# Chapter 2

# In vitro comparison of platelet storage in plasma and in four platelet additive solutions, and the effect of pathogen reduction

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## ABSTRACT

### Background

The introduction of platelet (PLT) additive solutions (PASs) and pathogen reduction (PR) technologies possibly allow extension of PLT shelf life. It was our aim to compare in vitro quality of white cell (WBC)-reduced PLT concentrates stored in various PASs with those in plasma during 8 days of storage. Also, the effect of PR was investigated. The study was performed in a nationwide, multicenter study design, where each center tested 4 of the 6 study conditions.

### Study design and methods

In paired experiments (n= 12 per center), 20 AB0-identical buffy coats were pooled and divided into 4 products, to which various storage media were added. Plasma was used as reference in all 4 centers. Two centers used InterSol followed by PR (InterSol+PR) and InterSol without PR; other investigated PASs were T-sol, SSP+ and Composol. A rating system was used to judge PLT quality based on CD62P expression, annexin A5 binding and lactate production: a rating of 6 for good quality and 0 for poor quality.

### Results

All PLT concentrates fulfilled release criteria (pH37°C>6.6; swirl present) until Day 8. Marked differences were seen for other in vitro parameters, including CD62P expression, which was 28±5; 31±7; and 39±9% for T-sol, Intersol+PR and without PR, respectively, which was significantly higher as the values found for Composol (12±3%), SSP+ (15±5%) and plasma (15±6%). Three in vitro parameters (CD62P, Annexin A5, and lactate concentration) were collapsed into one rating value; PLTs stored in plasma had a rating of 2.8±1.0, which was significantly higher as for PLTs in T-Sol (1.5±0.5), InterSol+PR (1.3±0.6) and without PR (1.7±0.5; all $p<0.001$ versus plasma). PLTs stored in potassium- and magnesium-containing PASs showed higher ratings as plasma, 4.3±0.5 for Composol and 3.8±0.8 for SSP+ ($p<0.05$).

### Conclusion

PLT concentrates in plasma, SSP+ and Composol scored better using an arbitrary rating system as PLTs stored in T-Sol or InterSol; PR further impaired rating parameters. The applicability of these differences in rating for clinical effects needs a clinical study.

## INTRODUCTION

Plasma is still the most widely-used medium for storage of platelet (PLT) concentrates. CPD plasma contains high levels of glucose as nutrient for the PLTs, and citrate to prevent clotting.[1] Other constituents are at near-physiological ranges, with the exception of a very low free calcium level, and a phosphate level approximately 3 times higher as in normal plasma.[2] Initially, a main motivation to investigate the use of PLT additive solutions (PASs) was an increased availability of plasma for fractionation of Factor VIII and other plasma-derived products. However, the use of PASs has more benefits. These include the fact that PASs can be manufactured sterile and pathogen-free, and have a standardized composition in contrast to donor-variations of plasma. Moreover, PLT concentrates in PAS contain less plasma proteins, reducing allergic reactions[3, 4], and have a lower titer of AB0 antibodies, thereby easier allowing AB0-mismatched PLT transfusions.[5] Theoretically, for the same reason of a 3- to 4-fold antibody dilution, PASs could decrease the risk of antibody mediated transfusion related acute lung injury (TRALI).[6, 7] Finally, PASs facilitate some pathogen reduction (PR) technologies that are inhibited by the presence of plasma proteins.[8] PASs can be introduced in currently used, plasma-based processing methods. PASs were initially developed in conjunction with the buffy coat method, where multiple (usually four to six) buffy coats are pooled, and instead of adding one unit of plasma, one bag with about 300 mL of PAS is added to this pool. After low speed centrifugation the PLT-rich supernatant is expressed to a PLT storage container. Due to the lower viscosity[9], PLT yield is generally a little less than when plasma is used. With apheresis, a highly concentrated PLT concentrate is collected which is diluted with PAS to produce a single-donor PLT concentrate that can be stored for 5 to 7 days.[10] With the PLT-rich plasma (PRP) method, the use of PASs is less easy to incorporate, although specific methods have been described.[11] All PASs require some residual plasma to maintain PLT quality and functionality.[12, 13]

Initial developments of PASs were done with PlasmaLyte-A, an infusion fluid licensed for use[14], and still under consideration for platelet storage.[15] One of the most widely used PAS in blood banks is T-sol, often referred to as PAS-II (Fenwal, Mont Saint Guibert, Belgium).[16] This solution is licensed for in vivo infusions in Europe, and it was developed for 5-day storage of PLT concentrates. With introduction of bacterial screening assays extension of the PLT storage time to seven days was allowed, but T-Sol could not always maintain the pH within acceptable limits.[17] Moreover, transfusions of PLTs stored in T-Sol resulted in lower increments compared to PLTs stored in plasma.[3, 4, 18] After T-Sol, a number of PASs have been developed that fulfilled in vitro quality requirements after 7-days PLT storage. Potassium and magnesium were added to some PASs to preserve PLT integrity throughout storage.[19] Also, specific PASs have been developed for PR technologies.[8] We compared a number of these newly marketed solutions, including: T-Sol, Composol-PS (Fresenius HemoCare, Emmer-Compascuum, The Netherlands), SSP+ (MacoPharma, Tourcoing, France) and InterSol (Cerus, Amersfoort, The Netherlands); plasma was used as reference. Because InterSol was specifically intended in combination with PR, we included PLT concentrates in InterSol after inactivation with amotosalen (Cerus, Concord, CA, USA). For this study, PLT concentrates derived from buffy coats were used. This in vitro study was conducted in conjunction with a phase III clinical trial in hemato-oncological patients, investigating the clinical effectiveness and safety of white cell (WBC)-reduced pooled random donor PLT concentrates, stored up to seven days in either PAS with and without PR, or in plasma.[20] For this preparative study many laboratory tests were applied to evaluate the quality of the new storage medium for PLTs in vitro, and run as a paired comparison with PLTs stored in approved containers.[21, 22] The benefits and pitfalls of such comparisons have been outlined before for recovery/survival studies[23]; in line with defining objective acceptance criteria for recovery and survival, we propose a rating system for in vitro PLT studies, which may allow a more objective interpretation of laboratory results.

## MATERIALS AND METHODS

All four blood centers in the Netherlands participated in this study, designated center 1, 2, 3 and 4. Each center used their own materials and methods compliant with the Dutch guidelines for preparation of platelet products, unless indicated otherwise.

### Blood collection and processing

Blood was collected in quadruple bag bottom-and-top systems with an inline red cell filter (from Fresenius HemoCare or from Baxter) on Day 0, and after rapid cooling to room temperature stored overnight at this temperature. On Day 1, after hard spin centrifugation, the units were separated into a unit of plasma, a buffy coat and a red cell concentrate using an automated separation device (Compomat, Fresenius HemoCare, or Optipress II, Baxter). Each center prepared 4 paired PLT concentrates by pooling 20 buffy coats in a large container, mixed well, and split in equal parts over four buffy coat pooling sets (from Terumo (Tokyo, Japan), Fresenius HemoCare, or from Baxter). All connections were made with a sterile connection device (Terumo). Each center prepared one unit PLT in plasma (derived from one of the whole blood units used for the buffy coat pool), and 3 others by adding one container of PAS to the content of the pooling bag according to the following scheme:

| | Center 1 | Center 2 | Center 3 | Center 4 |
|---|---|---|---|---|
| A. plasma | ● | ● | ● | ● |
| B. T-Sol (300 mL) | ● | - | - | ● |
| C. Composol (300 mL) | ● | ● | ● | - |
| D. SSP+ (300 mL) | ● | ● | - | - |
| E. InterSol (280 mL) | - | ● | ● | ● |
| F. InterSol, followed by a PR step | - | - | ● | ● |

The addition of the PASs resulted in a 35/65% ratio for plasma and PAS, respectively. After soft spin centrifugation (adapted to the use of plasma or PAS) the PLT rich supernatant was expressed through the WBC-reduction filter to the PLT storage container, both part of the buffy coat pooling set. The units A through E were placed on a flat bed shaker at room temperature in a climate-controlled cabinet at 60 strokes per minute. Units F underwent PR with Amotosalen according to the manufacturer's instructions, as described elsewhere.[24] These units were transferred to a storage container with a Compound Adsorption Device (CAD) and placed on the flat bed shaker. On Day 2, units F were transferred to the final storage containers present in the PR bag system. At that time, all units A through F were weighed, and sampled for in vitro analysis. Weighing and sampling was repeated on Day 6 and Day 8.

### In vitro analysis

The volume of the units was calculated from the net weight and specific gravity of the resuspension fluid (1.026 g/mL for plasma, 1.006 g/mL for PAS). The number of PLTs was counted on a hematology analyzer; residual WBCs were counted by flow cytometry. pH (measured and reported at 37°C), $PO_2$, and $PCO_2$ were measured with a blood gas analyzer, glucose and lactate were measured on a blood gas analyzer or enzymatically. CD62P expression[25] and annexin A5 binding[26] were determined with a flow cytometer. Swirl was judged visually on a scale from 0 to 3. Regular surveys with identical specimens were

performed between the labs to estimate intra- and inter-laboratory agreement (PLTs, WBCs, pH, blood gases). Specific optimizations were done to reduce intra- and inter-laboratory variations for CD62P and annexin A5.[25]

Dutch product specifications required that the units had a volume between 150 and 400 mL, contained >250 x$10^9$ PLTs/unit in >95% of the units and <1 x$10^6$ WBCs/unit in >90% of the units. $pH_{37°C}$ should remain between 6.6 and 7.2 throughout storage.[27]

## In vitro rating system

For the in vitro rating system we selected assays that could be performed shortly before transfusion of the PLTs, as endpoint measurement and to allow clinical evaluation of the rating system. We included three parameters in this rating system, each of which could play an independent role in predicting in vivo effectiveness of stored PLTs for reasons discussed below. First, we considered CD62P expression. This parameter of activation is, albeit contradicting, linked to PLT clearance[28-32] and there is evidence that a higher CD62P expression causes a faster PLT clearance. Impaired PLT survival in animals was found to be associated with the apoptosis marker annexin A5 binding[33,34] thus warranting inclusion of this parameter in our rating system. The third assay is the lactate concentration. The lactate production rate is considered as a good indicator of mitochondrial quality.[35] However, to calculate a lactate production rate over multiple days of storage, an additional baseline sample has to be taken immediately after production. In routine such sterile sampling before storage is not performed, making this (currently) unsuitable as endpoint measurement. The starting levels between units in plasma and PASs were slightly different (see Results), but the different production rates resulted in very different endpoint lactate concentrations, and so the lactate concentration prior to transfusion can be used as marker for lactate metabolism.

The in vitro outcomes of each of these three parameters were scored from 0 to 2, where 0 points would indicate a poor quality and 2 points a good quality. The combined rating then results in a value between 0 (poor quality) and 6 (excellent quality). For CD62P expression a value of 2 points was arbitrarily attributed to an expression <20%, 1 for 20-30% and 0 points for an expression >30%. For annexin A5, a value of 2 points for a binding <10%, 1 for 10-20% and 0 for all PLT concentrates with a binding >20%. Finally, lactate level >20 mM are known to indicate poor PLT quality [36], and we scored 2 points for a level <10 mM, 1 for concentrations between 10-20 mM and 0 points for a value >20 mM.

## Statistical analysis

The results were analyzed with Instat (version 3.06, GraphPad software, San Diego, CA, USA). Results between groups were compared with a repeated measures analysis of variance (ANOVA) followed by a Tukey Kramer post test, or, if data were not normally distributed, with Dunn's post test. Differences between storage days were also compared with a repeated measures ANOVA, followed by Dunnett's test to compare with Day 2 values. A p value <0.05 was considered to indicate a statistically significant difference.

## RESULTS

### Composition of platelet concentrates

The composition of the PLT concentrates on Day 2 is summarized in Table 1. The volumes of the PLT concentrates were significantly different amongst the groups, which was caused by the different volumes of plasma (current routine, approx. 330 mL) or PAS (T-sol, Composol and SSP+, 300 mL, InterSol, 280 mL) that had been added to the buffy coat pool. Additional volume loss due to the PR procedure was observed. The number of PLTs per unit differed significantly amongst the groups. The highest PLT counts were found in PLT concentrates in plasma, while the lowest were found in PLT concentrates in pathogen reduced InterSol concentrates. Though some units contained fewer than the required 250 x$10^9$ PLTs per unit (see Table 1), still >95% conformed to this requirement; these units were not excluded from evaluation.
None of the PLT concentrates in PAS contained >1 x$10^6$ WBCs/unit. On average, more residual WBCs were seen in the units in plasma. This was caused by one center that detected more WBCs in the units in plasma, but not in the units in PAS. Overall, 3/47 (6.4%) of the units in plasma contained >1 x$10^6$ WBCs per unit, and therefore standard product requirements were met.

**Table 1:** *Composition (on Day 2) and storage parameters of PLT concentrates in plasma and in four different additive solutions (one with additional pathogen reduction), used to compare storage characteristics (expressed as mean ± SD).*

| | Plasma | T-Sol | Composol | SSP+ | InterSol | InterSol+ PR |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| n | 47 | 23 | 23 | 35 | 36 | 24 |
| Volume, mL* | 380±30 | 354±23 | 367±16 | 352±16 | 303±17 | 275±11 |
| PL Ts, x$10^9$ † | 380±64 | 373±28 | 344±52 | 330±41 | 319±45 | 300±47 |
| PL T concentration, x$10^9$/ml ‡ | 1.03±0.16 | 1.06±0.12 | 0.94±0.11 | 0.94±0.10 | 1.06±0.17 | 1.09±0.16 |
| PL Ts <250x$10^9$/U | 0 (0%) | 0 (0%) | 0 (0%) | 1 (3%) | 1 (3%) | 3 (13%) |
| WBCs, $10^6$ \| | 0.23±0.43 | 0.03±0.06 | 0.04±0.05 | 0.06±0.06 | 0.05±0.05 | 0.08±0.06 |
| | | | | | | |
| *Storage data* | | | | | | |
| Glucose consumption, mmol/$10^{11}$ PLT/d \|\| | 0.08±0.05 | 0.08±0.03 | 0.05±0.01 | 0.06±0.02 | 0.09±0.02 | 0.10±0.02 |
| Lactate production, mmol/$10^{11}$ PLT/d** | 0.13±0.04 | 0.14±0.02 | 0.10±0.02 | 0.11±0.03 | 0.17±0.03 | 0.18±0.04 |

** all differences p<0.001 except A vs. CD, C vs. BD: n.s.*

*† B vs. D, C vs. F: p<0.05; A vs. C, B vs. E: p<0.01; A vs. DEF, B vs. F: p<0.001; all other differences n.s.*

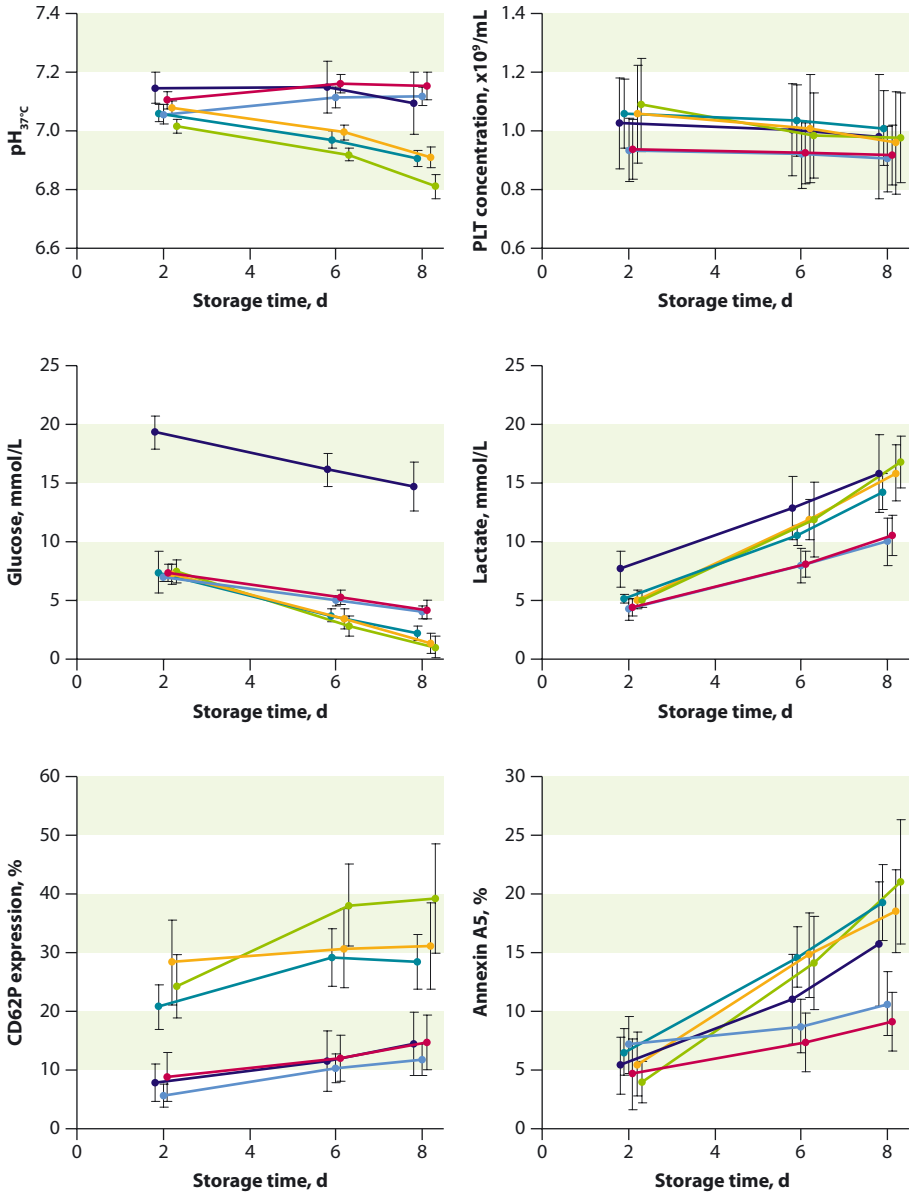
*‡ B vs. CD, C vs. E: p<0.05; C vs. F, D vs. E: p<0.01; D vs. F: p<0.001; all other differences n.s*

*| A vs. BD: p<0.05; A vs. E: p<0.01; all other differences n.s.*

*|| A vs. CD: p<0.05; C vs. EF, D vs. EF: p<0.001; all other differences n.s.*

*** A vs. C: p<0.05; B vs. D: p<0.01; A vs. EF, C vs. BEF, D vs. EF: p<0.001; all other differences n.s.*

**Figure 1:** *Figure 1 shows the various in vitro parameters of PLT concentrates in plasma and in 4 different additive solutions (one with additional pathogen reduction), stored for up to 8 days (shown as mean±SD; n: see Materials and Methods). Plasma ●, T-sol ●, Composol ●, SSP+ ●, Intersol ●, InterSol with pathogen reduction ●.*



## In vitro quality during 8-day storage

The results of various parameters during 8 days of storage are shown in Figure 1. All PLT concentrates were well able to maintain pH37°C >6.6. Plasma and the potassium- and magnesium-containing PAS (Composol and SSP+) showed constant pH throughout storage, while the others showed a decline over time. The PO2 increased during storage under all conditions, plasma having the lowest and InterSol+PR having the highest absolute levels (not shown). PCO2 decreased over time; throughout storage the PCO2 was highest for the units in plasma, while units in InterSol after PR had the lowest levels (not shown). Glucose levels in plasma were considerably higher in the PLT concentrates prepared with CPD-plasma as those made with PAS; all PLT concentrates showed a steady decline of glucose over the storage time.

The PASs with potassium and magnesium, Composol and SSP+, showed the lowest glucose consumption rates and lactate production rates, while InterSol (without and with PR) showed the highest. With respect to PLT activation, shown as CD62P expression, plasma, Composol and SSP+ had much lower expression rates throughout the storage period as the other three PASs tested. The initial PS expression (measured as annexin A5 binding) was similar for all tested conditions, but showed a steady incline for InterSol (without and with PR), T-Sol and for plasma; while PS exposure hardly changed for Composol and SSP+ over the whole storage time. Swirl remained present in all tested units irrespective of the storage solution used.

## Rating results

The rating results for the PLT concentrates stored in various solutions are given in Table 2. After overnight storage following PLT processing, on Day 2 the PLT concentrates in plasma, Composol and SSP+ had a similar rating, while PLT stored in T-Sol, InterSol without or with PR had already significantly lower scores. These differences were mainly due to higher CD62P expression in the latter groups. From Day 6 onwards these differences between products became more pronounced, as the lactate concentrations increased more in the T-Sol and InterSol groups. By Day 6, Composol and SSP+ ended with higher scores over plasma, reaching statistical significance by Day 8, mainly as a result from to lactate production and higher annexin A5 expression for PLTs stored in plasma (compare with Figure 1).

**Table 2:** *Results of a rating system, based on CD62P expression, annexin A5 binding and lactate concentrations during storage. Results are shown as mean±SD.*

| | n | Day 2 | Day6 | Day 8 |
|---|---|---|---|---|
| Plasma | 37 | 5.6± 0.6 | 3.7±1.0 | 2.8±1.0 |
| T-Sol | 17 | 4.2±0.6* | 2.2±0.4† | 1.5±0.5† |
| Composol | 12 | 5.7±0.5 | 5.1 ±0.7 | 4.3±0.5* |
| SSP+ | 25 | 5.4±0.6 | 5.0± 1.0* | 3.8±0.8* |
| InterSol | 31 | 3.9± 0.6† | 2.0± 0.5† | 1.7±0.5† |
| InterSol+PR | 24 | 4.0±0.8† | 2.1 ±0.5† | 1.3± 0.6† |

** p<0.05 as compared with plasma*

*† p<0.001 as compared with plasma*

## DISCUSSION

This nationwide, multicenter comparison of PLT storage solutions revealed that all tested solutions maintained in-vitro PLT quality, allowing release to hospitals, for at least 8 days after blood collection: $pH_{37°C}$ was maintained above 6.6, and the swirling phenomenon continued to be visible. PLT concentrates in InterSol, and those that had undergone additional PR also fulfilled these criteria at day 8 with this limited set of requirements, but occasionally had low glucose levels. Though most preparation conformed to Council of Europe guidelines[37], additional assays displayed probably relevant differences in in vitro parameters between the storage solutions, underscoring the FDA instructions[21] for additional assays to be performed when evaluating new processing or storage methods. Unfortunately, the lack of correlation between a particular test result with clinical efficacy remains a significant limitation of most in vitro parameters. In the current study, PLT yield amongst the products differed; units in plasma had higher PLT numbers per unit than those in PASs. We explain this by the lower viscosity of the PASs which hampers to find good centrifugation conditions.[9] PR did not cause a decrease in the number of PLTs (since lower numbers would suggest PLT lysis), the lower PLT numbers in the final product were caused by volume loss alone.
When comparing between the PASs, a clear difference emerged between presence and absence of potassium and magnesium on the in vitro parameters. PLTs stored in PASs without potassium and magnesium (T-sol and InterSol) and had a lower pH, showed higher glucose and lactate metabolism, had a higher CD62P expression and higher annexin A5 binding as compared to solutes containing potassium and magnesium (Composol and SSP+).
These results are consistent with other publications.[19, 38]
Glucose consumption and lactate production are stimulated by the presence of phosphate in the storage medium.[36] Consequently, plasma, T-Sol and InterSol showed significantly higher conversion rates as compared with Composol or SSP+. Because of the lower starting value for glucose in InterSol-units, this resulted in depletion of all glucose in part of the units on day 8. Composol contains no phosphate and showed lower conversion rates. Despite the presence of phosphate in SSP+, low conversion rates were seen similarly to those with Composol, indicating that presence of potassium and magnesium in this solution can counteract the effects of phosphate.[39] In T-Sol and in InterSol the higher production rate of lactate resulted in lower pH values during storage.

The units that underwent a PR procedure had lower pH, higher lactate levels, and higher CD62P and annexin A5 binding as control units that had not undergone this procedure suggesting additional PLT activation.
In general, all PLT preparations conformed to the requirements for release, despite detectable differences among other in vitro parameters. Usually, requirements are applied as pass/fail criterion, such as PLT number per pool above or below 250 $x10^9$ PLTs, pH at the end of storage below 6.6.[27] There is clinical support that some parameters are indeed dichotomous, for example a $pH_{22°C}$ value below 6.2 will result in poor recovery and survival of PLTs[40, 41], while any pH value above that level does not. However, other in vitro measures could have a more gradual effect. We therefore propose a rating system. This rating system is based on three parameters, reflecting different aspects of PLT storage, i.e. activation, apoptosis/cell death, and metabolism: CD62P expression, annexin A5 binding and lactate production. We considered all in vitro measures for inclusion into the rating model, but most were rejected. Blood gases were not included, because the levels are dependent both on PLT quality and on gas permeability of the storage container. For example, low $CO_2$ levels can indicate poor PLT quality, where little or no $CO_2$ is produced, but can also indicate that the gas permeability of the container is very high. The same applies for oxygen levels. As indicated earlier, pH is a dichotomous parameter, and thus not suitable for our rating. Furthermore, with current-generation PLT storage containers,

pH values <6.2 are rarely seen. As bicarbonate levels in blood are directly related to pH and $CO_2$, this parameter was also not included. For PLT storage, it is important that glucose becomes not depleted, and thus we considered glucose informative for inclusion in the rating. However, only levels <1 mM would indicate poor PLT quality[39], while any level above that is not indicative as quality marker. Moreover, as glucose is normally converted into lactate in a 1:2 ratio (as was the case in the current study), and lactate was already included, we decided not to include glucose. Swirl is believed to be a good predictor of PLT quality[32], and therefore in Dutch guidelines all PLT concentrates are checked for presence of swirl at the time of issue. We would therefore never find a PLT concentrate without swirl being transfused and thus it is not a useful marker in the PLT rating. Finally, hypotonic shock response shows good correlation with in vivo recovery and survival.[2] In the current study, two centers included HSR but we found large differences in absolute values, and therefore, before being included in our proposed rating system, further standardization of the test is necessary.
In our rating system all study groups were compared with PLT stored in plasma. As proposed by AuBuchon et al.[23], this gold standard was used to circumvent a "downward creep" when methods were compared amongst each other for recovery/survival studies. Validation of our proposed rating system against clinical outcomes is necessary, and this validation should indicate whether a trichotomous distribution is a good indicator of clinical efficacy, or that a continuous distribution is feasible. At this time, our rating system is based on conjecture and assumptions, and includes only a limited number of in vitro tests. It is intended as a starting point for discussing a combination of parameters, that are collapsed into one composite outcome. So far, there is no "ultimate in vitro test" for predicting in vivo recovery, survival and functionality, but possibly a combination of in vitro tests, as summarized in our rating system, may provide such a helpful tool.

In summary, this study shows that PLTs in plasma or in 4 different PASs, of which one included a PR step, all conformed to release requirements after 7 days of storage; additional biochemical and functional measurements do demonstrate differences amongst PLT preparations.
The results were reproducible and comparable amongst 4 different blood centers.
A rating system is proposed to incorporate additional in vitro measures to judge PLT quality with the aim to predict in vitro quality. This rating system summarizes multiple (activation, apoptosis/cell death, and metabolism) PLT storage characteristics into a single score, and facilitated interpretation of an otherwise complex study. For example, the outcome of the rating clearly demonstrated the benefit potassium and magnesium in the PAS. On the other hand, a number of questions remain: there is some evidence that the chosen parameters for the rating are related to platelet survival, recovery and functionality, but no formal prospective evaluation has taken place so far. Also, based on that evidence we postulate that there is a relation between the rating score and PLT recovery, survival and effectiveness, but we have to provide data to support this hypothesis. Therefore, validation studies for this rating system will be initiated; until that time, the rating system should be considered as a proposal that needs further support from clinical studies.

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