using PerCP-Cy5.5 labelled Annexin V in buffers containing 2 mM $CaCl_2$. As a negative control for this experiment, calcium was left out. Median fluorescence intensities were determined of 10 000 cells in the platelet gate which were selected by positive staining for CD61 (anti-CD61~allophycocyanin; Life Technologies) and forward scatter. The signals of the negative isotype antibody controls were used to set threshold gates including 0·5% of 10 000 negative events.

Platelet adhesion during blood perfusion in microfluidic conditions

Reconstituted blood was prepared using platelets from AF or PA concentrates combined with fresh red blood cells (RBC) and plasma from an ABO-matched peripheral blood sample taken from healthy non-medicated volunteers in

heparin vacutainers® (REF368480; BD Diagnostics, Franklin Lakes, NJ, USA). Measures for preserving platelet quiescence during phlebotomy were taken into account as published elsewhere [7]. The blood was centrifuged at 250 g to prepare platelet rich plasma (PRP) and packed RBC. The PRP and buffy coat were transferred to a new tube and centrifuged at 4500 g to yield platelet-poor plasma. This cell pellet was discarded. To reconstitute blood, the packed RBC and plasma from this fresh blood were mixed with platelets from AF and PA concentrates aiming at 40 per cent haematocrit and 250 000 platelets/μl as described [8].

Perfusion experiments were performed using Vena8 Fluoro+™ biochips (Cellix Ltd, Dublin, Ireland). The microfluidic channels were incubated with 5 IU/ml VWF (Wilfactin®, CAF-DCF, Brussels, Belgium) at 4°C overnight and next blocked with HBS containing 1·0% (w/v) bovine albumin and 0·1% (w/v) D-glucose at room temperature for 30 min. Blocking buffer and remainder coating solution were rinsed with 1 ml HBS buffer. The reconstituted blood was labelled with calcein-AM (5 μM final) (Life technologies). Perfusion was performed at 135 dynes/cm^2 (3000/s flow rate) using a Mirus Evo microfluidic pump (Cellix Ltd). Because the Wilfactin® fractionated VWF component contains trace fibrinogen, 50 ng/ml of tirofiban (aggrastat®; Sigma-Aldrich, St Louis, MO, USA) was added to all samples to inhibit integrin $\alpha_{IIb}\beta_3$ interactions and focus on the VWF–GPIbα interaction. Every 6 s, three side-by-side images were snapped in real time in each lane during 5 min using an inverted fluorescent microscope at 100× magnification equipped with a Colibri-LED fluorescent light source (488 nm) and high-resolution CCD camera (all Carl Zeiss, Oberkochen, Germany). One single image per time-point was generated by digitally stitching the three side-by-side images (ZEN2012 software (Carl Zeiss)). Surface coverage was determined by measuring the fraction of pixels positive for platelet deposition defined as having a fluorescence of 400 arbitrary units or higher. As expected this parameter was almost immediately steady state in function of time, because there is no thrombus build-up when only interactions with VWF are formed [9]. All image acquisition and analysis variables, including CCD camera exposure time, LED intensity, binning settings and threshold values, were kept constant throughout this study.

Immunosorbent assays

The platelet cytokines transforming growth factor beta (TGFβ1), chemokine (C-C motif) ligand 5 (CCL5 or RANTES) and platelet factor 4 (PF4 or CXCL4) were determined by commercial immunosorbent assays (ELISA) following the instructions of the provider (R&D Systems, Minneapolis, MN, USA). These assays were based on the sandwich principle and included validated calibration standards provided by the manufacturer.

VWF antigen (VWF:Ag) was measured as described [10]. Binding of VWF to human type III collagen (Sigma-Aldrich) was measured by ELISA according to the method described by Siekmann [11]. The level of activated VWF was determined by ELISA using a nanobody (kind gift from Dr. P.J. Lenting, Paris, France) specifically binding to the activated conformation of VWF [12]. To correct for variation caused by VWF:Ag, equal VWF concentrations were used for all samples in the assay. D-dimer (Raybiotech, Norcross, GA, USA) and fibrinogen (Molecular Innovations Inc, Novi, MI, USA) concentrations were determined using commercially available ELISA kits. Total protein concentration was measured by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA).

Model of premature VWF–GPIbα interaction

Platelet concentrates (n = 5) were prepared by pooling of six buffy coats, and storage in SSP+ was used for mimicking the effect of premature VWF–GPIbα interactions. Each single product was split in two equal volume daughter products. One was supplemented with 600 μg/ml of filter-sterilized ristocetin (in SSP+) and the other with only vehicle to serve as a paired control. Analysis of platelet concentrate quality in function of storage was on days 1, 2, 5 and 7 using small volume samples taken through a sterile connection and a syringe under a safety cabinet. Quality was assessed by the above described techniques. All products were assessed for bacterial contamination by inoculation of a 100 μl sample on agar plates on day 7 and incubation for 24 h at 37°C. None was infected.

Statistical analysis

Comparison of means of laboratory data was with t-test or Mann–Whitney testing for parametric or nonparametric datasets, respectively. A significance level of 0·05 was used to claim statistical difference. Analyses were performed with PRISM® software version 6.01 (GraphPad Software Inc, La Jolla, CA, USA). P-values are indicated on top of the panels, except when non-significant. Data are shown as mean with standard deviation or median with interquartile range for parametric or nonparametric datasets, respectively. Gaussian distribution was tested using the Shapiro–Wilk normality test.

Results

Platelets in PA concentrates (n = 11) had significantly more bound VWF (P = 0·009) compared to AF (n = 8)

(Fig. 1). This was not caused by increased VWF antigen levels in PA (n = 13) vs. AF (n = 7), on day 1 nor day 6 (Fig. 2a). VWF function is however not solely dependent on its concentration, and therefore, the level of activated VWF was measured (Fig. 2b), but no difference could be found. Notably, significantly lower amounts of activated VWF were found in all apheresis concentrates when compared to normal human pooled plasma (NHP, dotted horizontal line). VWF collagen binding and GPIbα expression levels were measured (Fig. S2) as well as total protein concentration, fibrinogen concentration and D-dimer concentration, but none of these parameters differed between study arms (Fig. S3).

Platelet adhesion to immobilized VWF under flow was neither different between PA (n = 7) and AF (n = 6) in day 1 samples but on day 6, significantly less (P = 0·009) VWF adhesion was seen in PA samples compared to AF controls (Fig. 3). Platelet counts of the reconstituted samples could not account for this as these were not significantly different (247 ± 11 for AF and 266 ± 18 for PA, P = NS).

P-selectin expression (P = 0·030) as well as cytokine concentrations ($P \leq$ 0·042) were increased on day 1 in PA samples (n = 13) compared to AF (n = 7) demonstrating degranulation early on in the process of collection, processing or storage of concentrates with PA (Fig. 4). Platelet concentrations in both arms were not significantly different, and therefore, cytokine concentration increase was specific.

To model the effects of VWF binding to platelets, ristocetin was added to platelet concentrates immediately after production and storage lesion parameters were measured in function of time. The model shows that binding of VWF to platelets on day 1 is increased compared to vehicle-treated controls (Fig. 5). The VWF-binding levels were similar to those found in apheresis products with PA (Fig. 1). During storage however VWF binding reversed, unlike the observation in PA concentrates. Of note, storage lesion parameters in function of storage time were not increased compared to vehicle-treated controls as measured by degranulation (Fig. 5b and Fig. S4), GPIbα

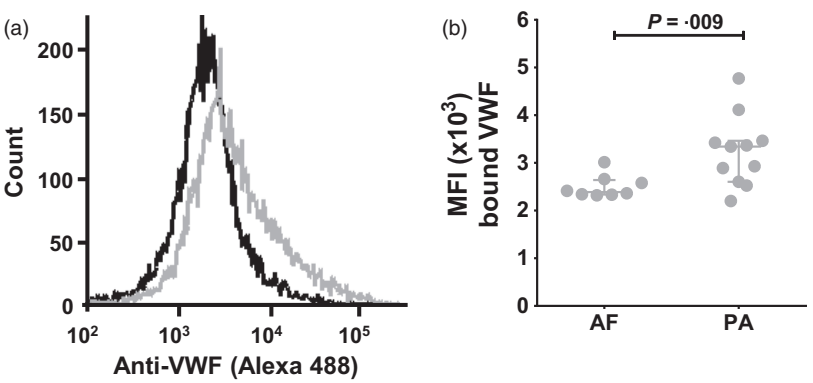

Fig. 1 Comparison of VWF binding to platelets in concentrates with PA compared to AF controls. (a) Flow cytometry histograms depicting 10 000 CD61 positive events taken from a representative AF (black) and PA concentrate (grey). (b) Median fluorescence intensities (MFI) of the anti-VWF signal were collected in AF (n = 8) and PA (n = 11) concentrates on day 1.

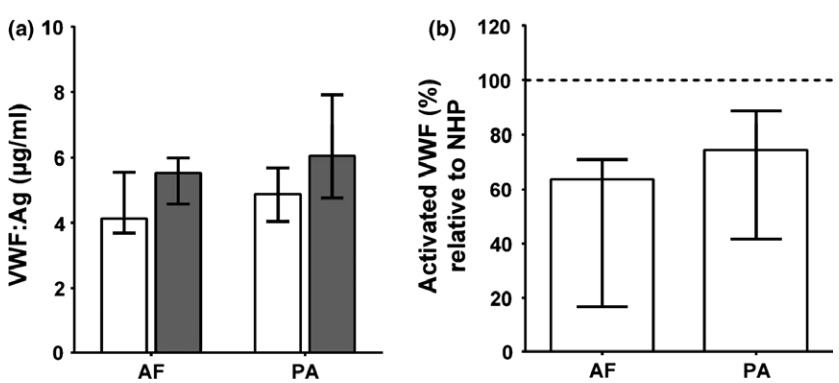

Fig. 2 VWF antigen and activated VWF in supernatants of concentrates with PA and AF controls. (a) VWF antigen (VWF:Ag) concentration was determined on day 1 (open bars, AF n = 7 and PA n = 13) and day 6 (shaded bars, AF and PA n = 13) postdonation. Concentrations were determined relative to that in NHP. (b) The levels of activated VWF were determined on day 1 using NHP as a standard arbitrarily set to 100% and indicated by the dotted horizontal line (AF n = 7 and PA n = 13). In both panels, median and interquartile range are depicted. Differences between groups were not statistically significant for both parameters tested.

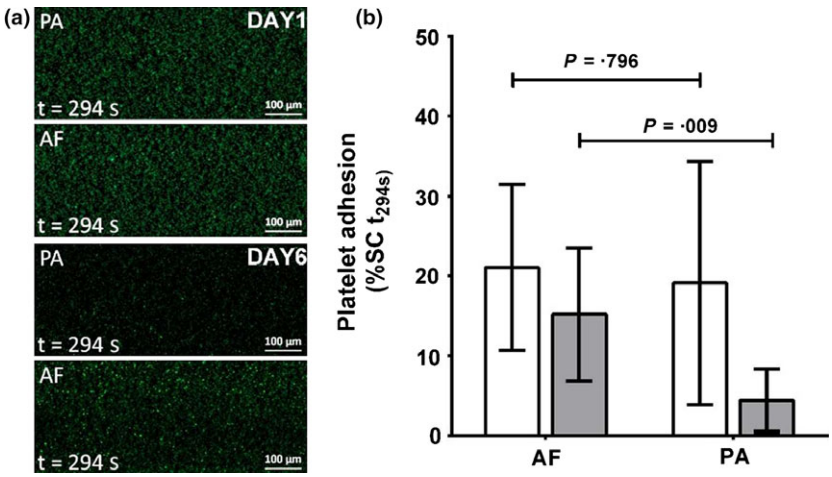

Fig. 3 Platelet adhesion to VWF of platelets from concentrates with PA and AF controls. (a) Representative snapshots taken at 294 s of perfusion depicting adhering fluorescently labelled platelets on day 1 (top panel) and day 6 (bottom panel). Scale bar indicates 100 µm. (b) Platelet adhesion at 294 s of perfusion (% SC t_{294s}) is shown as mean surface coverage with SD. Results are shown for unpaired AF (n = 6) and PA (n = 7) concentrates on day 1 (open bars) and day 6 (shaded bars).

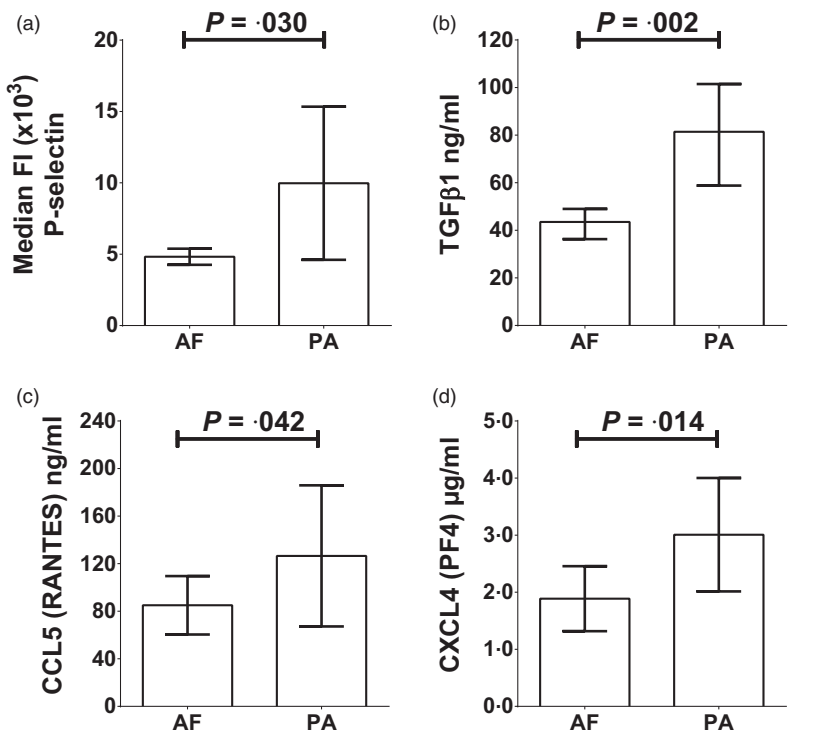

Fig. 4 Levels of degranulation in platelets from concentrates with PA and AF controls. (a) Median fluorescence intensities (MFI) indicate the median level of platelet degranulation using an anti-P-selectin antibody binding to 10 000 CD61 positive events in AF and PA concentrates. (b–d) The concentration of platelet related cyto- and chemokines was determined by immunosorbent assays in the supernatants of AF and PA concentrates. (b) TGFβ1 in ng/ml, (c) CCL5 in ng/ml and (d) CXCL4 in µg/ml. All measurements were done on material from day 1 postdonation. All data are shown as mean and SD except for TGFβ1 in panel B depicting median and interquartile range.

expression (Fig. 5c), phosphatidylserine exposure (Fig. 5d) nor platelet metabolism (Fig. S5).

Discussion

In this study, we demonstrate that platelets in PA concentrates had increased degranulation as well as increased binding of VWF compared to AF controls. Adhesion to immobilized VWF was decreased in PA products, but only on day 6 pointing to increased storage lesion. A model of chemically induced VWF binding was used to mimic increased VWF–platelet binding but could not show increases in platelet degranulation nor storage lesion.

The primary interaction between platelets and the damaged vessel wall is highly dependent on the prevailing fluid shear forces [9] and strengthens in proportion to the impacting drag [13]. Apheresis involves fluid aspiration and centrifugation of blood or its components, and this process equally evokes fluid shear and drag forces [3] that may facilitate platelet–platelet binding through GPIbα and sheared soluble plasma VWF [14]. Furthermore, our previous work on this topic indicated that certain donors

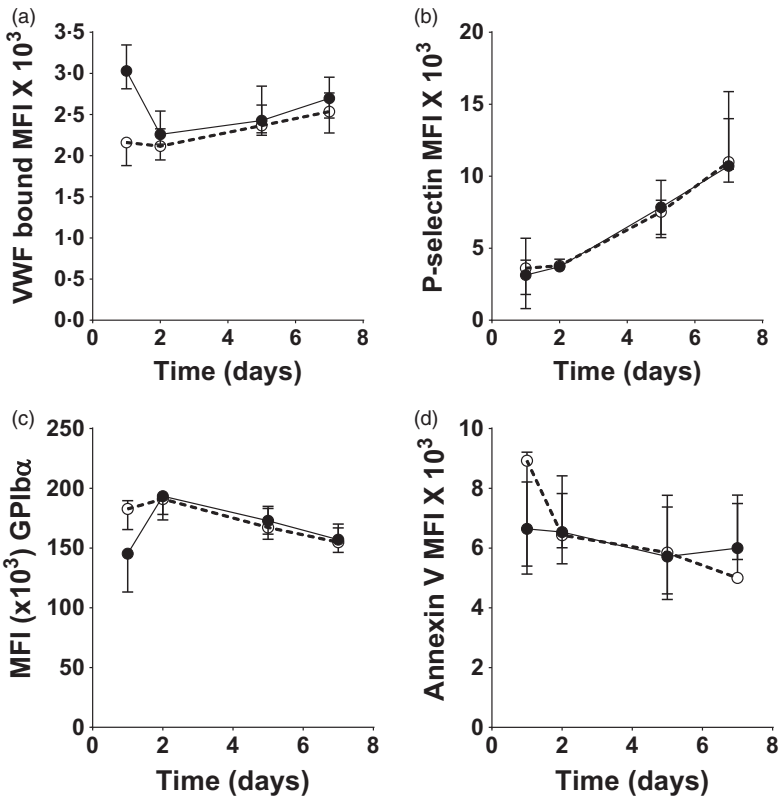

Fig. 5 Experimental model of increased VWF binding. (a) The amount of bound VWF, (b) degranulation, (c) GPIbα receptor expression and (d) apoptosis and/or activation. Median fluorescence intensities (MFI) are expressed as median and interquartile range (n = 5) for samples having been treated with 600 μg/ml ristocetin on day 1 to model increased VWF–GPIbα binding (●) vs. paired vehicle-treated control platelets (○, dashed line).

are more likely to routinely donate concentrates with PA [4]. In that study, we also showed that platelets from PA products are more sensitive to low-dose ristocetin. Both VWF and GPIbα are polymorphic molecules explaining biological variation in the quality and number of established interactions [15, 16]. Together, this supported the hypothesis that VWF–GPIbα interactions are established during apheresis and that these may form the basis for PA formation in a subset of donors.

The current study demonstrates that platelets in concentrates with PA indeed carry more bound VWF than AF controls which is in support of the hypothesis. Increased VWF binding is clinically relevant to transfusion medicine because it increases macrophage uptake of affected platelets and subsequent clearance from circulation [17, 18], potentially decreasing transfusion yields. Mutations in VWF or GPIbα can also cause increased binding in rare diseases like von Willebrand's disease type 2B and platelet-type von Willebrand's disease, respectively, each with varying degrees of thrombocytopenia and bleeding [19]. Additional research is needed to demonstrate whether transfusion of apheresis concentrates with PA or with higher VWF–platelet binding in general, results in lower count increments than with AF products.

Binding of VWF to platelets in healthy donors may show degrees of variation as a consequence of acquired or hereditary differences in either binding partners [20, 21]. We therefore assessed several VWF laboratory parameters as well as the GPIbα receptor expression levels in the AF and PA donor population. VWF antigen, collagen binding and activated VWF were not significantly different, indicating no measurable phenotypic variation explaining the difference between AF and PA on the basis of VWF. Of note, VWF:Ag levels rise in both conditions during storage, as platelets gradually release alpha-granules.

Moreover, we found no significant difference in GPIbα receptor expression levels, indicating that the number of receptors is not responsible for the observed increased VWF binding. However, this does not rule out the possibility of functional GPIbα differences between donors that could account for the observation. Functional GPIbα differences were not assessed in this study but may be related to GPIbα linkage with the cytoskeleton [22], to its affinity for VWF [23, 24] or to the signal transduction cascade that is linked with the GPIb-IX-V complex [25]. Finally, no differences in total protein concentration, fibrinogen levels nor D-dimer concentrations were found. The latter two are important because variable anticoagulation may variably induce thrombin formation which in turn can affect GPIb-IX-V complexes on platelets. Our data indicate that on the D-dimer level, there was no

measurable coagulation contributing to the phenomenon of PA formation compared to AF concentrates. This supports the finding that mere increases in the anticoagulant to plasma ratio has not eliminated the problem of PA in apheresis collection [26].

Because normal interaction with immobilized VWF may be compromised when platelets are 'coated' with VWF [27], platelet adhesion was analysed in flow chambers. We found no difference between AF and PA concentrates on day 1 indicating that adhesion of platelets from PA concentrates is normal in the early stages of storage. However, on day 6, platelets from PA concentrates were significantly less adhesive than AF indicating accelerated storage lesion. This corresponds to our previous work showing increased levels of P-selectin and lactic acid resulting in lower pH values following storage of PA concentrates [4]. Importantly, degranulation is not a consequence of storage itself because platelets have degranulated early on following apheresis on day 1. This allows speculation that established VWF–GPIbα interactions are required to stimulate platelets to release its granule content contributing to accelerated storage lesion.

To confirm or refute this hypothesis, we simulated increases of VWF–GPIbα binding in platelet concentrates prepared from buffy coats by adding low-dose ristocetin [28] early on in storage (day 1). Immediately after ristocetin supplementation, VWF binding increased significantly to levels comparable to those in concentrates with PA. In addition, macroscopic disseminated aggregates were formed instantaneously which visually resembled the characteristic transient aggregates collected during apheresis, but these disappeared within 10 min of storage on the orbital shaker. In line with this, the laboratory model of increased VWF–GPIbα binding did not result in increased storage lesion displaying normal metabolism, degranulation and phosphatidylserine exposure throughout storage compared with paired vehicle controls. It could be that molecular differences between the primary products, that is apheresis vs. buffy coat derived prevent exact mimicking of PA formation.

Yet, our data collectively indicate that PA formation is multifactorial, hence not dependent on initial VWF–GPIbα binding alone. Studies on signal transduction from GPIb-IX-V indeed demonstrate that binding to this particular receptor does potentiate signals from other receptors [29–32], but on itself is not sufficient to fully and irreversibly activate platelets. Because degranulation is nonetheless apparent on day 1 already, it is likely that the observed increase in VWF–GPIbα binding has potentiated other signals that have activated platelets during apheresis from certain donors and thereby causing PA.

In conclusion, this study demonstrates that products with PA have accelerated storage lesion. This coincides with increased VWF–platelet binding and platelet degranulation. The former phenomenon can be mimicked (transiently) by the addition of ristocetin while the latter cannot. Therefore, increased VWF binding to platelets is not sufficient to explain increased storage lesion. Yet to be identified additional stressors are probably required to boost platelet degranulation in products with increased VWF–GPIbα interactions. These are pivotal in speeding up storage lesion, and future research should indicate if this influences count increments and haemostasis quality of stored PA products.

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Authorship contributions

HBF and RD performed experiments and interpreted results; BVA interpreted results; PV and VC provided essential materials and supported the study; HBF and VC designed research and interpreted results; HBF wrote the manuscript. All authors critically reviewed the manuscript and agreed upon submission.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Fig. S1 Persistent aggregates in apheresis platelet concentrates.
Fig. S2 VWF binding to collagen and the expression of the collagen binding receptor GPIbα.
Fig. S3 Total protein and fibrinogen.
Fig. S4 cytokine concentrations in a model for increased VWF binding.
Fig. S5 Metabolism is normal in a model of storage lesion in platelet concentrates with increased VWF-platelet binding.