BURNS 50 (2024) 585–596

Available online at www.sciencedirect.com

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Non-severe burn injury causes sustained platelet hyperreactivity

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ARTICLE INFO

Article history:
Accepted 6 October 2023

Keywords:
Non-severe burn injury
Platelets
Cardiovascular disease
Burn survivors
Thrombosis
Flow cytometry

ABSTRACT

Individuals who present to a hospital for treatment of a burn of any magnitude are more frequently hospitalised for ischemic heart disease, even decades after injury. Blood platelets are key mediators of cardiovascular disease. To investigate platelet involvement in post-burn cardiovascular risk, platelet reactivity was assessed in patients at 2- and 6-weeks after non-severe (TBSA < 20%) burn injury, and in a murine model 30 days after 8% TBSA full-thickness burn injury. Platelets were stimulated with canonical agonists and function reported by GPIIb/IIIa PAC1-binding site, CD62P expression, and formation of monocyte-platelet aggregates. In vivo thrombosis in a modified Folts model of vascular injury was assessed. Burn survivors had elevated frequencies of circulating monocyte-platelet aggregates, and platelets were hyperreactive, primarily to collagen stimulation. Burn plasma did not cause hyper-reactivity when incubated with control platelets. Platelets from burn injured mice also demonstrated increased response to collagen peptides but did not show any change in thrombosis following vascular injury. This study demonstrates the persistence of a small but significant platelet hyperreactivity following burn injury. Although our data does not suggest this heightened platelet sensitivity modulates thrombosis following vascular injury, the contribution of sub-clinical platelet hyperreactivity to accelerating atherogenesis merits further investigation.

1. Background

Epidemiological data has provided evidence that burn survivors present with ischemic heart disease (IHD) at an incident rate ratio (IRR) of 1.73 vs controls [1]. This rate of IHD is greater in burn survivors than those with other non-burn trauma (IRR 1.39). The risk of CVD in burn survivors is increased for all levels of burn severity [1,2]. As the majority (84%) of all hospitalised burn injuries are non-severe burn injuries (NSBI) with a total body surface area (TBSA) involvement of < 10% [3], it is important to explore the mechanisms driving this increased risk in NSBI. The contribution of burn injury depth on CVD is more difficult to quantify, as injuries typically have variable depths across the area of the burn.

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https://doi.org/10.1016/j.burns.2023.10.011

The healing of burn injuries – like all wounds – begins with haemostasis. While bleeding is rarely present immediately following burn injury, vasoconstriction of blood vessels and maintenance of damaged endothelium is crucial to controlling fluid leakage at the wound site. The exposure of subendothelial collagen initiates a cascade of haemostatic pathways in nearby blood platelets, which in turn recruit additional platelets to the site of injury [4]. Activated platelets adhere and aggregate at the site of injury, stemming blood loss. Platelets also release chemokines and growth factors in situ, and adhere and signal to leukocytes, thus beginning the process of inflammatory wound repair [5].

The nature of burn injuries prolongs the inflammatory stage of repair: as per the Jackson model, necrotic tissue in the zone of coagulation is prone to infection, while oxygen deprived tissue in the zone of stasis risks progression to necrosis, or may become significantly inflamed in the event of a reperfusion injury [6]. While surgical debridement has been proven to significantly reduce time to heal, this intervention necessitates additional insult to the injury to ensure exposure of viable tissue.

These factors culminate in a prolonged inflammatory environment that increases platelet activation and consumption of platelets as they aggregate or leave the circulation. Platelet count is reduced to a nadir at day 3–4 post injury, followed by a reactive thrombocytosis peaking at day 21 [7–9]. Several studies have examined the impact of burn injuries on platelet function during the acute period – typically during a single timepoint from admission up to seven days [10–13]. These studies have demonstrated increased titres of the haematopoietic cytokine thrombopoietin (TPO), which is capable of priming platelets to subsequent stimuli [11], increased titres of cytokines and metabolites derived from activated platelets (β-TG, sCD40L, thromboxane metabolite TXB_2) [13–15], increased agonist-dependent expression of platelet activation markers (CD62P, CD63, heterotypic platelet aggregates) [10,11], and a paradoxical depression of platelet aggregation to *ex vivo* stimulation, dependent on burn severity and platelet agonist used [11,12,16].

While acute platelet activation during early wound healing is has been detailed, the mechanism, natural history and persistence of platelet activation and function after management of the acute injury has not yet been described.

Platelets have a well-established relationship with the late stage, thrombotic complications of CVD [17]. Ruptured atherosclerotic plaques are sites of superimposed platelet-rich thrombosis, resulting in the occlusion of blood vessels and ischemia. Thus, it has been suggested that platelet activation in the acute period following burn injury may contribute to the likelihood or the severity of cardiovascular events following the burn [2]. However, the risk of CVD has been found to remain elevated long after the acute period of burn injury has resolved. Epidemiological studies have demonstrated this risk persist for at least 30 years following injury [1]. The potential for ongoing, subclinical platelet activation in promoting plaque formation and instability should also be considered [20]. Endothelial cells activated in response to inflammatory stress from the burn injury upregulate receptors that bind and activate platelets rolling across their surface [18]. Activated platelets further recruit additional platelets and adjacent monocytes, forming monocyte platelet aggregates (MPAs) whereby platelets facilitate rolling across the surface of activated endothelium [19]. Monocytes may then taxis into the intima where they terminally differentiate into foam cells and establish atherosclerotic plaques [20,21].

An increase in circulating MPAs has been correlated with coronary artery disease, both with and without recent myocardial infarction [22]. Upon admission, patients with severe burn injuries have demonstrated increased frequencies of MPAs [11]. The duration of elevated circulating and inducible platelet activation, including the presence of circulating and inducible MPAs, has not been previously established beyond the acute period of the injury.

This study aims to investigate the natural history and significance of burn-mediated platelet activation in the post-acute period. Using platelet agonists which target specific, canonical pathways of platelet function and in vitro plasma exchange between burned and non-burned controls, this study aimed to explore the mechanism of increased platelet activation following burn injury. Using a mouse model of platelet-mediated arterial thrombosis, the impact of burn-induced platelet activation on clot formation and arterial perfusion was investigated. We hypothesise that in the post-burn period platelets circulate in a hyper-active state for several weeks. A combination of plasma factors and release of platelets hyper-sensitive to stimulation may contribute to this. The persistence of platelet activation may contribute to the magnitude and severity of arterial thrombosis.

2. Methods

2.1. *Collection of whole blood from humans*

Patients presenting with a NSBI at the Fiona Stanley Hospital burns unit (Murdoch, Western Australia) were recruited. Patients were over 18 years of age, had no prior history of cardiovascular disease or platelet disorders, and were not taking medicines which affect platelet function. Patients provided a blood sample at 2- and/or 6-weeks after their injury. 14 participants in the 2-weeks post-injury group and 15 participants in the 6-weeks post-injury group were taking 200 mg of celecoxib twice daily for a parallel clinical study. No other non-steroidal anti-inflammatory drugs (NSAIDs) were used by this group. An additional 9 participants at 2-weeks and 8 at 6-weeks were clinically prescribed celecoxib for less than 5 days following injury. Celecoxib, a COX-2 inhibitor, has been shown not to affect platelet function [23], and we found no significant difference in indices of platelet activation between these burn patients and others (Supplementary Data). All other participants provided verbal confirmation at the time of collection that they had not taken any NSAIDs in the previous 7 days. Flame burns and scalds composed the majority of injury aetiology, with > 80% of patients receiving surgical intervention, with a mean length of stay of approximately 5 days from admission (Table 1). Of 72 total patient recruitments, 20 individuals provided blood samples at both 2- and 6-weeks. Controls were recruited from age/sex-matched healthy, non-hospitalised controls (matches could

Table 1 – Demographics of patients in two- and six-week post-injury groups.

	Controls	Week 2	Week 6
Males, n (%)	27 (65.9)	33 (78.6)	39 (78)
Females, n (%)	14 (34.1)	9 (21.4)	11 (22)
Mean Age (SD)	34.38 (10.82)	37.88 (13.31)	37.78 (15.47)
Mean TBSA (SD)	-	3.02 (2.6)	3.74 (3.32)
Burn Aetiology n (%)			
Flame	-	18 (43.0)	18 (36.0)
Scald	-	15 (36.0)	16 (32.0)
Contact	-	2 (4.76)	5 (10.0)
Friction	-	1 (2.38)	1 (2.0)
Chemical	-	4 (9.52)	5 (10.0)
Radiation	-	0 (0)	3 (6.0)
Cold	-	1 (2.38)	1 (2.0)
Not Recorded	-	1 (2.38)	1 (2.0)
Surgical Intervention, n (%)	-	35 (83.33)	43 (86)
Mean Length of Stay (SD)	-	5.57 (3.99)	5.25 (3.99)
Received First Aid n (%)	-	26 (61.9)	27 (54)
Prescribed Celecoxib n (%)	-		
< 5 days		9 (21.43)	8 (16)
> 5 days	-	14 (33.33)	15 (30)

not be recruited for all controls). Overall demographics for patients and controls were similar (Table 1).

Blood was collected from patients and healthy volunteers by venepuncture of the antecubital vein. A 21-gauge butterfly needle was used to minimise sheer-activation of platelets, and the first 2 mL of blood was discarded. Blood was collected into vacutainers containing 3.2% sodium citrate and processed within 15 min of collection [24,25]. For plasma exchange experiments, blood was collected from the patient and an age/sex-matched healthy control in parallel. Two vacutainers of blood were collected from each individual for plasma exchange experiments and the order of collection clearly marked. Blood collection for this study was undertaken with informed consent and human ethics approval (RGS000000731, RGS0000004277, RA/4/1/5750, 2020/ET000289).

2.2. Platelet isolation and plasma exchange

In order to determine whether changes in platelet function were due to a factor in plasma, a plasma exchange experiment was performed in a subset of patients. All instruments and reagents were brought to room temperature before beginning experiments. The first vacutainer of blood collected from each individual was centrifuged at 1000 x g for 30 min and the platelet-poor plasma (PPP) was carefully decanted into siliconized (LoBind) tubes, ensuring the buffy coat was not disturbed. PPP was centrifuged for a further 10 min at 1200 x g for complete platelet depletion. Concurrently, the second tube from each individual was centrifuged at 150 x g for 15 min and the platelet-rich plasma (PRP) was collected and washed twice with citrate wash buffer (CWB) supplemented with 50 ng/mL PGE_1 and 1.2 U/mL apyrase VII, centrifuged at 1200 x g for 10 min each time. The platelet pellet was carefully resuspended by flick-mixing. The platelets were resuspended in 200uL HEPES-Tyrodes buffer, then aliquots were diluted 1:31 with either autologous or heterologous plasma. Diluted platelets were used immediately in downstream experiments.

2.3. Assessment of platelet activation by flow cytometry

Platelets were incubated with agonists and stained for surface markers of activation (PAC1) and exocytosis (CD62P) by diluting whole blood 1:4 with HEPES saline + 0.1% BSA, then further diluting 1:4 with an antibody cocktail of PAC1-FITC, CD62P-PE, and CD42b-PE-Cy5 (Table 2), and either a submaximal or threshold concentration for each agonist (Table 3a). Two concentrations of each agonist are required to detect both inhibition and augmentation of platelet function via canonical pathways. Submaximal and threshold concentrations were determined by dose response curve in triplicate using healthy controls for whole blood (human and murine), and isolated platelets (data not shown). Samples were gently mixed without vortex, and incubated for 15 min at ambient temperature, except for arachidonic acid (AA) reactions, which were conducted at 37 °C. Isolated platelets were treated identically except without the initial 1:4 dilution, and agonist concentrations are altered (Table 3b).

Table 2 – Antibody panels for measuring platelet activation by flow cytometry.

Panel	Reagent/Target	Clone	Fluoro-phore	Dilution	Manufacturer	Cat #
Platelet Surface Markers	PAC1	PAC1	FITC	1/8	BD	340507
	CD62P	AK-4	PE	1/6	BD	555524
	CD42b	HIP1	PE-Cy5	1/12	BD	551141
Platelet Surface Markers (Isotype Control)	PAC1	PAC1	FITC	1/8	BD	340507
	Eptifibatide			2.5 pg/uL	Integrilin	
	IgG1κ	MOPC-21	PE	1/50	BD	555749
	CD42b	HIP1	PE-Cy5	1/12	BD	551141
MPAs	CD14	M5E2	BV421	1/80	BioLegend	301830
	CD42b	HIP1	APC	1/80	BioLegend	303912
MPAs (Isotype Control)	CD14	M5E2	BV421	1/80	BioLegend	301830
	IgG1κ	MOPC-21	APC	1/80	BioLegend	400122
Platelet Surface Markers (murine)	CD61	2C9. G2	PE	1/8	BD	553347
	CD62P	Psel.KO2.3	APC	1/8	eBiosciences	17–0626–82
Isotype Control (Murine)	CD61	2C9. G2	PE	1/8	BD	553347
	IgG1k	MOPC-21	APC	1/8	BioLegend	400122

Table 3 – Concentrations of agonists used to assess platelet function.

Experiment	A Human Whole blood		B Human Plasma exchange		C Murine Whole blood	
Agonist	*Threshold*	*Submaximal*	*Threshold*	*Submaximal*	*Threshold*	*Submaximal*
TRAP-6[a]/AYPGKF[b]	2.0 μM	10 μM	5.0 μM	40 μM	25 μM	250 μM
ADP	0.05 μM	1.0 μM	0.1 μM	2.0 μM	5.0 μM	25 μM
Epinephrine (EPI)	0.1 μM	20 μM	0.1 μM	20 μM	-	-
xCRP	20 ng/mL	200 ng/mL	2.0 ng/mL	200 ng/Ml	1.0 μg/mL	2.0 μg/mL
Arachidonic Acid (AA)	100 μM	300 μM	-	-	100 μM	1.0 mM

[a] TRAP-6 is the PAR4 agonist used for human studies.
[b] AYPGKF is the PAR4 agonist used for murine studies. ADP – adenosine diphosphate. xCRP = cross-linked collagen-related peptide.

Activation and staining was quenched by incubation with 20x volume of 1% formaldehyde fixative in HEPES buffer at ambient temperature, gently resuspending samples without vortex [24,26].

Monocyte-platelet aggregates were assessed by incubating whole blood 1:4 with an agonist (Table 2) and an antibody cocktail consisting of CD14-BV421, and CD42b-APC. Tubes were mixed without vortex and incubated at ambient temperature for 15 min. Reaction was quenched by incubation with 10x volume of FACSLyse (BD, USA) at ambient temperature, gently resuspending with vortex [27].

Isotype controls were stained concurrently with all samples. The isotype cocktail for platelet surface markers was PAC1-FITC, CD42b-PE-Cy5, IgG1κ-PE, and eptifibatide. Isotypes were prepared at both ambient temperature and 37 °C. The cocktail for MPAs was CD14-BV421 and IgG1κ-APC.

Data was acquired with a BD FACSCanto II flow cytometer with daily calibration of voltages against CS&T beads. Low-rate fluidics were used to minimise coincident events [27]. A PE-Cy5 threshold applied for platelet analysis and a FSC threshold applied for MPAs. Analysis of flow cytometry data was performed using FlowJo software. Single platelets were identified using characteristic forward scatter, side scatter, and CD42b expression [24]. Autofluorescence and non-specific staining was accounted for using isotypic controls for PAC1-FITC and CD62P-PE [26] (Fig. 1a). A minimum of 10,000 single-platelet events were recorded for each sample. Monocytes were identified by characteristic forward scatter, side scatter, and CD14 expression [27]. Monocyte-platelet aggregate events were identified as CD42b+ monocytes, compared to isotype control (Fig. 1b). Compensation was calculated using single-stained Anti-Mouse IgGκ/Negative Control Compensation Particles (BD, USA).

2.4. *Mouse model of non-severe burn injury*

Animal experiments were undertaken with approval from the University of Western Australia animal ethics committee (RA/3/100/1032, RA/3/100/1697, and 2022/ET000138).

Six- to eight-week old mice on a C57BL/6J background were purchased from Animal Resources Centre (Perth, Western Australia). Mice were housed in individually ventilated cages of up to four animals per cage, at 22.5 °C (18–24 °C) and ambient humidity (30–70%). Enrichment was provided as paper towels, cotton nesting material, aspen gnawing blocks, and cardboard tubes. Mice are provided standard chow (20% protein, 4.8% fat; Specialty Feeds, Western Australia) and acidified water (pH 2.5–3) ad libitum. Following injury procedures, 500 mg/L of paracetamol was added to drinking water for five days as an analgesic, and soft food was provided to aid recovery.

All animals received subcutaneous analgesic (buprenorphine, 0.1 mg/kg) prior to procedures, and were anaesthetised using 4% isoflurane in oxygen at a flow rate of 2 L/min in an induction chamber. Once induced mice were positioned in a face mask and anaesthesia was maintained with 2% isoflurane in oxygen.

Animals in the burn group were shaved on the right dorsal flank, the skin was sterilised with a chlorhexidine scrub, and a brass rod (19 mm diameter) heated to > 90 °C was applied to the area for 10 s to induce an 8% TBSA full-thickness burn injury [2].

Animals in the excision (non-burn trauma) group had their backs shaved and sterilised with chlorhexidine. A section of skin 12 mm in diameter was excised (the lack of tension in mouse skin results in a wound area equivalent to the burn injury model) [28]. Animals in the sham group were treated similarly except without excision.

All animals were returned to their cages to recover. No dressings were applied as this increases animal discomfort. All mice were monitored for four weeks following injury procedures.

2.5. *Cardiac puncture and whole-blood platelet function assessment in mice*

Mice were anaesthetised with isoflurane and blood was collected via cardiac puncture using a 21-gauge needle and immediately added 1:9–3.2% sodium citrate. Citrated whole blood was diluted 1:5 with HEPES-saline + 1% BSA, then further diluted 1:3 with a cocktail of CD61-PE and either CD62P-APC or isotype (Table 2), and agonists (Table 3c). Samples were incubated for 10 min at ambient temperature. Incubation was halted and samples were fixed with 20x volume of HEPES-saline + 1% formaldehyde. Samples were analysed by flow cytometry, as described above for human whole blood samples, excepting that CD61 was substituted for CD42b, and exposure of the fibrinogen binding site was not assessed (Fig. 1c).

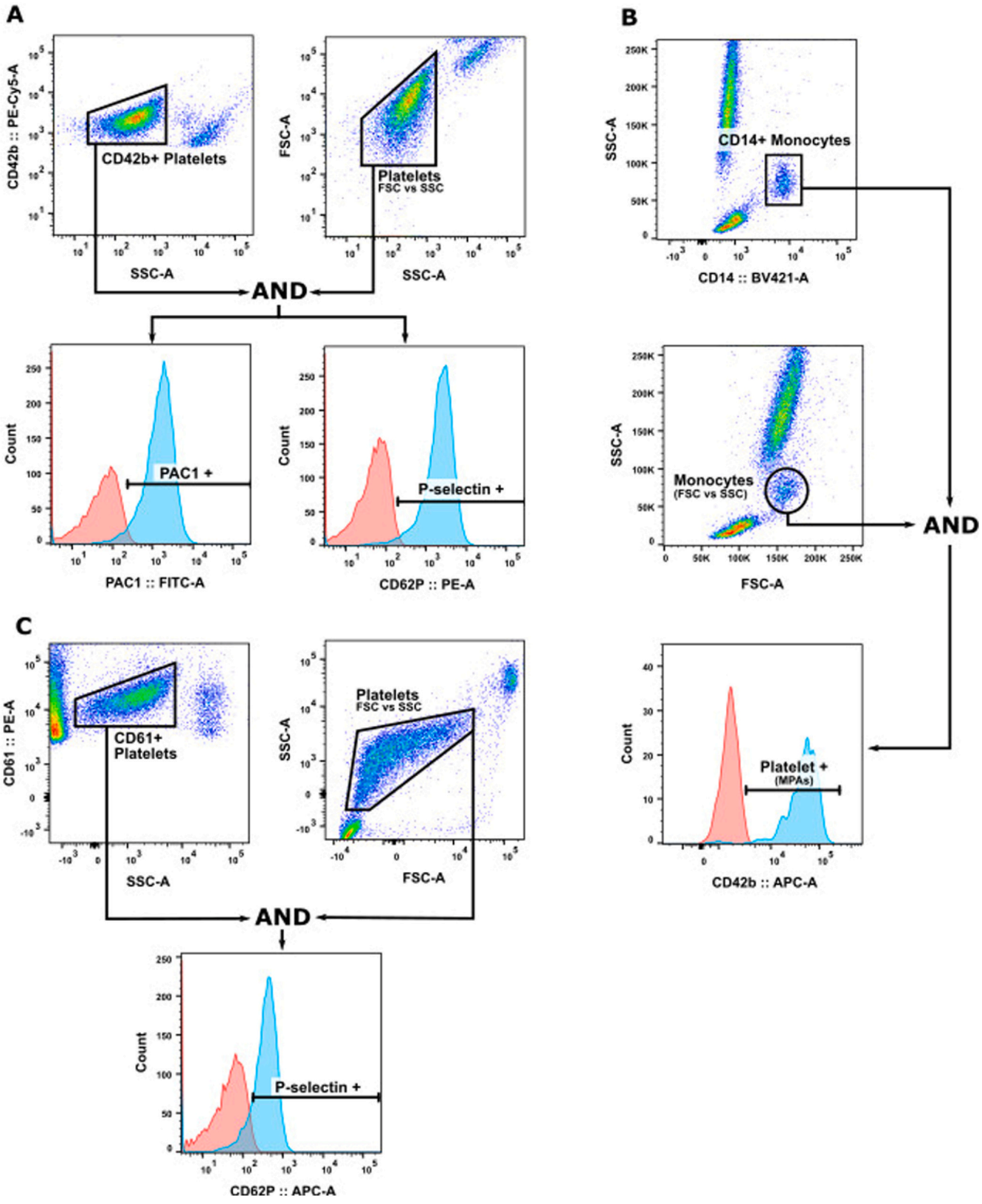

Fig. 1 – Flow cytometric detection of platelet activation markers. A) Human platelets were identified by characteristic forward and side scatter and expression of platelet-specific CD42b. Isotype controls (red) were used to set PAC1-positive expression and P-selectin-positive expression gates (0.95–1.00% positive events). Characteristic positive expression of markers following incubation with PAR-4 agonist TRAP-6 is shown (blue). B) Human monocytes were identified by characteristic forward and side scatter, and monocyte-specific CD14. Isotype controls were used to set gate threshold for identifying events displaying monocyte-platelet tethering, based on co-expression of CD42b (0.95%–1.00% positive events). Characteristic co-expression of CD42b on monocytes-platelet aggregates (MPAs) following incubation with TRAP-6 is shown. C) Murine platelets were identified based on forward and side scatter, and positive CD61 expression. Isotype controls (red) were used to set P-selectin-positive expression gates (0.95–1.00% positive events). Characteristic positive expression of P-selectin following incubation with PAR-4 agonist AYPGKF is shown (blue).

2.6. *In vivo assessment of thrombosis (ferric chloride-induced carotid injury model)*

Mice were anaesthetised by intraperitoneal injection with ketamine and xylazine (100:10 mg/kg). The carotid artery was exposed via midline cervical incision and isolated from the surrounding tissue [29]. A laser doppler flow probe (ADInstruments, Australia) was tethered in contact with artery using a custom-printed guide to ensure consistent readings between procedures. Baseline blood flow was established as saturation of the flow signal, and a piece of 2x4mm filter paper soaked in 12.5% ferric chloride was applied to the artery for one minute, then discarded. Blood flow was monitored for 30 min; time to 95% occlusion of the artery (where blood perfusion units (BPU) fell below 95% of the saturation value) and area under the curve (AUC; from injury to end of procedure) was recorded. After 30 min pentobarbitone was administered to euthanise the mouse while still under anaesthesia.

2.7. *Statistics*

Markers of activation as determined by flow cytometry were compared between controls and patient groups using mixed-effects ANOVA (Prism v9.3.1, Graphpad) with a random effect for subject/matched control. To allow for the multiple comparisons within each marker (i.e 2 weeks vs control and 6 weeks vs control), Dunnett's test was used and p values less than 0.05 were considered significant.

The effect of plasma exchange on platelet activation was calculated as the log2-fold change in platelet activation from platelets in autologous plasma to heterologous plasma. Comparisons between patient groups and controls were made as per the flow cytometry data.

Based on the effect size of circulating platelet activation in humans, it was determined that at least 15 animals per group would be required to assess in vivo platelet reactivity, with $\alpha = 0.05$ and $\beta = 0.2$.

In the arterial thrombosis model, time to occlusion was determined as the time from point of injury to the point where blood perfusion measurements dropped from saturation to a steady value < 5000BPU. Mean BPU was recorded as the average BPU value from time of occlusion. Conversely, time to 95% occlusion was calculated as the point where BPU fell below 250BPU. To assess the change in perfusion from the time of occlusion, a ten second average was taken for each animal from the occlusion time point. The average perfusion value for each ten second increment was subsequently compared to the initial average at occlusion, reported as log2-fold change. Reperfusion was determined using Log2-transformed unaveraged data, where a transient reperfusion was defined using Area Under the Curve (Prism v9.3.1, Graphpad) to identify peaks with a value > 2 over at least 5 consecutive points (seconds).

Categorical variables were analysed with Fischer's exact test, where p-values greater than 0.05 were considered significant.

3. Results

3.1. *Platelets show increased responsiveness to ex vivo stimulation*

Platelet activation and reactivity was assessed based on surface expression of activation markers with and without to *ex vivo* stimulation with platelet agonists, and the formation of MPAs. At two weeks following injury, circulating MPAs were elevated in burn patients, compared to healthy controls (1.80-fold, $p < 0.001$). This was despite no change in circulating platelet granule exocytosis (as demonstrated by CD62P expression) or inside-out signalling (as demonstrated by PAC1 binding) at that time point. Increased agonist-induced tethering of platelets to monocytes occurred at two weeks following stimulation with threshold doses of ADP (1.50-fold, $P < 0.01$), epinephrine (1.65-fold, $p < 0.001$), xCRP (1.83-fold, $p < 0.001$), and AA (1.96-fold, $p < 0.01$), and submaximal doses of TRAP (1.05-fold, $p = 0.043$), ADP (1.51-fold, $p < 0.01$), epinephrine (1.71-fold, $p < 0.01$), and xCRP (1.21-fold, $p < 0.01$). Increased MPA formation was also observed at 6-weeks for threshold concentrations of xCRP (1.43-fold, $p = 0.046$) (Fig. 2).

Platelet reactivity to agonists was generally elevated across the range of agonists, with significant increases in PAC1 binding observed at both time points for threshold doses of xCRP (2 weeks: 1.57-fold, $p < 0.001$; 6 weeks: 1.39-fold, $p = 0.028$) and submaximal doses at 6 weeks (1.13-fold, $p = 0.012$). PAC1 binding was also elevated for submaximal doses of ADP 6-weeks post-injury (1.08-fold, $p < 0.01$) (Fig. 2). Activation induced granule exocytosis (as measured by CD62P expression) was increased in burn patients following stimulation with threshold doses of xCRP at 2 weeks (1.41-fold, $p = 0.018$), and with submaximal doses at both 2- and 6-weeks (1.15-fold, $p = 0.044$; and 1.15-fold, $p = 0.04$, respectively). CD62P expression was increased following stimulation with AA at 2 weeks

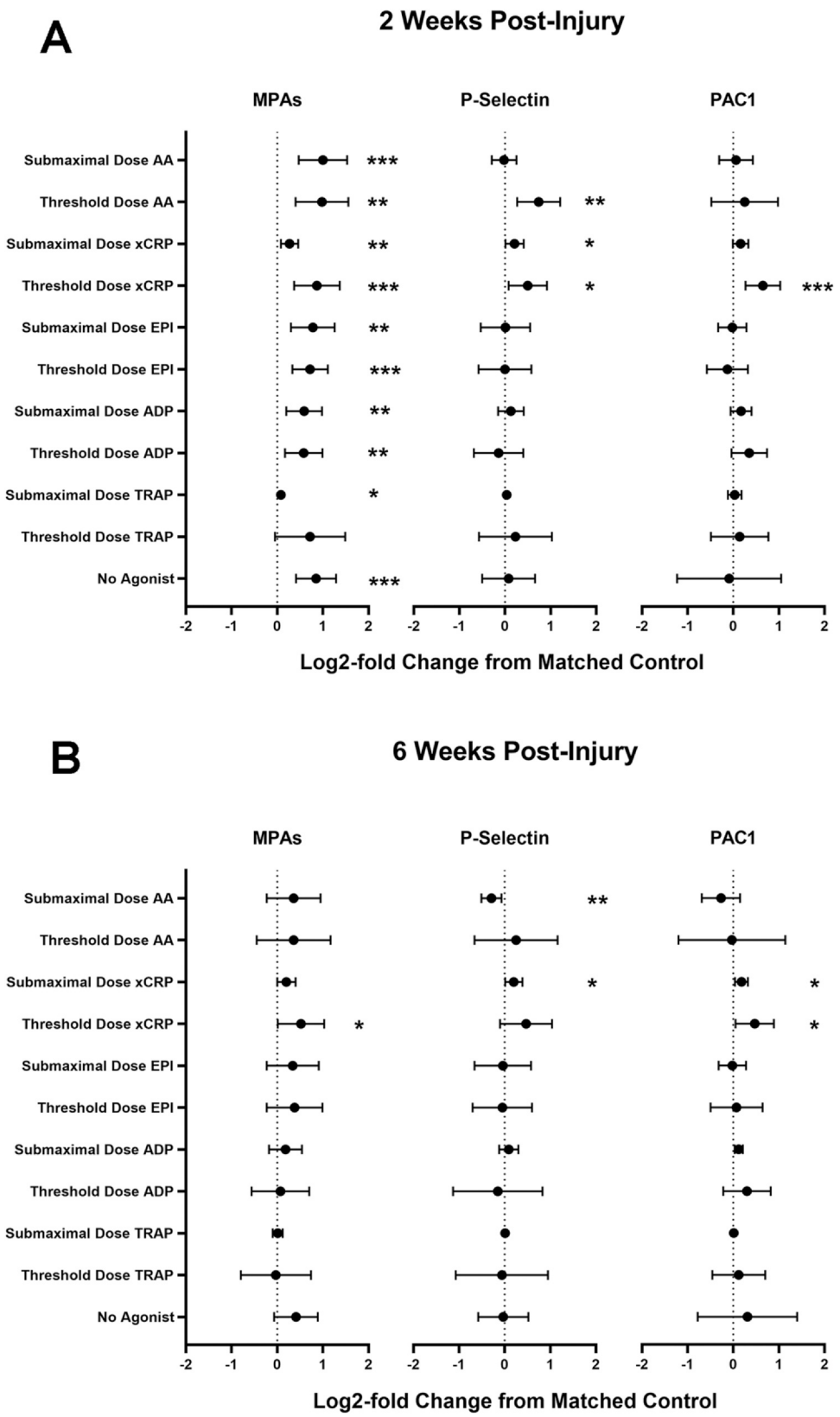

Fig. 2 – Platelet activation is increased following non-severe burn injury. Markers of platelet activation were measured by flow cytometry following stimulation with and without canonical platelet agonists, and the frequency of activated platelets was compared between controls and survivors of NSBI at A) 2-weeks and B) 6-weeks. Data is presented as log2 fold-change of means between patients and controls, with error bars showing the standard error of the mean. N controls = 41, n at week 2 = 41, n at week 6 = 50. MPA – monocyte-platelet aggregate. PAC1 – GPIIb/IIIa fibrinogen binding site antibody. P-selectin – CD62P. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

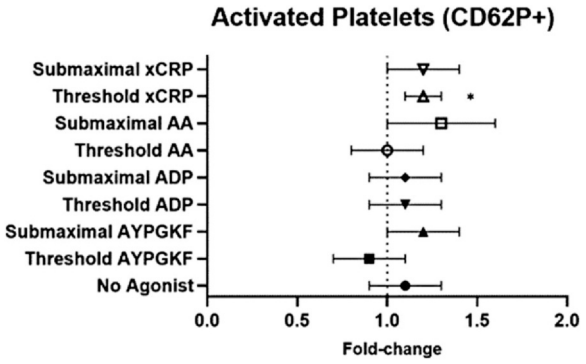

Fig. 3 – Platelet activation is increased in murine model of non-severe burn injury following ex vivo incubation with collagen. Platelet activation was quantified based on the frequency of platelets with surface expression of CD62P, measured via flow cytometry. Data is presented as fold-change of the mean compared to sham, with the standard error of the mean. xCRP – cross-linked collagen-related peptide. AA – arachidonic acid. ADP – adenosine diphosphate. AYPGKF – PAR4 agonist (murine equivalent to TRAP-6). * $p < 0.05$.

(1.67-fold, $p < 0.01$), and decreased in patient platelets on stimulation with submaximal AA at 6 weeks (0.82-fold, $p < 0.01$) (Fig. 2). Trends were also observed for PAC1 binding (threshold ADP, 2-weeks; and submaximal xCRP, 2-weeks), and MPA formation (threshold TRAP, 2-weeks; and submaximal xCRP, 6-weeks).

Consistent data were recorded for a murine model of burn injury, with a significant increase in platelet reactivity at 4 weeks following injury demonstrated by increased xCRP induced CD62P expression (1.2-fold, $p = 0.05$). While not statistically significant, increased responsiveness to submaximal doses of xCRP, AA, and AYPGKF were also observed in this model (Fig. 3).

3.2. *Platelets from burn patients are more reactive in control plasma*

Cross-incubation of patient and control platelets in the alternative's plasma was used to assess the contribution of circulating mediators on the altered platelet reactivity following burn injury. Incubation of burn platelets (two weeks post-injury) in control plasma resulted in an increased sensitivity to threshold doses of ADP, as reported by PAC1 binding (1.54-fold, $p = 0.034$) and CD62P (1.11-fold, $p = 0.023$) expression. A small decrease in control platelet activation following incubation in patient plasma contributed to this significance. A similar trend was observed with threshold doses of TRAP, EPI, and xCRP (Fig. 4).

Surprisingly, incubation of control platelets with plasma from burn patients two weeks post-injury decreased CD62P expression in the absence of exogenous stimulation, and increased sensitivity to TRAP was observed at six weeks post-injury, however the effect sizes are small.

A post-experimental comparison was made to determine whether ABO mismatching may be modulating these results [30]. Removing data from patient-control pairs with poor ABO matching diminished the significance of the findings. Threshold doses of ADP no longer resulted in a significant increase in PAC1-binding or CD62P surface expression following incubation of patient platelets in control plasma (Supplementary Data). However, the while the magnitude of the change was lower, the direction of the change was maintained.

3.3. *Burn injury has no effect on time to occlusion in vivo*

A murine model of arterial thrombosis was used to assess in vivo platelet function in response to endothelial damage. No change in time to occlusion was observed between burn, excision, or sham groups. No difference was observed in the frequency of occlusion between groups, mean BPU following occlusion, or the rates of reperfusion (Table 4).

A trend observed in the data was for the perfusion value to decrease in the burn group (and to a lesser extent the excision group) after 20 min, while the sham group remained consistent (Supplementary Data). Due to a smaller number of individuals contributing data at times further from initial occlusion, significance cannot be reliably determined.

4. Discussion

A small but significant increase in parameters of platelet function were demonstrated in both human burn patients at 2 weeks and 6 weeks after injury, and a murine model of non-severe burn injury at 4 weeks after injury. While previous studies have demonstrated formation of monocyte-platelet aggregates in circulation in the acute burn period [11], we have demonstrated that this phenomenon persists for at least two weeks following burn injury in humans, with agonist-inducible MPA formation remaining elevated at 6-weeks. While the effect size is small, it represents a significant and persistent thromboinflammatory response. Previous studies have demonstrated that similarly small but significant in vivo MPA formation is correlated with cardiovascular outcome in high risk patients [22]. Patients with stable coronary artery disease have increased in platelet reactivity anywhere from 20%– 150%, compared to other non-CAD controls [22,31,32]. One study found patients with acute myocardial infarction to have an 80% increase in circulating MPAs, which is a similar magnitude increase to our findings at 2-weeks post burn injury [33]. An increase in MPAs to levels consistent with those reported in CVD suggests burns induce a temporary microenvironment with conditions that could be clinically relevant to the progression of CVD.

Sensitivity of the collagen-activation pathway was observed to be increased at both 2- and 6-weeks following NSBI, and the data suggests that this heightened sensitivity is likely transient as increases relative to controls are lower at 6-weeks compared to 2-weeks (though not significantly). The platelet collagen receptor – GPVI – is known to have greater expression on young platelets, which in turn constitute a greater fraction of circulating platelets following thrombocytopenia [34]. While clinical thrombocytopenia (platelet count $< 150 \times 10^9$ platelets/L) is not expected to occur in most

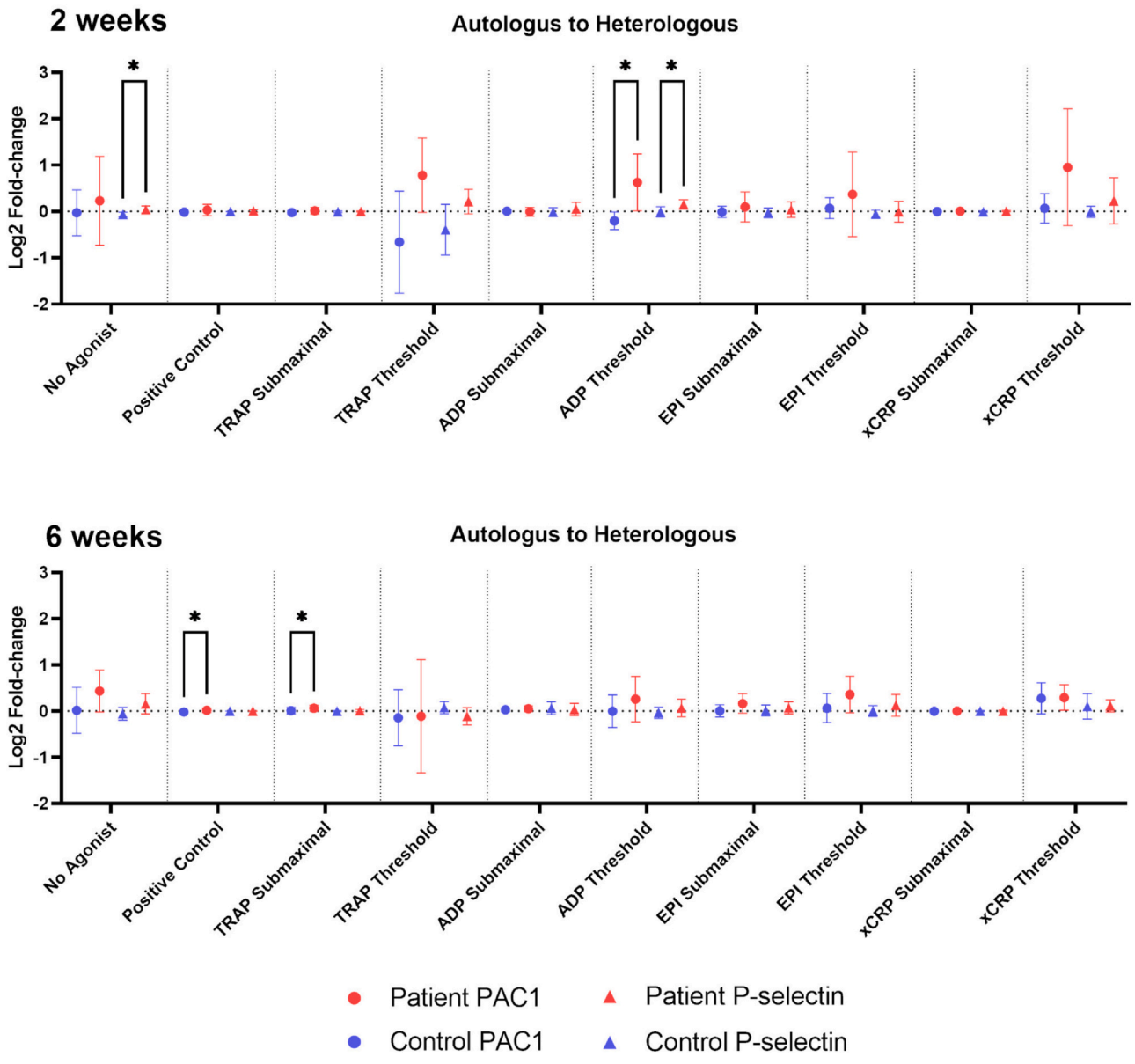

Fig. 4 – Fold-change of activated platelets by surface marker following agonist stimulation in heterologous plasma exchange. Mean fold-change of platelet activation at 2- and 6- weeks post NSBI was compared between patient platelets in healthy control plasma, and healthy control platelets in burn patient plasma. Fold-change was determined based on platelet activation in heterologous plasma compared to activation in autologous plasma. *p < 0.05.

patients with a NSBI, thrombocytosis occurs regardless of severity, whereby platelet counts may double compared to measurements taken around the time of injury [8]. GPVI-shedding is elevated in severe burn patients [35]; due to their shared cytosolic domain, activation of the FcγRIIA receptor is known to contribute to extracellular GPVI cleavage [36].

The mechanism driving altered platelet function in the acute and post-acute burn injury is complex and likely to be multifactorial. Sepsis impacts platelet counts and contributes to diminished reactive thrombocytosis. However, sepsis is extremely rare in non-severe burns and unlikely to contribute to the effect we report. Certain antibiotics, including cephalosporin, penicillin and sulfa-containing drugs may lower platelet counts in some people. But these are not prescribed prophylactically in our clinic. We have previously demonstrated that increased inflammatory cytokines persist long after the initial burn injury and may still be detected years later [37]. Platelet activation by thromboinflammatory factors in plasma has previously been established a variety of pathophysiological conditions [38–40]. There is also evidence for the priming of platelets by thrombopoietin in the post burn period [11].

Platelets from burn patients and the mouse model of burn injury sustained increased sensitivity to canonical agonists, consistent with broad-spectrum priming of platelets to subsequent activation by a thromboinflammatory milieu or thrombopoietin. However, our preliminary data suggests that this effect was marginally enhanced, rather than

Table 4 – Murine model of arterial thrombosis.

	Burn	Excision	Sham
Number of Animals	16	16	16
Number Reaching Occlusion (%)	16 (100%)	15 (93.75%)	14 (87.5%)
Number Reaching 95% Occlusion	9 (56.25%)	9 (56.25%)	9 (56.25%)
Time to Occlusion (SD)	10.53 min (2.48)	10.49 min (1.89)	9.56 min (1.97)
Mean BPU from Time of Occlusion (SD)	750.5 (916.08)	723.33 (570.03)	955.00 (962.82)
Number of Reperfusions*	6	4	4
Number of Animals with Reperfusions*	5	3	4
Peak AUC (SD)*	51,467.66 (96,065.09)	48,060 (43,480.57)	50,882 (95,186.06)
Ratio of Peak AUC to total AUC*	0.7	0.74	0.63

* Reperfusions determined using Log2-transformed data, where reperfusion was defined using Area Under the Curve (Prism v9.3.1, Graphpad) to define peaks with a value > 2 over at least 5 consecutive points (seconds).

ameliorated, when platelets from burn patients were incubated in control plasma. While burn platelets remain hyper-functional at 2 weeks following injury, the factors responsible for priming may no longer be present in plasma by this stage. Indeed, augmentation of burn platelet function by control plasma suggests the potential for compensatory inhibition of platelet function in burn plasma at 2 weeks, which is resolved by 6 weeks. While our study design precludes any residual exogenous anti-platelet therapy which might explain the inhibition of normal platelets by burn plasma, we cannot exclude biological variables, such as ABO or PLA incompatibilities as contributing to this phenomenon.

In the model of in vivo thrombosis, occlusion time was unchanged compared to sham and non-burn trauma, despite murine platelets demonstrating significantly elevated sensitivity to collagen-mediated activation. Platelets appear to respond appropriately to exogenously induced endothelial trauma following recovery from an acute burn injury, but a subclinical enhancement of platelet sensitivity could contribute to other more chronic CVD.

Increased MPA frequency is consistent with data from a previous study by Lupia et al., who demonstrated increased circulating MPAs < 72hrs following severe burn injury [11]. Our data suggests that this increase in MPAs also occurs following NSBI and persists out to at least 2 weeks. Elevation of circulating MPAs is associated with cardiovascular disease, particularly recent acute CVD events (ie: myocardial infarction) [22,41], and chronic conditions such as stable coronary artery disease [22,31,32]. The likelihood of NSBI resulting in acute CVD events or late-stage chronic disease in under two weeks is expected to be very low; however, the contribution of MPAs to precipitating long-term CVD risk observed in burn survivors warrants exploration.

Activated endothelial cells express von Willebrand factor (vWF) and integrins that bind and activate platelets rolling across their surface [18]. Subsequent recruitment of additional platelets and monocytes through inflammatory signalling (IL-1β, RANTES, MCP-1) incites the formation of MPAs, which roll across the endothelial surface. Platelets assist in tightly tethering monocytes to the endothelium via CD40/CD40L, inducing the expression of integrins on the endothelial surface which bind their respective ligands expressed on monocytes [19]. Monocytes may then taxis into the intima where they differentiate into macrophages, scavenging oxidised low-density lipoproteins and other lipids before further differentiating into plaque-resident foam cells [20,21].

While our findings overall suggest that persistence of a small increase in circulating platelet activation and non-specific responsiveness to stimulation does not contribute to more rapid or persistent clot formation when the vasculature is injured, it is possible that persistence of slightly hyper-activated platelets in burn survivors for many weeks could contribute to accelerated atherogenesis and vascular injury through well-established platelet-monocyte interaction and chronically elevated inflammatory cytokines [37,42]. These cells may then become resident and contribute to CVD years after the initial injury, consistent with epidemiological data [1]. This merits further investigation. Patients could be recruited into a prospective study that would assess their platelet function in the weeks following injury, then assessed at follow up for evidence of atherogenesis and endothelial dysfunction. Alternatively, our standard mouse model of NSBI could be applied to a mouse model of atherosclerotic disease, such as an apoE knockout model [43]. This model could be assessed for the rate of monocyte infiltration into the vascular intima and the progression of atherosclerotic lesions within a condensed timeframe, and may serve as a useful in vivo model for the pre-clinical assessment of therapeutic interventions.

4.1. *Limitations*

Due to disruptions to recruitment over the study period, complete pairing of patients with age and sex-matched controls could not be completed. The mixed effects modelling approach used did not allow for the closer association between results for a specific patient at 2 and 6 weeks, than between the patient and their matched control. However, analyses were repeated using a simplified approach of ANOVA modelling for 2 week results vs the overall control group (ignoring the matching) and separately for 6 weeks vs control, and similar results were obtained (Supplementary Data). The Dunnett's test considers each group independently while controlling for multiple comparisons to a single control group. Because significance changes were only determined between the control group and each time point (not between time points) the reported outcomes between

groups remain valid, however statistical power is lower. The reported outcomes are therefore more conservative, and future work with complete pairing may identify more nuanced outcomes between groups.

Our study design did not consider the effects of ABO or PLA incompatibilities on platelet activation during incubation in heterologous plasma. This should be addressed in future work to control for effects not dependent on the burn injury.

Data is not currently available for our cohorts detailing individuals who went on to develop cardiovascular morbidities. This limits our ability to determine which factors are directly associated with future disease. Long-term follow up is required to identify individual patients who develop CVD, and future studies should aim to establish protocols that allow patients to be consulted years after initial data collection. Multivariate analysis can then be used to assess if biomarkers can distinguish those who develop morbidities from those that do not.

5. Conclusion

This study reports for the first time platelet hypersensitivity to collagen at 2- and 6-weeks following a non-severe burn injury. Our findings extend previous findings noting increased frequency of circulating monocyte-platelet aggregates around the time of injury, demonstrating that circulating MPAs remain elevated for at least 2-weeks following injury. Furthermore, MPAs can be induced at higher rates following incubation with canonical platelet agonists 2-weeks post-injury, with heightened sensitivity to collagen-mediated activation contributing to significant *ex vivo* MPA formation at 6-weeks. This platelet hyperreactivity does not appear to affect physiological response to trauma, but further research should confirm whether this contributes to pathological thromboendothelial interactions that could explain the observed rates of CVD in burn survivors. A greater understanding of early post-acute thromboinflammatory milleu could guide early interventions with specific antiplatelet agents targeted to reducing agonist sensitivity (with a focus on the collagen-GPVI pathway) while preserving vitally important haemostatic thrombosis.

CRediT authorship contribution statement

Blair Johnson: Conceptualization, Methodology, Analysis, Laboratory, Writing. **Emily O'Halloran**: Laboratory. **Andrew Stevenson**: Methodology, Laboratory, Writing. **Fiona Wood**: Conceptualization, Supervision. **Mark Fear**: Conceptualization, Methodology, Analysis, Supervision, Writing. **Matthew Linden**: Conceptualization, Methodology, Laboratory, Analysis, Supervision, Writing.

Declaration of Competing Interest

The authors disclose that they have no conflicts of interest.

Acknowledgements

We would like to acknowledge the team at the Fiona Stanley Hospital burns unit for their support, particularly Ed Raby, Paul Gittings, and Natalie Morellini, who facilitated sample collections. Thank you to Nutan Chaudhari and the UWA animal care services team for their help with animal model experiments. We acknowledge the facilities support of the Centre for Microscopy, Characterisation, and Analysis (UWA), Harry Perkins Institute, and the University of Western Australia. Joanna Leadbetter provided consultation for statistical analyses.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.burns.2023.10.011.

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