

Haemostatic profile of riboflavin-treated apheresis platelet concentrates

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Background - The haemostatic activity of platelet concentrates (PCs) treated with pathogen reduction technology (PRT) remains a subject of debate. Our aim was to investigate the effect of Mirasol PRT on the haemostatic properties of PCs stored in plasma.

Material and methods - Untreated and Mirasol-treated platelets stored in plasma and derived from ten split double-dose apheresis PCs were evaluated in vitro on days 1, 3 and 5 post collection for functionality, microparticle procoagulation activity (MPA), endogenous thrombin potential (ETP), and haemostatic profile using rotational thromboelastometry (ROTEM).

Results - P-selectin expression was significantly higher in Mirasol-treated platelets compared with untreated counterparts on days 3 and 5 ($p=0.003$ and $p=0.002$, respectively). Clot strength, as shown by EXTEM maximum clot firmness (MCF), was significantly lower in the Mirasol-treated platelets at all time points (days 1, 3, 5) than in untreated platelets ($p=0.009$, $p<0.001$, $p<0.001$, respectively). There was a considerable increase in MPA over time ($p<0.001$) and this was significantly higher in the Mirasol-treated platelets on day 5 ($p=0.015$). A notable acceleration of decrease in ETP values was observed for Mirasol-treated PCs over time ($p<0.001$), with significant differences between PRT-treated and untreated PCs on days 3 and 5 ($p=0.038$ and $p=0.019$, respectively). Clot strength attenuation was significantly associated with pH reduction ($p<0.001$, Spearman's rho: 0.84), increased microparticle procoagulant activity ($p<0.001$, Spearman's rho: -0.75), and with decreased ETP ($p<0.032$, Spearman's rho: 0.41).

Discussion - Increased platelet activation induced by PRT treatment leads to a decrease in in vitro haemostatic capacity as seen by reduced clot strength and thrombin generation capacity over time. The clinical relevance of this needs to be investigated.

Keywords: platelet inactivation, Mirasol-treated apheresis platelets, haemostatic profile, clot strength, procoagulant activity.

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INTRODUCTION

Reducing the risk of pathogen transmission to transfusion recipients is of great concern in transfusion medicine. Pathogen reduction technology (PRT) systems are important

to minimise pathogen transmission. Photochemical treatment prevents replication of pathogens in platelet concentrates (PCs) by cross-linking nucleic acids and thus affects all cells containing DNA or RNA. Two main systems have been developed for PCs; psoralen light treatment and riboflavin (vitamin B2) light treatment. Among riboflavin-based systems that target pathogen nucleic acids, the Mirasol Pathogen Reduction Technology system (Mirasol PRT; Terumo BCT, Lakewood, CO, USA) has been well described⁴⁻⁵. The Mirasol PRT system for platelets and plasma uses riboflavin and UV light to inhibit both pathogen and white blood cell replication. Although PRT-treated platelets had significantly poorer post transfusion recovery and survival than untreated platelets (control), the PRT-treated platelets retained sufficient *in vivo* efficacy for clinical use^{6,7}.

PC functionality decreases during storage. Platelet storage lesions include activation, proteolysis, and changes in morphology, membrane glycoproteins, and surface receptor expression⁸. Loss of PC quality over time in storage is referred to as platelet storage lesion or platelet storage deficit⁹. Pathogen inactivation (PI) may accelerate or induce lesions, potentially accounting for reduced viability. The PRT-treated platelets show signs of increased cellular activation and increased metabolism. These platelets had elevated CD62P expression, lactate production, and glucose consumption rates after PRT treatment¹⁰⁻¹². In particular, Mirasol treatment has been found to exacerbate the effects of platelet storage lesion, resulting in increased glucose consumption and lactate production, and increased markers of platelet activation^{4,13-16}. Plasma membrane-derived microvesicles or microparticles (MPs) are sub-cellular vesicles released upon shear stress, cell activation, injury or apoptosis. MPs are an inherent part of all blood labile products delivered to transfused patients. Platelet-derived extracellular vesicles (P-MPs) are present in platelet apheresis concentrates and may influence PC quality. MPs derived from activated platelets contain membrane surface proteins and exhibit tissue factor activity that account for their high thrombogenic potential¹⁷⁻²⁰. Preparations of platelets stored under blood bank conditions appear to be enriched in MPs with high procoagulant activity. This suggests a potential role in initiating blood coagulation, which might be of clinical importance. The clinical relevance of

P-MPs in supporting coagulation has been documented in leukaemic and thrombocytopenic patients²¹. Despite their low platelet counts, these patients do not bleed, probably because of the high levels of circulating P-MPs. The potential role of P-MPs to support coagulation can be assessed by thrombin generation and microparticle procoagulant activity assays which are used to investigate plasma hyper- or hypo-coagulability through thrombin generation measurement^{22,23}. Moreover, viscoelastic tests, such as thromboelastography (TEG) and rotational thromboelastometry (ROTEM), provide information on the dynamics of clot development, stabilisation and dissolution²⁴.

Today, the haemostatic activity of PRT-treated PCs remains a subject of debate given that the overall survival of PI platelets seems to be somewhat reduced. *In vitro* measurements have identified some alterations in platelet function even though their clinical efficacy is not compromised. Our aim was to investigate the effect of Mirasol PRT on the functional and coagulation properties of PCs stored in plasma as compared to untreated PCs by assessing the haemostatic profile of these blood components.

MATERIALS AND METHODS

***In vitro* studies**

Ten split double-dose apheresis PCs stored in plasma were produced. Collection of PCs was performed with an apheresis collection device (Trima, Accel Terumo BCT, Lakewood, CO, USA). A protocol of 6.5×10^{11} platelets and 40 mL plasma was selected; this was also used for the ROTEM and other tests described below. The whole platelet units were kept undisturbed for 2 hours (h) at ambient temperature to allow dissociation of any platelet aggregates after which they were agitated for 10 minutes (min) before being divided into two equal-sized PC units (aliquots). In each experiment, one of the aliquots was PRT-treated using ultraviolet light after the addition of riboflavin (M) and the other remained untreated (control). A platelet dose (total platelet count) was determined in all platelet units at the time of production. PRT-treated platelets were prepared according to the manufacturers' instructions. Briefly, PC and riboflavin were transferred into an Illumination/Storage Bag. The bag was placed in the Illuminator and exposed to UV light with a linear agitation of 120 cpm and a product temperature of 37°C. After the

target energy of 6.24 J/mL had been delivered, the bag was removed. Control PCs were prepared in the same manner as the treated counterparts, except that no riboflavin was added and no PRT treatment was performed. The treated and control PCs were stored for an additional 5-day period at 20–24°C. On Storage days 1 (to examine the immediate effects of PRT treatment), 3 and 5 (to examine longer-term and storage effects), platelet samples were taken from the bag using an aseptic technique and analysis was completed within 4 h after sampling. Samples taken under sterile conditions were analysed for platelet count, blood gases (pH, pO₂ and pCO₂), metabolism (lactate, glucose, lactate dehydrogenase [LDH]), *in vitro* function (aggregation), activation (P-selectin expression), P-MP procoagulant activity, endogenous thrombin potential (ETP), and viscoelastic properties.

The study was approved by the “Attiko” University Hospital’s institutional review board (2nd/ 28-1-2020, 43). Before plateletpheresis, every blood donor was informed about the study protocol and invited to take part in the study. All blood donors gave written informed consent. Complete blood counts were performed on a Sysmex XE-2100 analyser (Roche, Lincolnshire, IL, USA) and blood gas analysis on a Cobas® b 123 POC blood gas analyser (Roche Diagnostics Ltd, Rotkreuz, Switzerland).

Microparticle procoagulant activity assay

Microparticle-associated procoagulant activity was measured by a colourimetric ELISA kit (Zymuphen, Hyphen BioMed, Neuville-sur-Oise, France), according to the manufacturers’ instructions, as previously described²³.

Thrombin generation assay

INNOVANCE ETP (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) is a haemostasis function test used to assess the ETP of plasma samples. The incubation of plasma with phospholipids and activator and calcium ions leads to initiation and propagation of the coagulation processes, eventually resulting in the generation of thrombin. Thrombin generation and the subsequent inactivation were recorded by monitoring the conversion of a specific slow-reacting chromogenic substrate at a wavelength of 405 nm over time. The assay was performed on a BCS XP haemostasis system (Siemens Healthcare Diagnostics), as previously reported²⁵. The estimated parameters of the thrombin generation curve included area under the curve (AUC), also referred to as ETP and

maximum thrombin generation depicted by peak height (C_{max}). PCs were centrifuged at 2,500 × *g* for 20 min. The supernatant was removed and then centrifuged again. Plasma was snap frozen in small portions and stored at –20°C until the assay was performed.

Thrombin/antithrombin III complex

An enzyme immunoassay was used for the determination of human thrombin/antithrombin III complex (TAT) in plasma. Enzygnost® TAT micro (Enzygnost® TAT micro Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) is a sandwich enzyme immunoassay for the *in vitro* determination of human TAT. Samples were immediately centrifuged at no less than 1,500 × *g* for at least 15 min and removed the supernatant plasma. Plasma was snap frozen in small portions and stored at –20°C until the assay was performed.

Platelet aggregation

The specimen was centrifuged at 2,000 × *g* for 10 min to obtain platelet-rich plasma (PRP). The remaining specimen was re-centrifuged at 2,000 × *g* for 15 min to obtain platelet-poor plasma (PPP). The platelet count was adjusted to between 200×10⁹/L and 300×10⁹/L with PPP. Aggregation was performed using a Biodata-PAP-4 aggregometer (Bio/Data Corporation, Horsham, PA, USA). The 100% line was set using PPP and a 0% baseline established with PRP before addition of the agonist. A panel of two different agonists was used: ADP 2.0×10⁻⁵ M, and epinephrine (EPI) 1.0×10⁻⁴ M (Bio/Data Corporation). The test was conducted as previously reported²⁶. Briefly, 0.45 mL PRP were transferred into a cuvette incubated at 37°C for 3 min. Then, 0.05 mL of the agonist was added into the PRP and the aggregation pattern was allowed to generate for 5 min.

Rotational thromboelastometry

Viscoelastic measurements were taken using rotational ROTEM (Tem Innovations GmbH, Munich, Germany). For direct measurements of platelet concentrates, Mirasol and control PCs were diluted 1:10 in plasma (ultracentrifuged and frozen at –40°C in aliquots that were discarded after a single defrosting) and were run according to the manufacturer’s instructions for whole blood²⁷. Recombinant tissue factor was used in the EXTEM tests to activate the extrinsic coagulation pathway, and cytochalasin D was added in the FIBTEM tests to inhibit

platelet contribution. The following parameters were estimated within 2 h after sample collection: (i) clotting time (CT, seconds [sec]), the time from the beginning of measurement until the formation of a clot 2 mm in amplitude; (ii) clot formation time (CFT, sec), the time from CT (amplitude of 2 mm) until a clot firmness of 20 mm was achieved; amplitude recorded at 10, 20 and 30 min (A10, A20 and A30, mm); and (iii) maximum clot firmness (MCF, mm), the final strength of the clot. Maximum clot elasticity was calculated using the following formula: $MCE = (MCF \times 100) / (100 - MCF)$.

Flow cytometry analysis of platelets

For the identification of platelet activation, P-selectin (CD62P) expression on the platelet surface was measured. Briefly, samples were incubated for 15 min in room temperature with anti-human CD62P-APC along with the platelet gating marker CD41a-PECy5. Flow cytometry analysis was performed by multicolour flow cytometry using a FACSCanto II cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis

The variables were described using median values with interquartile ranges (IQR). The two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to compare variables between Mirasol-treated PCs and control. Variable values on days 1, 3 and 5 were confirmed using the Kruskal-Wallis equality-of-populations rank test. Correlations were assessed based on Spearman rank correlation coefficient (Spearman's rho categories of correlation: $r < 0.20$, very weak correlation; $0.21 < r < 0.40$ weak correlation; $0.41 < r < 0.60$, moderate correlation; $0.61 < r < 0.80$, strong correlation; $r > 0.81$ very strong correlation).

$p < 0.05$ was considered statistically significant for all tests. Stata 16 was used for statistical analyses (Stata Corp., College Station, TX, USA).

RESULTS

The comparison of metabolic activities between Mirasol-treated and control PCs during the storage period is presented in **Table I**. In both treated and control platelets, the amount of glucose consumed and lactate produced significantly increased over time ($p < 0.001$), but both glucose consumption and lactate production rates were significantly higher in treated platelets compared

with control platelets on days 1, 3 and 5 ($p = 0.009$, $p < 0.001$, $p < 0.001$ and $p = 0.160$, $p = 0.013$, $p = 0.002$, respectively). The mean pH of Mirasol-treated PCs was significantly lower at all time points relative to control PCs ($p < 0.001$), but remained within acceptable limits. Statistical analysis indicated no significant difference in PO_2 levels between the control and treated PCs during the storage period, while PCO_2 significantly decreased with storage time in both groups, and were significantly lower in treated platelets compared with control platelets on day 5 ($p = 0.005$).

All haemostatic parameters in both groups over time are presented in **Table II**. The expression of P-selectin was significantly higher in Mirasol-treated platelets on days 3 and 5 than in control counterparts ($p = 0.003$ and $p = 0.002$, respectively). Clot strength (MCF) was significantly lower in Mirasol-treated platelets at all time points (days 1, 3, 5) than in untreated platelets ($p = 0.009$, $p < 0.001$, $p < 0.001$, respectively). There was no significant difference in either EXTEM CT or CFT between the two groups at any of the time points. On the other hand, microparticle procoagulant activity considerably increased over time and was significantly higher in Mirasol-treated platelets on day 5 ($p = 0.015$). Although TAT values in Mirasol-treated platelets exhibited significant elevation during storage ($p = 0.033$) and were higher in this group related to control platelets at all time points, the latter difference did not reach statistical significance. ETP values for Mirasol-treated PCs decreased over time, with significant differences being observed between treated and untreated platelets on days 3 and 5 ($p = 0.038$ and 0.019 , respectively). Aggregation with both agonists significantly decreased with storage time in both groups and was significantly lower in control PCs on days 3 and 5 ($p < 0.001$ in ADP-induced aggregation).

The main correlations between tests for Mirasol-treated and control platelets are presented in **Tables III** and **IV**. In both groups (Mirasol-treated and untreated), a significant association was detected between clot strength reduction with glucose consumption ($p < 0.001$), lactate production ($p < 0.001$), decrease in HCO_3^- ($p < 0.001$), microparticle procoagulant activity ($p < 0.001$), and P-selectin expression ($p < 0.001$ and $p = 0.029$, respectively). Of note, a significant correlation between clot strength attenuation with pH reduction ($p < 0.001$) and ETP decrease ($p < 0.001$) was shown only in Mirasol-treated PCs. In Mirasol-treated group,

other notable, significant associations observed were the increased platelet aggregation with decreased metabolic rate (pH: $p < 0.001$; lactate: $p < 0.001$), reduced microparticle procoagulant activity ($p < 0.001$), and increased clot strength ($p < 0.001$), the enhanced microparticle

procoagulant activity with increased metabolism (pH: $p < 0.001$; lactate: $p < 0.001$), and the high ETP with decreased metabolic rate (pH: $p = 0.009$; lactate: $p = 0.003$). These correlations were stronger in the Mirasol-treated group than in control.

Table I - Metabolic parameters on days 1, 3 and 5 in Mirasol-treated and control platelets

Parameter/day	Median (IQR)		p-value
	Mirasol	Control	
Volume (gr), 1	284 (279-290.5)	269 (264-272)	<0.001
Volume (gr), 3	263.5 (254-266.5)	248 (247-252)	0.034
Volume (gr), 5	239.3 (229.8-251.5)	232.3 (224.8-238.5)	0.156
p-value	<0.001	<0.001	
Concentration (10 ³ /uL), 1	1,072 (1,004-1,130)	1,191.5 (1,155-1,279)	0.005
Concentration (10 ³ /uL), 3	1,087 (1,045-1,143)	1,258 (1,209-1,327)	0.009
Concentration (10 ³ /uL), 5	1,150 (1,088-1,218)	1,240 (1,232-1,286)	0.024
p-value	0.218	0.573	
pH, 1	7.16 (7.14-7.20)	7.16 (7.13-7.22)	0.820
pH, 3	7 (6.98-7.03)	7.29 (7.17-7.32)	<0.001
pH, 5	6.55 (6.5-6.76)	7.19 (7.03-7.24)	<0.001
p-value	<0.001	0.189	
pO ₂ (mmHg), 1	37.85 (21.3-63.5)	32.25 (20.5-50.4)	0.791
pO ₂ (mmHg), 3	46.3 (39.5-70.3)	51.6 (37.9-64.1)	0.691
pO ₂ (mmHg), 5	74.5 (56.2-106.9)	85.2 (45.2-86.9)	0.354
p-value	0.056	0.117	
pCO ₂ (mmHg), 1	43.1 (37.4-46.4)	52.4 (42.5-58.7)	0.034
pCO ₂ (mmHg), 3	25.2 (22.9-26.4)	24.2 (22.5-26.7)	0.825
pCO ₂ (mmHg), 5	17.8 (16.1-18.8)	21.4 (20.8-22)	0.005
p-value	<0.001	<0.001	
Glucose (mg/dL), 1	283.5 (257-298)	310.5 (298-335)	0.009
Glucose (mg/dL), 3	199 (191-21)	275 (260-285)	<0.001
Glucose (mg/dL), 5	118 (98-136)	243 (222-246)	<0.001
p-value	<0.001	<0.001	
Lactate (mmol/L), 1	2.4 (1.8-2.5)	2.7 (1.9-3)	0.160
Lactate (mmol/L), 3	10.8 (10.6-11)	7.1 (6.8-9.4)	0.013
Lactate (mmol/L), 5	17.8 (17.3-18.8)	11.2 (10.7-12.5)	0.002
p-value	<0.001	<0.001	
HCO ₃ (mmol/L), 1	14.8 (13.9-15.5)	17.7 (16.6-19.1)	0.007
HCO ₃ (mmol/L), 3	5.8 (5.3-6.6)	10.4 (9.7-11.5)	<0.001
HCO ₃ (mmol/L), 5	2.6 (2.5-3)	7.8 (6-8.7)	0.003
p-value	<0.001	<0.001	
LDH (IU/L), 1	80 (66.5-90.5)	123.5 (95.5-143)	0.031
LDH (IU/L), 3	95.5 (75.5-162.5)	143 (110-194.5)	0.345
LDH (IU/L), 5	137 (103-153)	121 (110-133)	0.654
p-value	0.026	0.694	

p-values in **bold** show statistical significance ($p < 0.05$). IQR: interquartile range; LDH: lactate dehydrogenase.

Table II - Haemostatic parameters on days 1, 3 and 5 in Mirasol-treated and control platelets

Parameter/Day	Median (IQR)		p-value
	Mirasol	Control	
LTA ADP(%), 1	30.5 (24-47)	27.5 (10-57)	0.623
LTA ADP(%), 3	14 (9-20)	3 (2-3)	<0.001
LTA ADP(%), 5	11 (11-14)	2 (1-3)	<0.001
p-value	0.002	0.002	
LTA EPI(%), 1	23 (21-40)	16 (9-26)	0.150
LTA EPI(%), 3	13 (12-28)	9 (6-11)	0.019
LTA EPI(%), 5	14 (12-16)	7 (7-10)	0.002
p-value	0.008	0.016	
P-selectin(%), 1	3.5 (2.4-20.1)	0.6 (0.3-3.5)	0.055
P-selectin(%), 3	17.9 (12.8-34.7)	6.6 (2.2-6.8)	0.003
P-selectin(%), 5	22.8 (19.7-35)	6.4 (2.7-12.1)	0.002
p-value	0.026	0.016	
ETP AUC(%), 1	86.5 (79-91)	86 (80-96)	0.596
ETP AUC(%), 3	78 (66-80)	87 (82-91)	0.038
ETP AUC(%), 5	65 (46-81)	86 (76-93)	0.019
p-value	0.037	0.863	
ETP Cmax(%), 1	79.5 (73-85)	93.5 (87-104)	0.019
ETP Cmax(%), 3	73 (68-77)	98 (83-106)	<0.001
ETP Cmax(%), 5	58 (44-66)	89 (85-99)	<0.001
p-value	<0.001	0.741	
TAT(µg/L), 1	3.06 (2.80-3.31)	2.89 (2.80-3.20)	0.623
TAT(µg/L), 3	3.65 (3.44-4.53)	3.22 (2.63-3.64)	0.059
TAT(µg/L), 5	3.8 (3.6-4.4)	3.2 (2.9-4.2)	0.102
p-value	0.033	0.529	
MPA(nM), 1	28.67 (23.83-33.23)	34.71 (28.49-37.4)	0.1152
MPA(nM), 3	70.76 (41.41-83.3)	43.51 (35.52-58.80)	0.208
MPA(nM), 5	85.42 (76.82-87.16)	61.97 (53.59-78.46)	0.015
p-value	<0.001	0.004	
CT EXTEM(sec), 1	53 (48-65)	59 (51-66)	0.535
CT EXTEM(sec), 3	50 (47-65)	55 (49-60)	0.757
CT EXTEM(sec), 5	55 (43-63)	54 (45-61)	0.930
p-value	0.759	0.698	
A10 EXTEM(mm), 1	59 (59-62)	66 (64-66)	0.004
A10 EXTEM(mm), 3	42 (38-46)	59 (56-61)	<0.001
A10 EXTEM(mm), 5	27 (23-38)	53 (52-60)	<0.001
p-value	<0.001	0.003	
A10 FIBTEM(mm), 1	22 (20-23)	23 (22-26)	0.399
A10 FIBTEM(mm), 3	22 (21-24)	22 (20-24)	0.929
A10 FIBTEM(mm), 5	21 (18-21)	20 (19-21)	0.858
p-value	0.395	0.150	

Parameter/Day	Median (IQR)		p-value
	Mirasol	Control	
A20 EXTEM(mm), 1	63 (63-66)	68 (68-69)	0.010
A20 EXTEM(mm), 3	39 (33-41)	61 (56-63)	<0.001
A20 EXTEM(mm), 5	27 (24-34)	51 (48-58)	<0.001
p-value	<0.001	<0.001	
A30 EXTEM(mm), 1	62 (62-65)	67 (66-67)	0.010
A30 EXTEM(mm), 3	36 (32-37)	58 (52-60)	<0.001
A30 EXTEM(mm), 5	27 (25-31)	47 (43-54)	<0.001
p-value	<0.001	<0.001	
CFT EXTEM(sec), 1	53 (49-57)	47 (45-55)	0.199
CFT EXTEM(sec), 3	55 (50-58)	50 (39-62)	0.691
CFT EXTEM(sec), 5	57 (53-77)	46 (42-58)	0.144
p-value	0.405	0.973	
MCF EXTEM(mm), 1	63 (63-66)	68 (68-69)	0.009
MCF EXTEM(mm), 3	44 (41-46)	61 (57-63)	<0.001
MCF EXTEM(mm), 5	34 (30-43)	53 (52-60)	<0.001
p-value	<0.001	<0.001	
MCF FIBTEM(mm), 1	23 (21-25)	25 (22-28)	0.535
MCF FIBTEM (mm), 3	24 (22-25)	24 (22-26)	0.965
MCF FIBTEM (mm), 5	23 (21-23)	20 (20-22)	0.397
p-value	0.660	0.201	
MCE EXTEM, 1	169 (167-191)	212 (208-218)	0.009
MCE EXTEM, 3	79 (70-86)	153 (133-170)	<0.001
MCE EXTEM, 5	52 (43-76)	115 (110-152)	<0.001
p-value	<0.001	<0.001	

p-values in **bold** show statistical significance (p<0.05).
 IQR: interquartile range; LTA: light transmission aggregometry;
 ETP: endogenous thrombin potential; AUC: area under the curve; Cmax:
 peak thrombin generation; TAT: thrombin-antithrombin complex;
 MPA: microparticle procoagulant activity; CT: clotting time; A10: amplitude
 10 minutes (min) after CT; A20: amplitude 20 min after CT; A30: amplitude
 30 min after CT; CFT: clot formation time; MCF: maximum clot firmness;
 MCE: maximum clot elasticity.

Table III - Spearman correlation coefficients for pairwise comparisons in Mirasol-treated platelets

	Volume	Concentration	pH	pO ₂	pCO ₂	Glucose	Lactate	LDH	HCO ₃	LTA ADP	LTA EPI	P-selectin	ETP AUC	ETP Cmax	TAT	MPA	MCF EXTEM	MCE EXTEM
Volume	1																	
Concentration	0.03	1																
pH	0.81	-0.33	1															
pO ₂	-0.42	0.14	-0.25	1														
pCO ₂	0.83	-0.33	0.81	-0.54	1													
Glucose	0.81	-0.38	0.91	-0.39	0.83	1												
Lactate	-0.82	0.32	-0.91	0.47	-0.87	-0.96	1											
LDH	-0.39	0.38	-0.52	0.39	-0.51	-0.55	0.51	1										
HCO ₃	0.85	-0.05	0.87	-0.41	0.94	0.85	-0.89	-0.48	1									
LTA ADP	0.48	-0.27	0.69	-0.32	0.56	0.62	-0.68	-0.53	0.57	1								
LTA EPI	0.39	-0.34	0.59	-0.09	0.50	0.51	-0.56	-0.60	0.60	0.73	1							
P-selectin	-0.61	0.23	-0.63	0.44	-0.65	-0.63	0.66	0.15	-0.56	-0.42	-0.28	1						
ETP AUC	0.35	-0.59	0.49	-0.20	0.59	0.51	-0.53	-0.45	0.34	0.33	0.33	-0.32	1					
ETP Cmax	0.50	-0.43	0.69	-0.33	0.71	0.74	-0.72	-0.42	0.58	0.34	0.39	-0.57	0.70	1				
TAT	-0.57	-0.15	-0.57	0.01	-0.37	-0.45	0.50	0.33	-0.56	-0.42	-0.37	0.56	-0.25	-0.26	1			
MPA	-0.64	0.30	-0.81	0.43	-0.68	-0.82	0.79	0.72	-0.73	-0.80	-0.61	0.56	-0.52	-0.63	0.51	1		
MCF EXTEM	0.69	-0.38	0.84	-0.43	0.81	0.81	-0.84	-0.49	0.80	0.67	0.59	-0.67	0.41	0.65	-0.35	-0.75	1	
MCE EXTEM	0.69	-0.37	0.84	-0.43	0.80	0.81	-0.84	-0.49	0.79	0.68	0.59	-0.68	0.40	0.64	-0.35	-0.76	1.00	1

When a p-value for a certain correlation is less than 0.05, the corresponding coefficient is in bold.

LDH: lactate dehydrogenase; LTA: light transmission aggregometry; EPI: epinephrine; ETP: endogenous thrombin potential; AUC: area under the curve; Cmax: peak thrombin generation; TAT: thrombin-antithrombin complex; MPA: microparticle procoagulant activity; MCF: maximum clot firmness; MCE: maximum clot elasticity.

Table IV - Spearman correlation coefficients for pairwise comparisons in control platelets

	Volume	Concentration	pH	pO ₂	pCO ₂	Glucose	Lactate	LDH	HCO ₃	LTA ADP	LTA EPI	P-selectin	ETP AUC	ETP Cmax	TAT	MPA	MCF EXTEM	MCE EXTEM	
Volume	1																		
Concentration	-0.10	1																	
pH	0.01	0.01	1																
pO ₂	-0.36	0.15	0.69	1															
pCO ₂	0.63	-0.13	-0.50	-0.65	1														
Glucose	0.82	-0.20	0.18	-0.24	0.53	1													
Lactate	-0.88	0.19	-0.22	0.24	-0.55	-0.91	1												
LDH	0.22	0.28	0.24	0.17	-0.02	0.18	-0.04	1											
HCO ₃	0.80	-0.26	0.10	-0.25	0.74	0.80	-0.89	-0.08	1										
LTA ADP	0.53	0.02	-0.29	-0.39	0.55	0.48	-0.53	-0.09	0.51	1									
LTA EPI	0.46	-0.14	-0.12	-0.32	0.50	0.55	-0.55	-0.03	0.53	0.54	1								
P-selectin	-0.62	0.02	0.02	0.20	-0.59	-0.61	0.59	-0.24	-0.68	-0.51	-0.46	1							
ETP AUC	0.03	-0.44	0.42	0.32	-0.15	0.20	-0.20	0.01	0.22	-0.09	-0.32	-0.12	1						
ETP Cmax	0.00	-0.46	0.41	0.25	-0.15	0.29	-0.20	0.16	0.17	-0.15	-0.16	-0.10	0.81	1					
TAT	-0.21	0.10	-0.25	-0.06	-0.03	-0.34	0.26	-0.06	-0.24	-0.18	-0.20	0.25	-0.26	-0.14	1				
MPA	-0.72	0.22	-0.16	0.28	-0.36	-0.57	0.73	0.13	-0.65	-0.47	-0.62	0.36	-0.05	0.02	0.33	1			
MCF EXTEM	0.79	-0.29	0.16	-0.24	0.50	0.84	-0.90	0.02	0.82	0.47	0.62	-0.49	0.08	0.20	-0.10	-0.65	1		
MCE EXTEM	0.79	-0.27	0.16	-0.23	0.49	0.83	-0.89	0.02	0.81	0.47	0.64	-0.49	0.06	0.18	-0.11	-0.65	1.00	1	

When a p-value for a certain correlation is less than 0.05, the corresponding coefficient is in **bold**.
 LDH: lactate dehydrogenase; LTA: light transmission aggregometry; EPI: epinephrine; ETP: endogenous thrombin potential; AUC: area under the curve; Cmax: peak thrombin generation;
 TAT: thrombin-antithrombin complex; MPA: microparticle procoagulant activity; MCF: maximum clot firmness; MCE: maximum clot elasticity.

DISCUSSION

In the current study, PRT treatment was found to have an important influence on apheresis platelet functionality considering both metabolic activity and haemostatic capacity as assessed by *in vitro* assays, during a 5-day storage period. A progressive decrease in clot strength and thrombin generation capacity was noted during storage of PRT-treated PCs stored in plasma; this was significantly greater than that detected in the respective control PCs. Notably, clot strength attenuation was significantly associated with microparticle procoagulant activity and with a decrease in thrombin generation capacity. Moreover, platelet activation was found to be significantly higher in PRT-treated platelets than in control counterparts and was also associated with a decline in clot strength. It seems that PRT-treated PCs stored in plasma undergo considerable platelet activation leading to time-dependent enhanced microparticle and plasma procoagulant activity; this results in reduced clot development capacity and a decrease in thrombin generation potential over storage time.

As far as the blood gas analysis is concerned, a time-dependent decrease in pH levels was observed, and this was greater in PI platelets. This decrease was apparent on day 3 reaching the lowest value on day 5, when all Mirasol-treated platelets demonstrated pH values <7.0. This was attributed to the elevated glycolysis during storage, which led to an increased production of lactate and to a concurrent fall in pH. A similar observation was made by Janetzko *et al.*²⁸ who, unlike previous studies^{7,29,30}, reported significantly lower pH values in PI PCs, even though in all studies platelets were stored under the same conditions. Nevertheless, in our study, pH values were always above the limit of 6.4 set by the Council of Europe in their guidelines for product release³¹. It is also important to note that comparisons of pH levels across different studies should be made with caution because these measurements are not always performed in the same temperature conditions³².

Furthermore, we observed a time-dependent decrease in PCO₂ levels during storage in both groups, while no significant changes were seen in PO₂ levels. Other authors reported similar observations, even though different storage media and conditions were used^{15,30,33}. However, the findings were not consistent in all cases.

Ostrowski *et al.* noted similar PCO₂ levels between PRT treated and untreated platelets, which did not change significantly over the storage period¹³. On the contrary, they found that inactivated platelets showed reduced PO₂ levels on day 2, and this was a persistent finding throughout the storage period. Once again, results of blood gas analysis should be interpreted with caution because of the different temperature conditions in which measurements are taken³². The type of analyser used is also an important factor to be taken into consideration, as in some analysers riboflavin interferes with PO₂ measurement³⁴. Moreover, we observed a gradual decline in HCO₃⁻ values in all PCs over time, but mainly in Mirasol-treated platelets which were affected immediately after treatment, reflecting the impaired buffer capacity previously reported^{15,28}.

Regarding metabolic markers, in the current study, Mirasol-treated platelets showed increased glucose consumption and lactate production rates compared to their untreated counterparts over time, indicating higher metabolic activity after PRT treatment. It has been well established that PI platelets show signs of increased metabolism resulting in lactate production and glucose consumption^{4,10-12,33}. As reported, lactate production and pH levels are correlated with survival time, along with platelet recovery⁶ and, consequently, these parameters are extremely important. LDH is a marker of platelet membrane integrity and LDH levels are also correlated with platelet survival³⁵. No differences in LDH values were observed between control and Mirasol-treated platelets, in agreement with findings from previous studies^{4,10,28}. Platelet aggregation is known to be reduced even after the first day of storage when low concentrations of ADP or EPI are used^{36,37}. This was observed in both groups in the current study but, quite surprisingly, even though Mirasol-treated platelets showed a reduction in aggregation capacity over time, they displayed higher responses with both agonists when compared to control, especially during the late storage period. A similar finding was reported in a previous study investigating ADP-induced aggregation in Intercept-treated platelets at late storage time, but the underlying pathophysiologic mechanism could not be identified and was attributed to the very similar aggregation capacity to that of untreated platelets³⁸. Ostrowski *et al.* observed an immediate decrease in ADP-induced aggregation in PI platelets

compared to control platelets. After that immediate effect, no significant changes in either of the test groups were observed³⁹. Nevertheless, most studies describe no differences in ADP-induced aggregation between control and PI platelets, while this does not apply to other agonists^{15,40}. It is important to bear in mind that the use of platelet additive solutions imposes a marked reduction in fibrinogen and von Willebrand factor (adhesive proteins) levels, which are necessary for aggregation. Thus, when attempting to compare the findings in different studies, one must consider that different storage media and aggregation protocols using variable concentrations of several agonists might contribute to this inconsistency.

In this study, EXTEM measurements clearly showed that PRT treatment accelerates the development of platelet storage lesions resulting in a significant decrease in clot strength over time in the Mirasol group as compared to control. Therefore, PRT treatment influenced clot stability, as reflected by A10, A20 and MCF, and this was greater as time passed. There have been only two previous studies on the evaluation of haemostatic function with thromboelastography in PI platelets. To the best of our knowledge, the current study is the first to assess the haemostatic profile with the use of ROTEM (EXTEM and FIBTEM assays). Ostrowski *et al.* reported only a mild reduction in maximum amplitude (MA) no earlier than day 8 of storage³⁹. Significantly lower MA values were also observed in PI platelets by Ballester-Servera *et al.*⁴¹ but, once again, this was observed only at the late storage period, more specifically on days 7 and 14. In the same study, untreated platelets had only undergone a minor decline in MA even on day 14. On the contrary, our clot strength measurements in Mirasol-treated platelets were significantly affected at a much earlier storage time point. It has been reported that the development of platelet storage lesions over time can not be reflected by TEG in untreated platelets, indicating that it lacks sensitivity⁴². Furthermore, the two aforementioned studies^{39,41} referred to platelets in additive solution (PAS); but this is unlikely to be the reason for the observed inconsistency with our findings as previous studies revealed that there was no difference in TEG-derived parameters between reconstituted whole blood PAS-C-platelets and plasma platelets⁴³. Finally, a comparison between TEG and ROTEM in untreated platelets showed no differences

between the two methods when kaolin was used in TEG⁴². Thus, our findings are probably to be attributed exclusively to PRT-treatment and may be indicative of an important impairment of haemostatic capacity in PI platelets. The fact that TEG induces activation of the intrinsic coagulation pathway with kaolin, while EXTEM uses tissue factor to initiate the extrinsic clotting cascade, might account for this inconsistency. The extrinsic pathway is the main contributor to physiological activation of coagulation *in vivo*, while MPs lead to increased procoagulant activity due to the presence of anionic phospholipids and the procoagulant protein tissue factor⁴⁴. Thus, it can be argued that the EXTEM assay is more suitable to assess the haemostatic profile in platelet concentrates. Moreover, the fact that MCE values, a parameter used to provide a better representation of clot strength, also differ significantly between treated and untreated PCs, while FIBTEM measurements did not show any difference between the two groups, supports the hypothesis that PRT treatment leads to a deterioration in platelet function during storage. ETP measurements in the current study revealed significantly compromised thrombin generation capacity in PI platelets as storage time passed. This haemostatic deficit could indicate an "exhaustion" of the coagulation process caused by the more intense platelet activation and microparticle procoagulant activity due to PRT treatment. A reduction in thrombin generation was greater in the Mirasol group than in control and, furthermore, the longer the storage time, the greater the effect on PI-treated platelets. Consequently, ETP values were significantly affected in the Mirasol group on day 3, with the major difference between the two groups being observed on day 5. It is noteworthy that the significant correlation between clot strength attenuation and decrease in ETP was only detected in Mirasol-treated PCs.

TAT complex is also a marker of thrombin generation. Mirasol-treated platelets showed elevated TAT levels compared to control platelets, but this difference did not reach statistical significance. However, the increase in TAT levels in the Mirasol group over time was statistically significant. Regarding TAT formation, it seems that storage time mainly influences PI-treated platelets, revealing the activation of the coagulation cascade in this group and consequently leading to functional "exhaustion" of haemostatic capacity

(decreased clot strength and thrombin generation potential).

During platelet preparation and storage, MPs are released⁴⁵, while platelet activation leads to α -granule degranulation, which is reflected by the elevated CD62P expression^{4,10-12}. The higher P-selectin expression in Mirasol-treated platelets and the significant association of clot strength reduction with enhanced microparticle procoagulant activity and P-selectin expression further support the hypothesis of the increased platelet activation associated with increased coagulation cascade activity resulting in a decrease in haemostatic capacity. The significant increase in CD62P expression over time, which was more evident in Mirasol-treated platelets, is in line with most of the similar studies performed previously^{4,10,12,13,15,27,30}.

Finally, in the current study, measurements of microparticle procoagulant activity showed elevated levels in Mirasol group, which were observed by day 3 and which reached statistical significance on day 5. Time had a cumulative effect on both groups leading to increased procoagulant activity levels in parallel with reduced clot strength at the late storage period, especially in PRT-treated PCs. This finding supports the role of MPs in activating the coagulation cascade during platelet PRT treatment and storage.

This study has some limitations. These include the relatively small number of samples and the fact that the *in vivo* effects of the PRT-treated *versus* untreated PCs on the haemostatic profile of transfused patients were not investigated. However, to the best of our knowledge, this is the first study to assess both platelet function and the coagulation profile of PI-treated PCs stored in plasma. A further limitation of this study is that only plasma PCs were examined, without consideration of other PC preparation processes.

CONCLUSIONS

PRT is an established practice used to extend the storage period to up to 7 days. It is of great importance that PI-treated platelets preserve their procoagulant activity in order to be efficient for the recipient⁴⁶. Thus, whether platelets maintain *in vivo* their ability to adhere to damaged endothelium and can effectively activate and aggregate must be confirmed. Our findings revealed increased

platelet activation along with accelerated metabolism induced by PRT treatment, leading to a decrease in clot strength and reduced thrombin generation capacity with longer storage time. It can be assumed that Mirasol treatment and storage affect several aspects of the haemostatic capacity of PCs, indicating that extending storage time to 7 days might influence their *in vivo* efficacy. Hence, these data could raise the question as to whether PRT-treated platelets should be transfused as soon as possible after PRT treatment in order to enhance both the safety and efficacy of the product. On the other hand, a possible enhanced procoagulant effect of PRT treatment on PCs stored in plasma, as suggested by elevated TAT complex and microparticle procoagulant activity levels over time, could play a role in counterbalancing the diminished *in vitro* haemostatic capacity of PRT-treated platelets. The mechanism of how the PRT-treated platelets and their high thrombogenic potential act *in vivo* on the haemostatic function of the transfused patients remains unknown. More evidence is needed to identify the clinical effect of these findings regarding the increment in platelet counts or the risk of bleeding after transfusion. Thus, further investigation on their clinical relevance is warranted.

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AUTHORSHIP CONTRIBUTIONS

EP, AGK and AET conceptualised the project. EP, AET, AGK, SK and AG designed the methodology. AGK, GKN, KP, EL, HTG, AGT, EM, SM and ER were involved in data collection, analysis, and interpretation. EP, AET and GKN wrote the manuscript. All the co-Authors critically revised and approved the final version of the manuscript.

The Authors declare no conflicts of interest.

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