# Random aggregates in newly produced platelet units are associated with platelet activation and release of the immunomodulatory factors sCD40L and RANTES

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**BACKGROUND:** In connection with platelet (PLT) production, random but transient aggregates sometimes form in the newly produced units. The underlying mechanisms as well as the impact on cellular level of this phenomenon are unknown. Hypothetically, random occurrence of aggregates may induce biochemical changes leading to PLT activation and release of immunomodulatory factors from the PLTs.

**STUDY DESIGN AND METHODS:** PLTs were aliquoted and prepared with an automated system for PLT pooling (OrbiSac, Terumo BCT) for a three-arm nonpaired study design (n = 8). Initially aggregated PLT units in SSP+ were selected by visual inspection and compared to unaffected PLT units stored in SSP+ or 100% plasma. Cellular, metabolic, and functional variables were analyzed, including the concentrations of RANTES, sCD40L, and sTWEAK in the bags during a 9-day storage period.

**RESULTS:** Isolated aggregated PLTs show signs of spontaneous activation and respond less efficiently to TRAP stimulation. RANTES, sCD40L, and sTWEAK accumulated in the various PLT units during storage but sCD40L and RANTES accumulated in the initially aggregated PLT units to higher concentrations than the reference units and PLTs stored in 100% plasma ($p < 0.001$). Over time, the levels of sTWEAK increased more in the plasma storage environment compared with PLT units stored in SSP+ ($p < 0.001$).

**CONCLUSION:** Our data indicate that random occurrence of aggregates may lead to higher activation level and increased release of immunomodulatory factors from the PLTs.

Febrile nonhemolytic transfusion reactions (FNHTRs) associated with platelet (PLT) transfusion therapy may be caused by cytokines accumulating in the storage bag during storage. Prestorage leukofiltration has proven effective in lowering the cytokine content in storage bags,[1-3] but despite this procedure, which is performed routinely in most blood centers, FNHTRs still occur.[4] It has been suggested that PLTs themselves secrete immunomodulatory factors during storage, because many factors accumulate in the bags with time.[5,6] This suggestion also includes PLTs stored in plasma-free solutions.[7-9] In addition, PLT washing in saline before transfusion further reduces the risk for FNHTR, suggesting that released factors play a role.[10]

Several factors associated with PLT production have been proposed to play a major role in FNHTR. Of those, CD40 ligand (CD40L, CD154) and its soluble counterpart sCD40L have attracted great attention.[11-16] Other substances with proposed similar effects include tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK), another member of the TNF-α family of cytokines,[11,17-19] regulated upon activation of normal T-cells expressed and secreted (RANTES), a factor with

**ABBREVIATIONS:** ESC = extent of shape change; FNHTR(s) = febrile nonhemolytic transfusion reaction(s); HSR = hypotonic shock response reactivity.

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proinflammatory and chemoattractive functions,[20,21] β-thromboglobulin, PLT factor 4, and interleukin-7 (IL-7).[7,9]

Because of the potentially harmful effects of PLT-derived factors, such as the ones mentioned here, in FNHTR, identifying conditions that affect their release by PLTs during storage is of major importance. One such condition may be the spontaneous occurrence of random aggregates in newly produced PLT units from buffy coats. In our blood bank, approximately 15% of all produced units display such aggregates, characterized by fragmentary layer of visually detectable flakes or lumps. Most of those units show a normal appearance the day after production day, suggesting that the initially formed aggregates dissolve with time. In the current routine, these units are used for transfusion like the unaffected units.

To evaluate the possible effects of random aggregation on metabolic, phenotypic, and cytokine-secreting properties, we set up a study comparing PLTs from aggregated and unaffected units suspended in 70% SSP+. As a third arm, we also included nonaggregated PLTs stored in 100% plasma. All variables were followed over a 9-day storage period. Our data show that while the metabolic variables remained largely unaffected, initially aggregated PLTs showed signs of increased activation reflected by cell surface expression of CD62P. Concentrations of CD40L and RANTES were strongly increased in the bags with initially aggregated PLTs. TWEAK accumulated over time in all PLT bags but did not increase as a consequence of increased activation and aggregation. We conclude that initially aggregated PLT units may increase the risk of causing adverse negative events after PLT transfusion.

## MATERIALS AND METHODS

### Preparation and storage of PLTs

PLTs were collected from normal blood donors who met standard donation criteria and gave written, informed consent according to institutional guidelines. In an attempt to reduce the risk of interruption in the production line for blood component preparation, a total of 450 mL of whole blood was drawn into either a CPD/SAG-M quadruple-bag blood container system (Fenwal, La Châtre, France) or the NPT 6280LE blood bag system (MacoPharma, Mouvaux, France). After storage at room temperature for 2 to 6 hours, whole blood units were centrifuged ($2700 \times g$) for 10 minutes at 22°C. Automatic equipment was used for the preparation of blood components (Optipress, Fenwal or Macopress, Smart, MacoPharma), including buffy coat. Buffy coats were prepared with plasma inclusion of 20 to 25 mL to allow suspension of PLTs either in SSP+ (MacoPharma) or in 100% plasma. Five buffy coats were pooled to produce 1 PLT unit by a PLT pooling processing system (OrbiSac, Terumo BCT, Zaventem, Belgium).[22] PLTs were stored in PLT storage bags (OrbiSac Standard BC set, storage bag, Terumo BCT, Inc., Lakewood, CO) made of polyvinyl chloride plastic with a citrate-based plasticizer (BTHC), intended to contain up to $5 \times 10^{11}$ PLTs in 400 mL of plasma. The study design is as follows:

(A) PLTs stored in 70% SSP+ (reference PLTs), n = 8.
(B) Initially aggregated PLTs stored in 70% SSP+ (aggregated PLTs), n = 8.
(C) PLTs stored in 100% plasma (PLTs in plasma), n = 8.

To avoid disintegration and adverse negative effects on the PLTs, the air and foam were excluded from all units.[23] The selection of PLT units included in the (B) arm was based on visual visible aggregates immediately after production with the OrbiSac device. All PLT units were stored on a flatbed agitator in a temperature-controlled cabinet at 22 ± 2°C (Model PC900i, Helmer, Noblesville, IN). The samples were drawn aseptically on Days 2, 5, 7, and 9.

### Analysis of activation status and responsiveness of aggregated PLTs

Samples from units with visible aggregates were transferred to 1.5-mL polypropylene tubes. After sedimentation of the aggregates most of the supernatant was removed and the aggregates were dissolved by pipetting vigorously. Nonaggregated PLTs from the supernatant and PLTs from a normal unit were treated in the same way before flow cytometric analysis; untreated PLTs from a normal unit were used as a reference.

The expression of the two activation markers CD62P (P-selectin/GMP-140/PADGEM; clone CLB Thromb/6) purchased from Immunotech (Beckman Coulter, Marseilles, France) and the expression of a conformational epitope on the GPIIb/IIIa complex of activated PLTs, assessed by use of the fluorescein isothiocyanate–conjugated monoclonal antibody (MoAb) PAC-1, immunoglobulin M (BD340507, Becton Dickinson, San Jose, CA) was measured by flow cytometry (LSRFortessa cell analyzer, BD Biosciences, San Jose, CA) on PLTs from all four groups with and without TRAP stimulation.

### RANTES, sCD40L, sTWEAK, and intracellular and cell surface expression of TWEAK

Samples from the PLTs and randomly collected plasma units (n = 8) were collected in citrate theophylline adenosine dipyridamole tubes. The samples from the collected PLTs and plasma units were centrifuged at $2500 \times g$ (Eppendorf 5810R, Westbury, NY) for 30 minutes at +10°C and the supernatants were stored at –80°C pending analysis. RANTES detection was performed as earlier described.[8] sCD40L and soluble TWEAK (sTWEAK) concentrations were determined with enzyme-linked

immunosorbent assay (ELISA) commercial kits (Quantikine, CD40 ligand immunoassay DCDL40 and eBioscence, human TWEAK instant ELISA) in accordance with the manufacturer's (R&D Systems, Inc., Minneapolis, MN; and Bender MedSystems GmbH, Vienna, Austria) recommendations. All measurements were performed in duplicate on an HT3 microtiter plate reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at 466 nm and the results for the sCD40L and sTWEAK concentrations are given in pg/mL. In an attempt to trace the origin of TWEAK, intracellular and cell surface expression of TWEAK from the reference samples with or without ADP and TRAP stimulation were determined by using a cell analyzer and commercial kits (LSRFortessa and BD Cytofix/Cytoperm, respectively) and anti-Tweak PE clone (CARL-1) in accordance with the manufacturer's recommendations (BD Biosciences).

### Analysis of cellular, metabolic, in vitro functional, and PLT surface variables

The in vitro cellular and metabolic variables evaluated included PLT counts ($10^9$/L and $10^9$/unit) and mean PLT volume (MPV) using a hematology analyzer (CA 620 Cellguard, Boule Medical, Stockholm, Sweden). The volume (mL) was calculated by weighing the contents of the storage bag, in grams, on a scale (Mettler PB 2000, Mettler-Toledo, Albstadt, Switzerland) and the result, in grams, was divided either by 1.01 (1.01 g/mL is the density of the storage medium composed of approx. 70% SSP+ and 30% plasma) or by 1.03 (1.03 g/mL is the density of the storage medium composed of 100% plasma). By use of routine blood gas equipment (ABL 800, Radiometer, Copenhagen, Denmark) we also measured the pH (37°C), $pCO_2$, $pO_2$ (kPa at 37°C), glucose (mmol/L), and lactate (mmol/L). Bicarbonate (mmol/L) was calculated from pH and $pCO_2$ partial pressure measurements. The pH of all samples was measured at 37°C. Therefore, Rosenthal's factor of 0.0147 unit/1°C was used to correct pH to the temperature of sampling (22°C). This factor gives an approximation to the change in pH of the sample per degree centigrade when it is warmed anaerobically from the collecting temperature 22°C to 37°C.

The assessment of swirling was done by inspection and grading according to Bertolini and Murphy.[24] The white blood cell (WBC) count on Day 1 was determined with a Nageotte chamber and a microscope (Zeiss, standard, Carl Zeiss, Chester, VA).[25] Hypotonic shock response reactivity (HSR) as well as extent of shape change (ESC) measurements were performed using a dedicated microprocessor-based instrument (SPA 2000, Chronolog, Havertown, PA) with the modifications of these tests described by VandenBroeke and coworkers.[26] The total adenosine triphosphate (ATP) concentration, (μmol/$10^{11}$ PLTs) was determined with a luminometer (Orion microplate luminometer, Berthold Detection Systems GmbH, Pforzheim, Germany) on the basis of principles described by Lundin.[27] Lactate dehydrogenase (LDH) activity, a marker for disintegration of PLTs, was measured with a spectrophotometric method (Sigma Aldrich kit 063K6003; spectrophotometer Jenway 6500, Jenway, Staffordshire, UK).[28] MoAb labeling and flow cytometry analysis were performed as recently described.[23]

### Statistical analysis

The mean values and standard deviations (SDs; n = 8) are usually given unless otherwise indicated. A repeated-measures analysis of variance including post hoc test Bonferroni's adjustment was performed. Three different groups, SSP+ and plasma, were studied over time (Days). "Days" was the repeated factor and "group" was a between factor. The results of Bonferroni's test are presented in Tables 1 and 2. The p value represents the differences between groups at specific time points and was considered significant at p values of less than 0.001. The analyses were carried out using computer software (Statistica version 9, StatSoft, Inc., SPSS, Chicago, IL).

## RESULTS

### Initial PLT aggregation

During production of PLTs from pooled buffy coats using the OrbiSac device, we noted visible PLT aggregation immediately after production in some units (Fig. 1). The number of units with initial aggregation was approximately 600 during a 5-month period, which represents approximately 15% of all produced units. Of eight numbers of initially aggregated units, eight spontaneously resolved and none remained aggregated after 24 hours. Units that spontaneously resolved were included in the (B) arm.

### Aggregated PLTs show signs of spontaneous activation and respond less efficiently to TRAP stimulation

Initially, we hypothesized that aggregation was connected to PLT activation. To test this, PLT aggregates were isolated by sedimentation. Because we assumed that the aggregation of PLTs in one unit might affect the nonaggregated PLTs in the same bag we also analyzed PLTs from the supernatant. The aggregates were dissolved mechanically by pipetting vigorously before they were stained for flow cytometry. Because shear stress has an impact on the activation status of PLTs,[29] we wanted to exclude that any observed differences between aggregated and nonaggregated samples were due to the treatment. Therefore, nonaggregated PLTs from the supernatant of the aggregates (referred to as "nonaggregated PLTs" in the following) and PLTs from a normal unit (referred to as "control") were

**TABLE 1. Metabolic and cellular in vitro effects on PLTs (n = 8) stored for 9 days in SSP+ and plasma***

| | Day | | | |
|---|---|---|---|---|
| Variable | 2 | 5 | 7 | 9 |
| MPV (fL) | | | | |
| (A) Reference PLTs | 9.1 ± 0.6 | 9.3 ± 0.6 | 9.2 ± 0.5 | 9.4 ± 0.6 |
| (B) Aggregated PLTs | 8.4 ± 0.4 | 8.6 ± 0.6 | 8.7 ± 0.5 | 8.8 ± 0.5 |
| (C) PLTs in plasma | 8.5 ± 0.5 | 8.7 ± 0.5 | 8.8 ± 0.5 | 9.1 ± 0.6 |
| LDH (extracellular % of total) | | | | |
| (A) Reference PLTs | 4.3 ± 2.3 | 4.0 ± 1.2 | 4.6 ± 1.8 | 5.2 ± 1.5 |
| (B) Aggregated PLTs | 3.8 ± 2.0 | 4.9 ± 1.5 | 4.9 ± 2.2 | 5.5 ± 2.1 |
| (C) PLTs in plasma | 7.3 ± 1.2 | 8.5 ± 2.3†‡ | 7.3 ± 2.2 | 9.1 ± 2.4†‡ |
| Glucose (mmol/L) | | | | |
| (A) Reference PLTs | 6.0 ± 0.3 | 4.7 ± 0.4 | 3.8 ± 0.4 | 2.6 ± 0.4 |
| (B) Aggregated PLTs | 4.3 ± 0.8 | 3.1 ± 0.8 | 2.1 ± 0.8 | 0.9 ± 0.9 |
| (C) PLTs in plasma | 16.2 ± 0.7†‡ | 14.0 ± 1.1†‡ | 12.3 ± 1.3†‡ | 10.5 ± 1.7†‡ |
| Lactate (mmol/L) | | | | |
| (A) Reference PLTs | 6.7 ± 1.0 | 9.4 ± 0.7 | 11.4 ± 0.8 | 13.5 ± 1.0 |
| (B) Aggregated PLTs | 7.4 ± 0.9 | 9.7 ± 1.0 | 11.6 ± 1.1 | 13.9 ± 1.3 |
| (C) PLTs in plasma | 8.8 ± 0.9 | 12.2 ± 1.1†‡ | 15.0 ± 1.6†‡ | 18.1 ± 2.2†‡ |
| pH (22°C) | | | | |
| (A) Reference PLTs | 7.216 ± 0.039 | 7.324 ± 0.039 | 7.344 ± 0.037 | 7.344 ± 0.034 |
| (B) Aggregated PLTs | 7.187 ± 0.043 | 7.275 ± 0.051 | 7.285 ± 0.061 | 7.267 ± 0.075 |
| (C) PLTs in plasma | 7.347 ± 0.044†‡ | 7.411 ± 0.074†‡ | 7.347 ± 0.076 | 7.241 ± 0.106 |
| $pCO_2$ (kPa at 37°C) | | | | |
| (A) Reference PLTs | 3.92 ± 0.21 | 2.78 ± 0.14 | 2.56 ± 0.1 | 2.39 ± 0.1†‡ |
| (B) Aggregated PLTs | 3.54 ± 0.21 | 2.37 ± 0.20 | 2.13 ± 0.1 | 1.85 ± 0.2 |
| (C) PLTs in plasma | 6.11 ± 0.43†‡ | 3.78 ± 0.36†‡ | 3.48 ± 0.28†‡ | 3.14 ± 0.26†‡ |
| Bicarbonate (mmol/L calculated) | | | | |
| (A) Reference PLTs | 6.8 ± 0.3 | 6.3 ± 0.3 | 6.0 ± 0.4 | 5.6 ± 0.3 |
| (B) Aggregated PLTs | 5.8 ± 0.6 | 4.8 ± 0.7 | 4.4 ± 0.7 | 3.7 ± 0.9 |
| (C) PLTs in plasma | 14.5 ± 0.6†‡ | 10.6 ± 0.9†‡ | 8.3 ± 1.1†‡ | 5.9 ± 1.3†‡ |
| ATP (μmol/1011 PLTs) | | | | |
| (A) Reference PLTs | 7.95 ± 0.34 | 8.32 ± 0.36 | 8.20 ± 0.19 | 8.03 ± 0.26 |
| (B) Aggregated PLTs | 8.14 ± 0.51 | 8.26 ± 0.48 | 8.08 ± 0.54 | 7.80 ± 0.57 |
| (C) PLTs in plasma | 8.07 ± 0.37 | 8.37 ± 1.29 | 8.56 ± 0.82 | 7.98 ± 0.63 |

* Values are reported as mean ± SD.
† $p < 0.001$ vs. (A).
‡ $p < 0.001$ vs. (B).

**TABLE 2. In vitro functional effects and glycoprotein expression on PLTs (n = 8) stored for 9 days in SSP+ and plasma***

| | Day | | | |
|---|---|---|---|---|
| Variable | 2 | 5 | 7 | 9 |
| HSR (%) | | | | |
| (A) Reference PLTs | 63.9 ± 8.2 | 57.4 ± 4.2 | 49.5 ± 4.3 | 48.4 ± 6.0 |
| (B) Aggregated PLTs | 54.6 ± 8.5 | 50.3 ± 7.7 | 47.5 ± 5.4 | 44.2 ± 4.3 |
| (C) PLTs in plasma | 61.4 ± 10.4 | 63.7 ± 5.9 | 58.3 ± 8.6 | 53.8 ± 7.9 |
| ESC (%) | | | | |
| (A) Reference PLTs | 23.2 ± 2.9 | 15.0 ± 3.5 | 13.6 ± 2.3 | 13.3 ± 3.2 |
| (B) Aggregated PLTs | 21.7 ± 4.8 | 14.8 ± 4.2 | 13.0 ± 4.2 | 9.4 ± 5.3 |
| (C) PLTs in plasma | 22.0 ± 1.0 | 23.2 ± 1.8 | 20.7 ± 2.3†‡ | 14.1 ± 3.5 |
| CD42b (%) | | | | |
| (A) Reference PLTs | 95.02 ± 3.09 | 91.49 ± 3.02 | 91.38 ± 3.33 | 88.43 ± 3.07 |
| (B) Aggregated PLTs | 96.77 ± 3.15 | 91.14 ± 2.28 | 86.40 ± 2.10 | 85.28 ± 4.82 |
| (C) PLTs in plasma | 95.82 ± 2.22 | 89.88 ± 1.87 | 92.08 ± 4.56 | 88.68 ± 5.59 |

* Values are reported as mean ± SD.
† $p < 0.001$ vs. (A).
‡ $p < 0.001$ vs. (B).

treated in the same way. PLTs from a normal unit without this treatment were used as reference samples. PLTs from disrupted aggregates showed clear signs of activation, as measured by increased expression of CD62P and αIIbβ3 in the open confirmation detected by PAC-1 (Figs. 2A and 2B). In addition, nonaggregated PLTs expressed higher levels of activation markers compared to control and reference PLTs. In case of CD62P, the expression level was still lower than that of aggregated PLTs, while PAC-1 expression was equal on nonaggregated and aggregated PLTs (Figs. 2A and 2B).

When the PLTs were incubated with the thrombin analog TRAP, we found that relative to their initial activation state, aggregated PLTs responded poorly compared to the other PLTs (Fig. 2C). This was particularly evident using PAC-1 as readout (Fig. 2C, bottom panel). When the fold change in expression of activation markers was determined, it was clear that both nonaggregated and aggregated PLTs showed a compromised responsiveness to TRAP compared to control and reference PLTs (Fig. 2D).

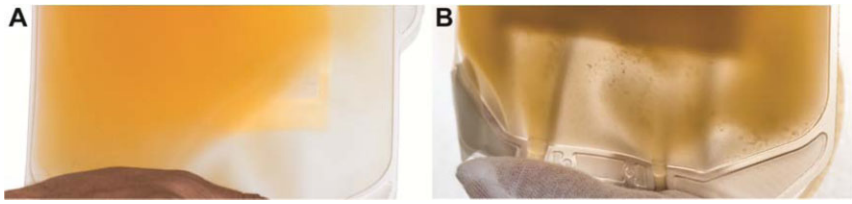


**Fig. 1. Example of a normal concentrate (A) and one that contains aggregates (B) directly after production.**

### Difference in responsiveness between PLTs from normal and initially aggregated units persists during storage

The lower increase in PAC-1 expression after stimulation of aggregated PLTs was of interest since it suggested a potentially compromised responsiveness of those PLTs to fibrinogen.[30] To test if this difference would persist during storage, we analyzed the expression of activation markers from normal and aggregated units over a period of 9 days after production. Because the aggregates had dissolved within the first 24 hours, PLTs analyzed after that time point were made up by a mixture of aggregated and non-aggregated PLTs.

Consistent with the analysis in Fig. 2, we observed a lower expression of PAC-1 after stimulation in the initially aggregated units compared to reference units, although it was only statistically different on Day 2 after production (Fig. 3). Thus, despite the fact that aggregates dissolve within 24 hours after production, PAC-1 up regulation after stimulation remains poorer compared to reference PLTs over 9 days of storage. The expression of CD62P was higher on PLTs from initially aggregated units compared to reference units at all time points (Fig. 3B). These data are consistent with data from Fig. 2 and suggest that also regarding CD62P expression, the differences imposed by initial aggregation persist during storage.

### Initially aggregated PLT units show enhanced secretion of RANTES and sCD40L

Given the evidence for spontaneous activation in aggregated units, we tested if the PLTs had been triggered to release higher levels of immunomodulatory factors compared to reference units and if there would be continuous release during storage. To evaluate the contribution of the plasma component in SSP+ for the amount of cytokine in the bags, we also included units in the (C) arm, containing 100% plasma as storage medium.

The concentration of RANTES increased in all types of units over time but was significantly higher in the initially aggregated units at all time points compared with reference units and units with plasma only (Fig. 4A). The levels of RANTES were higher in PLTs in plasma compared to PLTs in SSP+ from Day 5, suggesting that factors in plasma promote secretion of RANTES.[31] sCD40L showed an even larger difference in initially aggregated units compared to reference PLTs (Fig. 4). In contrast to RANTES, sCD40L was equally low in plasma as in units with SSP. Thus, we conclude that PLT activation caused by reversible aggregation leads to significantly enhanced secretion of RANTES and sCD40L in the storage bags.

### Soluble TWEAK accumulates in the storage bag with time but is not affected by initial aggregation

TWEAK (TNF-like weak inducer of apoptosis) is a member of the TNF-α family of cytokines.[11,18] It is an immunomodulatory factor that regulates cell growth, cell survival, and angiogenesis.[19] It can be found at the cell surface of PLTs but also exists in a soluble form.[19] We consistently failed to detect TWEAK at the cell surface of either resting or activated PLTs from our PLT concentrates (Fig. 5A). However, TWEAK was present intracellularly both in resting and in activated PLTs (Fig. 5A) and activated PLTs contained more TWEAK at all time points analyzed (Figs. 5A and 5B). A significant increase in the concentration of sTWEAK over time was seen, suggesting that TWEAK is continuously released by PLTs during storage (Fig. 5). Interestingly, no differences between the three groups were seen, showing that initial aggregation had no effect on the release of TWEAK. It was also of interest to note that sTWEAK was quite abundant in plasma (Fig. 5C, dashed line) providing an explanation for the higher levels of sTWEAK in PLTs stored in 100% plasma compared to SSP (Fig. 5C). Despite this, it was possible to see an increase of TWEAK in the storage bag also in the units containing 100% plasma, strengthening the conclusion that TWEAK is released from PLTs during storage.

### Biochemical, metabolic, and cellular properties of PLTs in the three arms

Given their differential state of activation and secretion of mediators, it was important to evaluate the biochemical, metabolic, and cellular properties of the PLTs in the three arms. No significant differences in PLT counts ($\times 10^9$/L) between the (A) units, 973 ± 73; (B) units, 937 ± 96; and (C) units 1011 ± 114, or content ($\times 10^9$/unit) in the (A) units, 355 ± 36; (B) units, 341 ± 30; and (C) units, 336 ± 31, were detected on Day 2 as well as throughout storage (data not shown). The WBC content on Day 2 was less than $0.2 \times 10^6$/L in all units (data not shown).

The MPV increased slightly in all groups but without any significant difference between them (Table 1). The extracellular LDH in percentage of total remained stable at

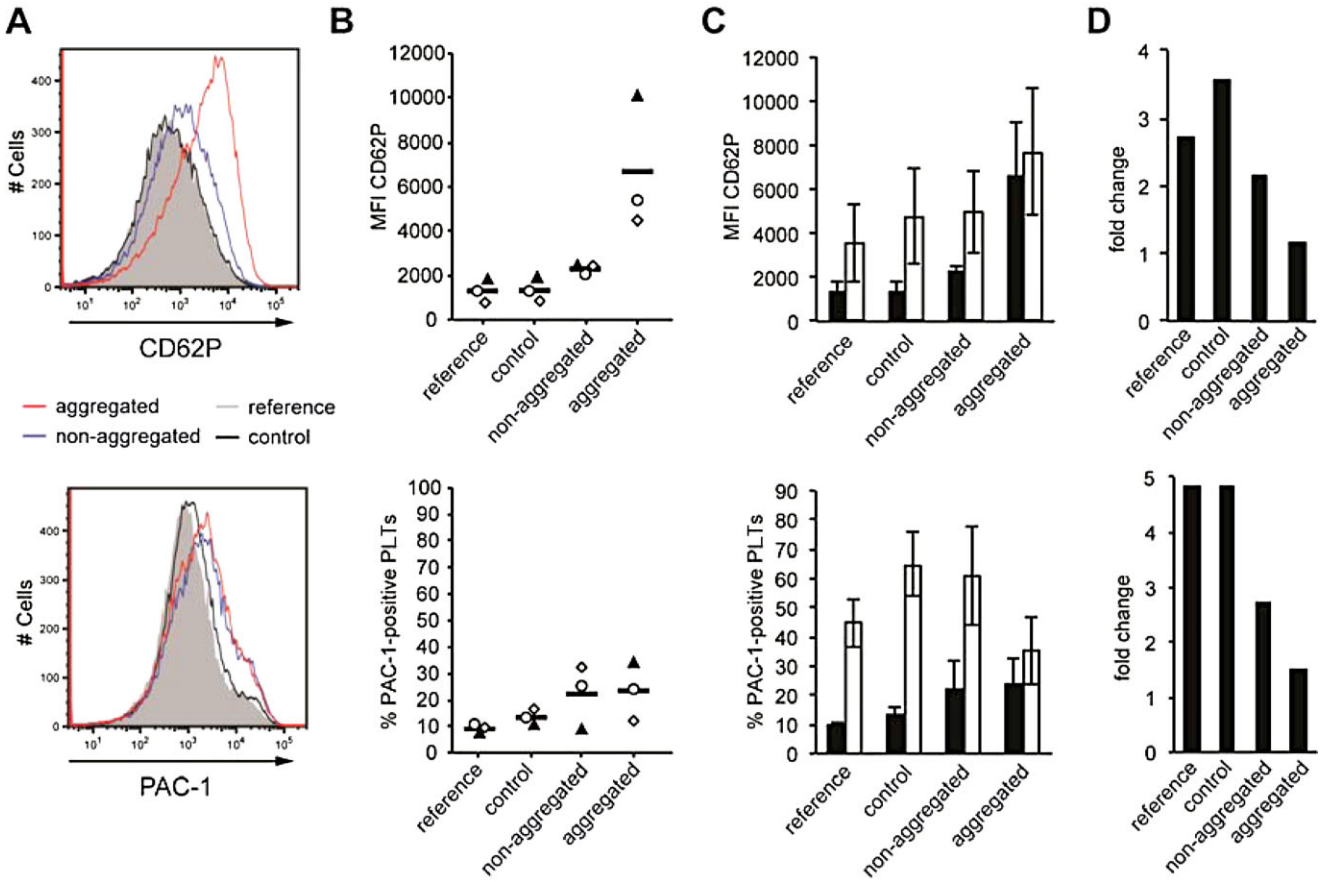


**Fig. 2. Activation status and responsiveness of PLTs from aggregated units measured by surface expression of CD62P and PAC-1. PLT aggregates were isolated and dissolved mechanically. Nonaggregated PLTs from the supernatant of the aggregates and PLTs from a normal unit (control) were treated in the same way. Reference PLTs are nontreated PLTs from a normal unit. The expression of the two activation markers was measured by flow cytometry on PLTs with and without TRAP-stimulation. (A) Representative stainings from one experiment. (B) MFI values or percentage of positive PLTs on nonstimulated PLTs from three experiments. The bars indicate mean values. (C) Mean values of nonstimulated (■) and TRAP-stimulated (□) PLTs. (D) Fold change induced by stimulation was calculated from the means.**

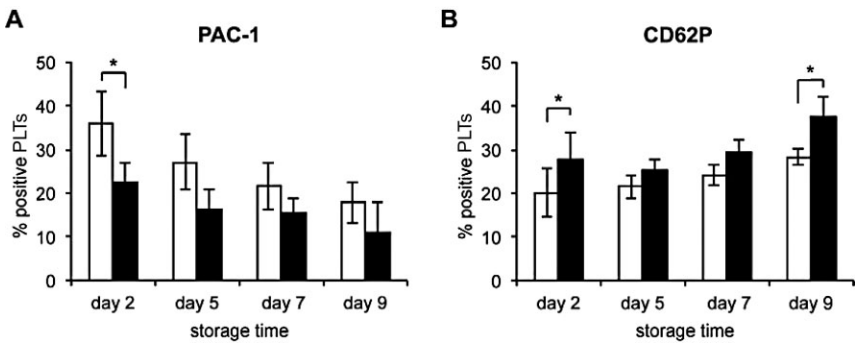


**Fig. 3. Responsiveness of PLTs during storage as measured by surface expression of the activation markers PAC-1 (A) and CD62P (B) after ADP stimulation. The bars represent the mean percentage ± SD of positive PLTs on the indicated days of storage for eight samples in each group. *p < 0.001 vs. (aggregated). (□) References; (■) aggregated.**

low levels in all SSP+ units but was somewhat higher in the plasma units ($p < 0.001$ Days 5 and 9).

Throughout storage, the glucose concentration ($p < 0.001$ from Day 2) and the production of lactate ($p < 0.001$ from Day 5) was higher in the (C) units compared with the (A) and (B) units (Table 1). The glucose consumption rate was higher in the (C) unit ($1.2 \pm 0.04$ mmol/day/$10^9$ PLTs vs. $0.49 \pm 0.01$ in the (A) unit and $0.60 \pm 0.01$ in the (B) unit), as was the lactate production rate ($0.20 \pm 0.07$ mmol/day/$10^9$ PLTs vs. $0.09 \pm 0.01$ in the (A) unit and $0.11 \pm 0.03$ in the (B) unit; Table 1). During storage, the glucose and lactate rate was found to be consistent in all

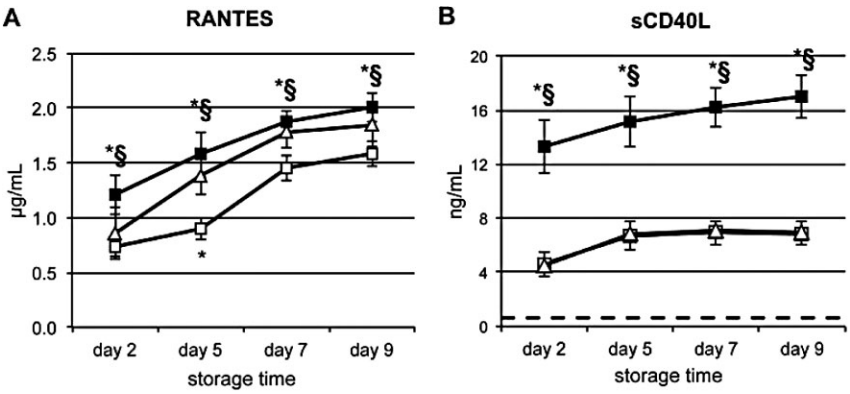


**Fig. 4. Accumulation of RANTES (A) and sCD40L (B) in reference PLTs in SSP+, initially aggregated PLTs, and PLTs in plasma stored for 9 days: Values are reported as mean ± SD of eight samples in each group. *p < 0.001 vs. (reference); §p < 0.001 vs. (aggregated) + p < 0.001 vs. (plasma). (□) Reference units; (■) aggregated units; (△) plasma units; (- - -) concentration in plasma.**

units. Initially, the pH was significantly higher in the plasma units ($p < 0.001$). Carbon dioxide and the calculated bicarbonate decreased in all units throughout storage but was significantly higher in the plasma units at all time points ($p < 0.001$). The ATP levels remained stable in all units at all times without any significant difference between the groups (Table 1).

The HSR reactivity, the response to ESC, and glycoprotein CD42b expression of PLTs over 9 days of storage decreased over time but, with exception of ESC on Day 7, were not significantly different between groups (Table 2). Swirling remained at the highest level (score, 2) in all the units at all times (data not shown).

## DISCUSSION

One of the major properties of PLTs is their capacity to aggregate quickly to sites of vessel injury and secrete factors that promote blood clotting and tissue regeneration.[32] To secure this function, PLTs must be easily triggered when they encounter damaged or aberrant surfaces.[32] As a consequence, when PLTs are removed from the blood and are put in an artificial environment, a substantial degree of PLT activation can be demonstrated.[33] Production and storage of PLTs for transfusion represent no exception to this notion.[34-36] The extent to which these manipulations compromise PLT function is not fully known, but it is nevertheless important to characterize situations in which aberrant PLT activation may occur during PLT production.

One such potential situation that caught our attention was a randomly occurring aggregation of PLTs during production. This phenomenon does not seem to be center specific, because aggregation with similar production systems and additive solution (AS) has been observed in several laboratories in Sweden and Europe. These observations, discussed collegially, have not generated any scientific data on this phenomenon. In our center, the appearance of aggregates in automated PLT production is not a new phenomenon, but has been observed since the development of the first Swedish OrbiSac prototype in the early 2000s. However, the increased demand for PLTs, in combination with the implementation of new ASs, have made us aware that the problem may have increased. Over a period of 5 months, approximately 15% of all units produced from buffy coats in our facility showed some degrees of transient macroscopic aggregates that subsequently dissolve at room temperature. The appearance of aggregates is an unexplored area without clear links to specific donors or blood groups and seems to occur randomly. Which mechanisms might be responsible for this aggregation? And why is it seen in some of the storage bags but not in others? It is notable that we observe aggregates in the use of SSP+ as storage medium, which is surprising since this solution contains both potassium and magnesium, two ions that in previous studies have been shown to prevent activation and aggregation.[37,38] We note, however, that the favorable effects on the PLTs by these two ions have only been evaluated during the storage time and not during the time of production itself. One possibility is also that serum-derived factors from one or several of the buffy coats used to make one PLT unit may play a role. Such factors could be physiologic PLT-activating molecules, but also drugs, antibodies, or environmental factors, such as differences in pH[39] could be of importance.

Irrespective of the reason for aggregation, we found that the PLTs in the aggregated units seemed to be activated to a higher degree as evidenced by elevated CD62P expression. Even after stimulation, the expression levels on PLTs from aggregated units were higher than those on reference PLTs. The higher degree of activation was also indicated by an increased expression level of the αIIbβ3 integrin in an open conformation on PLTs from the aggregated units, as shown by staining with PAC-1. Surprisingly, the increase in PAC-1 staining after stimulation was much lower on the PLTs from the aggregates compared both to nonaggregated PLTs in the same bag and to PLTs from control units. One possibility to explain this finding is that PLTs that have aggregated are primed to more efficient release of fibrinogen upon stimulation. This fibrinogen could then potentially bind the αIIbβ3 integrin at the PLT cell surface in an autocrine loop, resulting in blockage of the PAC-1 epitope for the antibody. Another explanation is that the signaling pathways that link TRAP and ADP

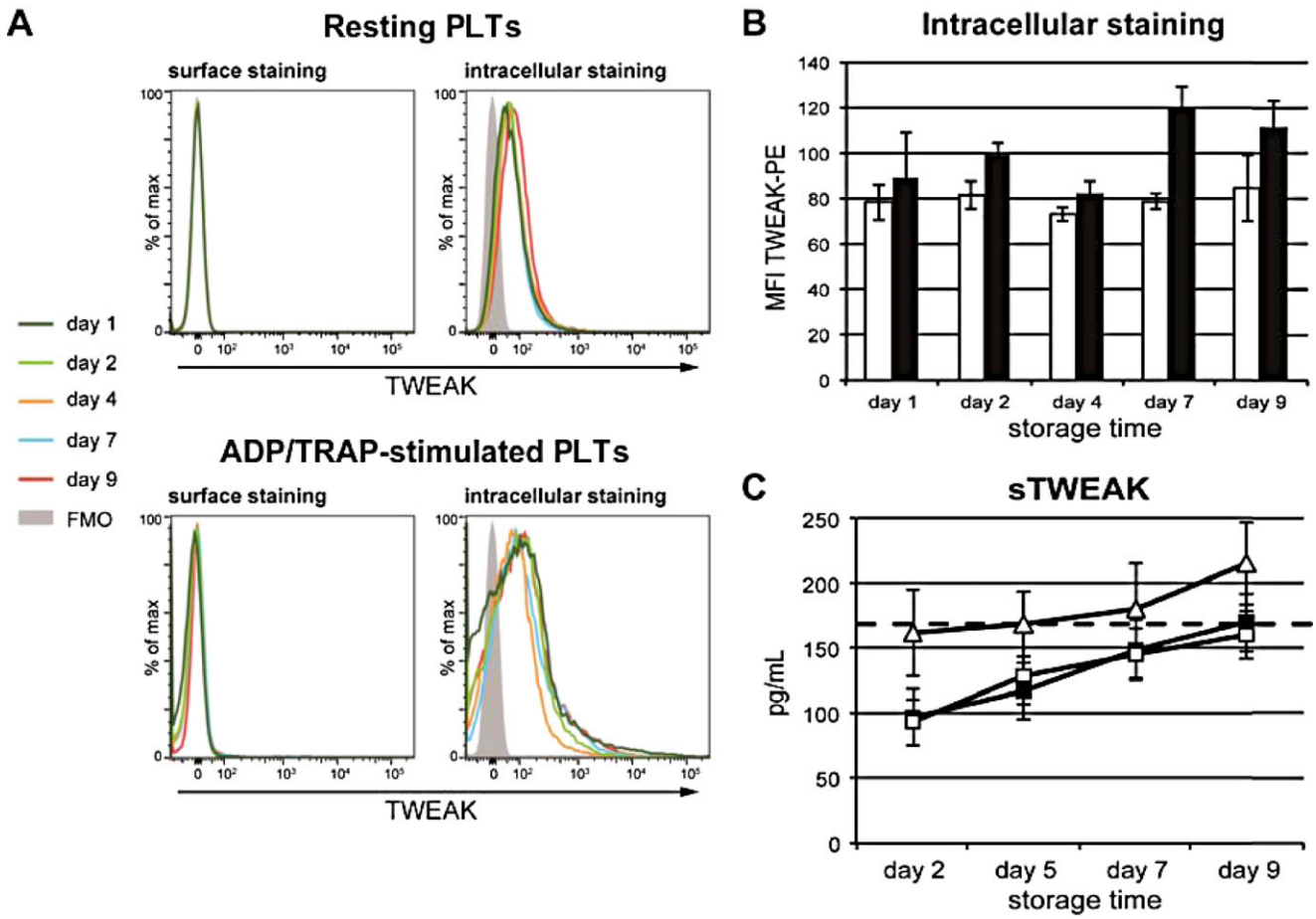


**Fig. 5. Levels of surface and intracellular TWEAK in normal PLTs during 9 days of storage. PLTs were stained in the resting state and after stimulation with ADP and TRAP. (A) Representative stainings on PLTs from one unit. (B) Mean values of the MFI of intracellular stainings from three units ± SD. (□) Resting PLTs; (□) ADP- and TRAP-stimulated PLTs. (C) Accumulation of sTWEAK in reference PLTs in SSP+, initially aggregated PLTs, and PLTs in plasma stored for 9 days. Values are reported as mean ± SD of eight samples in each group. (□) Reference units; (■) aggregated units; (△) plasma units; (- - -) concentration in plasma.**

stimulation to the conformational change of the αIIβIII integrin are disturbed or dampened due to excessive signaling during aggregation. Irrespective of the mechanism, it is noteworthy that this phenotype persisted even after the spontaneous dissolution of the aggregates, suggesting that some of the changes caused by aggregation may be difficult to revert during storage.

Vigorous pipetting to disrupt visible aggregates was used to allow flow-cytometric analysis of aggregated PLTs. Even though pipetted control samples did not become activated, we cannot exclude that the forceful detachment of aggregated PLTs by pipetting is more activating than pipetting PLTs in single-cell suspensions. This limitation must be kept in mind when interpreting the data. However, we believe that the biologic differences between the two samples at least partly depend on aggregation, because differences in the expression of activation markers were observed also after the aggregates had resolved, on Day 2 after production, in which case no mechanical stress was applied (Fig. 3, Day 2). In addition, the increased expression of activation markers on the nonaggregated fraction from units containing aggregates might also indicate an overall higher level of activation status in units containing aggregated PLTs.

The data presented demonstrate that three independently analyzed cytokines accumulated in the various PLT units during storage. A large variety of factors are present in PLT concentrates and several studies have shown that PLT-derived factors such as CD40L, RANTES, C5a, and BDNF are biologically active in experimental systems.[14,40] It has been more difficult to directly correlate individual factors to the risk for transfusion reactions, and both positive and negative findings can be found in the literature.[15,16,40-42] One reason for the conflicting data is likely the critical role for recipient factors in transfusion reactions, which act in concert with the transfused blood components and donor serum to cause reactions.[43-45] Such recipient factors can be genetically controlled host

response elements and also properties caused by various diseases, most of which are unknown. While waiting for more studies to clarify these questions, it nevertheless makes sense to consider proinflammatory factors in the storage bags potentially harmful and to try to minimize their release during storage.

Although sTWEAK does not appear to respond to similar signaling pathways as RANTES and sCD40L, the finding that PLTs release sTWEAK into the storage bag raises important questions about its possible role in PLT transfusion therapy. In contrast to sCD40L, sTWEAK seems to be present at relatively high concentrations already in the plasma and further accumulated from the PLTs. Consequently, sTWEAK concentrations may be reduced by using SSP+ as storage medium instead of plasma. However, any possible biologic significance of transfusing sTWEAK remains uncertain and merits further study.

At this stage, our results do not prove a causal relationship between initial aggregation, poor metabolic and functional storage conditions, and subsequent poor recovery and survival after transfusion. Such questions must be addressed using additional experimental approaches, including new functional analyses.[46] It has been suggested that febrile responses in recipients of PLT donors may depend on the level of sCD40L[15] and RANTES.[4] Given this notion, it is conceivable that initially aggregated PLT units might increase the risk of adverse negative events after PLT transfusion. Future studies should focus on the underlying causes of PLT aggregation in newly produced units. In addition, clinical studies should also be performed to ask if initially aggregated PLTs are more likely to cause negative side effects after transfusion compared to unaffected units. There is also little insight into the possible impact of aggregation on the hemostatic behavior of PLTs after transfusion, warranting an increased focus on new experimental focusing on PLT recovery and survival in vivo.

### CONFLICT OF INTEREST

None.

## REFERENCES


1. Muylle L, Peetermans ME. Effect of prestorage leukocyte removal on the cytokine levels in stored platelet concentrates. Vox Sang 1994;66:14-7.
2. Muylle L, Wouters E, Peetermans ME. Febrile reactions to platelet transfusion: the effect of increased interleukin 6 levels in concentrates prepared by the platelet-rich plasma method. Transfusion 1996;36:886-90.
3. Wadhwa M, Seghatchian MJ, Dilger P, Sands D, Krailadisiri P, Contreras M, Thorpe R. Cytokines in WBC-reduced apheresis PCs during storage: a comparison of two WBC-reduction methods. Transfusion 2000;40:1118-26.
4. Kluter H, Bubel S, Kirchner H, Wilhelm D. Febrile and allergic transfusion reactions after the transfusion of white cell-poor platelet preparations. Transfusion 1999;39:1179-84.
5. Bubel S, Wilhelm D, Entelmann M, Kirchner H, Klüter H. Chemokines in stored platelet concentrates. Transfusion 1996;36:445-9.
6. Fujihara M, Ikebuchi K, Wakamoto S, Sekiguchi S. Effects of filtration and gamma radiation on the accumulation of RANTES and transforming growth factor-beta1 in apheresis platelet concentrates during storage. Transfusion 1999; 39:498-505.
7. Sandgren P, Hansson M, Gulliksson H, Shanwell A. Storage of buffy-coat-derived platelets in additive solutions at 4 degrees C and 22 degrees C: flow cytometry analysis of platelet glycoprotein expression. Vox Sang 2007;93: 27-36.
8. Sandgren P, Shanwell A, Gulliksson H. Storage of buffy coat-derived platelets in additive solutions: in vitro effects of storage at 4 degrees C. Transfusion 2006;46:828-34.
9. Shanwell A, Falker C, Gulliksson H. Storage of platelets in additive solutions: the effects of magnesium and potassium on the release of RANTES, beta-thromboglobulin, platelet factor 4 and interleukin-7, during storage. Vox Sang 2003;85:206-12.
10. Vo TD, Cowles J, Heal JM, Blumberg N. Platelet washing to prevent recurrent febrile reactions to leucocyte-reduced transfusions. Transfus Med 2001;11:45-7.
11. Chicheportiche Y, Bourdon PR, Xu H, Hsu YM, Scott H, Hession C, Garcia I, Browning JL. TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. J Biol Chem 1997;272:32401-10.
12. Cognasse F, Boussoulade F, Chavarin P, Acquart S, Fabrigli P, Lamy B, Garraud O. Release of potential immunomodulatory factors during platelet storage. Transfusion 2006;46:1184-9.
13. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 1998;391:591-4.
14. Khan SY, Kelher MR, Heal JM, Blumberg N, Boshkov LK, Phipps R, Gettings KF, McLaughlin NJ, Silliman CC. Soluble CD40 ligand accumulates in stored blood components, primes neutrophils through CD40, and is a potential cofactor in the development of transfusion-related acute lung injury. Blood 2006;108:2455-62.
15. Phipps RP, Kaufman J, Blumberg N. Platelet derived CD154 (CD40 ligand) and febrile responses to transfusion. Lancet 2001;357:2023-4.
16. Phipps RP, Koumas L, Leung E, Reddy SY, Blieden T, Kaufman J. The CD40-CD40 ligand system: a potential therapeutic target in atherosclerosis. Curr Opin Investig Drugs 2001;2:773-7.
17. Burkly LC, Michaelson JS, Hahm K, Jakubowski A, Zheng TS. TWEAKing tissue remodeling by a multifunctional

cytokine: role of TWEAK/Fn14 pathway in health and disease. Cytokine 2007;40:1-16.
18. Winkles JA. The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. Nat Rev Drug Discov 2008;7:411-25.
19. Zimmermann M, Koreck A, Meyer N, Basinski T, Meiler F, Simone B, Woehrl S, Moritz K, Eiwegger T, Schmid-Grendelmeier P, Kemeny L, Akdis CA. TNF-like weak inducer of apoptosis (TWEAK) and TNF-alpha cooperate in the induction of keratinocyte apoptosis. J Allergy Clin Immunol 2011;127:200-7. 207 e1-10.
20. Song A, Chen YF, Thamatrakoln K, Storm TA, Krensky AM. RFLAT-1: a new zinc finger transcription factor that activates RANTES gene expression in T lymphocytes. Immunity 1999;10:93-103.
21. Uchide N, Ohyama K, Bessho T, Takeichi M, Toyoda H. Possible roles of proinflammatory and chemoattractive cytokines produced by human fetal membrane cells in the pathology of adverse pregnancy outcomes associated with influenza virus infection. Mediators Inflamm 2012;2012: 270670.
22. Larsson S, Sandgren P, Sjodin A, Vesterinen M, Gulliksson H. Automated preparation of platelet concentrates from pooled buffy coats: in vitro studies and experiences with the OrbiSac system. Transfusion 2005;45:743-51.
23. Sandgren P, Saeed K. Storage of buffy-coat-derived platelets in additive solution: in vitro effects on platelets of the air bubbles and foam included in the final unit. Blood Transfus 2011;9:182-8.
24. Bertolini F, Murphy S. A multicenter inspection of the swirling phenomenon in platelet concentrates prepared in routine practice. Biomedical Excellence for Safer Transfusion (BEST) Working Party of the International Society of Blood Transfusion. Transfusion 1996;36:128-32.
25. Moroff G, Eich J, Dabay M. Validation of use of the Nageotte hemocytometer to count low levels of white cells in white cell-reduced platelet components. Transfusion 1994;34:35-8.
26. VandenBroeke T, Dumont LJ, Hunter S, Nixon J, Murphy S, Roger J, Herschel L, AuBuchon JP, Gulliksson H, Dengler T, Hornsey V, Prowse C; Biomedical Excellence for Safer Transfusion Working Party of the International Society of Blood Transfusion. Platelet storage solution effects on the accuracy of laboratory tests for platelet function: a multilaboratory study. Vox Sang 2004;86:183-8.
27. Lundin A. Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. Methods Enzymol 2000;305:346-70.
28. Vanderlinde RE. Measurement of total lactate dehydrogenase activity. Ann Clin Lab Sci 1985;15:13-31.
29. Kroll MH, Hellums JD, McIntire LV, Schafer AI, Moake JL. Platelets and shear stress. Blood 1996;88:1525-41.
30. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. J Biol Chem 1985;260:11107-14.
31. El-Sharkawy H, Kantarci A, Deady J, Hasturk H, Liu H, Alshahat M, Van Dyke TE. Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. J Periodontol 2007;78:661-9.
32. Broos K, De Meyer SF, Feys HB, Vanhoorelbeke K, Deckmyn H. Blood platelet biochemistry. Thromb Res 2012;129:245-9.
33. Daugirdas JT, Bernardo AA. Hemodialysis effect on platelet count and function and hemodialysis-associated thrombocytopenia. Kidney Int 2012;82:147-57.
34. Ali SF. Platelet activation of platelet concentrates derived from buffy coat and apheresis methods. Transfus Apher Sci 2011;44:11-3.
35. Gulliksson H. Defining the optimal storage conditions for the long-term storage of platelets. Transfus Med Rev 2003; 17:209-15.
36. Sandgren P, Hild M, Sjodin A, Gulliksson H. Storage of buffy-coat-derived platelets in additive solutions: in vitro effects on platelets prepared by the novel TACSI system and stored in plastic containers with different gas permeability. Vox Sang 2010;99:341-7.
37. Gawaz M, Ott I, Reininger AJ, Neumann FJ. Effects of magnesium on platelet aggregation and adhesion. Magnesium modulates surface expression of glycoproteins on platelets in vitro and ex vivo. Thromb Haemost 1994;72: 912-8.
38. Gulliksson H, AuBuchon JP, Vesterinen M, Sandgren P, Larsson S, Pickard CA, Herschel I, Roger J, Tracy JE, Langweiler M; Biomedical Excellence for Safer Transfusion Working Party of the International Society of Blood Transfusion. Storage of platelets in additive solutions: a pilot in vitro study of the effects of potassium and magnesium. Vox Sang 2002;82:131-6.
39. Rogers AB, Des Prez RM. The effect of pH on human platelet aggregation induced by epinephrine and ADP. Proc Soc Exp Biol Med 1972;139:1100-3.
40. Sahler J, Spinelli S, Phipps R, Blumberg N. CD40 ligand (CD154) involvement in platelet transfusion reactions. Transfus Clin Biol 2012;19:98-103.
41. Apelseth TO, Hervig TA, Wentzel-Larsen T, Bruserud O. Cytokine accumulation in photochemically treated and gamma-irradiated platelet concentrates during storage. Transfusion 2006;46:800-10.
42. Wakamoto S, Fujihara M, Kuzuma K, Sato S, Kato T, Naohara T, Kasai M, Sawada K, Kobayashi R, Kudoh T, Ikebuchi K, Azuma H, Ikeda H. Biologic activity of RANTES in apheresis PLT concentrates and its involvement in nonhemolytic transfusion reactions. Transfusion 2003;43:1038-46.
43. Savage WJ, Savage JH, Tobian AA, Thoburn C, Hamilton RG, Schroeder JT, Ness PM. Allergic agonists in apheresis platelet products are associated with allergic transfusion reactions. Transfusion 2012;52:575-81.
44. Savage WJ, Tobian AA, Fuller AK, Wood RA, King KE, Ness PM. Allergic transfusion reactions to platelets are

associated more with recipient and donor factors than with product attributes. Transfusion 2011;51:1716-22.
45. Savage WJ, Tobian AA, Savage JH, Wood RA, Schroeder JT, Ness PM. Scratching the surface of allergic transfusion reactions. Transfusion 2012;53:1361-71.
46. Ostrowski SR, Bochsen L, Windelov NA, Salado-Jimena JA, Reynaerts I, Goodrich RP, Johansson PI. Hemostatic function of buffy coat platelets in additive solution treated with pathogen reduction technology. Transfusion 2011;51:344-56.