Pathogen inactivation with amotosalen plus UVA illumination minimally impacts microRNA expression in platelets during storage under standard blood banking conditions

Niels Arni Arnason,[1] Freyr Johannson,[2] Ragna Landrö,[1] Björn Hardarsson,[1] Johannes Irsch,[3] Sveinn Gudmundsson,[1] Ottar Rolfsson,[2] and Olafur E. Sigurjonsson[1,4]

BACKGROUND: To reduce the risk of transfusion transmission infection, nucleic acid targeted methods have been developed to inactivate pathogens in PCs. miRNAs have been shown to play an important role in platelet function, and changes in the abundance of specific miRNAs during storage have been observed, as have perturbation effects related to pathogen inactivation (PI) methods. The aim of this work was to investigate the effects of PI on selected miRNAs during storage.

STUDY DESIGN AND METHODS: Using a pool and split strategy, 3 identical buffy coat PC units were generated from a pool of 24 whole blood donors. Each unit received a different treatment: 1) Untreated platelet control in platelet additive solution (C-PAS); 2) Amotosalen-UVA-treated platelets in PAS (PI-PAS); and 3) untreated platelets in donor plasma (U-PL). PCs were stored for 7 days under standard blood banking conditions. Standard platelet quality control (QC) parameters and 25 selected miRNAs were analyzed.

RESULTS: During the 7-day storage period, differences were found in several QC parameters relating to PI treatment and storage in plasma, but overall the three treatments were comparable. Out of 25 miRNA tested changes in regulation of 5 miRNA in PI-PAS and 3 miRNA U-PL where detected compared to C-PAS. A statistically significant difference was observed in down regulations miR-96-5p on Days 2 and 4, 61.9% and 61.8%, respectively, in the PI-PAS treatment.

CONCLUSION: Amotosalen-UVA treatment does not significantly alter the miRNA profile of platelet concentrates generated and stored using standard blood banking conditions.

Platelets play a central role in hemostasis and represent an integral part of transfusion medicine. Platelet concentrates (PCs) are normally stored at room temperature for a maximum of 5 to 7 days, depending on country-specific regulations.[1,2] One of two main reasons for this limited storage time is the potential for bacterial growth during storage.[3,4] The risk of transfusion-transmitted bacterial infections (TTBIs) due to contaminated PCs is persistent despite improved donor selection protocols, phlebotomy techniques, and PC bacterial screening. Life threatening septic transfusion reactions (STRs) related to platelet transfusions still occur.[4,5] The currently available bacterial detection assays are not sufficient to prevent TTBI.[6] The rate of STRs due to PC transfusion has likely been underestimated, as highlighted in a recent 7-year retrospective study of PC transfusions using passive and active surveillance.[7]

The second reason for the relatively short shelf life of stored platelets is platelet storage lesion (PSL). PSL is a collective term for a variety of factors that describe the deterioration of platelet quality during storage.[8,9] The onset and acceleration of PSL during storage is likely to affect platelet post transfusion recovery and survival.[10,11] The onset and accumulation of PSL is related to the activation of or damage to the platelets as result of their preparation or storage conditions, and can eventually

From the [1]The Blood Bank, Landspitali - The National University Hospital of Iceland, the [2]Department of Medicine, University of Iceland, the [4]School of Science and Engineering, Reykjavik University, Reykjavik, Iceland; and the [3]Cerus B.V., Amersfoort, The Netherlands.

Address reprint requests to: Olafur E. Sigurjonsson, Principal Investigator, The Blood Bank, Landspitali - The National University Hospital of Iceland, Snorrabraut 60, 105 Reykjavik, Iceland; e-mail: oes@landspitali.is.

Sources of support: Landspíali Research fund grant number A-218-058.

Received for publication July 25, 2019; revision received September 15, 2019, and accepted October 3, 2019.

doi:10.1111/trf.15575

TRANSFUSION 2019;59;3727–3735

lead to further effects. All aspects of platelet processing can contribute to platelet alterations, including collection, post collection manipulation, and storage.[12–17] The majority of PCs are used as prophylaxis of bleeding in patients with low platelet count, e.g., as a result of bone marrow failure due to hematological malignancy and radio- or chemotherapy, a relatively frequent scenario in older populations.[18]

Countries with aging populations have seen a rise in the demand for PC transfusions, making meeting harvesting and storage demands ever more challenging.[19] To address this increase in demand, measures aimed at increasing transfusion safety while prolonging the standard 5-day shelf life of PCs to 6 or 7 days have been or are being developed. Several technologies for pathogen inactivation (PI) are currently available. These utilize ultraviolet (UV) light of different wavelengths with or without photoactive chemicals.[20,21] Amotosalne-UVA (INTERCEPT Blood System, Cerus) represents the most studied and most widely used of these methods. Amotosalen-UVA has been in routine use in many centers for more than a decade and is being used nationwide in several countries. The technology utilizes a synthetic psoralen (amotosalen) and UVA light. UVA light has been shown to have a much lower impact on platelet quality than UV light of shorter wave-lengths, as utilized with some other technologies. Amotosalen can intercalate into nucleic acids and upon UVA light treatment forms permanent adducts and crosslinks at high frequency, inhibiting strand separation and thus proliferation of infectious pathogens and residual white blood cells. The INTERCEPT method is effective against a broad spectrum of known and emerging viral, bacterial, and protozoan pathogens and potentially against unrecognized blood-borne pathogens as well.[22,23]

Some studies have shown a decreased quality of amotosalen-UVA-treated platelets at the laboratory level during storage compared to untreated platelets. These reports describe an increase in the activation of treated PCs.[24–26] Other studies, however, have not shown detrimental effects for the technology.[27–29] These differences may be due to the large variations between studies in test methods and parameters used to define platelet quality in vitro. There is currently no consensus regarding how the potential impacts of a PI treatment on in vitro platelet quality actually reflect clinical platelet function. Though numerous clinical studies have been conducted successfully over more than 15 years,[30–37] and over a million INTERCEPT-treated PCs safely transfused, there is still ongoing debate about the clinical value of implementing INTERCEPT pathogen inactivation technology.[38,39] Given recent reports on routine experience with INTERCEPT-treated PCs in clinical settings, the importance of in vitro findings needs further consideration.[40,41]

Recently, microRNAs (miRNAs) have been identified as potentially playing a role in post-transcriptional gene-expression regulation in platelets.[42–47] miRNAs are small, non-coding RNA molecules with a length of 19 to 24 nucleotides.[48] These small molecules serve as post-transcriptional regulators of gene function by binding to messenger RNA (mRNA) and facilitating translation inhibition or degradation of mRNA.[44,49] It has been estimated that miRNAs regulate up to 60% of all human protein coding genes.[50,51] Studies based on microarray technology and next generation sequencing methods revealed that human platelets harbor transcriptomes representing many of the human protein coding genes.[52–54] Up to 28% of all annotated human miRNA have been detected in platelets,[55] as have all key components needed to make use of the miRNA post-transcriptional control pathway.[48,56] A few publications have focused on miRNA in stored platelets, showing differences in the regulation of miRNAs during storage and describing the effects of post-collection processing methods like PI.[57–60] It has even been suggested that specific miRNA patterns could predict the quality of stored PCs and that manipulation of the miRNA gene regulation pathway could lead to more effective storage of platelets.[61,62] Research on miRNAs in stored PCs could contribute to better quality and prolonged shelf life of stored platelets.[63]

We analyzed the potential impact on miRNAs of three different PC preparations, including PI, using a pool and split design. The three treatments were: 1) a non-pathogen inactivated PC stored in platelet additive solution (PAS) (control); 2) a pathogen-inactivated (INTERCEPT) PC in PAS; and 3) a non-pathogen inactivated PC stored in donor plasma, to evaluate the effects of PAS. Platelet quality and miRNA profiles were monitored over 7 days of storage.

MATERIALS AND METHODS

Collection, processing, and storage

Double-dose PCs were obtained from a pool of eight healthy blood donors by the buffy coat method at the Blood Bank, Landspitali - The National University Hospital of Iceland. Buffy coats (BCs) were prepared from whole blood units by centrifugation at 3578 × g for 11 minutes at 20°C (Sorvall RC12BP, Thermo Fisher Scientific) and separated by Optipress II (Fenwal) following the Blood Bank's standard operating procedures. Using the double train method, eight ABO-matched BCs were pooled using Fenwal BC pooling sets (code K4R7039). This was done three times to create three BC pools. These three BC pools were further combined and mixed by genital rolling for 10 seconds in a 1.5-L intermediate collection bag obtained from the INTERCEPT (Cerus Europe BV) processing set, and split again to generate three identical single pools originating from 24 whole blood (WB) donors.

To two of the BC pools, 300 mL PAS (Storage Solution for Platelets SSP+, Macopharma) was added, creating PCs in 35% plasma and 65% PAS. To one BC pool, 300 mL of ABO-matched donor plasma was added, creating a PC in 100% donor plasma. At this point, each unit had a total volume of 660 mL. BC pools rested for 1 hour at room temperature before PC isolation by centrifuging. A double-dose PC unit (average volume 402.3 ± 24.1 mL; range 336.1-432.2 mL

and platelet count 509.7 × 10^9; range 351 × 10^9-579× 10^9) was isolated from each PAS BC pool by split centrifuging at 40 × g for 2 minutes followed by 463 × g for 6.5 minutes (Sorvall RC12BP, Thermo Fisher Scientific). The PC produced with 100% donor plasma was isolated by centrifuging at 987 × g for 7 minutes (Sorvall RC12BP, Thermo Fisher Scientific). All PCs were leukocyte-filtered and separated from the BC pool by Optipress II (Fenwal) following the Blood Bank's standard operating procedures.

One PAS double-dose PC received INTERCEPT PI treatment (Cerus Europe BV). PI treatment included addition of 17.5 mL of 3 mM amotosalen solution, UVA (340 to 400 nm) light exposure with total energy dose delivered 3.9 J/cm^2, and incubation with a compound absorption device for 6 to 16 hours in a flat-bed temperature-controlled incubator at 22°C ± 2°C (PC900H, Helmer) under light agitation. All double-dose PCs were then split into two single doses, as used for patient infusion. One single-dose unit was used for further analysis and the second single-dose unit was discarded or used in other research projects.

PCs were stored in a platelet incubator at standard blood bank storage conditions of 22 ± 2°C with gentle agitation (PC900H, Helmer) and sampled on Day 1 (data not shown), and Days 2, 4, and 7. The experimental setup is depicted in Fig. 1.

The treatments in this study are referred to herein as follows: 1) non-pathogen inactivated PC in PAS (control), C-PAS; 2) INTERCEPT pathogen inactivated PC in PAS, PI-PAS; 3) non-pathogen inactivated PC in donor plasma, U-PL.

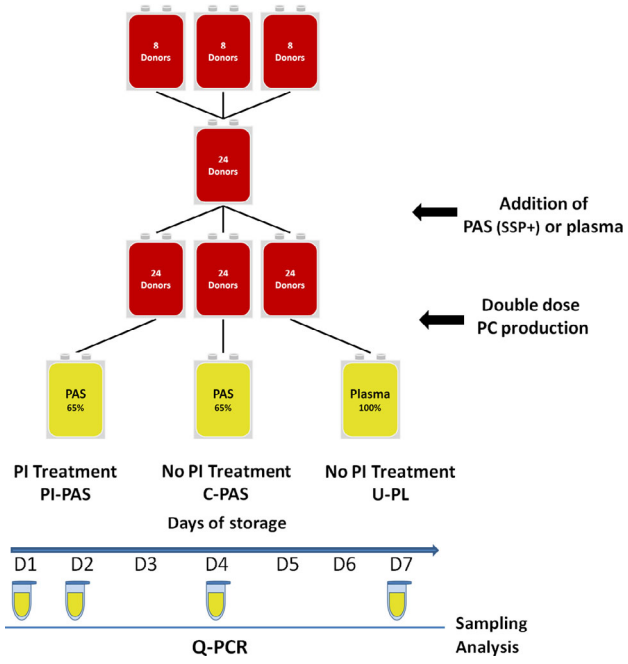

Fig. 1. Summary of experimental treatment and control preparation, n = 8. [Color figure can be viewed at wileyonlinelibrary.com]

Sampling and quality control

Sampling of PC units was done in a closed sterile system. A sample of 10 mL was collected from a single-dose unit. The 10 mL sample was further split into a 6.8-mL sample for analysis of miRNA by qPCR and a 3.2-mL sample for quality control (QC) parameters. Immediately after QC sample collection, the following parameters were analyzed: pH, pO_2, pCO_2, and concentrations of total glucose and lactate on a blood gas analyzer (ABL90, Radiometer); platelet count, mean platelet value (MPV), and platelet distribution width (PDW) on a hematology analyzer (Sysmex XN-1000); and Annexin V, glycoprotein IIb (GPIIb), GPIbα, P-selectin, and the CD63 gene by flow cytometry (FacsCalibur, BD Biosciences).

A platelet pellet for q-PCR was generated by separating the cells from spent media by centrifugation at 1730 × g and room temperature for 10 minutes (Sorvall RC5C, Thermo Fisher Scientific). The supernatant was retained for analysis by ELISA. Both pellets and supernatant were stored at −80° C until further analysis.

White blood cell depletion

Prior to RNA isolation and miRNA analysis, all samples were white blood cell (WBC) depleted using CD45 dynabeads (Thermo Fisher Scientific). Samples were incubated with the CD45 dynabeds for 15 minutes and any residual WBCs were removed using a magnet.

Flow cytometry

Flow cytometric analyses (FacsCalibur, BD Biosciences) were performed to determine the mean fluorescence intensity (MFI) of the following proteins in PCs during storage: Annexin V conjugated to PE for binding of membrane exposed phosphatidylserine; CD41 antibody against the CD41 antigen (also known as platelet glycoprotein IIb or IIb integrin) conjugated to FITC; CD42b antibody against the CD42b antigen (also known as platelet glycoprotein Ib alpha chain) conjugated to PE; and CD62p antibody against the CD62p antigen (also known as P-selectin, platelet activation-dependent granule-external membrane [PADGEM] protein, or granule membrane protein GMP-140) conjugated to PE. All antibodies were obtained from BD Biosciences.

Each PC sample was diluted 1:10 by mixing 100 µL of the sample with 900 µL of room-temperature HEPES-buffered saline (HBS). A diluted sample of 5 µL was stained with 10 µL CD41-FITC and 20 µL of CD42b-PE and CD-62p-PE for 20 minutes in the dark. Prior to analysis, 2 mL of cold HBS was added. Samples for Annexin V analysis were stained with 10 µL of CD41-FITC for 15 minutes in the dark before adding 10 µL of Annexin V following 10 minutes incubation in the dark. Added to these samples prior to analysis was 2 mL of 3.5 mM calcium HBS.

ELISA

Quantikine ELISA (enzyme-linked immunosorbent assay) kits (R&D Systems) were used to determine the concentrations of soluble CD40 ligand (sCD40L), sCD62P/sP-Selectin, and CXCL4/platelet factor 4 (PF4) in PC supernatants during storage, according to the manufacturer's instructions. PC supernatants (controls and treatments) sampled on Days 1, 2, 4, and 7 were removed from −80°C storage and thawed on ice in the dark for 1 hour. ELISA kits and samples were allowed to reach room temperature (~22°C) before use. Sample measurements were performed in duplicate on a Multiskan Spectrum v1.2 microplate reader (Thermo Fisher Scientific).

RNA isolation

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen). To control the isolation, synthetic spike-ins were added to the samples (UniSp2, UniSp4, and UniSp5). In short, platelet samples were lysed with 700 µL of Qiazol lysis reagent in a TissueLyser (Qiagen) with one 5 mm stainless steel bead. RNA was extracted using chloroform, ethanol, and spin columns. RNA was stored in RNase-free water at −80°C until analysis.

miRNA real-time qPCR

Based on previous miRNA results (unpublished data) and literature on their roles in platelet biology and storage, 25 miRNA were selected for analysis.

Using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon), 10 ng RNA was reverse transcribed in 10 µL reactions. Each RNA sample was reverse transcribed into cDNA and tested for the expression of 5 control miRNAs (miR-23a, miR-30c, miR-103, miR-142-3p, miR-451) and one synthetic spike-in RNA (UniSp6). cDNA was diluted 100-fold and assayed in a 10 µL PCR reaction according to the manufacturer's protocol for miRCURY LNA Universal RT microRNA PCR. Each miRNA was assayed once by qPCR on a custom Pick & Mix panel (Exiqon). No template controls (NTCs), excluding the template from the reverse transcription reaction, were included as negative controls. Amplification was performed in a LightCycler® 480 Real-Time PCR System in 384-well plates. Amplification curves were analyzed using Roche LC software to determine quantitation cycle (Cq) and melting curves.

Data analysis

All assays were inspected for distinct melting curves and the melting temperatures were confirmed to be within known specifications for the assay. In order to be included in data analysis, individual assays had to be above the detection limit and within the range of 3 Cq below the negative control to 37 Cq. Cq was calculated as the second derivative. Using NormFinder (Arhus University Hospital), the best normalizer was determined; all data were normalized to the average of assays detected in all samples (average–assay Cq).

Statistical analysis

Fold changes in miRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. For paired t-tests, differences were considered significant if p-values remained below 0.05 after applying the Benjamini-Hochberg method for false positive discovery rate.

RESULTS

Platelet count and size

Swirling was observed at all time points in all treatment groups. No aggregates were detected in any tested unit. Platelet count decreased on average significantly more over the 7-day storage period in PI-PAS (19.8%; p-value = 0,0132) and U-PL (20.4%; p-value = 0,0165) vs. C-PAS (16.7%). No significant difference was observed in MPV or PDW in either treatment group compared to control.

Metabolic activity of PCs

Metabolic activity was assessed by monitoring the following parameters in the three different PC products: acidity, glucose consumption, lactate production, and pO_2 and pCO_2 pressures (Table 1). The pH was significantly different at all time-points during storage between C-PAS and the other treatments. All groups had elevated pH between Days 2 and 4, with a decrease from Day 4 to the end of storage at Day 7. The pH remained above 6.4 in all treatments, thus maintaining compliance with European Directorate for the Quality of Medicines requirements.

All treatments showed decreasing glucose and pCO_2 values over time, as well as rising lactate and pO_2 values, which are typical trends during PC storage. Significant differences were observed in glucose, lactate, and pCO_2 levels in U-PL compared to C-PAS during the 7-day storage period (Table 1). pCO_2 was significantly different in PI-PAS than in C-PAS on Day 2 (20.01 $\pm$ 1.20 mmHg for PI-PAS vs. 17.47 $\pm$ 0.89 mmHg for C-PAS; p-value = 0.029) and Day 4 (9.51 $\pm$ 0.41 mmHg vs. 10.09 $\pm$ 0.33 mmHg; p-value = 0.016).

Markers of platelet activation

Surface expression of P-selectin and GPIbα and degree of Annexin V binding were monitored as markers of platelet activation and degree of PSL. A gradual increase was detected for P-selectin during storage in all PCs. Platelets stored in 100% plasma (U-PL) expressed significantly more P-selectin at all time points than did C-PAS (Table 1). P-selectin expression was significantly higher in PI-PAS

TABLE 1. Results of QC analyses over a period of 7 days

Parameter	Day	C-PAS	PI-PAS	U-PL
PDW (fL)	2	9.75 ± 0,35	9.96 ± 0,36	9.49 ± 0,33
	4	9.41 ± 0,30	9.75 ± 0,30	9.44 ± 0,40
	7	9.47 ± 0,49	9.79 ± 0,49	9.56 ± 0,46
MPV (fL)	2	9.69 ± 0,2	9.86 ± 0,20	9.55 ± 0,20
	4	9.60 ± 0,15	9.80 ± 0,19	9.56 ± 0,21
	7	9.84 ± 0,21	9.84 ± 0,21	9.74 ± 0,25
Plt count (10^9)	2	227.3 ± 65,2	230.6 ± 55,1	224.1 ± 61,0
	4	208.2 ± 61,9	211.3 ± 52,6	207.8 ± 59,3
	7	196.9 ± 57,5	196.0 ± 47,7	193.9 ± 54,3
pH 22°C (–)	2	7.14 ± 0.03	7.04 ± 0.01	7.25 ± 0.03
	4	7.31 ± 0.02	7.23 ± 0.01	7.40 ± 0.02
	7	7.28 ± 0.01	7.17 ± 0.02	7.17 ± 0.03
pCO_2 (mmHg)	2	17.5 ± 0.9	20.0 ± 1.2	27.4 ± 1.2
	4	10.1 ± 0.3	9.51 ± 0.41	14.6 ± 0.6
	7	9.23 ± 0.20	8.80 ± 0.14	12.4 ± 0.18
pO_2 (mmHg)	2	108 ± 7	105 ± 8	98.4 ± 5.8
	4	118 ± 7	122 ± 7	103 ± 6
	7	117 ± 6	128 ± 5	104 ± 3
Glucose (mmol/L)	2	6.36 ± 0.32	5.69 ± 0.12	17.1 ± 0.5
	4	5.23 ± 0.32	4.64 ± 0.14	15.0 ± 0.5
	7	2.88 ± 0.32	2.13 ± 0.25	11.0 ± 0.7
Lactate (mmol/L)	2	7.49 ± 0.43	7.17 ± 0.36	9.11 ± 0.52
	4	9.44 ± 0.37	8.93 ± 0.36	12.4 ± 0.5
	7	13.8 ± 0.5	13.4 ± 0.6	19.0 ± 0.8
P-selectin (% gated)	2	7.69 ± 1.15	8.27 ± 1.17	10.2 ± 0.8
	4	11.6 ± 0.9	14.9 ± 1.7	16.9 ± 1.3
	7	21.2 ± 2.4	26.5 ± 2.1	22.6 ± 1.7
GPIbα low (% gated)	2	1.15 ± 0.29	1.55 ± 0.21	1.79 ± 0.47
	4	2.41 ± 0.26	3.61 ± 0.27	2.18 ± 0.18
	7	5.08 ± 0.57	7.86 ± 0.98	4.92 ± 0.44
Annexin V (% gated)	2	9.94 ± 1.76	6.45 ± 1.16	11.5 ± 1.2
	4	7.25 ± 1.50	4.57 ± 0.98	9.27 ± 2.36
	7	15.2 ± 2.1	11.1 ± 2.2	18.5 ± 2.0
sCD40L (ng/mL)	2	1.74 ± 0.19	1.93 ± 0.17	1.01 ± 0.11
	4	2.70 ± 0.22	3.33 ± 0.29	1.71 ± 0.15
	7	3.84 ± 0.26	4.34 ± 0.58	2.73 ± 0.17
sP-selectin (ng/mL)	2	42.2 ± 5.3	43.1 ± 2.3	70.5 ± 3.3
	4	54.0 ± 2.6	65.4 ± 4.3	89.2 ± 2.8
	7	90.7 ± 4.1	99.2 ± 4.8	124 ± 5
PF-4 (μg/mL)	2	2.29 ± 0.47	3.30 ± 0.64	4.50 ± 0.77
	4	5.10 ± 0.35	7.75 ± 0.63	7.80 ± 0.57
	7	9.27 ± 0.50	13.1 ± 0.8	10.7 ± 0.6

Results are presented as mean ± standard deviation. n = 8.
Day 1 data is not shown.

(26.53 ± 2.06% gated) than in C-PAS (21.22 ± 2.35% gated) on Day 7.

There was a steady increase in the proportion of platelets with decreased GPIbα expression in all PCs. On Days 4 and 7, GPIbα low population was significantly higher in PI-PAS (3.61 ± 0.27% gated and 7.86 ± 0.98% gated, respectively) than in C-PAS (2.41 ± 0.26% gated and 5.08 ± 0.57% gated, respectively).

All groups displayed similar patterns in Annexin V binding, with an initial increase from baseline (data not shown), followed by a decrease from Day 2 to Day 4 and a second increase from Day 4 to Day 7. Annexin V binding was highest in U-PL at all time points. On Days 2 and 4, Annexin V binding was significantly lower in PI-PAS than C-PAS (6.45 ± 1.16% gated and 4.57 ± 0 .98% gated vs. 9.94 ± 1.76% gated and 7.25 ± 1.50% gated, respectively). No significant differences were observed between U-PL and C-PAS.

Release of platelet factors

Accumulation of platelet factors in the PC storage media also indicates activation and PSL. The concentration of four factors released from platelets via secretion or receptor shedding were monitored: CD40L, sP-selectin, and PF4 (Table 1). No significant differences were observed on Days 2 and 7 for CD40L in PI-PAS versus C-PAS. On Day 4, however, the CD40L concentration was significantly higher in PI-PAS (3.33 ± 0.29 ng/mL) than in C-PAS (2.70 ± 0.22 ng/mL). Platelets stored in plasma had significantly lower CD40L concentrations during the entire storage period than did control platelets.

PI-PAS units had significantly higher levels of PF4 on Days 4 and 7 (7.75 ± 0.63 μg/mL and 13.06 ± 0.82 μg/mL, respectively) than C-PAS (5.10 ± 0.35 μg/mL and 9.27 ± 0.50 μg/mL). On Day 4, the PF4 concentration was significantly higher in U-PL (7.80 ± 0.57 μg/mL) than in C-PAS (5.10 ± 0.35 μg/mL).

sP-selectin levels were significantly higher on Day 4 in PI-PAS (65.42 ± 4.31 ng/mL) than in C-PAS (54.02 ± 2.61 ng/mL), and concentrations were significantly higher at all time points in U-PL compared to C-PAS.

miRNA profiling with real-time qPCR

Out of the 25 miRNA analyzed, Student's t-tests showed that only five showed differences in regulation during storage at one or more time points. On Day 2, upregulations of 16.7% and 10.8% were detected for miR-326 and miR-146a-5p, respectively, in U-PL as compared to C-PAS. Also in U-PL, downregulations of 55% and 52% were observed for miR-96-5p on Days 2 and 4, respectively.

In the PI-PAS treatment, there was a 20.6% upregulation of miR-1260b on Day 7 compared to C-PAS, and a 7.9% upregulation of miR-146a-5p was observed on Day 2 (Table 2). Downregulations of 14.8% and 13.5% were observed in PI-PAS for miR-17-3p on Days 2 and 4, respectively, as well as a 7.1% downregulation of miR-20a-5p on Day 7 (Table 2). miR96-5p was down-regulated throughout the storage period (61.9%, 61.8%, and 51.4% on Days 2, 4, and 7, respectively). After applying methods to detect possible false positives due to multiple testing, the only statistically significant differences observed were downregulations of miR-96-5p on Days 2 and 4 (Table 2). Principal component analysis revealed no specific clustering of samples related to different treatments (Fig. 2).

TABLE 2. Differently regulated miRNA over a 7 day storage period

		% difference from C-PAS*	
miRNA	Day	PI-PAS	U-PL
hsa-miR-17-3p	2	+15.0†	ns
	4	+ 15,5†	ns
	7	ns	ns
hsa-miR-96-5p	2	−61.9†,‡	−55.1†
	4	−61.8†,‡	−51.9†
	7	−51.4†	ns
hsa-miR-146a-5p	2	−7.9†	−10.8†
	4	ns	ns
	7	ns	ns
hsa-miR-326	2	ns	ns
	4	ns	ns
	7	ns	+16.7†
hsa-miR-1260a	2	ns	ns
	4	ns	ns
	7	+20.0†	ns

Note that no significant differences were observed in this study for the other 20 miRNAs investigated.
(+) Positive differences represent upregulation compared to the C-PAS control.
"ns" means there were no significant differences between the C-PAS control and the treatment.
(−) Negative differences represent downregulation compared to the C-PAS control.
* Differences shown are based on means.
† t-test – p-values <0.05 statistical analysis using paired student t-test.
‡ BH FDR – p-values <0.05 after applying Benjamini Hochberg false discovery rate method.

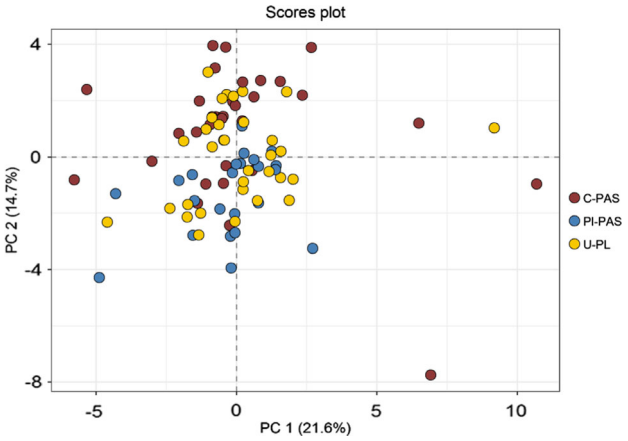

Fig. 2. Principal component analysis. PC 1 shows clustering of samples related to the expression of 25 individual miRNAs, with all treatments and sampling days included. No clustering can be seen related to treatment. [Color figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

It is important to understand the effect PSL has on platelet function during storage. In this paper, we looked at the expression of 25 miRNAs, chosen based on previous array results from our lab (unpublished data) and/or with specific relevance to platelet biology. The main goal was to evaluate whether PI using the INTERCEPT method had any effect on these miRNAs during standard storage. Using buffy coat PCs and a pool and split design, the effect of PI was compared to untreated PCs during a 7-day storage period. The results indicated that PI did not have a significant impact on the miRNA profile of PCs during storage; limited effects were observed, with only a single miRNA out of the 25 analyzed demonstrating a significant differential regulation in the PI-treated PC.

To back up results from miRNA analysis extensive in vitro data on platelet quality was collected during this study. No difference was detected in PDW, MPV, or swirling between different treatment groups. A difference was observed in lower platelet count during the 7-day storage in P-PAS possibly relating to additional steps in the PI treatment protocol (Table 1). Previous in vitro studies of PI-treated PCs have shown some indication of increased activation and acceleration of PSL during storage compared to standard untreated PCs.[24–26] In our data, we also saw some indication to this effect, with increased shedding of the GPIba receptor and surface expression of P-selectin, and concentration changes in sP-selectin, PF-4 and CD40L in the storage solution. CD40L concentrations were higher at all time points in PCs stored in PAS than in plasma; this might indicate that some degree of activation is attributable to the PAS storage solution.

The specifics of the onset and acceleration of PSL on the molecular level are not fully understood. As the miRNA post-transcriptional control pathway is active in platelets, the effects of post-collection processing and storage on the

miRNA profile of PCs are of interest. It has been suggested that specific miRNA expression patterns can be used to monitor cellular damage related to PSL.[62] Studies looking at miRNAs during platelet storage have shown high levels of miRNAs associated with apoptosis. Some of these pro-apoptotic miRNAs seem to be further upregulated during storage, while others are downregulated.[57,59]

There is limited published data on the potential effects of PI treatment on platelet miRNAs. One research group compared PI (INTERCEPT and Mirasol) and irradiation of single donor aphaeresis PCs. The authors speculated that the PI treatments had a perturbation effect on both platelet miRNA and mRNA and, further, that this observed effect was due to an increased activation state as result of the INTERCEPT process inducing microparticle release, containing miRNA and mRNA.[58,60] INTERCEPT treatment seemed to have an effect on 6 out of the 11 miRNAs included in these analyses. In the current study, all 11 of these miRNAs were included, but we did not see the same effect. Only one miRNA, miR-96-5p, was significantly reduced by the PI treatment. These different outcomes are likely related to differences in study designs. In both studies by Osman et al.,[58,60] single donor aphaeresis PCs were used without a pool and split design; thus, miRNAs from different donors were compared in the different treatments. This may be an issue because variations in the regulation and expression of miRNAs between individuals have been demonstrated and can be related to factors such as sex, age, and race.[46,64] The pool and split design used in the current study reduced the potential effects of donor variation. In one of the Osman et al. studies,[58] INTERCEPT had a more pronounced impact on the investigated miRNAs than did Mirasol. Again, donor variation could have had an effect. In addition, storage media were not the same in each treatment; Mirasol PCs were resuspended in 100% donor plasma, while INTERCEPT PCs were resuspended in 65% PAS and 35% donor plasma. In our study design, the PI-PAS treatment and C-PAS control were stored in identical storage media in order to minimize the effects of different preparation and storage of the treatments, while the U-PL treatment (resuspended in 100% plasma) was compared to the C-PAS control to detect potential effects of PAS.

Osman et al. further analyzed the effects of INTERCEPT on mRNAs using RNA sequencing and found that INTERCEPT markedly deregulated the platelet mRNA transcriptome.[60] We did not specifically look at mRNAs or proteins in this study, but if PI treatment affects platelet mRNAs on a large scale, this effect does not seem to be translated into the proteome, where studies have indicated a weak impact of such treatment on the platelet proteome.[65,66] Research into how miRNAs regulate platelet mRNAs is important to further our knowledge of the molecular mechanisms of stored platelets.

Based on the results of this study, PI did not have a significant impact on the miRNA profile of PCs during storage. Down regulation of miR-96-5p in PI-PAS compared to C-PAS might be related to accelerated granule release in the PI-PAS as demonstrated by increased concentration of PF-4, sP-selectin, and CD40L especially on Day 4. All these factors are released by platelet α-granules.[67] The release of platelet factors from there granules is under the control of v-SNEARE complex containing endobrevin/Vamp8.[68] miR-96-5 has been indicated in the post transcriptional control of Vamp8 and down regulation of miR-96-5 associated with hyperactive platelets.[42]

Despite evidence that the manipulation involved in the processing steps of Intercept treatment effects the biology of stored platelets[24–26,69–71] numerous clinical trials and hemovigilance reports indicate Intercept treated platelets being safe and to be none inferior to standard platelets when it comes to transfusion efficacy.[30,31,35,36,40,41] A recent clinical study using WHO bleeding scores in patients with hematological malignant neoplasm concluded Intercept treated PC being non-inferior to standard platelets stored in PAS III (Intersol) but inferior to standard platelets stored in plasma,[37] an indication of this particular PAS contribution to PSL.

Although there is still an ongoing debate on INTERCEPT-treated PCs not performing as well as standard PCs, it must be taken into consideration what is achieved by implementing this technology, which drastically lowers the risk of potentially life-threatening septic reactions due to TTBIs, versus a reduction CCI tests or potential slight increase in non-life-threatening bleeding complications.[72] Countries and regions that have already implemented PI have seen a drop in both TTBIs and adverse reactions.[26]

ACKNOWLEDGMENTS

The authors would like to thank the staff of the Blood bank in Iceland and Kristine Wichuck.

CONFLICTS OF INTEREST

Cerus B.V. paid for part of the QPCR products. Johannes Irsch is an employee of Cerus B.V.

REFERENCES

1. Hogman CF. Storage of blood components. Curr Opin Hematol 1999;6:427-31.
2. Vasconcelos E, Figueiredo AC, Seghatchian J. Quality of platelet concentrates derived by platelet rich plasma, buffy coat and Apheresis. Transfus Apher Sci 2003;29:13-6.
3. Schrezenmeier H et al. Bacterial contamination of platelet concentrates: results of a prospective multicenter study comparing pooled whole blood-derived platelets and apheresis platelets. Transfusion 2007;47:644-52.
4. Pietersz RN et al. Bacterial contamination in platelet concentrates. Vox Sang 2014;106:256-83.
5. Kleinman S, Reed W, Stassinopoulos A. A patient-oriented risk-benefit analysis of pathogen-inactivated blood components:

application to apheresis platelets in the United States. Transfusion 2013;53:1603-18.
6. Spindler-Raffel E et al. Enlargement of the WHO international repository for platelet transfusion-relevant bacteria reference strains. Vox Sang 2017;112:713-22.
7. Hong H et al. Detection of septic transfusion reactions to platelet transfusions by active and passive surveillance. Blood 2016; 127:496-502.
8. Kocazeybek B et al. Prospective evaluation of platelets prepared by single and random methods during 5 days of storage: aspects related to quality and quantity. Transfus Apher Sci 2002;26:29-34.
9. Akay OM et al. Platelet function testing during 5-day storage of single and random donor plateletpheresis. Transfus Apher Sci 2007;36:285-9.
10. Murphy S, Sayar SN, Gardner FH. Storage of platelet concentrates at 22 degrees C. Blood 1970;35(4):549-557. Blood 2016; 128:1155.
11. Chernoff A, Snyder EL. The cellular and molecular basis of the platelet storage lesion: a symposium summary. Transfusion 1992;32:386-90.
12. Devine DV, Serrano K. The platelet storage lesion. Clin Lab Med 2010;30:475-87.
13. Shrivastava M. The platelet storage lesion. Transfus Apher Sci 2009;41:105-13.
14. Singh S et al. Platelet storage lesion in interim platelet unit concentrates: a comparison with buffy-coat and apheresis concentrates. Transfus Apher Sci 2017;56:870-4.
15. Shams Hakimi C et al. In vitro assessment of platelet concentrates with multiple electrode aggregometry. Platelets 2015;26: 132-7.
16. Waters L et al. Refrigeration, cryopreservation and pathogen inactivation: an updated perspective on platelet storage conditions. Vox Sang 2018;113:317-28.
17. Rijkers M et al. Evaluation of the role of the GPIb-IX-V receptor complex in development of the platelet storage lesion. Vox Sang 2016;111:247-56.
18. Charlton A et al. Where did platelets go in 2012? A survey of platelet transfusion practice in the North of England. Transfus Med 2014;24:213-8.
19. Shah A, Stanworth SJ, McKechnie S. Evidence and triggers for the transfusion of blood and blood products. Anaesthesia 2015; 70(Suppl 1):10-9, e3-5.
20. Mohr H et al. A novel approach to pathogen reduction in platelet concentrates using short-wave ultraviolet light. Transfusion 2009;49:2612-24.
21. Pelletier JP, Transue S, Snyder EL. Pathogen inactivation techniques. Best Pract Res Clin Haematol 2006;19:205-42.
22. Lin L et al. Photochemical inactivation of viruses and bacteria in platelet concentrates by use of a novel psoralen and long-wavelength ultraviolet light. Transfusion 1997;37:423-35.
23. Wollowitz S. Fundamentals of the psoralen-based Helinx technology for inactivation of infectious pathogens and leukocytes in platelets and plasma. Semin Hematol 2001;38(4 Suppl 11): 4-11.
24. Johnson L et al. In vitro assessment of buffy-coat derived platelet components suspended in SSP+ treated with the INTERCEPT Blood system. Transfus Med 2013;23:121-9.
25. Apelseth TO et al. In vitro evaluation of metabolic changes and residual platelet responsiveness in photochemical treated and gamma-irradiated single-donor platelet concentrates during long-term storage. Transfusion 2007;47:653-65.
26. Kaiser-Guignard J et al. The clinical and biological impact of new pathogen inactivation technologies on platelet concentrates. Blood Rev 2014;28:235-41.
27. Sandgren P, Diedrich B. Pathogen inactivation of double-dose buffy-coat platelet concentrates photochemically treated with amotosalen and UVA light: preservation of in vitro function. Vox Sang 2015;108:340-9.
28. Hechler B et al. Preserved functional and biochemical characteristics of platelet components prepared with amotosalen and ultraviolet A for pathogen inactivation. Transfusion 2013;53: 1187-200.
29. Abonnenc M et al. In vitro evaluation of pathogen-inactivated buffy coat-derived platelet concentrates during storage: psoralen-based photochemical treatment step-by-step. Blood Transfus 2015;13:255-64.
30. van Rhenen D et al. Transfusion of pooled buffy coat platelet components prepared with photochemical pathogen inactivation treatment: the euroSPRITE trial. Blood 2003;101:2426-33.
31. McCullough J et al. Therapeutic efficacy and safety of platelets treated with a photochemical process for pathogen inactivation: the SPRINT Trial. Blood 2004;104:1534-41.
32. Nussbaumer W et al. Patient outcomes and amotosalen/UVA-treated platelet utilization in massively transfused patients. Vox Sang 2017;112:249-56.
33. Estcourt LJ et al. Pathogen-reduced platelets for the prevention of bleeding. Cochrane Database Syst Rev 2017;7:Cd009072.
34. Cid J, Escolar G, Lozano M. Therapeutic efficacy of platelet components treated with amotosalen and ultraviolet A pathogen inactivation method: results of a meta-analysis of randomized controlled trials. Vox Sang 2012;103:322-30.
35. Slichter SJ et al. Platelets photochemically treated with amotosalen HCl and ultraviolet A light correct prolonged bleeding times in patients with thrombocytopenia. Transfusion 2006;46:731-40.
36. Lozano M et al. A multi-centre study of therapeutic efficacy and safety of platelet components treated with amotosalen and ultraviolet A pathogen inactivation stored for 6 or 7 d prior to transfusion. Br J Haematol 2011;153:393-401.
37. Garban F et al. Comparison of the hemostatic efficacy of pathogen-reduced platelets vs untreated platelets in patients with thrombocytopenia and malignant hematologic diseases: a randomized clinical trial. JAMA Oncol 2018;4:468-75.
38. Garraud O, Lozano M. Pathogen inactivation/reduction technologies for platelet transfusion: where do we stand? Transfus Clin Biol 2018;25:165-71.
39. Garraud O. Pathogen reduction or inactivation technologies for platelet components: does decision making have to await further clinical trials? Transfus Apher Sci 2018;57:797-8.

40. Amato M et al. Impact of platelet pathogen inactivation on blood component utilization and patient safety in a large Austrian Regional Medical Centre. Vox Sang 2017;112:47-55.
41. Knutson F et al. A prospective, active haemovigilance study with combined cohort analysis of 19,175 transfusions of platelet components prepared with amotosalen-UVA photochemical treatment. Vox Sang 2015;109:343-52.
42. Kondkar AA et al. VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost 2010;8:369-78.
43. Nagalla S et al. Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood 2011;117:5189-97.
44. Edelstein LC, Bray PF. MicroRNAs in platelet production and activation. Blood 2011;117:5289-96.
45. Edelstein LC et al. MicroRNAs in platelet production and activation. J Thromb Haemost 2013;11(Suppl 1)):340-50.
46. Edelstein LC et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. Nat Med 2013;19:1609-16.
47. Basak I et al. miR-15a-5p regulates expression of multiple proteins in the megakaryocyte GPVI signaling pathway. J Thromb Haemost 2019;17:511-24.
48. Provost P. Platelet microRNAs. In: Michelson AD, editors. Platelets. London: Academic; 2013. p. 91-101.
49. Guo H et al. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 2010;466:835-40.
50. Friedman RC et al. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19:92-105.
51. Miranda KC et al. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 2006;126:1203-17.
52. Gnatenko DV et al. Transcript profiling of human platelets using microarray and serial analysis of gene expression. Blood 2003;101:2285-93.
53. Bugert P et al. Messenger RNA profiling of human platelets by microarray hybridization. Thromb Haemost 2003;90:738-48.
54. Rowley JW et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. Blood 2011;118:e101-11.
55. Ple H et al. The repertoire and features of human platelet microRNAs. PLoS One 2012;7:e50746.
56. Landry P et al. Existence of a microRNA pathway in anucleate platelets. Nat Struct Mol Biol 2009;16:961-6.
57. Kannan M et al. Membrane array-based differential profiling of platelets during storage for 52 miRNAs associated with apoptosis. Transfusion 2009;49:1443-50.
58. Osman A et al. Effects of pathogen reduction systems on platelet microRNAs, mRNAs, activation, and function. Platelets 2015;26:154-63.
59. Yu S et al. Detection of apoptosis-associated microRNA in human apheresis platelets during storage by quantitative real-time polymerase chain reaction analysis. Blood Transfus 2014;12:541-7.
60. Osman A et al. Differential Expression Analysis by RNA-Seq reveals perturbations in the platelet mRNA transcriptome triggered by pathogen reduction systems. PLoS One 2015;10:e0133070.
61. Edelstein LC, Bray PF. Small RNAs as potential platelet therapeutics. Handb Exp Pharmacol 2012;210:435-45.
62. Pontes TB et al. The miRNA profile of platelets stored in a blood bank and its relation to cellular damage from storage. PLoS One 2015;10:e0129399.
63. Dahiya N et al. Platelet MicroRNAs: an overview. Transfus Med Rev 2015;29:215-9.
64. Simon LM et al. Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. Blood 2014;123:e37-45.
65. Prudent M et al. Proteome changes in platelets after pathogen inactivation—an interlaboratory consensus. Transfus Med Rev 2014;28:72-83.
66. Prudent M et al. Proteomic analysis of Intercept-treated platelets. J Proteomics 2012;76:316-28.
67. Sut C et al. The non-hemostatic aspects of transfused platelets. Front Med (Lausanne) 2018;5:42.
68. Ren Q et al. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. Mol Biol Cell 2007;18:24-33.
69. Van Aelst B et al. Riboflavin and amotosalen photochemical treatments of platelet concentrates reduce thrombus formation kinetics in vitro. Vox Sang 2015;108:328-39.
70. Van Aelst B et al. Psoralen and ultraviolet A light treatment directly affects phosphatidylinositol 3-kinase signal transduction by altering plasma membrane packing. J Biol Chem 2016;291:24364-76.
71. Feys HB, Van Aelst B, Compernolle V. Biomolecular consequences of platelet pathogen inactivation methods. Transfus Med Rev 2019;33:29-34.
72. Devine DV. Pathogen inactivation strategies to improve blood safety: let's not throw pathogen-reduced platelets out with their bath water. JAMA Oncol 2018;4:458-59.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Summary of sample preparation for flow cytometry analysis.
Table S2. Relevance of miRNA included in these analyses.