Optimization of blood component preparation

Processing and donor influence

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CHAPTER 8

Platelet storage performance is consistent by donor: a pilot study comparing "good" and "poor" storing platelets

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CHAPTER 8

ABSTRACT

Background
In retrospective studies, it has been shown that differences in storage variables of platelet (PLT) concentrates (PCs) are partially donor dependent. It was our aim to prospectively determine the donor effect on PLT quality.

Study design and methods
Based on quality control data of outdated apheresis PCs, male donors were selected with at least one PC with a pH value of more than 7.0 (‘good’, n=6) or one PC with a pH value of less than 6.7 (‘poor’, n=6) on Day 8. These donors donated a PC (Trima Accel, Terumo) and completed a short questionnaire about their health and lifestyle. PCs were stored for 12 days and analyzed at regular intervals for in vitro quality.

Results
Donor characteristics were comparable, except that zero of six ‘good’ and four of six ‘poor’ donors reported high blood pressure and/or high cholesterol/fat and/or use of medicines. Lactate production in ‘good’ PCs was lower than in ‘poor’ PCs (0.09±0.03 vs 0.13±0.04 mmol/day/10^{11} PLTs, p<0.05) resulting in a higher pH from Day 5 onward. At the end of storage, the ‘good’ PCs showed lower CD62P expression, lower phosphatidylserine exposure and higher mitochondrial membrane potential. PLT functional properties were only slightly different. Despite having lower pH, also the ‘poor’ PCs fulfilled European Guidelines during 7 day storage.

Conclusion
Platelet storage performance is consistent when donors are dichotomized as having good or poor storing PLTs. Metabolic differences are perhaps due to different functionality of the mitochondria. More research is needed to establish the underlying causes and the implications for donors and blood products.
INTRODUCTION

Platelet (PLT) concentrates (PCs) are stored at room temperature for a short period of 5 to 7 days because a gradual loss of in vitro quality takes place, which is called storage lesion. Storage time is also restricted to minimize the risk of bacterial growth. Much research has been done to improve PLT preparation and storage. Among others, important conditions for storage are PLT count and number, the gas permeability of the plastic container (material, surface area), temperature (20-24°C) and the storage medium (plasma, PLT additive solutions).\(^1\)

Besides technical and physical conditions, there is a donor effect, resulting in biologic variance of in vitro PLT quality, as was previously shown investigating single donor PCs.\(^2\) After a 12 day storage period, a broad range of in vitro data showed some PCs with very good and some with very poor quality (pH, hypotonic shock response [HSR], thromboelastography) while other conditions, including PLT number and concentration, were approximately the same. Donors could be classified as having PLTs with good, average or poor storage properties. Dumont and colleagues showed that lactate production of apheresis PCs can differ with a factor of 3 and they suggested that this may be related to donor-specific factors.

In search for an explanation of donor-related PLT storage properties, we hypothesized that mitochondrial function would be very important for maintaining in vitro quality of PLTs. In a study comparing first- and second-generation storage containers, significant contributions to pH were not only determined by glucose consumption and lactate production, but also by mitochondrial membrane potential (MMP) and intracellular superoxide levels.\(^4\) Also the difference in thromboelastographic (TEG) clot retraction results, as found in our 2013 study,\(^2\) suggests an important role for mitochondria because clot retraction depends on mitochondrial energy production, as shown recently by Misztal and coworkers.\(^5\) Further, it is known that dysfunction of mitochondria plays an important role in several diseases like neurodegenerative (Parkinson’s, Alzheimer’s) diseases, migraine headaches and Type 2 diabetes.\(^6\) Mitochondrial defects have also been associated with age and age-related diseases.\(^7\)

When examining the grey area between sickness and health, insulin resistance, one of the characteristics of metabolic syndrome and a strong predictor for Type 2 diabetes,\(^8\) has been reported to be related with mitochondrial dysfunction.\(^9\) A study of our own institute revealed that 23% of the whole blood donors have metabolic syndrome,\(^10\) characterized by elevated blood pressure, fasting glucose, triglycerides, waist circumference and/or reduced
high-density lipoprotein (HDL) cholesterol. Taken together, partial dysfunction of donor PLT mitochondria due to symptoms of metabolic syndrome could be a cause of poor PLT storage properties.

We hypothesized that PLT quality, as reflected by storage variables, are in part determined by the donor characteristics, and that these donor characteristics affect the ability to store the PLTs under blood bank conditions. It was our aim to prospectively determine the donor effect on PLT quality, comparing apheresis PCs from donors that were classified as ‘good’ or ‘poor’ based on historical quality control (QC) data of outdated PCs. For that purpose, 12 male donors, six of whom were expected to have ‘good’ and six of whom were expected to have ‘poor’ PLTs, were invited to donate a single apheresis PC and to fill out a short questionnaire. Various PLT quality variables were determined over a 12-day storage period to compare the two groups.

**MATERIALS AND METHODS**

**Donor selection and apheresis procedure**

Donors were selected based on historical QC data of their outdated apheresis PCs collected over a 3-year period (September 2011 – August 2014). This QC database contains measurements of volume, PLT count and pH on the day after expiry, usually on Day 8. Data of 908 PCs were collected from 371 donors, about 75% of our total PLT donor population. Because the majority of apheresis PCs are collected with Trima Accel (Terumo BCT, Lakewood, CO) and to avoid differences induced by the apheresis machine and/or storage container, only units collected by the Trima were taken into account. To avoid possible sex-related differences, female donors were excluded, because the majority of PC donors are male. Finally, donors had to meet a third selection criterion of having at least 2 outdated PCs in the database, with on at least one occasion a pH$_{37^\circ C}$<6.7 (‘poor’) or pH$_{37^\circ C}$ >7.0 (‘good’).

PCs were collected after written informed consent from the donor with Trima Accel [Software Version 6] as single units with collection settings at 318 mL and 3.5x10$^{11}$ PLTs. A sample of whole blood was taken from the blood deviation pouch in a sodium citrate tube (9 ml, 3.2%, Ref. 455322, Greiner Bio-One, Alphen aan den Rijn, the Netherlands) and donors were asked to fill out a short questionnaire with health and life-style related questions. Questions about high blood pressure, high cholesterol and/or lipids and diabetes were objective because these traits had to be diagnosed by a practitioner and when a donor used medicines, the relevant diagnosis was also asked. As checked by the Medical Ethical
Platelet storage performance is consistent by donor.

Committee (Academic Medical Center, Amsterdam), approval for this questionnaire was not necessary. On the day of collection (Day 0), PCs and blood samples were routinely shipped from the collection site to the central processing site at room temperature. PCs were stored overnight on a flatbed shaker (20-24°C) and sampled routinely for bacterial screening on Day 1. Blood samples were also held overnight at room temperature and were used for complete blood count (Sysmex XT2000i, TOA, Tokyo, Japan) from an EDTA tube (4.5 mL, Ref. 454223, Greiner).

In vitro analysis of PCs
On Day 1, the PCs were weighed and sampled aseptically using a swan-lock adapter (Ref.16.4200, Codan, Lensahn, Germany) and syringe. Sampling was repeated on Day 5, 8 and 12. Immediately after sampling pH, blood gases, glucose and lactate were determined at 37°C using a blood gas analyzer (ABL90, Radiometer, Copenhagen, Denmark). PLT counts were determined with the blood cell analyzer (Sysmex XT2000i) and low-level white blood cells were counted using a counting kit (LeukoCount, Ref 340523, BD Biosciences, San Jose, CA) on a flowcytometer (FacsCalibur, BD Biosciences).

CD62P expression as activation marker of the PLTs and annexin A5 binding as a marker for phosphatidylserine (PS) exposure, were assessed using flow cytometric assays as described earlier.2,11 The MMP was measured with the fluorescent dye JC-1.12 Samples were diluted in PLT additive solution (PAS, SSP+, MacoPharma, Tourcoing, France, ratio 1:33) and incubated with JC-1 dye (final concentration 0.5 µmol/L) at 37°C for 20 min in a temperature controlled mixer at 800 rpm (Thermomixer Comfort, Eppendorf, Hamburg, Germany). After dilution 10x with PAS, analysis was performed by a flowcytometer (FACSCalibur) and by calculating the mean fluorescence intensity (MFI) ratio of the FL2 and FL1 detectors as a measure for the MMP.

To determine morphological variables, the swirling effect was judged on a scale from 0 (no swirl) to 3 (excellent swirl), the mean PLT volume (MPV) was measured with the cell analyzer (Sysmex XT2000i), and the method of Kunicki and colleagues13 for PLT morphology was applied using a phase-contrast microscope (BX60F, Olympus, Zoeterwoude, the Netherlands).

Nucleotide content of the PLTs was assessed as follows: PC samples (120 µL) were diluted in phosphate-buffered saline (PBS), centrifuged for 1 minute at 16,873 x g, and after the supernatant was removed, the pellet was extracted with 300 µL of ice-cold 0.4 N perchloric acid and kept on ice for 15 to 30 minutes. After a second centrifugation run the extract was neutralized with 5 mol/L K₂CO₃. Extracts from Day 1 and 8 samples were
analyzed with high-performance liquid chromatography (HPLC) using a cartridge column (Partisphere SAX column, Whatman International Ltd.) and NaH$_2$PO$_4$·H$_2$O/acetoniitrile mixtures as running buffer at 254 nm extinction.

Total cholesterol, HDL cholesterol and triglycerides were measured on a modular analyzer (Roche Cobas 8000 c502 or c702, Roche Diagnostics, Almere, the Netherlands) from Day 1 supernatants, using standard colorimetric methods. Low-density lipoprotein (LDL) cholesterol was calculated using the formula of Friedewald.

**PLT function assays**

Several functional properties of the PLTs were determined. HSR was measured with an optical aggregometer (CH490, Chrono-log, Havertown, PA) using 200 µL PC after adding 100 µL of PBS (reference) or water. TEG measurements (TEG5000, Haemoscope Corp., Niles, IL) were performed by diluting PC samples 1:9 in solvent/detergent plasma (Omniplasma, Octapharma, Vienna, Austria) as described earlier.\(^2\) Previously,\(^2\) clot retraction in TEG as determined by a sudden change in the amplitude and expressed by the LY30 value, was considered to be a discriminating functional property between ‘good’ and ‘poor’ PLTs. To overcome some shortcomings of the LY30 value and TEG measurement, clot retraction was also determined from a static clotting assay in glass tubes. The same PLT suspension as used in TEG was pipetted (0.5 mL) in an aggregometer tube and the coagulation reaction was started after addition of 30 µL 0.2 mol/L CaCl$_2$ by contact activation with glass, which also activates the intrinsic pathway. After 1 hour the clot was removed from the tube, and weighed, and clot retraction was calculated using also the weight of the empty and filled tube. Reactivity of PLTs was determined by measuring the agonist-induced CD62P expression on Day 1 and 8, a modified procedure of a method published by Middelburg and colleagues,\(^{14}\) using serial dilutions of convulxin (0.0024-39 nmol/L) instead of collagen-related peptide for activation of the collagen receptor glycoprotein VI.

**Mass spectrometry analysis of platelets**

On Day 1, about 100x10$^6$ PLTs were lysed in 8 mol/L urea in 100 mmol/L Tris·HCl (pH 8). Disulfide bonds were reduced with 10 mmol/L dithiothreitol for 60 minutes at 20°C, alkylated with 55 mmol/L iodocetamide for 45 minutes at 20°C, and samples were digested overnight at 20°C with mass spectrometry (MS)-grade trypsin (Promega). Peptides were desalted and concentrated using C18 StageTips (Empore, 3M) and eluted with 0.5% (vol/vol) acetic acid, 80% (vol/vol) acetonitrile. Sample volume was reduced by a concentrator (SpeedVac,
Platelet storage performance is consistent by donor

ThermoFisher) and supplemented with 2% acetonitrile and 0.1% trifluoroacetic acid to a final volume of 5 µL. The tryptic peptides (3-mL sample) were separated by nanoscale C18 reverse phase chromatography coupled online to a mass spectrometer via a nanoelectrospray ion source (Orbitrap Fusion Tribrid, Thermo Fisher Scientific). Instrument settings as well as the employed elution gradient are described in Gazendam and coworkers.15

Statistical analysis
The dichotomous answers of the questionnaire (yes or no) were analyzed with a Fisher's exact test using computer software (SPSS, Version 23, SPSS, Chicago, IL). Differences in composition of whole blood samples and PCs were analyzed with a two-sided unpaired t test. Based on the hypothesis of superiority of ‘good’ PCs, differences during storage were analyzed with a one-sided unpaired t test (Excel 2010, Microsoft Corp.). Changes in storage variables within each group were analyzed with a repeated-measures analysis of variance followed by a Tukey-Kramer posttest (GraphPad, Instat, San Diego, CA). A p value of less than 0.05 was considered as significant for all tests. For the MS analysis, the raw MS files were processed with the MaxQuant 1.5.2.8 computational platform, as described elsewhere.15,16 Label Free Quantification was used to evaluate the difference in protein level between the PLTs of both groups. Identified proteins were filtered for potential contaminants, reverse hits and ‘only identified by site’ using computer software (Perseus 1.5.1.6). The label-free quantification values were transformed in log2 scale and proteins were filtered for having at least six valid values in one experimental group. Missing values were imputed by normal distribution (width, 0.3, shift, 1.8). The adapted permutation-based false discovery rate t test in Perseus software was used to assess quantitative significance between the groups using false discovery rate of less than 0.05 and $S_0 = 0$.

RESULTS

Selected donors
All ‘good-PC’ donors met the selection criterion of having at least one PC with a pH value of more than 7.0 in the database. One of the ‘good-PC’ donors was also characterized as a ‘good-PC’ donor in our previous study.2 Five ‘poor-PC’ donors met the selection criterion of having at least one PC with a pH value of less than 6.7 whereas 1 ‘poor-PC’ donor was found after a concession on maximum pH (<6.8), but with 3 units below this value. Coincidentally,
one of the donors who was initially selected for the ‘poor-PC’ group, was excluded from donating just prior to the study, because the donor had to start using insulin.

Donors in both groups were comparable in age, body mass index (BMI) and measured blood pressures (Table 1). The questionnaire revealed that one ‘good-PC’ donor smoked, and four ‘poor-PC’ donors had health-related issues. One was diagnosed by a doctor for high blood pressure, one for high cholesterol and/or other lipids, one used medication (esomeprazole against stomach acid) and one donor positively answered all these three questions using simvastatin for cholesterol treatment (Table 1). One ‘poor-PC’ donor did not fill out the questionnaire, despite several attempts to obtain this information. Statistical analysis showed that the differences in answers to the health questions were not significant (p≥0.18).

Table 1: Characteristics of male donors (mean±SD) and results donor questionnaire (number answered yes). All p-values were >0.05.

<table>
<thead>
<tr>
<th></th>
<th>‘good-PC’</th>
<th>‘poor-PC’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>Age, years</td>
<td>57±14</td>
<td>52±11</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27±3</td>
<td>27±4</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>140±15</td>
<td>142±11</td>
</tr>
<tr>
<td>Diastolic</td>
<td>80±6</td>
<td>85±10</td>
</tr>
<tr>
<td>Donor questionnaire*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>0/6</td>
<td>2/5</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
<td>Periodontal disease</td>
<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
<td>Medicines</td>
<td>0/6</td>
<td>0/5</td>
</tr>
<tr>
<td>Smoking</td>
<td>1/6</td>
<td>0/5</td>
</tr>
<tr>
<td>Sporting activities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 hour/week</td>
<td>1/6</td>
<td>1/5</td>
</tr>
<tr>
<td>≥1 hour/week</td>
<td>5/6</td>
<td>4/5</td>
</tr>
</tbody>
</table>

*available for 11/12 donors

The mean time of day of collection (13:15±3:46 vs 11:04±2:17 h:min) was similar. Whole blood samples showed comparable counts of all cell types including PLTs (226±50 vs 232±53 x10⁹/L), in both groups prior to the apheresis procedure. Hemoglobin concentrations (9.7±0.8 vs 9.4±0.4 mmol/L) were also comparable but a small difference was detected in mean corpuscular volume (MCV) of red cells (89.1±4.1 vs 94.3±3.2 fl, p<0.05). Day 1 supernatants of PCs showed comparable cholesterol (total, HDL, LDL) and triglyceride levels between both donor groups, which were all within the normal range (data not shown).
Comparison of metabolic characteristics of the platelet concentrates

PCs in both groups were of comparable volume on Day 1 (295±6 vs 294±15 mL), but ‘good’ PCs had higher PLT concentrations (1.46±0.11 vs 1.27±0.13, p<0.05) and, by consequence, higher absolute PLT numbers (Table 2). Contamination with white blood cells was low in both groups (<0.22×10⁶ per unit).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 1</th>
<th>Day 5</th>
<th>Day 8</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>P</td>
<td>G</td>
<td>P</td>
</tr>
<tr>
<td>PLT, x10⁶</td>
<td>429 ± 29</td>
<td>375 ± 45§</td>
<td>392 ± 25</td>
<td>370 ± 27¶</td>
</tr>
<tr>
<td>MPV, fL</td>
<td>8.9 ± 0.8</td>
<td>9.3 ± 0.5</td>
<td>8.7 ± 1.0</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>pH (37°C)</td>
<td>7.15 ± 0.05</td>
<td>7.16 ± 0.03</td>
<td>7.06 ± 0.03*</td>
<td>6.90 ± 0.09¶</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>16.9 ± 1.0</td>
<td>18.4 ± 1.1§</td>
<td>14.6 ± 0.7†</td>
<td>12.5 ± 0.7†</td>
</tr>
<tr>
<td>Lactate, mM</td>
<td>2.9 ± 0.8</td>
<td>3.6 ± 0.7</td>
<td>7.4 ± 0.7†</td>
<td>11.0 ± 0.9†</td>
</tr>
<tr>
<td>pO₂, mm Hg</td>
<td>42 ± 23</td>
<td>56 ± 22</td>
<td>41 ± 20</td>
<td>50 ± 22</td>
</tr>
<tr>
<td>pCO₂, mm Hg</td>
<td>50 ± 7</td>
<td>42 ± 6</td>
<td>34 ± 3</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>JC-1, ratio FL2/FL1</td>
<td>11.5 ± 3.8</td>
<td>9.0 ± 2.0</td>
<td>11.7 ± 2.6</td>
<td>9.9 ± 1.4</td>
</tr>
<tr>
<td>CD62P expression, %</td>
<td>5.8 ± 3.0</td>
<td>6.4 ± 3.3</td>
<td>10.5 ± 2.5*</td>
<td>12.4 ± 3.2†</td>
</tr>
<tr>
<td>Annexin A5 binding, %</td>
<td>2.9 ± 0.8</td>
<td>4.3 ± 2.2</td>
<td>8.0 ± 2.8†</td>
<td>9.7 ± 2.6†</td>
</tr>
<tr>
<td>HSR, %</td>
<td>88 ± 18</td>
<td>82 ± 24</td>
<td>91 ± 17</td>
<td>81 ± 17</td>
</tr>
<tr>
<td>R-time, min</td>
<td>11.4 ± 1.7</td>
<td>9.9 ± 1.3</td>
<td>10.9 ± 2.0</td>
<td>10.3 ± 1.3</td>
</tr>
<tr>
<td>Max Amplitude, mm</td>
<td>68.0 ± 4.2</td>
<td>70.3 ± 2.3</td>
<td>66.3 ± 3.5</td>
<td>69.9 ± 1.5</td>
</tr>
</tbody>
</table>

Table 2: Storage variables of PCs with good (G) and poor (P) in vitro quality (mean±SD, n=6).

* p<0.05, † p<0.01, ‡ p<0.001 versus Day 1
§ p<0.05, ¶ p<0.01, † p<0.001

During storage, pH decreased in the ‘poor’ PCs more steeply, which resulted in significantly lower pH values in this group on Day 5 and thereafter (Table 2). All ‘good’ PCs but only one of the ‘poor’ PCs had a pH value of more than 7.0 on Day 8 and more than 6.7 on Day 12, but all PCs fulfilled European requirements after the current shelf life of 7 days. On Day 12, one of six ‘poor’ PCs had a pH<sub>37°C</sub> below the limit of 6.3. Initially, glucose concentrations were higher in ‘poor’ PCs but were lower on Day 12 than ‘good’ PCs. Lactate concentrations were lower in ‘poor’ PCs throughout the study period.
in ‘poor’ PCs were, in accordance with the decreasing pH, higher on Day 5, 8 and 12. As shown in Figure 1, the significant differences in glucose consumption (53-83%) and lactate production (53-67%) revealed a higher glycolytic rate in the ‘poor’ PCs. For each time period also the ratio of lactate production over glucose consumption (L/G) was calculated, which shows values nearby 2 in the ‘good’ PCs between Day 1 and 5 (1.89±0.27, Figure 1), indicating almost full conversion of the glucose taken up into lactate. After Day 5, the L/G ratio in the ‘good’ PCs leveled off to the lower ratio in the ‘poor-PC’ group (1.60±0.63 vs 1.66±0.45).

**Figure 1:** Glycolysis rates during each storage period of 3 or 4 days in ‘good’ (□) and ‘poor’ (■) platelet concentrates.

Comparison of O₂ and CO₂ partial pressures did not show significant differences between the groups (Table 2). On Day 1, the mean pO₂ values were lower in the ‘good’ PCs, while the mean pCO₂ values were higher, respectively, than in the ‘poor’ PCs. This was in accordance with the higher PLT numbers in this group, because on Day 1 a good correlation was found between PLT number and pO₂ (n=12, R²=0.61).
Differences in nucleotide levels did not reach statistical significance, but mean levels of AMP, ADP and ATP were somewhat higher in the ‘poor-PC’ group on both sampling days: ATP on Day 8 47.7±6.9 vs 52.4±11.9 pmol/10^6 PLTs, ‘good’ vs ‘poor’ (other data not shown).

**Flow cytometric, proteomic, morphologic and PLT function assays**

The activation parameter CD62P expression and PS exposure due to decreasing PLT membrane asymmetry (measured as annexin A5 binding), increased in both groups, but slightly more in the ‘poor’-PC group, resulting in significantly higher values in this group on Day 8 and/or Day 12 (Table 2). Determination of the MMP with the fluorescent dye JC-1, showed a rather constant level of the FL2/FL1 ratio in the ‘good’ PLTs throughout storage but more variable levels in the ‘poor’ PLTs, being significantly lower on Day 12. Remarkably, the PLT mitochondria of the smoker showed the lowest MMP on Day 1 (FL2/FL1 ratio:6.3), followed by a gradual increase until Day 12 (FL2/FL1 ratio:12.3). MS data revealed no significant differences in peptide or protein levels between the groups (Figure S1).

Swirl and morphology score in both groups (data not shown) were similar and of sufficient level throughout storage, except on Day 12 in the ‘poor’ unit with pH<sub>37°C</sub> value of less than 6.3 (swirl 1, morphology score <200). However, in MPV more pronounced differences were seen between ‘good’ and ‘poor’ PLTs: the first showed a slightly increasing volume following a decreasing volume between Day 1 and 5, whereas the latter showed a large increase in MPV after Day 8.

With respect to in vitro PLT function, HSR on Day 12 was somewhat lower in the ‘poor’-PC group. TEG measurements showed a slightly lower maximum amplitude on Day 5 in the ‘good’ PCs which was accompanied with higher LY30 values (‘good’, n=4: 2.4-11.3% vs. ‘poor’, n=1: 3.2%, other values <0.5%), most likely due to a higher clot retraction. However, determination of clot retraction by the static clotting assay did not reveal differences between the groups. Also PLT reactivity to the agonists ADP, convulxin and TRAP was comparable between the groups (data not shown). A higher baseline level of CD62P expression was observed on Day 8 in the ‘poor-PC’ group, in accordance with the standard assessment of this PLT activation marker without using agonists.
CHAPTER 8

DISCUSSION

In this pilot study, it was prospectively shown that PLTs from donors whose PCs had a high or low pH at the end of shelf life in previous donations, indeed had different glycolysis rates resulting in relative ‘good’ or ‘poor’ storage properties, thereby confirming retrospective studies. The obtained information about the donors was associated with the ‘poor’-PC donors as the group having more health issues, but our study was not designed to show a causal relationship.

In the PCs collected, PLT concentrations and numbers were different between the two groups of donors, but these are very likely caused by differences in the apheresis devices used. Three of the ‘good’ and one of the ‘poor’ PCs were collected with one device, which yielded significantly higher PLT concentrations when compared to the other eight PCs collected with six other devices. However, it cannot be excluded that donor effects also affect this parameter, albeit unlikely.

The more than 50% higher glucose consumption and lactate production in ‘poor’ PCs was detected as the main difference between both groups, indicating large differences in PLT metabolism and a higher demand for ATP in ‘poor’ PLTs. Although not significant until Day 8, the JC-1 ratio was always on average lower in ‘poor’ PLTs during the whole storage time. Hence, we surmise that partial dysfunction of the ‘poor’ PLT mitochondria will certainly play a role. As known for a long time, PLTs use glucose as the main fuel, but several other substrates such as amino acids and fatty acids may be used as well. The latter substrates depend on oxidative steps taking place in the mitochondria. Mitochondrial oxidation is also important for glucose consumption, because pyruvate, the end-product of glycolysis, can be either converted into lactate regenerating NAD+, or taken up by the mitochondria and converted into acetyl-CoA. Another metabolic pathway that could play a role is the pentose phosphate pathway, shunting glucose 6-phosphate into oxidation to pentose phosphates, a pathway used by the cells to maintain a high ratio of NADPH/NADP+ and a high ratio of reduced to oxidized glutathione to prevent free radical damage, also producing CO₂.

Theoretically, when all glucose is converted into lactate, and thus no pyruvate or glucose 6-phosphate is oxidized by the mitochondrial oxidative pathways or pentose phosphate pathway respectively, the ratio of lactate over glucose (L/G) is 2. So, when the ratio of L/G is rather high, as was seen in the ‘good’ PLTs in the initial period of storage, pyruvate is predominantly converted into lactate. Mitochondria use mainly other substrates for ATP synthesis and only small amounts of glucose are consumed by the pentose phosphate pathway. As
derived from Baker and colleagues\textsuperscript{20}, the consumption of glucose in PLTs is roughly 30x to 100x higher in glycolysis compared to the pentose phosphate pathway, depending on pH. This means that actual L/G ratios are underestimated compared to the measured L/G ratios, because the actual glucose consumption in anaerobic glycolysis is slightly lower, and may deviate to a maximum of 0.06. On the other hand, the ‘poor’ PLTs, which showed a rather low ratio L/G in combination with a higher glycolysis rate, hence, with higher intra-cellular pyruvate levels, use probably much more pyruvate in the mitochondria than ‘good’ PLTs as well as more glucose 6-phosphate in the pentose phosphate pathway. The first assumption is supported by observations of Michno and coworkers\textsuperscript{21} who reported higher activities of pyruvate dehydrogenase and higher acetyl-CoA content in PLTs of diabetic subjects. In addition, our study showed that ‘poor’ PLTs contained somewhat higher average values of nucleotides than ‘good’ PLTs, which again is in agreement with PLT studies in Type 2 diabetes patients.\textsuperscript{22,23} Taken together, it is likely that ‘poor’ PLTs are not only characterized by increased glycolysis but also by increased rates of the citric acid cycle and oxidative phosphorylation. Unfortunately this metabolic differences could not be related to different protein levels of the PLTs, and a metabolomics study should be undertaken to elucidate this.

As a result of the higher decrease in pH, indicators of the storage lesion like CD62P expression and PS exposure were more pronounced in ‘poor’ PCs at the end of storage. As described by Zharikov and Shiva,\textsuperscript{6} PLT activation, apoptosis and MMP are closely linked, and the mechanism would be as follows. The average highest JC-ratio on Day 5 in the ‘poor’ PLTs are indicative for an increase in MMP, known as hyperpolarization. The higher activity of the electron chain transfer can result in more leakage of electrons out of the chain and subsequent superoxide and hydrogen peroxide generation. These reactive oxygen species may lead to more damage of the ‘poor’ PLT mitochondria and subsequent loss of MMP. However, because differences were not significant and we did not measure reactive oxygen species, we can only speculate about the underlying mechanism.

The very low MMP of PLTs from the smoking donor in the ‘good-PC’ group was perhaps due to lower activity of complex I (or NADH-Q-oxidoreductase) in the mitochondrial membrane. Smith and coworkers\textsuperscript{24} observed a 24% decrease of complex I activity, when they studied PLT mitochondria of healthy smoking individuals compared to non-smoking healthy controls.

No differences were seen in functional behavior of PLTs as measured in the agonist-induced CD62P, TEG, clotting and HSR assays, during the normal shelf life of 7 days. Until now, HSR is the only functional property which showed a high correlation with in vivo recovery of
PLTs,\textsuperscript{25} so it is not likely that PCs would have different \textit{in vivo} results. Holme\textsuperscript{25} also reported a high lactate production per PLT as a strong predictor for survival, which was confirmed by results of Goodrich and colleagues,\textsuperscript{26} who studied a small number of riboflavin and UV light-treated PCs versus controls. However, an effect of differences in lactate production was not reported by Dumont and coworkers.\textsuperscript{3} The \textit{in vitro} quality of ‘poor’ PLTs may be more compromised when, in contrast to our study, less optimal, storage conditions are used.

Demographic characteristics of the donors were not significantly different, but there is a suggestion that donors with a history of ‘poor’ PCs were more likely to have symptoms of metabolic syndrome and/or Type 2 diabetes than donors with a history of ‘good’ PCs. This suggestion is first of all given by the results of the questionnaire pointing to the ‘poor’-PC donors as the group having more health issues, despite their lower mean age. Second, it is supported by two individual cases: one selected ‘poor-PC’ donor could not be included in the study because he had to start using insulin (and thus no longer qualified as blood donor in the Netherlands), and one other ‘poor’-PC donor used in a previous study by our group\textsuperscript{2} was diagnosed with diabetes in 2014 (and continued as plasma donor). However, this is only suggestion and not proof, and therefore a larger study including more subjects, also including insulin-dependent diabetics, is needed to establish a causal relationship between donor health and PLT storage properties.

In conclusion, this pilot study shows that PLT storage performance is consistent by donor when donors are dichotomized as having ‘good’ or ‘poor’ storing PLTs, and reveals large metabolic but small functional differences. More research is needed to establish the underlying causes with regard to health status and mitochondrial dysfunction and the implications for blood products and donors.
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AUTHOR CONTRIBUTIONS

IB designed the study, performed the experiments, analysed the results and wrote the article. PvdM, AV and DdK designed the study and wrote the article. KvdH analysed the donor questionnaire and revised the article.

CONFLICT OF INTEREST

There are no conflicts of interest.
REFERENCES

Platelet storage performance is consistent by donor.


SUPPLEMENTAL INFORMATION

Figure S1: Mass spectrometry analysis revealed no significant proteomic differences between ‘good’ and ‘poor’ storing platelets, as illustrated by this volcano plot.