PLATELET PHYSIOLOGY AND FUNCTION IN NEONATES AND CHILDREN ON EXTRACORPOREAL MEMBRANE OXYGENATION (ECMO)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work had not been submitted previously, in whole or in part, to qualify for other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research programme; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Signed:

Hui Ping Yaw

Date: 26th April 2019

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Abstract

Extracorporeal membrane oxygenation (ECMO) is a modified form of heart-lung machine that aims to provide short- to-medium length of support to patients with cardiac and/or respiratory dysfunction. Children represent the majority of the ECMO population. While increasing experience and technical improvements for ECMO over the years have seen some improvement in outcomes, the rates of morbidity and mortality remain high in this population and many complications are related to bleeding and thrombosis. Platelets are a key element of the coagulation system. Platelet dysfunction can cause coagulopathy in adults on ECMO, however, the association between modification of platelet function and coagulopathy remains unknown for children.

This study hypothesised that there are platelet-specific differences: I.) for paediatric patients on ECMO according to their age, pathway onto ECMO and duration of ECMO that could be associated with the development of bleeding or thrombosis during ECMO and II.) at different sites in a paediatric ECMO system. This study aimed to characterize the molecular indices of circulating platelets in the paediatric ECMO population using whole blood flow cytometry approach:

1) To examine and compare the effect of a patient's pathway onto ECMO on platelet phenotype and function and their associations with the development of bleeding or thrombosis during ECMO.
2) To examine and compare the effect of a patient's age on platelet phenotype and function and their associations with the development of bleeding or thrombosis during ECMO.
3) To examine and compare the effect of a patient's duration of ECMO on platelet phenotype and function and their associations with the development of bleeding or thrombosis during ECMO.

4) To examine and compare the site-specific differences for platelet phenotype and function in the paediatric ECMO system.

A total of 22 paediatric patients [median (interquartile range): 0.34 (0.01 – 3.38) years] were included in this study. Citrated whole blood samples were collected and a whole blood flow cytometry method was developed for the evaluation of platelet phenotype and function in the setting of ECMO.

The platelet assays were standardized to ensure minimal pre-analytical activation. By using multiple thrombin receptor activator peptide 6 (a thrombin mimic) concentrations, the platelet panels also showed sensitivity to detect subtle changes in platelet response. The multifaceted flow cytometry panels allowed simultaneous evaluation of platelets for their phenotype, function and interactions with monocytes and neutrophils. Such approach to investigate platelet-specific changes from different aspects suits well for the ECMO population, representing a complex group of patients. These results showed that the whole blood flow cytometry assay is a reliable and useful platelet function test for paediatric ECMO patients.

Results from the analysis for platelet-specific markers within the first 24 hours showed no difference in platelet phenotype and function between patients from different pathways onto ECMO and different ages. However, the association of platelet-specific changes and the development of clinical events during ECMO were different according to a patient's age and pathway onto ECMO. Patients who had cardiopulmonary bypass before coming onto ECMO and had bleeding had increased platelet GPIIb/IIIa receptor expression and reduced circulating neutrophil-platelet aggregates level compared to patients who had no bleeding during ECMO. In contrast, patients who had no cardiopulmonary bypass before coming onto ECMO and developed bleeding had reduced platelet response compared to those who had no bleeding during ECMO. Conversely, increased lysosome release was observed for children with

thrombosis and may indicate the presence of a protective mechanism against increased thrombus formation.

Duration of ECMO had been recognized as an important factor affecting the outcome of paediatric ECMO patients. The results showed an increased level of von Willebrand factor (VWF) receptor and reduced platelet response for granule exocytosis with increasing number of days on ECMO (Day 2 vs. Day 5). Most importantly, such platelet-specific changes that involved GPIb/IX/V receptor and granule release with increasing duration of ECMO were only observed in patients who had bleeding but not in patients without bleeding after five days on ECMO. In addition, elevated circulating monocyte-platelet aggregates level was only observed in patients who had thrombosis but not for those without thrombosis. Together, these results suggested a link between pathway onto ECMO/age/duration of ECMO, bleeding/thrombosis and platelet dysfunction. Hence, markers relevant to the platelet-specific changes could be used as the indicators for increased bleeding or thrombosis risk for paediatric patients during ECMO.

Platelet phenotype and function were also compared at different sites in the ECMO system to identify the site-specific differences of platelet-specific changes that have not previously been investigated in a paediatric ECMO system. In the setting of mechanical circulatory support, shear and oxidative stress are known to modify platelet phenotype via integrin αIIbβ3 and GPIb/IX/V receptors and increase platelet response via multiple platelet activation pathways. The results demonstrated that platelet phenotype and function were different at different sites in a paediatric ECMO system. The platelet-specific changes observed included the modification of platelet phenotype via increased VWF and integrin αIIbβ3 receptor expression, increased platelet activation through the activation of the integrin αIIbβ3 receptor and higher platelet responsiveness at the post-oxygenator site compared to the pre-oxygenator site.

However, the exact cause of the site-specific differences of platelet phenotype and function remained to be identified.

In summary, this study demonstrated the feasibility of using whole blood flow cytometry method with multifaceted platelet-specific panels as a reliable platelet function test in paediatric patients on ECMO. Platelet-specific changes could be associated with the development of bleeding or thrombosis during ECMO. In addition, platelet phenotype and function were different at different sites in a paediatric ECMO system. Together, this study provides new insights for the circuit-related platelet-specific changes and the understanding of how modifications of platelet phenotype and function that are dependent on patient's factors may be associated with coagulopathy in children on ECMO.

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ACT	Activated clotting time
ADP	Adenosine diphosphate
APC	Allophycocyanin
APTT	Activated partial thromboplastin time
AUC	Area under the curve
BD	Becton Dickinson
BV421	Brilliant Violet 421
BV711	Brilliant Violet 711
CPB	Cardiopulmonary bypass
ECD	PhycoErythrin-Texas Red
ECLS	Extracorporeal Life Support
ECMO	Extracorporeal membrane oxygenation
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FSC	Forward-scattered light
GPIb	Glycoprotein Ib
GPIIb/IIIa	Glycoprotein IIb/IIIa
GPVI	Glycoprotein VI
IQR	Interquartile range

LPA	Leukocyte-platelet aggregates
MCS	Mechanical circulatory support
MFI	Median fluorescence intensity
MPA	Monocyte-platelet aggregates
NPA	Neutrophil-platelet aggregates
PE	Phycoerythrin
PICU	Paediatric Intensive Care Unit
PSGL-1	P-selectin glycoprotein ligand-1
RCH	The Royal Children's Hospital
SD	Standard deviation
SSC	Side-scattered light
TRAP-6	Thrombin receptor activator peptide-6
VA	Veno-arterial
VAD	Ventricular assist device
VV	Veno-venous
VWF	von Willebrand factor

1 Introduction

1.1 Extracorporeal membrane oxygenation (ECMO)

1.1.1 History and development of ECMO

The idea of Extracorporeal life support (ECLS) originates from the effort to maintain normal cardiopulmonary function under pathophysiological conditions [1]. The development of ECLS started around year 1930 when John Gibbon MD and his wife, Mary invented a freestanding roller pump device with the size of a spinet piano [2]. However, the first clinical use of such device started 16 years later and was trialed on an 18-year-old patient with an atrial septal defect.

In 1954, C. Walton Lillehei MD performed the first cardiac surgery with a bubble oxygenator that he co-invented with Richard DeWall [3, 4]. However, in the absence of a proper blood-gas interface, the usage of such oxygenator was limited due to high incidence of haemolysis and multiple organ failure. Hence, subsequent development of ECLS focused on creating devices that could provide cardiopulmonary support for an extended period of time, without disrupting the haemostatic system.

A huge advance for ECLS devices took place in 1957 with the discovery of silicone rubber by Kammermeyer [5]. Incorporation of the silicone rubber membrane into the oxygenator with its unique feature of being able to withstand hydrostatic pressure, while retaining its permeability to gas transfer (between the blood and the oxygen in the oxygenator) allowed prolonged bypass support outside the operating room [6, 7]. The application of the silicone membrane oxygenator also signified the beginning of the usage of the term Extracorporeal Membrane Oxygenation (ECMO). ECMO is a modified form of Cardiopulmonary Bypass (CPB) which aims to support heart and/or lung functions from days to weeks based on the patient's condition as opposed to

a number of hours in CPB. Often, patients who are unable to wean-off CPB during cardiac surgery were placed on ECMO for recovery.

Encouraging outcomes of ECMO in children with congenital heart disease following the usage of the silicon membrane oxygenator initiated its application worldwide. The first prolonged usage of the extracorporeal circuit outside of the operating room was reported by Dr. JD Hill in 1972 [8]. The patient, an adult male with post-traumatic respiratory distress syndrome survived after receiving venoarterial support for 75 hours [8]. Following this successful case, there was expanding use of ECMO in adults, however, with a low survival rate.

ECMO application in neonates and children advanced with the pioneering works of Dr. Robert Bartlett, the father of modern ECLS from the University of Michigan, USA and his collaboration with engineers. Multiple advancement in ECMO devices at that time allowed successful ECMO application in children with cardiac failure in 1972, followed by that in a neonate with respiratory failure in 1975 [9, 10]. Together with the growing interest and experience, beneficial outcomes shown by various randomized, controlled trial (RCT) and active collaboration among the ECLS medical community expanded the usage of ECMO worldwide. Formation of multiple ECLS centres further supported the establishment of Extracorporeal Life Support Organization (ELSO) in 1989.

Initially, ELSO was formed with the initiative to collect ECMO-related data (e.g. outcomes) and to act as a platform for the medical community to exchange ideas for optimal ECMO usage. The role of ELSO then expanded to the development of guidelines, manuals and textbook publication for use across different clinical centres. These efforts were supported by the initiation of the ELSO Registry [1], an international patient database to track and evaluate the outcomes of the ECMO population from different centres worldwide; supplemented with an

annual ELSO meeting for the ECLS personnel to meet and exchange ideas of their ECMO experience.

According to the ELSO data, in 2006, 131 centres used ECMO in 2346 cases, whilst in 2016, 329 centres had used ECMO in 9127 cases worldwide (Figure 1.1) [11, 12]. This large increase in the use of ECMO can be related to the increasing understanding and technical improvements with the usage of ECLS in both the adults [13-16] and children [10, 17, 18]. However, the requirement for intensive care, frequent laboratory testing and considerable usage of blood products to manage bleeding complications make ECMO one of the highest costs in clinical care especially for children. For example, the average cost involved for the management of one child on ECMO is $ 519,450 compared to the bone marrow transplantation ($ 207,212), liver ($ 231,755) or kidney transplantation ($ 82,008) [19].

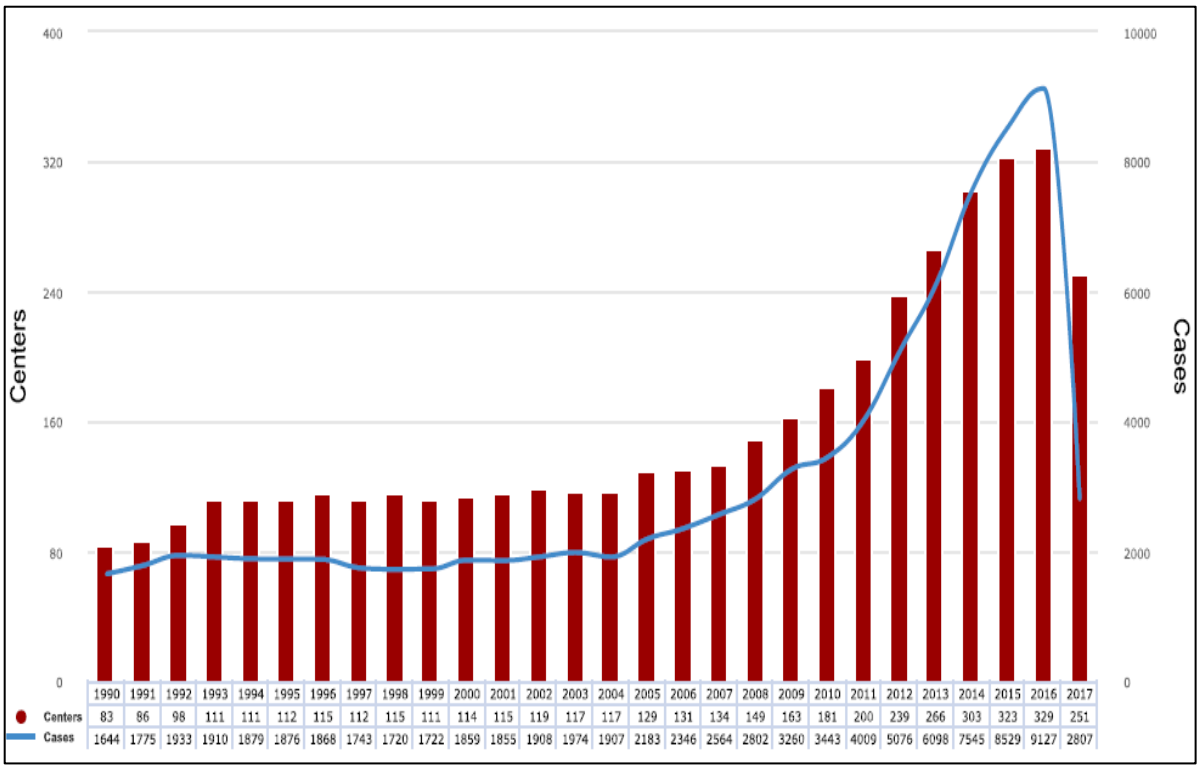


Figure 1.1 Summary of ELSO centres and cases worldwide 2017.

Total number of ELSO centres and cases worldwide in January 2017 [11].

1.1.2 Patient selection criteria for paediatric ECMO

Ever since its first successful application reported by Hill and colleagues in 1972 [8], ECMO has been the predominant supportive system for patients with cardiac and/or respiratory failure due to a variety of medical surgical conditions (Table 1.1) [20].

Table 1.1 Conditions that may be supported by ECMO.

ECMO patients from different age groups [20].

Age groups		
Neonates	**Children**	**Adults**
• Persistent pulmonary hypertension • Meconium aspiration syndrome • Sepsis or infection • Pneumonia • Congenital diaphragmatic hernia • Congenital heart disease / Post-op Shock Lung • Hyaline membrane disease	• Post-op cardiac repair • Myocarditis • Sepsis • Pneumonia • Aspiration pneumonia • Asthma • Near drowning • Hydrocarbon ingestion (lighter fluid, turpentine)	• Underlying diseases with reasonable likelihood of reversal • Septic shock • Pneumonia • Pulmonary failure

At The Royal Children's Hospital in Melbourne, a patient will be placed on ECMO if he/she fulfils the following criteria:

I.) He/She had reversible acute cardiac or pulmonary disease.

II.) He/She had no pre-existing major disability that requires the need for dependent care.

III.) He/She can achieve normal quality of life with no major disability resulting from the cardiac/respiratory disease.

IV.) He/She had predicted 80% mortality rate.

1.1.3 ECMO circuit design and mode of ECMO

An ECMO circuit is made up of a mechanical blood pump and membrane oxygenator that are linked together via the circuit tubing as shown in Figure 1.2 [21]. In an ECMO system, deoxygenated blood is removed from the venous circulation via an access cannula. The blood then passes through an oxygenator that allows the addition of oxygen and removal of carbon dioxide via a semi-permeable membrane. Lastly, the oxygenated blood is returned to the patient via a return cannula.

ECMO can be categorized based on the site of the return cannula insertion [22]. VA and VV-ECMO are the most common types of ECMO used and the general features for different mode of ECMO were listed as followed:

I.) VA-ECMO is used when both cardiac and respiratory supports are required. The deoxygenated blood is drained from the venous circulation and returned to the arterial circulation of the patient.

II.) VV-ECMO is used when only respiratory support is needed. The blood is both drained and returned via the venous circulation and the efficacy of ECMO relies entirely on the intrinsic cardiovascular system.

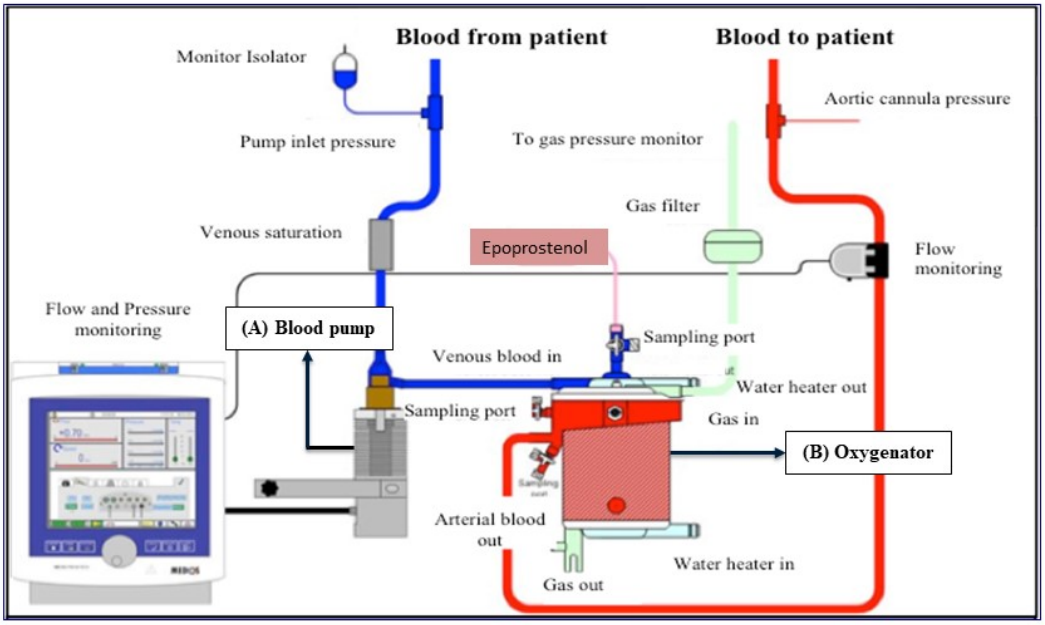


Figure 1.2 The ECMO circuitry currently used at The Royal Children's Hospital, Melbourne.

The most important components of an ECMO circuitry are the (A) blood pump and (B) oxygenator.

1.1.3.1 Blood pump

A blood pump serves to replace the cardiac pumping function in ECMO patients (Figure 1.3). The pump generates force to drain venous blood from the patient, which then passes through the inlet line and propels blood to the oxygenator through the outlet line. Such force dictates pressure difference at various sites of an ECMO circuitry, which corresponds to *in vivo* venous and arterial flow in an intact circulatory system (Figure 1.3).

The blood pump generates force that propels blood into the oxygenator through the inlet line (A). Presence of a semipermeable membrane separates space within the oxygenator (B) into a blood and a gas compartment. Gas exchange occurs across the membrane when blood interacts with the fresh gas. A gas blender allows the adjustment of the proportion of air and oxygen delivered to the oxygenator. After oxygenation and removal of carbon dioxide from the blood, the arterialized blood is returned to the patient through the artery via an inlet line. P1, P2 and P3 represent changes in pressure at different sites of an ECMO circuitry: P1- Negative suction pressure from the inlet found between the inflow cannula and pump; P2- Positive ejection pressure between the outlet line of a pump and inlet of the oxygenator; P3- Positive ejection pressure of the outflow line, between the outlet of the oxygenator and the outflow cannula. P2-P3 represents gradient of the pressure pre- and post-oxygenator, an indicator of oxygenator's functionality [23].

The normal blood pump function depends on its inlet and outlet pressures represented by P1 and P3 respectively in Figure 1.3. The inlet pressure measures the pressure generated from venous blood draining from the patient through the inlet line while the outlet pressure records the pressure of blood returning to the patient via the outlet line. The suction exerted by pump is recorded as a function of negative pressure ranging from negative 100 to 300 mmHg. Under extreme negative pressure, cavitation (removal of dissolved gases such as oxygen and carbon

dioxide from blood) causes haemolysis in the associated tubing or pump head. Hence, it is important to measure the inlet pressure for the control of the pump speed.

On the other hand, the outlet pressure is expressed as a function of the positive pressure with an upper limit of 400 mmHg. The outlet pressure has no direct impact on the state of red blood cells; however, an extreme positive pressure generates excessive heat. Such increased heat production changes the integrity of blood tubing connectors which can affect the functionality of the whole pump system [24]. Over the years, although multiple types of blood pumps with improved functions had been developed, the roller and centrifugal pumps remain as the two most commonly used blood pumps in the ECMO circuits. Each of the roller and centrifugal pumps has its own advantages and disadvantages (Table 1.2) and the currently available studies aiming to compare their strengths remain inconclusive.

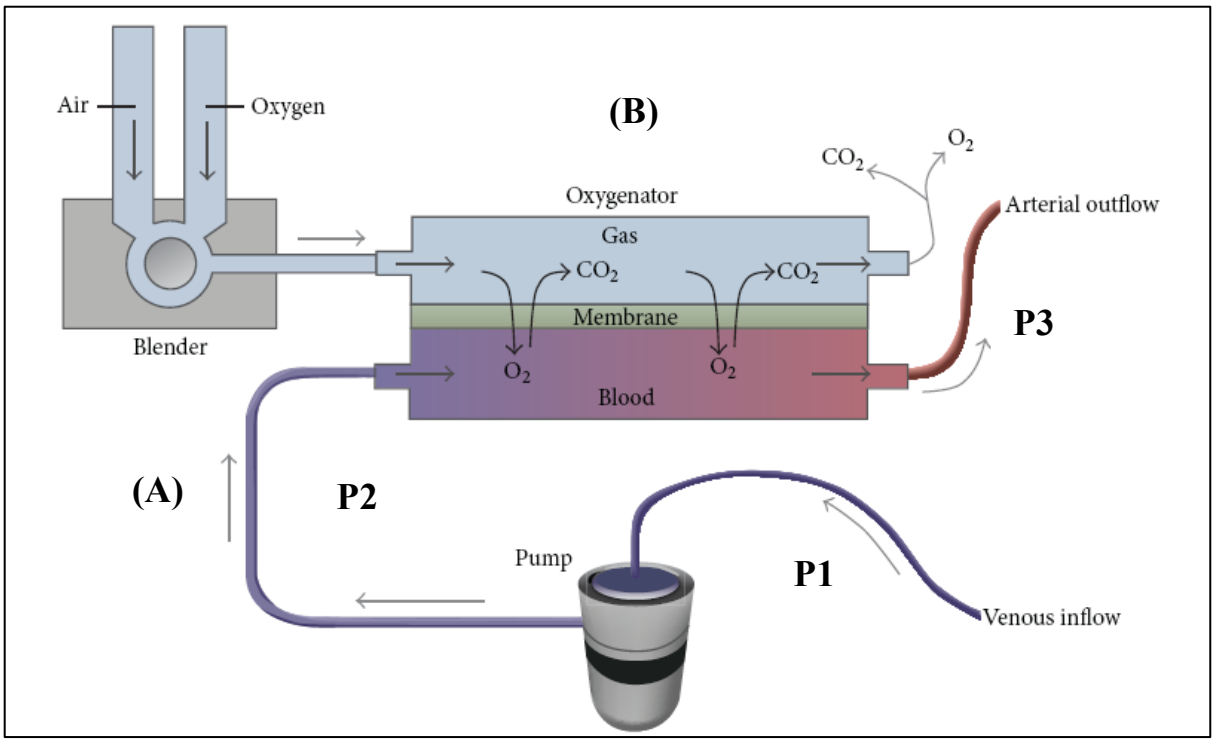


Figure 1.3 The role of the blood pump-sequential events that take place in an ECMO circuitry to maintain normal blood flow in the patient.

Table 1.2 Primary features, advantages and disadvantages of roller and centrifugal pumps.

Pumps	Roller	Centrifugal
Feature	- Also known as the 'semi-occlusive pump' - A positive displacement pump that generates direct suction on the venous catheter	- A spinning rotor generates centrifugal force to create flow and pressure that directs blood to a dedicated outlet, expressed as revolutions per minute (RPM)
Advantages	- Useful when low blood flow is required	- Small and light motor - Durable circuit components - Inlet pressure limited by RPM hence a low risk of circuit rupture
Disadvantages	- Big and heavy motor - Tubing in the pump head can wear or rupture - No limit of inlet pressure hence a risk of circuit rupture	- Presence of a fixed shaft and incorporation of seal within the blood path creates a stagnant blood zone, together with high RPM generates heat that could lead to: I.) Thrombus formation under low flow or the event of occluded outlet line II.) Cavitation and haemolysis when inlet line is occluded
Modifications made to improve their functions	- Incorporation of a collapsible bladder to reduce the speed or turn off the pump when the threshold suction is reached	- Presence of a hole in the center of pump head allows continuous washing of the area around the rotor to reduce stagnant area

1.1.3.2 Oxygenator

The oxygenator functions to replace the lung as the site of gas exchange in an ECMO circuit. Before the discovery of the semi-permeable silicone membrane by Kammermayer in 1957 [5], the direct contact between blood and oxygen resulted in haemolysis and plasma leakage. Incorporation of the semi-permeable membrane created a blood-gas interface that has allowed continuous flow of fresh gas and venous blood in a counter-current direction to generate a diffusion gradient that allows the addition of oxygen and removal of carbon dioxide from the blood. Over the years, various biomaterials such as the silicone membrane, polypropylene, polymethylpentene (PMP), polyvinylchloride, polyurethane and stainless steel have been used as the membrane for optimum gas exchange. Kolobow silicone and PMP are the two most commonly used materials for oxygenators with PMP being the more popular option due to its multiple advantages over the silicone membrane (Table 1.3).

The efficiency of gas exchange in an oxygenator depends on the diffusion gradient and surface area of the membrane in contact with gas. The maximum oxygenation capacity is defined as the amount of desaturated (75 %) blood that can be nearly saturated (95 %) per minute. The efficiency of oxygenation in an oxygenator is measured as the fraction of delivered oxygen (F_DO_2) and the rate of blood flow. F_DO_2 can be controlled by the gas blender attached to an oxygenator that mixes gas and oxygen that is able to provide a range of F_DO_2. Increase in blood flow increases the volume of blood surface exposed to the surface of membrane. Fresh gas flow to the membrane is controlled by a flow meter. An increase in fresh gas flow decreases the concentration of carbon dioxide in the fresh gas. Following this is an increase in diffusion gradient hence an increase in carbon dioxide elimination thus a reduction in partial pressure of carbon dioxide. The functionality of the membrane can therefore be determined by comparing pre- and post-oxygenator blood samples i.e. an increase in partial pressure of oxygen and a decrease in partial pressure of carbon dioxide [25].

Table 1.3 Comparisons of primary features, advantages and disadvantages of the silicone and polymethylpentene (PMP) membranes [21, 367].

Membrane	Silicone	Polymethylpentene (PMP)
Feature	- Standard oxygenator used for ECMO for near 50 years	- Made up of hollow fibre
Advantages	- Efficient for gas exchange	- Very efficient for gas exchange - Minimal plasma leakage - Low resistance to blood flow hence easy to prime - Well-suited for centrifugal pump - Low prime volume, one device for all size, can left assembled and crystalloid-primed, can support implementation within min - New model with integrated heat exchanger
Disadvantages	- Need different size for patients of different size - Requires separate heat exchanger - High resistance to flow- less suitable for centrifugal pump - High resistance- takes longer time and more difficult to prime, more difficult to transport	- Less efficient for gas exchange especially for carbon dioxide removal hence requires a higher sweep gas flow

1.1.3.3 Heat exchanger

Constant exposure to the artificial surface of the ECMO circuit causes heat loss from the ECMO patient. Hence, a heater unit is incorporated as part of the ECMO circuitry to maintain the body temperature within a specific range of between 33 – 39 °C for normal homeostasis.

1.1.3.4 Vascular cannula

Vascular cannulas are the catheters placed directly into the blood vessels for the purpose of ECLS to differentiate them from all of the other catheters. In general, there are two types of cannulas including the single- or dual-lumen cannulas that serve to provide the arterial and/or venous access for the patients according to their mode of ECMO (Table 1.4).

Table 1.4 General features of the cannulas.

<table>
<tr><th colspan="2" rowspan="2">Features</th><th colspan="2">Types of cannula</th></tr>
<tr><th>Single lumen</th><th>Dual-lumen</th></tr>
<tr><td colspan="2">Mode of ECMO and arterial/venous access</td><td>I.) Used for patients receiving VA-ECMO to provide arterial and venous access
II.) Used for patients receiving VV-ECMO with multiple venous access sites</td><td>Used to provide VV-ECMO support via a singular jugular venous access site</td></tr>
<tr><td colspan="2">Size and common property</td><td colspan="2">6 - 51 French (2 – 17 mm diameter) with wire-reinforced bodies to withstand pressure</td></tr>
<tr><td rowspan="2">Tip configurations</td><td>Single end-hole or a short fenestrated tip</td><td colspan="2">Arterial or venous vascular access</td></tr>
<tr><td>Long, multi-fenestrated flexible tips</td><td colspan="2">Jugular and femoral venous drainage</td></tr>
</table>

[VA-ECMO, veno-arterial extracorporeal membrane oxygenation; VV-ECMO, veno-venous extracorporeal membrane oxygenation]

1.1.4 Epidemiology of ECMO

1.1.4.1 Current clinical outcomes for ECMO patients

Over the years, although the advancement in ECMO-related technologies have resulted in an improved survival rate of the ECMO population particularly for patients with cardiac diagnosis in all age groups, the survival rate for neonates and children on ECMO with respiratory diagnosis has remained static from 2008 to 2018 (Table 1.5) [12]. Particularly, bleeding and clotting complications have been identified as the primary cause of the high mortality rate in the paediatric ECMO population [26]. For the overall ECMO population, the proportion of deaths resulted from bleeding and clotting complications has increased from 46 % in 1990 to 62 % in 2012 [27]. Specifically, the rate was increased from 48 % to 71 % for neonates and from 39 % to 84 % for children. Such high rate of mortality associated with bleeding and clotting complications observed for the paediatric ECMO population could be associated with the complexity of patient's existing condition which makes thorough understanding of respiratory, haemodynamic, metabolic and coagulation vital for the management of patients on ECMO.

An ECMO circuitry is made up of multiple compartments with flow rates that vary across different sites of the system. Shear stress and artificial surface originating from such system are able to induce changes in the coagulation system to either a hypercoagulable or hypocoagulable state [28]. Continuous consumption of plasma proteins and platelets associated with coating onto the ECMO circuitry can often lead to bleeding in the ECMO patients via different mechanisms. Meanwhile, together with shear stress and artificial surface originating from an intact ECMO circuitry, activation of the coagulation system in patients on ECMO is not limited to the circuitry but also to the patient's vasculature. This could lead to intravascular thrombus formation that could eventually develop into stroke. Further recruitment of platelets onto the existing clot in turns exacerbate the bleeding condition. These events often contribute

to the coexistence of bleeding and clotting in patients on ECMO. Such coagulative feature of ECMO thus makes ongoing anticoagulation a vital part of management for patients on ECMO. However, anticoagulation often exacerbates the already deranged haemostatic system in this population due to the existing coagulopathy such as post-surgical trauma or disseminated intravascular coagulation from sepsis. Since the survival rate and clinical outcome of neonates and children on ECMO vary based on the patient's age, disease aetiology and duration of ECMO; and a high cost is involved for their management; it is crucial to be able to identify the changes that take place in the coagulation system for effective management of ECMO patients so as to reduce the relevant clinical costs and most importantly to improve the clinical outcomes in this population.

Table 1.5 Age, diagnosis and survival rate for patients on ECMO from 2008 to 2018

Summary for the survival rate of paediatric patients from different age groups [12].

Category	Age	Diagnosis/ Type of ECMO	Survival rate (%)	
			2008	2018
Neonates	0 - 28 days	Cardiac	37	54
		Respiratory	66	69
Children	> 28 days and < 18 years	Cardiac	41	58
		Respiratory	52	67
Adult	18 years and over	Cardiac	25	45
		Respiratory	47	62

1.2 Haemostasis

Haemostasis is the physiological process of maintaining the well-regulated coagulation and anticoagulation activities to prevent excessive bleeding or clotting [29]. Under normal physiological condition, blood composed of the erythrocytes (red blood cells), leukocytes (white blood cells), platelets and plasma flows in the circulatory system to carry out three main important functions:

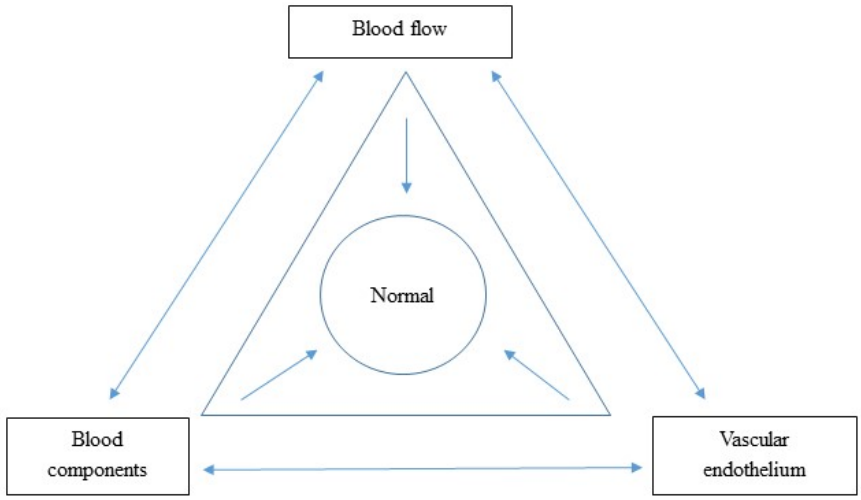
I.) Transportation: One of the most important roles of blood is for gas-exchange. This role is carried out by the erythrocytes with their unique oxygen-carrying ability. The oxygen is transported to the lung to be taken up by the body cells with concomitant carbon dioxide removal. Besides, blood can also carry nutrients, hormones and removes waste products from the body through liver, kidneys and the digestive system.

II.) Regulation: The blood with its buffering capacity (with the presence of various solutes) helps to maintain the body temperature and pH within a narrow range. This is mediated through multiple regulatory mechanisms to maintain the ideal body function.

III.) Protection: Our body is protected from coagulopathy and pathogens by the well-regulated role of different blood proteins and cellular components such as the platelets and leukocytes of the coagulation and immune systems.

Normal blood flow can be disrupted by the presence of foreign substance or a change in cellular interaction between blood and its surroundings for example blood vessels that could ultimately lead to a diseased state. In 1856, German Physician Rudolf Virchow proposed a well-known model known as the Virchow's triad (Figure 1.4) to demonstrate the inter-relationship between different components of the vascular system that could lead to thrombosis.

(A)



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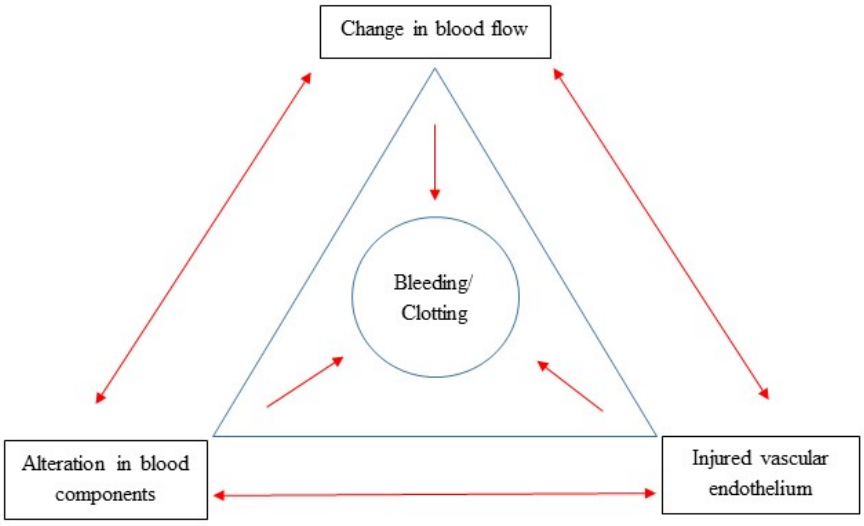

Figure 1.4 The Virchow's Triad.

The inter-relationships between blood flow, blood components and vascular endothelium are the important determinant of *in vivo* thrombus formation. (A) Under normal physiological conditions, all three components are maintained at a well-balanced condition to maintain normal blood flow. (B) Under pathophysiological condition, changes in any of the three components can contribute to bleeding or clotting.

1.2.1 The haemostatic system

In the modern or 'cell-based' model, haemostasis is viewed as a series of inter-related mechanisms that involves the interaction between the endothelium, platelets and various coagulation factors (Figure 1.5) [30]. In this model, the haemostatic system consists of primary, secondary and tertiary haemostasis as follows:

1.2.1.1 Cell-based model of haemostasis

1.2.1.1.1 Primary haemostasis

Primary haemostasis is the first event that takes place in the clotting process which involves the interaction between the platelets and endothelium [31]. Under normal physiological condition, platelets circulate freely in the bloodstream without attaching to the intact endothelium. The adhesion of platelets to the endothelial cells upon injury initiates the platelet plug formation, an important initiator of the coagulation process [32].

1.2.1.1.2 Secondary haemostasis

During initiation, coagulation factor VII (FVII) in the circulation reacts with the tissue factor (TF) found on the fibroblast and/or endothelial injury site. The resulting FVIIa/TF then activates both FX and FIX. FXa then reacts with FVa to activate prothrombin to produce thrombin which is a potent activator of platelets.

Thrombin generated on TF-bearing cells enhances the initial procoagulation signal by activating platelets. Upon activation, there is an increased expression of phosphatidylserine (PS) on the outer surface of platelets to provide an anionic surface, which is ideal for the the coagulant complex formation. Furthermore, platelets release FV from the alpha granules in which together with FVIII are activated by thrombin. The activated platelets, together with active form of FV and FVIII found on their surface initiate the subsequent procoagulant complex formation and the generation of a large amount of thrombin.

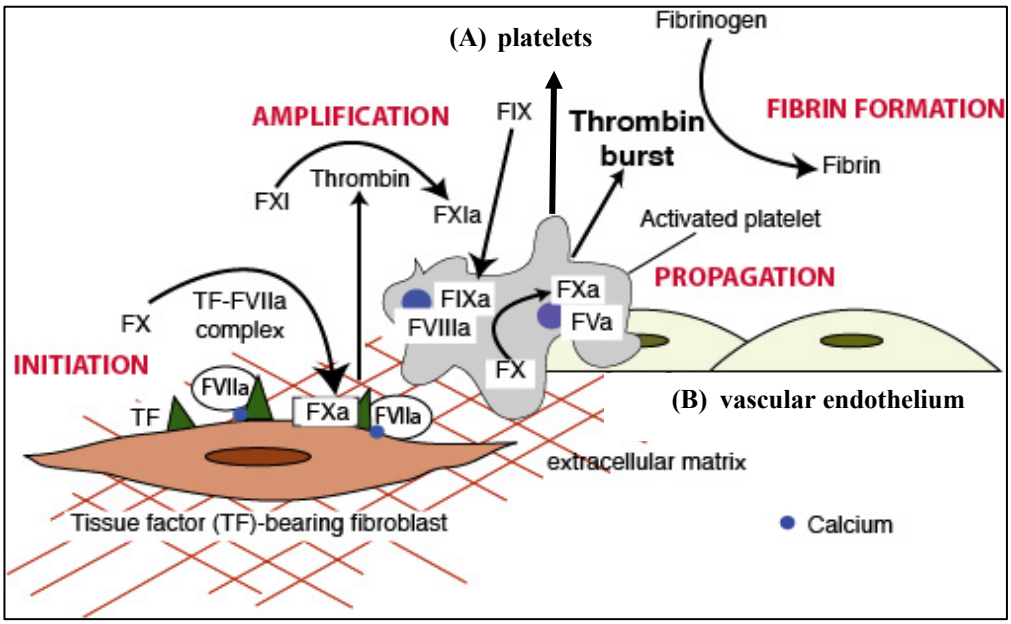

Figure 1.5 Cell-based model of coagulation.

The interactions between (A) platelets, (B) vascular endothelium and various coagulation proteins [33]. [FX, Factor X; FXa, Factor Xa; FVIIa, Factor VIIa; FIXa, Factor IXa; FVIIIa, Factor VIIIa; FXI, Factor XI; FX, Factor X; FIX, Factor IX; TF, tissue factor]

During the propagation phase, there is formation of the tenase (FVIIIa/IXa) and prothrombinase (FXa/Va) complexes, which are essential for thrombin burst, a vital process for further recruitment and activation of adjacent platelets for hemostatic plug formation. Meanwhile, there is the conversion of fibrinogen, a ligand that links the adjacent platelets to fibrin. This step is followed by the polymerization of fibrin strand that eventually forms the fibrin mesh that binds the platelets together and forms a stable blood clot.

1.2.1.1.3 Tertiary haemostasis

Activation of the fibrinolytic system dissolves the clot formed during the process of coagulation allowing blood vessels restoration. Plasminogen, the molecule involved in fibrinolysis is converted to plasmin by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Plasmin degrades the fibrin within the clot into soluble degradation products for re-utilization by the body.

1.2.1.2 Developmental haemostasis

Developmental haemostasis is a concept developed in late 1980s and early 1990s. This concept describes the physiological changes of the coagulation system with age across different stages of life, from neonates to children, adults and geriatrics [34, 35]. Previously, developmental haemostasis-related research has focused on the role of various plasma coagulation whereas age-related aspects of platelet function have only gained increased attention in recent years. Multiple studies have demonstrated the changes of coagulation proteins level with age and they are significantly different between children and adults [36-38]. On the other hand, very little is known for the changes in platelet function with age due to technical limitations such as to use only minimal sample volume for the younger age group e.g. neonates where sampling volume is often a limiting factor for research.

Platelets are equipped with multiple types of receptors and granules that are important for their phenotype and function. Despite having similar platelet numbers and volume [39], the differences in platelet structure and function between children and adults have been reported in multiple studies [40, 41]. In addition, platelet proteome and releasate in healthy children were also shown to be different from their adult cohort [49]. For receptors that are important for platelet adhesion and aggregation, although a 15 – 20 % reduction of platelet GPIIb/IIIa receptor (integrin αIIbβ3) was observed in neonates compared to the older children and adults, the level of von Willebrand factor (VWF) receptor (GPIb/X/V) was reported to be similar across all age groups [42-45]. Such reduction in the platelet surface receptors expression have been associated with platelet hyporesponsiveness seen for children compared to the adults.

In addition to the platelet surface membrane glycoproteins, the differences in platelet response to various physiological agonists were also reported between children and adults. Neonatal platelet aggregation in response to arachidonic acid was reported to be similar to adults whereas reduced platelet aggregation in response to adrenaline was associated with decreased number

of alpha adrenergic receptors [368, 369]. On the other hand, reduced activation in response to thrombin, TRAP and nitric oxide (NO) were reported for neonatal platelets [368-372]. Platelet hyporesponsiveness has also been associated with a lower level of marker important for platelet activation such as P-selectin (indicator of α-granule release) compared to the adults [46]. However, the reduced agonist-induced platelet granule release was associated with the immature signal transduction pathways but not the deficiency in granule contents [47, 48].

1.2.1.3 Important elements of the haemostatic system and their roles in haemostasis

A well-balanced haemostatic system depends on a series of inter-related mechanisms that involve the endothelium, platelets and various components of the coagulation system. Specifically, the interaction between these components during the initial stage of coagulation have been identified as the key determinant of the coagulation pathway.

1.2.1.3.1 Vascular endothelium

The vascular endothelium is a single layer of endothelial cells that form the inner cellular lining of blood vessels (arteries, veins and capillaries) and the lymphatic vessels that has a direct contact with all the cellular components in the circulation. The vascular endothelium has multiple physiological roles especially in haemostasis. Besides acting as an effective barrier between circulating blood components and subendothelial layers, the vascular endothelium constantly synthesise basement membrane and extracellular matrix where important coagulation proteins such as VWF, collagen, fibronectin, vitronectin can be found. In particular, the Weibel-Palade body is an endothelial-specific secretory organelle, the main producer of VWF. Specifically, VWF and collagen are important ligands for platelets during the initiation step of coagulation pathway [50]. In addition, the endothelium constantly release NO and prostacyclin which are the *in vivo* modulators of platelet activation.

1.2.1.3.2 Platelets

Under normal physiological conditions, haemostasis is initiated by binding of platelets to the injured endothelial wall, followed by further recruitment of platelets to form platelet plug, an important initiator of subsequent coagulation event that finally leads to stable clot formation. Platelets are the fragmentation products of the megakaryocyte that have an important role in the thromboregulatory pathway. Platelets have gained increased attention with recent advances that reveal their multiple roles that are not only limited to haemostasis but also inflammation and immunity [51, 52]. Upon stimulation by thrombopoietin (the main growth factor that controls platelets production), bone marrow produces megakaryocyte that sheds platelets from their cytoplasm. In recent years, lungs have been shown to be another major site of platelet production besides bone marrow [53]. Platelets circulate in the human body (normal range: 150-400 x 10^9 platelets/L) with a lifespan of approximately 7 days followed by sequestration in the spleen [54, 55]. In a resting state, platelet has a discoid shape with measurement of 2.0 - 5.0 μm in diameter and 0.5 μm in thickness [54-57] (Figure 1.6).

A unique feature of platelets is the absence of a nucleus. Over the years, although studies have showed that platelets are able to synthesise proteins from mRNAs, platelet physiology and function are largely-dependent on the existing proteins such as various receptors and their interactions with the corresponding ligands [361].

Upon activation, there is a series of changes in platelet morphology such as the shape and the presence of protrusions known as the filopods (Figure 1.7) followed by the release of various receptors or mediators that are vital for haemostasis. For this purpose, platelets are equipped with multiple types of storage organelles that include platelet-specific α- and dense granules and lysosomes where pre-synthesised molecules that are released only upon platelet activation can be found (Table 1.6).

The α–granule is the most abundantly found organelle in the platelet. The α–granules store multiple types of glycoprotein receptors that are expressed on the platelet surface (e.g. integrin αIIbβ3, GPIb/IX/V and (GPVI) receptors and P-selectin), adhesion proteins (e.g. fibrinogen and VWF) and coagulation factors (e.g. Factor XIII and antithrombin), the important regulators of various platelets physiological functions [58-61]. Dense granules, on the other hand, store adenosine diphosphate (ADP), serotonin, divalent cations which are the important molecules for amplification of platelet responses [62]. Platelet surface receptors including the integrin αIIbβ3and GPIb/IX/V receptors have also been found in the dense granule membranes [63]. Different from the α- and dense-granules, platelet lysosomes store various types of enzymes that are important for proteins, carbohydrates and lipid degradation [64]. However, very little is known for the physiological importance of platelet lysosomes except for their reported role in thrombus resolution [65].

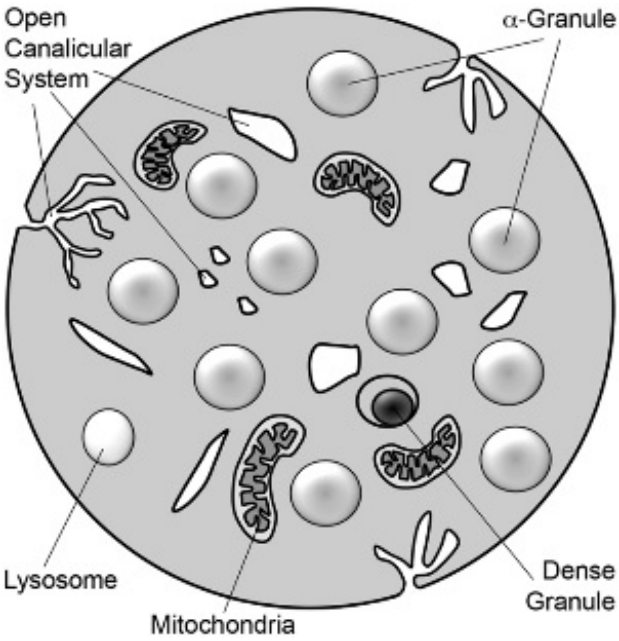

Figure 1.6 Schematic diagram of platelet structure.

Platelet is a discoid shape cell, mainly made up of the α-granule, dense granule, lysosome and mitochondria [66].

(A) (B**)**

Figure 1.7 Scanning electron microscopy of the resting and activated platelets.

(A) Resting platelets have a discoid shape. (B) Acivated platelets change shape and have protruding filopods that facilitate attachment of platelets to the contact surface [67].

Table 1.6 Platelets α- and dense granules contents.

Various contents for platelet α- and dense granules [62].

α-granule contents	
Adhesion molecules	P-selectin, von Wilebrand factor, thrombospondin, fibrinogen, integrin αIIbβ3, integrin αvβ3, fibronectin
Coagulation pathway	Factor V, multimerin, factor VIII
Fibrinolytic pathway	α_2-macroglobulin, plasminogen, plasminogen activator inhibitor 1
Chemokines	Platelet basic protein [platelet factor 4 and its variant (CXCL4) and β-thromboglobulin], CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL17, CXCL1 (growth-regulated oncogene-α), CXCL5 (ENA-78), CXCL8 (IL-8)
Immunologic molecules	B1H Globulin, factor D, c1 inhibitor, IgG
Growth and angiogenesis	Basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor 1, transforming growth factor β, vascular endothelial growth factor-A, vascular endothelial growth factor-C, platelet-derived growth factor
Other proteins	Albumin, α_1-antitrypsin, Gas6, histidine-rich glycoprotein, high molecular weight kininogen, osteonectin protease nexin-II (amyloid beta-protein precursor)
dense granule contents	
Ions	Calcium, magnesium, phosphorus, pyrophosphate
Nucleotides	Adenosine triphosphate, guanosine triphosphate, adenosine diphosphate, guanosine diphosphate
Membrane proteins	CD63 (granulophysin), lysosome-associated membrane protein 2
Transmitters	Serotonin

The primary role of platelets in coagulation which is for stable clot formation to stop bleeding depends on the well-regulated platelet receptor-ligand interaction in adhesion, aggregation and activation (Table 1.7). The receptors that are important for platelet function include: I.) GPIb/IX/V and GPVI receptors for platelet adhesion, II.) - integrin αIIbβ3receptor for platelet aggregation, III.) P-selectin, CD63 and activated integrin αIIbβ3 receptors, a group of indicators for platelet activation and IV.) P-selectin-PSGL-1 interactions for monocyte-platelet and neutrophil-platelet aggregates formation (Figure 1.8).

Table 1.7 Important platelet receptors in haemostasis.

Receptors and ligands important for platelet function (adapted from [68]).

Receptors	Ligands	Characteristics/Functions
GPIIb-IIIa	- fibrinogen - VWF - fibronectin - vitronectin - thrombospondin-1	- Most abundant receptors on platelets - Unique expression on platelets - Mediate platelet aggregation through cross-binding of divalent fibrinogen or multivalent VWF of adjacent platelets
GPIb/IX/V	- VWF - thrombospondin-1 - Mac-1 - P-selectin - α-thrombin	- Initiator and propagator of platelet aggregation which lead to thrombus formation - Important receptor for interaction between platelets and leukocytes
Protease activated receptors (PAR) - **PAR1** - **PAR4**	- Thrombin	Activated by thrombin-mediated cleavage of N-terminal to expose short peptide sequence for ligand binding within PAR
P2Y1 **P2Y12**	- Adenosine diphosphate (ADP)	ADP is an important autocrine activator of platelet activity. Binding of ADP towards the P2Y1 receptor induces reversible aggregation and further release of ADP from the dense granules whereas binding of ADP towards P2Y12 can trigger irreversible platelet aggregation
GPVI **α2β1**	- Collagen	Presence of both is vital for collagen-induced platelet aggregation
Thromboxane (TX) receptors	- Thromboxane A2	Thromboxane A2 is a strong platelet activator released as a by-product from the cyclo-oxygenase pathway. Cyclo-oxygenase pathway is inhibited by aspirin to prevent platelet activation.

[GPVI, glycoprotein VI; P2Y1, purinergic receptor P2Y1; P2Y12, purinergic receptor P2Y12; VWF, von Willebrand factor]

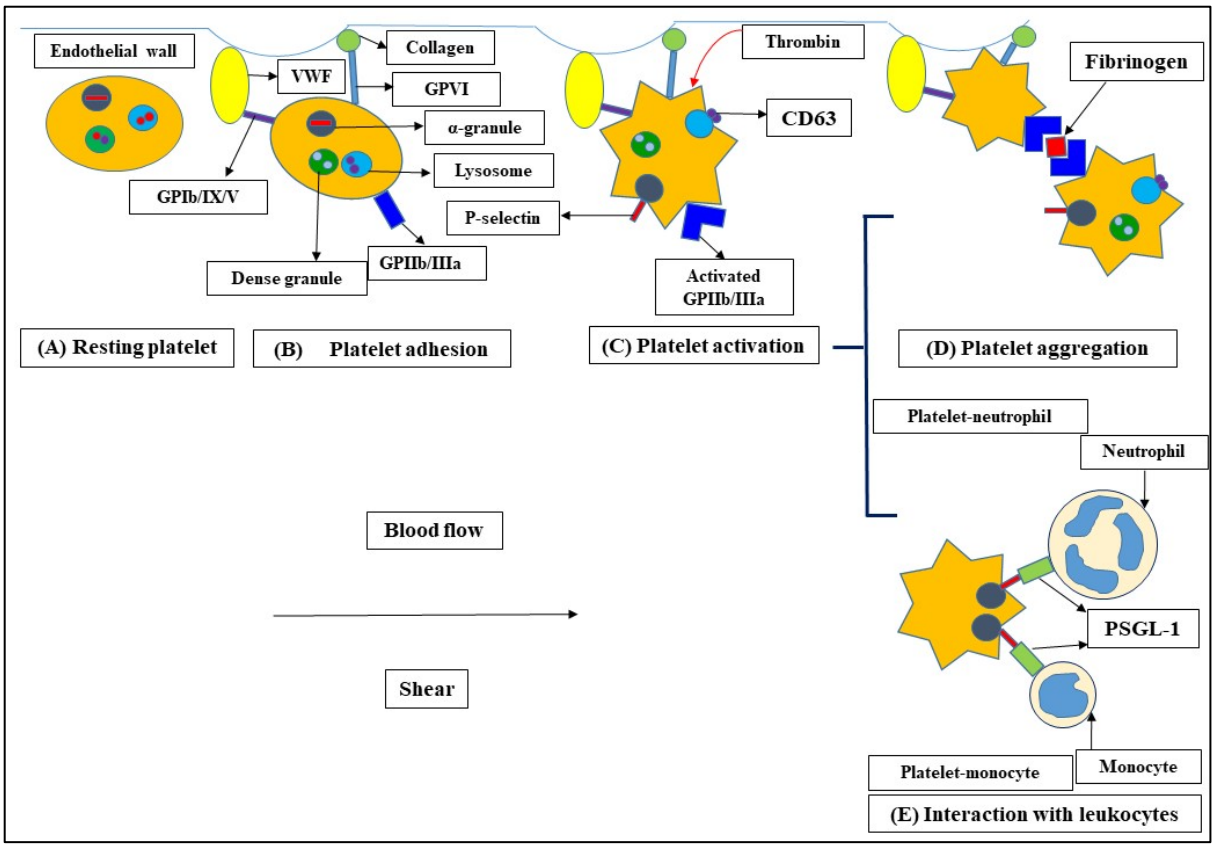

Figure 1.8 Key platelet-receptor ligand pair and their role in platelet function.

(A) Resting platelet maintains a discoid shape. (B) Platelet adheres to the endothelium mainly via the GPIb/IX/V-VWF and GPVI-collagen receptor-ligand pair interactions. (C) Upon activation, platelet changes shape accompanied by the activation of receptor and the release of various intragranular content. (D) Activated integrin αIIbβ3interact with fibrinogen and facilitate platelet aggregate formation. (E) Activated platelet can interact with monocyte and neutrophil to form heteroaggregates [69]. [GPIb/IX/V, glycoprotein Ib/IX/V; GPVI, glycoprotein VI; VWF, von Willebrand factor; PSGL-1, P-selectin glycoprotein ligand-1]

1.2.1.3.3 Platelets and shear stress

As one of the cellular components of blood, which is an environment with constant fluid motion, platelet function can be greatly affected by fluid dynamics. Shear stress, which is the force per unit areas between layers of material in contact, has been identified as the main mechanical force that determines platelet-mediated haemostasis and thrombosis [70]. Non-physiological shear stress (NPSS) induced platelet thrombus formation has been associated with various pathological conditions such as coronary artery stenosis and thrombosis [71, 72].

Changes in platelet morphology, reduction of platelet receptors and increased activation state have been associated with NPSS induced platelet dysfunction [73]. Specifically, NPSS induced platelet receptor shedding provides the mechanisms as followed: I.) down-regulation of the surface expression of platelet receptors, II.) loss of ligand binding, III.) reducing the surface density and IV.) affecting receptor cross linking and signalling that could contribute to platelet dysfunction [74]. Hence, NPSS which is often used in various settings of mechanical circulatory support (MCS) such as ECMO, CPB and ventricular assist device has been suggested to be the main cause of haemostatic dysfunction seen in patient on MCS [75, 76]. Specifically, the NPSS-induced platelet dysfunction may be mediated through platelet-specific changes that involve multiple platelet receptors of which their interactions with ligands had been shown to be shear- and activation-dependent (Table 1.8 and Table 1.9). Each of the receptor-ligand pair will be further discussed in details in the following section.

Table 1.8 Key receptor-ligand pair for platelet adhesion and activation.

Key Process/ Feature		(I) Resting platelet	(II) Adhesion		(III) Activation	
		- Discoid shape cell equipped with multiple surface receptors and storage granules	- Binding of platelets to surface in contact such as the endothelial wall		- Change in shape with extension of filopods for adherence and release of storage granule contents - Thrombin as the most potent activator	
Key Receptor		-	GPIb/IX/V	GPVI	P-selectin	CD63
Location		-	Surface		α-granule	Dense granule and lysosome
Ligand		-	VWF	Collagen	PSGL-1	integrin αIIbβ3-CD9 complex
Characteristic		-	Shear dependent receptor-ligand interaction		Released upon activation	
Receptor Expression	**Resting state**	-	Ubiquitously expressed		Absence or minimal	
	Changes upon activation	-	Down-regulation due to internalization	Up-regulation	Up-regulation	

[VWF, von Willebrand factor; PSGL-1, P-selectin glycoprotein ligand-1]

Table 1.9 Key receptor-ligand pair for platelet aggregation and interactions with leukocytes.

Key Process/ Feature		(I) Aggregation	(II) Interaction with leukocytes
		- Platelet-platelet interaction	- Heteroaggregate formation with leukocytes
Key Receptor		Integrin αIIbβ3	P-selectin
Location		Surface	α-granule
Ligand		Fibrinogen	PSGL-1 (on leukocytes)
Characteristic		Shear dependent receptor-ligand interaction	Released upon activation
Receptor Expression	**Resting state**	Ubiquitously expressed	Absence or minimal
	Changes upon activation	- Up-regulation - Confirmational change results in exposure of fibrinogen-binding site - Enhanced interactions with ligands via inside-out and outside-in signalling pathway	- Up-regulation - Increased heteroaggregate formation with leukocytes particularly monocytes and neutrophils

[PSGL-1, P-selectin glycoprotein ligand-1]

1.2.1.3.3.1 Important receptors, ligands and their interactions in platelet phenotype and function

1.2.1.3.3.1.1 GPIb/IX/V complex and their role in platelet physiological function

GPIb/IX/V is a non-covalently bound complex made up of GPIbα (CD42bα), GPIbβ (CD42bβ/CD42C), GPIX (CD42a) and GPV (CD42d) in the ratio of 1:2:1:1 [405]. Each of the GPIbα, GPIbβ, GPIX and GPV subunits is made up of 610, 181, 160 and 544 amino acid residues respectively [77-79]. GPIb/IX/V complex is a member of the leucine-rich repeat protein family. GPIb/IX/V complex presents at approximately 50,000 copies per platelet, the second most abundant receptor after integrin αIIbβ3. Absence or deficiency of the GPIb/IX/V complex causes Bernard-Soulier syndrome, the second most common platelet receptor-linked bleeding disorder [77]. The common features of individuals with Bernard-Soulier syndrome include prolonged bleeding time, enlarged platelets and thrombocytopenia [77].

In addition to its primary role in facilitating platelet adhesion to the subendothelial matrix, GPIb/IX/V complex can also regulate the procoagulant activity on the surface of the activated platelets and affects the activity of the other platelet receptors such as the integrin αIIbβ3via multiple signalling pathways. GPIb/IX/V complex can interact with multiple ligands such as Mac-1, P-selectin and α-thrombin for its adhesive property in relation to the haemostatic function. The main ligand for GPIb/IX/V is VWF where the ligand-specific binding site can be found on the GPIbα subunit.

<u>von Willebrand factor (VWF)</u>

The VWF is a multimeric form of the adhesive glycoprotein that can be found in the plasma and subendothelial matrix. Mature VWF comprises of 2050 residues with a molecular weight of approximately 275 kDa. VWF is synthesised in the Weibel-Palade bodies of the endothelial cells as ultra-large VWF (ULVWF), a disulphide multimer with the size of approximately 20,000 kDa. Upon stimulation, ULVWF is secreted and transported along with P-selectin that

serves to anchor it to the endothelial surface [80]. The release and activation of VWF into the plasma requires the proteolysis of ULVWF by ADAMTS-13 (a distintegrin and metalloproteinase with thrombospondin) [81]. This enzyme acts upon the motif Tyr 1605/Met1606 within the A2 domain of ULVWF [82]. Dysfunction or deficiency in VWF causes bleeding as seen in the von Willebrand disease. On the other hand, increased plasma level of ULVWF due to deficiency in ADAMTS-13 contributes to a prothrombotic state such as that seen in thrombotic thrombocytopaenic purpura. In addition to the endothelium, VWF is also released together with P-selectin from the α-granules of the activated platelets to promote platelet aggregation through the activation of integrin αIIbβ3 [83].

Shear stress, VWF and GPIb/IX/V

The interaction between VWF and GPIb/IX/V complex takes place at the A1 domain of VWF and the N-terminal globular domain of GPIbα, a region rich in leucine-rich repeats and anionic peptide sequence with tyrosine sulfate residues. Interaction between VWF and platelet GPIb-IX-V requires conformational changes of either the ligand and/or the receptor. *In vivo*, the presence of shear stress or immobilization of VWF onto collagen or the other components of the subendothelial matrix increase binding of VWF to GPIb/IX/V with high affinity [84]. At the arterial shear rates, a balance between ADAMTS-13-dependent conformational change in VWF multimers and the activation of VWF binding to GPIbα determines the initiation and development of thrombus. Such control mechanism prevents unwanted interaction between circulating plasma VWF and GPIbα, thus inhibiting pathogenic thrombus formation (Figure 1.9). *In vitro,* GPIb/IX/V-VWF interaction can be induced using modulators such as ristocetin (a vancomycin-like antibiotic) or botrocetin (a snake venom protein) under static conditions [366].

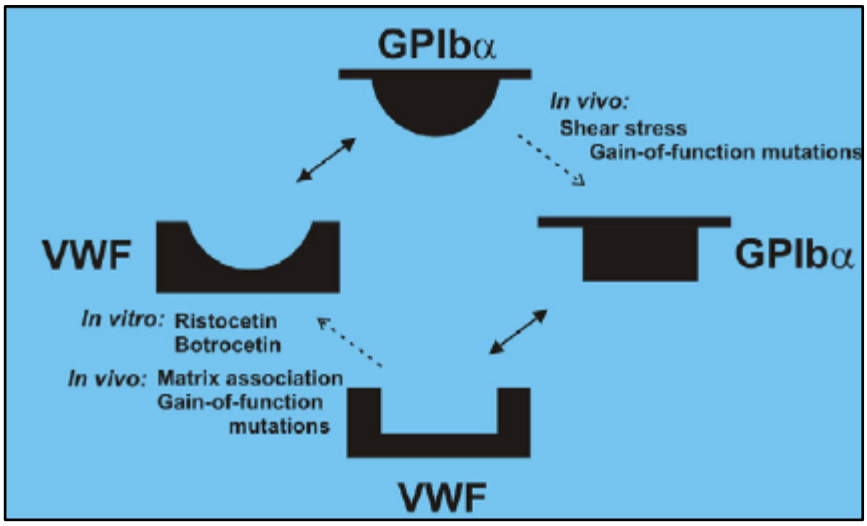

Figure 1.9 Interaction between VWF and GPIb/IX/V.

Activation of the receptor (GPIb/IX/V) and/or ligand (VWF) induce modifications required for their interactions [364]**.** [VWF, von Willebrand factor]

1.2.1.3.3.1.2 GPVI, collagen and their role in platelet physiological function

GPVI is the dominant receptor for collagen found exclusively in platelets (approximately 4000-6000 copies per platelet) and megakaryocytes. GPVI is a 60 – 65 kDa protein composed of 319 amino acids that belongs to the immunoglobulin (Ig) superfamily [365]. The structure of GPVI is detailed in Figure 1.10. Binding of collagen induces cross-linking of two GPVI complexes that leads to the activation of the GPVI/FcRγ-chain complex. Following this is the phosphorylation of the Immune Tyrosine Activation Motif (ITAM) by Src family kinases, Lyn and Fyn [85]. This generates signal that stimulates intracellular signalling through the activation of tyrosine kinase Syk that initiates platelet adhesion and aggregation [86]. Integrin α2β1 and GPVI are two types of receptors that are involved in the interaction between collagen and platelet [87, 88] with GPVI plays a dominant role in platelet activation..

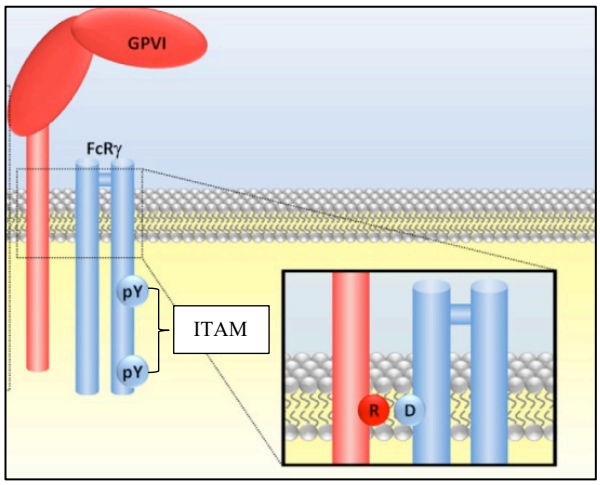

Figure 1.10 GPVI- FcRγ-chain signalling pathway.

Each GPVI is made up of 2 extracellular immunoglobulin domains linked by a single peptide strand. GPVI is linked to the Fc receptor (FcR)γ-chain on platelet surface through a salt bridge formed between an arginine (R) and an aspartate (D) locate within the transmembrane domains of the two proteins, respectively. Each FcRγ-chain is a covalently-linked homodimer with the presence of an immunoreceptor tyrosine-based activation motif (ITAM). FcRγ-chain is important in securing GPVI position on platelet surface as absence of FcRγ-chain prevents platelet activation by collagen (adapted from [87, 89]).

1.2.1.3.3.1.3 Integrin αIIbβ3and their role in platelet physiological function

Following adhesion, changes in platelet physiology resulting from the platelet activation promote further recruitment of platelets to initiate platelet plug formation. The platelet plug then serves as the surface for binding of coagulation factors and coagulation complex formation. Such role of platelets depends on their ability to aggregate, a mechanism mediated by integrin αIIbβIII. Due to its important role in platelet function, integrin αIIbβ3 antagonists (abciximab, eptifibatide, and tirofiban) have been used as the antiplatelet agents for cardiovascular diseases [90].

Integrin αIIbβIII (also known as GPIIb/IIIa) is the most frequently occurring receptor on the platelet surface, at approximately 80,000 - 100,000 copies [91]. Absence or deficiency of the integrin αIIbβ3 causes Glanzmann thrombasthenia. The integrin αIIbβ3 is a heterodimer composed of a αII and βIII subunit linked through non-covalent bond (Figure 1.11). The αIIb subunit (GPIIb or CD41) contains 1008 amino acids while the mature βIII unit (GPIIIa or CD61) is composed of 762 amino acids [92, 93]. The optimal role of integrin αIIbβ3 in platelet aggregation relies on its capacity to transit from low-affinity to high-affinity state, a process vital for integrin αIIbβ3 response to extracellular ligands [94, 95]. Such activation of integrin αIIbβ3 is vital for its binding towards various soluble ligands that are responsible for its physiological function i.e. platelet aggregation.

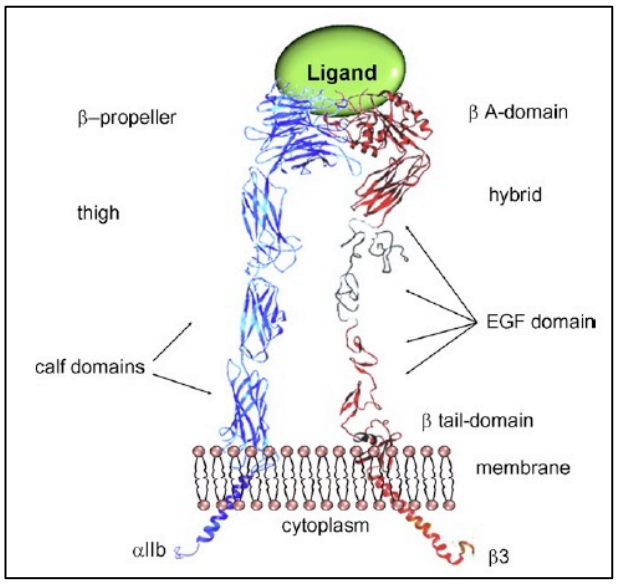

Figure 1.11 The integrin αIIbβ3.

An integrin αIIbβ3 is made up of the αIIb(blue) and β3(red) subunits [96].

Interaction of integrin αIIbβ3 with ligands- inside-out vs. outside-in signalling pathway

Interaction between the integrin αIIbβ3 and its agonists including VWF and collagen initiates a series of intracellular signalling events that converge at the cytoplasmic tails (CT) of integrin αIIbβ3. Such interaction activates and transforms integrin αIIbβ3 into a high affinity state via the inside-out signalling pathway (Figure 1.12) [94, 97]. Specifically, binding of collagen to GPVI leads to the mobilization of Ca^{2+} and activation of protein kinase C (PKC) [86] while integrin αIIbβ3 activation via VWF-GPIb/IX/V interaction is shear-stress dependent (Figure 1.13) [70, 98].

Besides VWF and collagen, mediators such as thrombin, ADP and thromboxane A_2 are also the agonists for integrin αIIbβ3 activation via specific pair of receptor-ligand interactions detailed as follows. For example, activation of integrin αIIbβ3 by thrombin is mediated through the protease-activated receptor [99, 100]. In relation to the ADP receptors, $P2Y_1$, $P2Y_{12}$ and $P2X_1$ are required for platelet activation. Particularly, $P2Y_1$ is involved in the change in shape and calcium mobilization while $P2Y_{12}$ is vital for integrin αIIbβ3 activation and stabilization of platelet aggregates. Lastly, the two isoforms of thromboxane A2 receptor (TXA_2) that can be found in the platelets are TPα and TPβ [101]. All of the agonists mentioned above interact with the platelets via the G-protein mediated signalling pathway.

Activation of integrin αIIbβ3 via the inside-out signalling pathway enhances its affinity towards soluble ligands such as fibrinogen, VWF, fibronectin and thrombospondin [102]. Integrin αIIbβ3 recognizes Arg-Gly-Asp (RGD) found in most of its ligands and the two most important ligands for integrin αIIbβ3, fibrinogen and VWF have RGD in their protein sequence [103, 104]. Binding of integrin αIIbβ3 to the divalent fibrinogen and multivalent VWF allows cross-linking of two different platelets [94, 97]. This bridging process promotes further platelets recruitment for the aggregate formation.

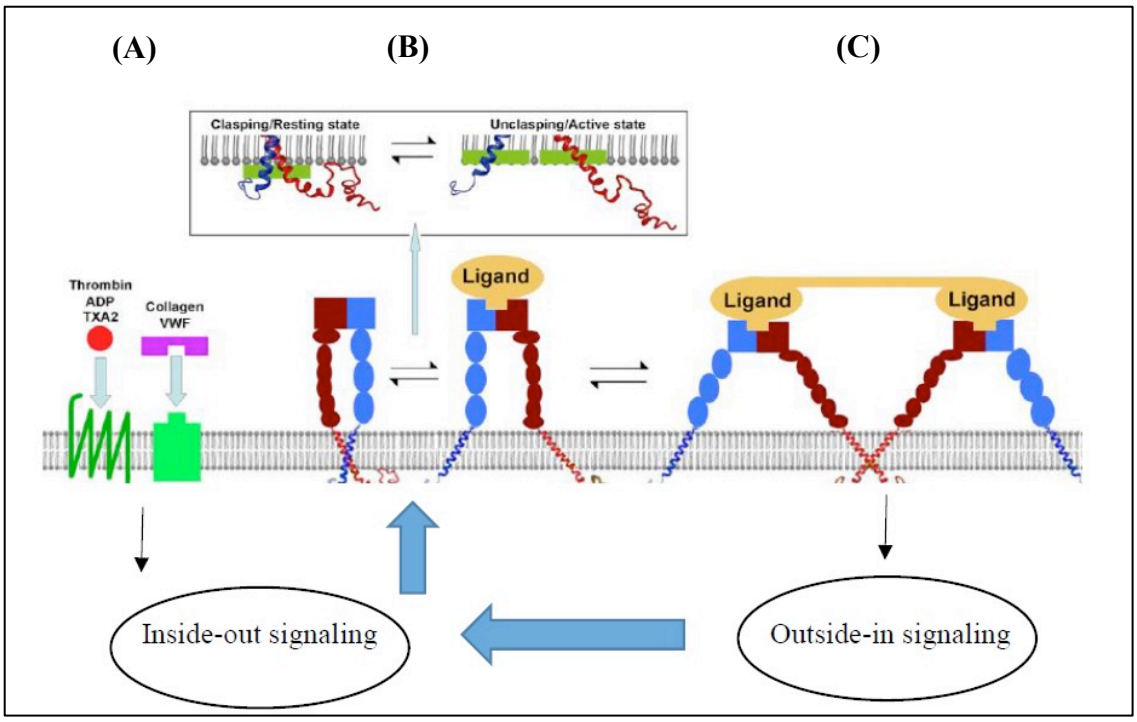

Figure 1.12 Inside-out vs outside-in signalling pathway of integrin αIIbβ3.

(A) Binding of agonists such as ADP and collagen induces inside-out signalling via various pathways that reach the cytoplasmic tail of αIIbβIII induces (B) conformational change of αIIbβIII that converts from an inactive to active state that allows it to bind soluble ligands such as fibrinogen. (C) This in turns generating outside-in signalling that further amplifies the inside-out signalling. [ADP, adenosine-diphosphate; TXA_2, thromboxane-A2; VWF, von Willebrand factor]

Interaction between the activated integrin αIIbβ3 and ligands also induce integrin-specific outside-in signalling pathway (Figure 1.13). Ligand binding leads to the conformational changes of CT enabling interaction with cytoskeletal and signalling proteins. Specifically, binding of fibrinogen to integrin αIIbβ3 is vital for activation of kinases from the Src and Syk family [105, 106]. Studies in knock-out mice deficient of these kinases demonstrate their importance in spreading and contraction of platelets (mechanisms essential for platelet activation) and irreversible aggregation, a step vital for stable clot formation [107].

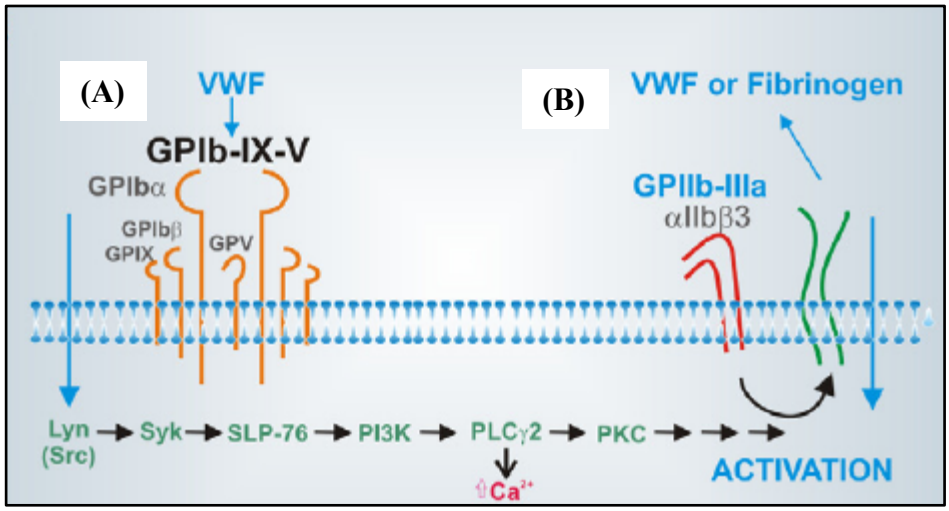

Figure 1.13 Interaction between GPIb/IX/V, VWF and fibrinogen.

Binding of (A) VWF to GPIb/IX/V induces (B) activation of integrin αIIbβ3 followed by conformational change that allows it to bind with its ligand such as VWF and fibrinogen with increased affinity. [PI3K, phosphoinositide 3 kinase; PKC, protein kinase C; PLCγ2; VWF, von Willebrand factor]

1.2.1.3.3.2 Platelet activation

Changes in various signalling proteins, particularly those involved in the activation of cytoskeleton-dependent processes can stimulate structural and conformational changes in platelets. This induces platelet activation and initiates contraction and spreading of platelets, a process vital for further amplification of platelet adhesion and aggregation. The next event is the elongation of filopods with concomitant change in charge of the surface membrane. In an inactive state, the anionic phospholipid phosphatidylserine (PS) only presents in the inner leaflet of the plasma membrane of platelets. Upon activation, immediate translocation of PS to the outer leaflet provides the anionic surface for optimal binding of coagulation factors and coagulation complex formation [108].

Subsequently, there is release of the storage granule content, a dominant event that signifies platelet activation. The surface of each platelet is covered with the membrane infoldings known as the open canalicular system (OCS), a network of selective-permeable membrane channels. The OCS serves as the source of membrane for the fusion of granules and cells spreading in the event of platelet activation. Upon activation, clotting factors such as I.) Factor V [109], II.) platelet activators e.g. factor-4 (PF-4) and beta-thromboglobulin (β-TG) [110], and III.) receptors e.g. P-selectin, GPIb/IX/V and integrin αIIbβ3 are released and amplify the coagulation process.

Among the content of platelets, the release of P-selectin, a membrane bound glycoprotein receptor from the α-granule is one of the most well-recognized platelet-activation dependent events. P-selectin (also known as CD62P and previously known as PADGEM or GMP-140) is a type-I membrane protein with an N-terminal C-type lectin domain. In addition to the α-granule, P-selectin can also be found in the Weibel-Palade bodies of the endothelium. PSGL-1 is the receptor for P-selectin that can be found on the neutrophils, monocytes, and subclasses of lymphocytes. PSGL is also present in the platelets for the binding of P-selectin found on the

endothelial cells. P-selectin-PSGL is the main receptor-ligand pair responsible for the monocyte-platelet interaction and essential for the initial tethering and rolling of leukocytes on the surfaces with P-selectin e.g. endothelium. Such interaction promotes fibrin formation and thrombus growth via procoagulant TF found on the activated platelet surface.

P-selectin had been used as the dominant marker for platelet activation. However, studies showed that the activated platelets lose their P-selectin rapidly after degranulation while remaining circulating and functional as an activated platelet [111, 112]. Thus, the platelet surface P-selectin had a limited role in being used for the detection of circulating degranulated platelets. Hence, new markers have been identified as the better indicators of platelet activation such as PAC-1 (activated form of integrin αIIbβ3) and monocyte-platelet aggregates. In addition to the above, CD63, a membrane protein found in the dense-granule and lysosomes which are expressed only on the activated platelets has also been proposed as an activation marker. CD63 regulates platelet spreading and phosphorylation on the immobilized fibrinogen without affecting platelet adhesion. Although it is more resistant towards proteolysis than P-selectin, the presence of CD63 at a lower concentration may require greater extent of platelet activation for it to be detected [113].

In addition to the various receptor-ligand interactions, recent study shows that activated platelets can also mediate thrombus formation via increased platelet-derived microparticle (PMP) release [114, 115]. PMPs are vesicles made up of fragmented platelet membrane with size of 0.1 - 1.0 μm and activated platelets-associated PMP release is a calcium-dependent event. PMP act as the surface for coagulation complex such as ‘tenase’ and ‘prothrombinase’ formation mainly involve FVIII [116] and TF [117]. Material-induced PMP generation has been found in the CPB [118] and ECMO setting and demonstrated good correlation with increased platelet-leukocyte aggregates formation [119, 120].

The extent of platelet activation varies depending on the type of agonists and their ability to initiate the calcium-dependant signalling pathway. Thrombin, with its ability to initiate spontaneous and powerful surge in cytosolic Ca^{2+} concentration at concentrations as low as 0.1 nM [121] has thus been recognized as the most potent activator of platelets [122, 123].

Thrombin and platelet activation

Thrombin is a serine protease derived from its precursor prothrombin that converts fibrinogen to fibrin, the final step for stable clot formation [121-123]. Protease activated receptors (PAR) are the main group of receptors responsible for thrombin-induced platelet activation, with four different types of PAR being identified including PAR1, PAR2, PAR3 and PAR4 [123]. The features of PARs are summarized in Table 1.10.

Table 1.10 Types of protease activated receptors (PAR).

The types and features of PAR [124].

Protease activated receptors	Feature
PAR 1	main thrombin receptor found in human and primates but not in mice and the other animals
PAR 2	found on the endothelium, neutrophils, smooth muscle cells, and epithelial cells but not platelets
PAR 3	can only be found in mouse but not human platelets
PAR 4	low-affinity thrombin receptor that can form a heterodimer with PAR 1

[PAR, protease activated receptors]

PAR1 is the first receptor identified for thrombin and is activated when thrombin cleaves its N-terminal exodomain at a single specific cleavage site at the amino acid sequence of LDPR ↓ SFLLR [125, 126]. Such cleavage by thrombin generates the tethered ligand SFLLRN which is also known as the thrombin receptor activating peptide (TRAP), a strong platelet agonist [122, 125, 126]. SFLLRN then undergoes a conformational change and binds intramolecularly to the body of the receptor and initiates transmembrane signalling. The SFLLRN can activate PAR1 without thrombin [122] and hence has been synthesised synthetically (also known as 'TRAP-6') and widely used to mimic the *in vivo* effect of thrombin on platelets.

In addition to PAR, the potential involvement of the other platelet receptors in thrombin stimulation of platelets had also been investigated and an important receptor is the GPIb/X/V complex. The importance was flagged by the observation that individuals with Bernard-Soulier syndrome (absence of GPIb in platelet) had reduced platelet aggregation in the presence of thrombin [127]. A high-affinity binding site for thrombin was then located to the GPIbα residue, which is also the main binding site of VWF [128]. This observation was further supported by studies where removal of the extracellular domain or direct blockade of the GPIbα residue reduced the response of platelets to thrombin [129-131]. It was later shown that the binding of thrombin with GPIb facilitates the cleavage of PAR1, a main step of PAR-mediated platelet activation by thrombin [132]**.** Such synergistic effect of dual thrombin receptor i.e. PAR and GPIb activation may thus contributing to the role of thrombin as the most potent platelet agonist. Besides, the interaction between GPVI and polymerized fibrin was also found to be able to amplify thrombin generation and promote recruitment of circulating platelets to existing clots [363].

Upon stimulation by thrombin via the PAR receptor, local platelet activation can induce further platelet recruitment hence supporting thrombus formation and the release of platelet-derived agonists including ADP, serotonin and thromboxane A_2 [375]. Concomitantly, thrombin can also induce the release of platelet inhibitor NO and prostacyclin from the endothelium and NO from the activated platelets. Such release of NO prevents platelet adhesion to the endothelium and platelet activation hence acting as the negative feedback mechanism for thrombus formation and propagation [373, 374].

1.2.1.3.4 Interaction of platelets with leukocytes and their role in inflammation

Leukocytes are a vital cellular component of the inflammatory response, the important defence mechanism of our body's immune system. Under normal physiological condition, most leukocytes present freely in circulation. Studies of whole blood in healthy human adult showed that approximately 5 - 10% of leukocytes form aggregates with platelets [133, 134]. However, such interaction between leukocytes and platelets is transient and under the constant regulation by various *in vivo* antiplatelet agents such as nitric oxide, matrix metalloproteinases and prostaglandin [135] and most importantly, shear stress [136].

In response to the pathological conditions such as in the event of endothelial disruption, extravasation of leukocytes allows recruitment of various type of leukocytes to the site of injury for their multifunctional roles where the main one being to combat against pathogens thus creating an ideal environment for wound healing. This multiple-step process is mediated by various types of molecules found on the exposed subendothelial layer that serves not only as the binding surface of platelets (for initiation of coagulation process hence to stop bleeding) but also for leukocytes via both the direct i.e. leukocyte-endothelial and indirect i.e. leukocyte-platelet (LPA) interactions. Interaction between leukocytes and platelets under such condition is often irreversible and can lead to the activation of leukocytes. Following this is further recruitment of leukocytes with concomitant recruitment and activation of platelets in the

circulation. In healthy adults, P-selectin on the activated platelets and PSGL-1 on the leukocytes has been suggested as the main receptor-ligand pair involved for LPA formation. On the other hand, a P-selectin independent mechanism had been proposed to be responsible for the elevated monocyte-platelet aggregates formation seen in healthy children compared to the adult cohort [137].

Interaction between platelets and various leukocytes including monocyte-platelet aggregates (MPA), neutrophil-platelet aggregates (NPA) and lymphocyte-platelet aggregates have been implicated in various thrombotic diseases such as artherosclerosis [138-140]. Although their roles and the underlying mechanisms in these pathophysiological processes remain elusive, the procoagulant characteristic of LPA found on the *in vivo* site of local injury [139] has been associated with the role of TF, an integral membrane protein that acts as the co-factor for FVII [139, 141]. The resultant FVIIa/TF complex increases the affinity of FVIIa towards FIX and FX and initiates the clotting process. The origin of surface-bound TF on leukocytes remains elusive, however, the interaction between leukocytes and platelets such as the formation of LPA has been shown to be an important vehicle of surface-bound TF expression found on the leukocytes via P-selectin-PSGL-1 interaction [141].

Interaction between leukocytes and platelets via P-selectin-PSGL-1 receptor-ligand pair stimulates downstream mechanisms and promote firm adhesion between platelets and leukocytes via I.) direct attachment of integrin receptor Macrophage antigen-1 or Mac-1 (CD11b/CD18) to GPIb or II.) indirect attachment to GPIb-bound high molecular weight kininogen and/or fibrinogen bound on to integrin αIIbβ3 [142] [52, 143, 144]. Mac-1, a receptor that mediates the adhesion of leukocytes to and hence determine their interaction with the endothelium and platelets is up-regulated upon leukocyte activation [145]. CD40 ligand (CD40L) is a thromboinflammatory molecule from the member of the tumor necrosis factor

family that interacts with Mac-1. Similar to P-selectin, CD40L is only released from the intraplatelet store upon activation via the integrin αIIbβ3 outside-in signalling pathway [146].

Specifically, platelet activation (as indicated by increased P-selectin and PAC-1 binding) was found to have direct correlation with MPA formation using whole blood flow cytometry method [147, 148]. MPA have also been proposed to be a sensitive marker of *in vivo* platelet activation. Despite both monocytes and neutrophils showing significant association with platelet activation, MPA has been a preferred indicator of platelet activation due to the higher tendency of monocytes to form aggregate with the activated platelets and the longer half-life of MPA than NPA in the circulation [148].

1.3 Platelets, ECMO and activation of the haemostatic system

Under the normal physiological condition, haemostasis is initiated by the binding of circulating platelets to the subendothelial layer, followed by a series of events that leads to stable clot formation, which in turn serves to prevent further blood loss. In the ECMO circuitry, changes in the haemodynamics and structure originating from shear stress and artificial surfaces different to that of an *in vivo* circulatory system can activate the coagulation system and induces clot formation in both the circuit and the patient and often lead to coagulopathy in ECMO patients. Platelets, as the core element of the coagulation system have been proposed to be the key determinant of the deranged haemostatic system in the ECMO population. In an intact coagulation system, platelet adhesion and activation are mainly dependent on the interaction between platelets and the subendothelial proteins such as VWF and collagen. Besides fibrinogen, VWF and fibronectin can also absorb to the artificial surface but they have minimal effect on mediating platelet adhesion [149, 150]. However, increased surface area of subendothelial layer originating from multiple wounds such as that in cannulation, where these mediators are commonly found, the pathophysiological state of patients on ECMO often promotes coagulopathy by itself.

ECMO-induced coagulopathy (EIC) is the pathophysiologic state of haemostasis in an individual on ECMO where the exposure of platelets to the foreign surface and mechanical force (e.g. turbulence in the oxygenator) contribute to excessive bleeding and/or clotting events. These further exacerbate the already deranged haemostatic system in the critically ill ECMO patients with existing coagulopathy and contribute to the high morbidity and mortality rates in this population (Figure 1.14) [151]. Development of EIC involves a series of inter-related events from protein adsorption, adhesion of cellular components particularly platelets and finally activation of the coagulation cascade and complement system that eventually contribute to clot formation.

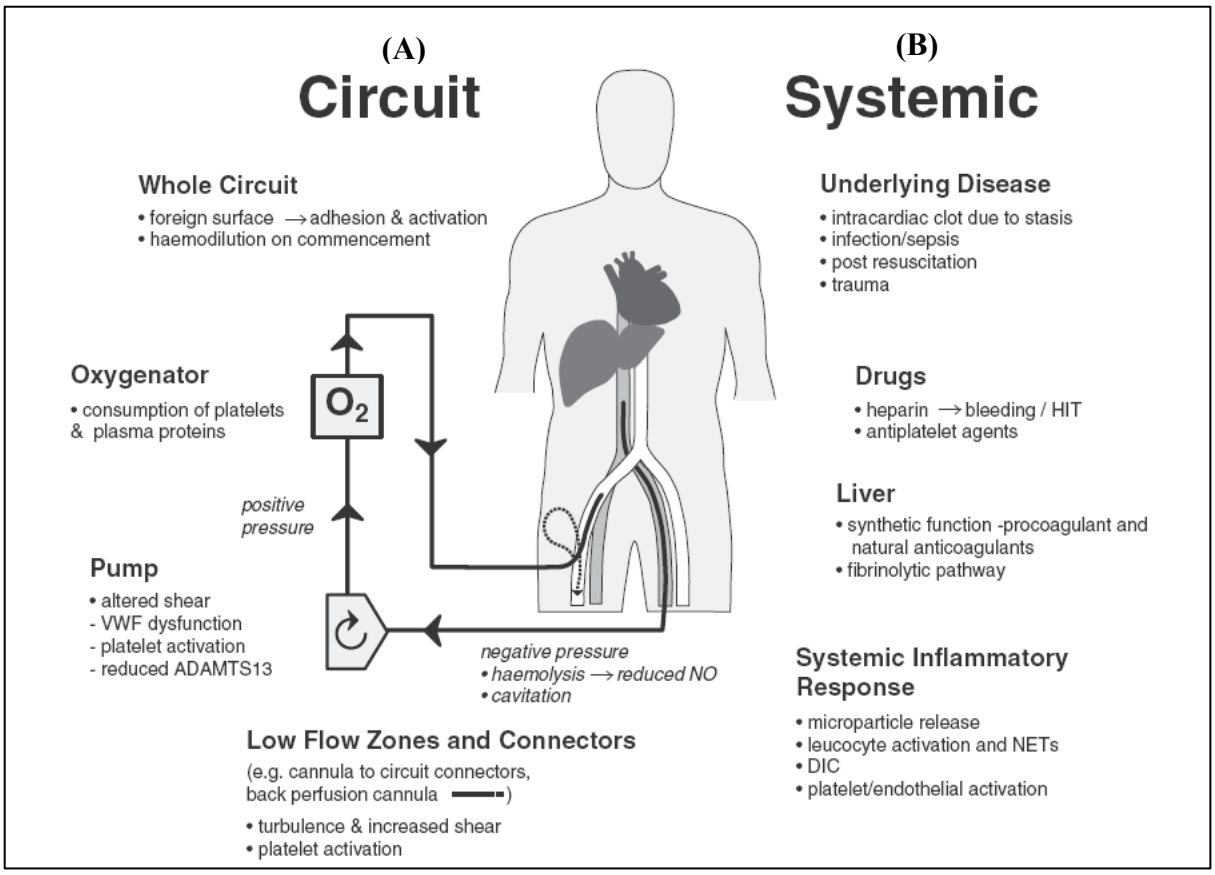

Figure 1.14 Potential effects of ECMO on the coagulation system.

Various factors originated from the (A) circuit (mechanical effect) and/or (B) systemic (patient-dependent) levels that can contribute to the activation of the coagulation system and lead to coagulopathy in patients on ECMO [152]. [ADAMTS13, A disintegrin and metalloproteinase with thrombospondin type I motif, 13; DIC, disseminated intravascular coagulation; HIT, heparin-induced thrombocytopenia; NETs, neutrophil extracellular traps; NO, nitric oxide; VWF, von Willebrand factor]

1.3.1 Pathogenesis of coagulopathy in ECMO

1.3.1.1 Platelets and their interaction with the haemostatic system in the setting of ECMO

Platelets and coagulation proteins

Upon exposure of blood to the ECMO circuitry, there is immediate coating of protein onto the artificial surface. This protein layer serves as the base for various receptor-ligand interaction between platelets and coagulation proteins to take place which then contribute to the clot formation [13]. Among the different type of coagulation proteins, fibrinogen is one of the first plasma proteins to be coated onto an artificial surface [153]. Fibrinogen interacts with the platelets via integrin αIIbβ3and initiates platelet adhesion and aggregation [154, 155]. This is followed by fibrin (cross-linked fibrinogen polymer) formation that serves as the base for the platelet aggregate formation in a thrombus [61]. Circulating platelets require conformational changes to bind to fibrinogen. However, surface-bound fibrinogen has been shown to have altered structure that has the potential to bind the resting platelets through high affinity interaction [156].

In addition, the affinity of platelets towards different coagulation proteins can be affected by local flow dynamic. For example, a low to medium shear rate promotes the adhesion of platelets to surface-bound fibrinogen while a higher flow rate promotes platelets adhesion to VWF [157]. To prevent platelet adhesion, the ECMO circuit is pre-coated with an inert protein such as albumin. However, the non-thrombogenic effect of albumin can only be achieved with at least 98 % of the surface coated by albumin whereas coverage of surface as little as 2 % by fibrinogen is shown to be sufficient for the initiation of platelet activation [158]. Concomitantly, the attachment of the other coagulation protein such as FXII and FXI from the contact activation pathway can further accelerate thrombus formation via the generation of thrombin, a potent activator of platelet activation.

1.3.1.2 Platelets and leukocytes

Patients that require ECMO for life support are often those who are in a critical condition. Together with an ongoing need of cannulation, the large surface area of a disrupted endothelial layer such as those originating from the multiple surgical wounds often subjects this population to continuous inflammatory state. LPA formation is associated with the development of biomaterial-associated thrombosis such as in the extracorporeal circuitry [159, 160] through various mechanisms [160-168], including:

I.) Up-regulation of membrane receptors:

- CD11b which facilitates the adherence of leukocytes to adsorbed fibrinogen
- PSGL-1 to interact with P-selectin on the activated platelets and form leukocyte-platelet aggregate
- TF expression as the initiator of coagulation

II.) Increased release of the inflammatory mediators that induce further recruitment and the activation of leukocytes and platelets

III.) Induction of oxidative burst followed by the release of oxidative products such as hydrogen peroxide and superoxide that have cell-damaging effects and are able to neutralize anticoagulant protein

IV.) Increased adhesive capacity to foreign surface e.g. endothelium

Modification of platelet function induced by the changes in the ECMO system creates a favourable environment for the interaction between platelets and different components of the haemostatic system such as the coagulation proteins and leukocytes and promote clot formation within the ECMO circuits. Activation of different parts of the coagulation system as discussed below have been proposed as the main pathways that lead to coagulopathy in the ECMO patients.

1.3.1.3 Activation of the coagulation cascade and complement system in patients on ECMO

1.3.1.3.1 Contact activation (intrinsic) pathway

Contact activation (intrinsic) pathway has been recognized as the primary contributor of thrombosis in the setting of artificial surfaces such as that in an ECMO system. Despite its indirect role in physiologic hemostasis, activated factor XII (FXIIa) of the contact system was found to play a significant role in the development of pathologic thrombosis as indicated by the FXII-deficient animal model which was protected from thrombosis [169]. Furthermore, an inhibitory antibody targets on FXIIa was shown to exert thromboprotective effect on the rabbit model of ECMO [170].

1.3.1.3.2 Tissue factor (extrinsic) pathway

In an intact coagulation system, FVII in the circulation reacts with TF found on the fibroblast and/or endothelial injury site. In patient on ECMO, persistent cannulation and wound originated from the underlying disease such as cardiac surgery or others allows continuous exposure of blood to the subendothelium hence providing the source of TF. In addition, the extrinsic system can also be activated by the increased inflammatory state under ECMO. This is mediated through the various roles of leukocytes via direct and/or indirect mechanism by complement activation as discussed below.

1.3.1.3.3 Complement activation

The complement system plays an important role in non-specific immune response for the elimination of foreign substances via acute inflammatory response. Similar to the coagulation cascade, the complement system is made up of a series of pro-enzymes that undergo series of proteolytic cleavage events for their activation. The activation of complement proteins is initiated via three different pathways i.e. the classical, alternative and lectin pathways. Studies showed that activation of the complement system takes place in various type of extracorporeal

circuitry [171, 172]. In the context of artificial surfaces e.g. the ECMO circuitry, the complement system is activated via the classical and alternative pathways. FXII activates complement 3 (C3) and complement 5 (C5), the main components of the complement system that become deposited on the artificial surface. This generates complement 3a (C3a) and complement 5a (C5a) which are the potent chemoattractants of leukocytes followed by the subsequent activation of the coagulation system via various mechanisms such as increased expression of TF on leukocytes and contribute to subsequent activation of the coagulation system and thrombosis [173, 174].

Activation of the coagulation system via multiple mechanisms contribute to coagulopathy seen in patients on ECMO. This makes anticoagulation a vital and integrated part of the management of patients on ECMO.

1.3.2 Management of coagulation during ECMO

Ideally, an anticoagulant should be able to prevent the activation of platelet and the coagulation system within the ECMO circuit with minimal effect on the endogenous coagulation activity. Although each ECMO centre adopts different anticoagulation protocols, the default anticoagulant for ECMO remains the unfractionated heparin (UFH) with concomitant regular blood tests to monitor the haemostatic state of the patient [175].

1.3.2.1 Unfractionated heparin (UFH)

UFH is a negatively charged anticoagulant that works with the antithrombin to inhibit the principal coagulant- thrombin and FXa [176]. The persistent use of UFH in modern ELSO can be associated with its effectiveness against thrombus formation, easy monitoring by various assays and ready-reversibility by protamine sulphate. Despite its multiple advantages, UFH also has multiple difficulties. The most important one being there are limited data on UFH dosage to be used on patients according to their age and disease aetiology. Particularly, there

has not been a single clinical trial for the determination of optimal clinical efficacy and safety of UFH in the setting of neonates and children. Most of the currently available guidelines for the clinical management of UFH in patients on ECMO have been derived from the UFH therapy used in patients with thromboembolic disease [177]. According to the ECMO handbook, a bolus of 50 - 100 U/kg of UFH was recommended for patients across all age groups. The kinetics of UFH in children is different from adults, for example, the volume of distribution and clearance of UFH is much higher in neonates and children than in adults [178]. Together, these contribute to the unpredictable anticoagulant response of UFH in neonates and children significantly [179] and thus increase the risk of anticoagulation-related complications.

Currently, multiple types of whole blood and plasma-based tests such as the activated clotting time, activate partial thromboplastin time and anti-factor Xa activity are available for the monitoring of UFH usage in patients on ECMO. However, each of these tests has its respective limitations. The common one being they are device and reagent-dependent and there is a lack of standardization for the assay protocols and reference ranges across all ECMO centres. Whole blood thrombin generation has been proposed as the alternative for monitoring of anticoagulation [180]. Lastly, as most of the monitoring tests target on the clotting proteins, the role of the other important element of coagulation such as the platelet may be overlooked. This is especially important as the paradoxical effect of UFH on platelet function [181] in addition to its known thrombocytopenia inducing effect [182, 183] has gained increased attention in recent years. Specifically, UFH was found to induce platelet activation and increase their responsiveness to agonists via the integrin αIIbβ3 signalling pathway [184].

Despite its effectiveness and persistent usage as an anticoagulant, UFH, similar to the other anticoagulants is unable to differentiate pathogenic thrombosis from thrombin generation required for normal haemostasis. This exposes patients on ECMO to the risk of excessive bleeding, in addition to thrombosis. Hence, the main challenge for anticoagulant usage in the

setting of ECMO remains maintaining a balance between an effective antithrombotic role and a meaningful anticoagulant effect.

Platelet activation along with thrombin and plasmin has been proposed as the primary cause of the haemostatic complications during ECMO [27]. Hence, UFH targeting at specific coagulation factors via an indirect inhibitory action may be with limited capacity to control the complex haemostatic system especially during ECMO. Recently, antiplatelet agents such as nitric oxide and prostacyclin had gained interest to be used along with UFH as part of the management of coagulation in the ECLS setting.

1.3.2.2 *In vivo* thromboregulation and antiplatelet agents

Upon injury, a well-balanced prothrombotic and antithrombotic processes is important to prevent either haemorrhage or thrombosis. In general, this involves modulation of cellular interactions, cytokine/hormone concentrations and the environmental factors. Specifically, the interactions between coagulation proteins, platelets and endothelial cells are the main determinant of such regulation. In particular, the endothelium plays an important role in thromboregulation, a process of limiting growth of haemostatic plug and thrombus or even with the ability to reverse platelet reactivity through inhibition of platelet activities such as platelet adhesion, aggregation and activation by releasing mediators such as nitric oxide and prostacyclin [185, 186].

Although both the anticoagulant and antiplatelet agents are able to prevent clot formation or stop existing clot from continuous growth, the latter targets specifically on platelets by inhibiting their adhesion or aggregation via different mechanisms. For the important role of platelets in haemostasis, the usage of antiplatelet agents for various clinical conditions have gained increased interests in recent years. The main antiplatelet agents used in children are aspirin, integrin αIIbβ3 antagonists, thienopyridines, dipyridamole and ticagrelor [187].

Aspirin is the irreversible inhibitor of platelet cyclooxygenase. Following this is the inhibition of thromboxane A_2 release and prostaglandins production, the important mediators of platelet activation [379]. Similar to most of the antiplatelet agents, the paediatric dosage of aspirin is not derived from studies in children [380]. A low dose of 1 - 5 mg/kg/day is recommended for paediatric usage [381]. Some of the common adverse effects of the aspirin include bleeding and gastrointestinal toxicity e.g. gastric ulcer and vomiting [381]. Aspirin is also associated with the Reye's syndrome (a rare form of hepatic encephalopathy) which is dose-dependent (in anti-inflammatory dosage of aspirin (> 40 mg/kg) rather than with the lower dose used for the antiplatelet therapy) [382].

The thienopyridines group comprised of clopidogrel and ticopidine. They inhibit platelet activation via inhibition of the $P2Y_{12}$ ADP receptor [383]. Some of the reported side effects for thioenopyridine include bleeding, gastrointestinal toxicity and thrombotic thrombocytopaenic purpura. The side effects are more frequently associated with clopidrogel than ticopidine and this makes clopidrogel the more commonly used thienopyridine [384]. The PICOLO study was conducted to identify the dosing of clopidrogel-mediated platelet inhibition in infants and children and a dose of 0.20 mg/kg/day was suggested for the paediatric population [385]. Ticagrelor is another antiplatelet agent that targets on the $P2Y_{12}$ receptor. A current ongoing study evaluating the safety and efficacy of ticagrelor in children reported no safety concern in the Phase 2 study [386].

Dipyridamole inhibits platelet activation via multiple mechanisms including an increase in cGMP-dependent phosphodiesterase-5 and enhance the effect of NO. A dosage of 2 - 5 mg/kg/day was suggested for use in children [381, 387]. Some of the dipyridamole-related side effects include bleeding, nausea and vomiting. Another group of the antiplatelet agent is integrin αIIbβ3 antogonists that include abciximab, eptifibatide and tirofiban [388]. This group

of drugs act via the inhibiton of fibrinogen binding to integrin αIIbβ3. The major adverse events are bleeding, thrombocytopenia and pseudo-thrombocytopenia [388].

Although there is increasing information available for the antiplatelet agents usage in children, majority of them are often empirical and extrapolated from adults [187, 188]. These could limit the effectiveness of antiplatelet agents and raise the issue of safety concern related to their usage in paediatrics. Most importantly, to date, there is no existing evidence for the efficacy and safety of antiplatelet agents usage in the paediatric ECMO population, representing a group of complex and vulnerable patients. Given the known role of nitric oxide and prostacyclin as the *in vivo* inhibitor of platelet activities, these two antiplatelet agents had been incorporated as part of the management of patients on ECMO at RCH.

1.3.2.2.1 Nitric oxide (NO)

Biosynthesis of nitric oxide involves nitric oxide synthases (NOS), a synthase family comprised of the endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) [189]. eNOS can be found in the endothelial cells, cardiomyocytes, megakaryocytes and platelets. Specifically, NO release from the platelets share similar feature to that of eNOS [190]. NO binds to the heme-containing enzyme soluble guanylyl cyclase (sGC) in the platelets and this is followed by a decrease in intracellular calcium concentration through multiple signalling pathways [191]. Such reduction in Ca^{2+} levels mediated via the cGMP route can lead to the inhibition of platelet activities via different mechanisms [189, 192]:

I.) Direct inhibition of integrin αIIbβ3 transition from low- to high-affinity state, the conformational change vital for their interaction with fibrinogen

II.) Inhibits release of mediator such as arachidonic acid

III.) Inhibits platelet activation by preventing the interaction between platelet receptor and its activator, the TXA_2 receptor for thromboxane and down-regulation of P-selectin expression

In addition, NO is also important in the autoregulatory function of platelet. In a resting state, platelet release NO in the nanomolar range [190]; upon activation, platelets release large amounts of NO i.e. in the micromolar range to prevent further platelet adhesion and aggregation [193, 194]. NO had previously been incorporated into CPB in combination with the other antiplatelet agents and was shown to improve platelet dysfunction [195].

1.3.2.2.2 Prostacyclin

Prostacyclin is a derivative of the C-20 unsaturated fatty acid, arachidonic acid [196]. Biosynthesis of prostacyclin is catalyzed by prostacyclin synthase (PGIS) in the endothelium. Prostacyclin acts on the high-affinity prostacyclin receptor (IP) found on the platelet surface [189]. Upon binding to IP, prostacyclin increases cAMP levels and inhibits platelets activity via different mechanisms that include: I.) direct inactivation of integrin αIIbβ3 and II.) direct inhibition of platelet cytoskeletal-dependent activities e.g. granule release hence preventing the release of mediators for platelet activation and promote disaggregation of existing platelet aggregates [197, 198].

Epoprostenol is a synthetic prostacyclin primarily used as a vasodilator in pulmonary hypertension and an inhibitor of platelet activation. Mutiple *in vitro* studies have shown that epoprostenol can inhibit platelet aggregation [389] and the interactions between platelets and leukocytes [390] and could be associated with reduced CD62P (platelet activation marker) expression in the platelets [200]. In the ECLS setting, epoprostenol showed significant effects on reducing platelet consumption but has no effect on platelet activation [199]. However, currently available information are either based on *in vitro* studies or derived from the adults

and there is no existing information for epoprostenol-mediated platelet inhibition in the paediatric ECMO population.

1.3.2.3 Assessing platelet function in ECMO

Platelet disorders could be due to I.) an increase (thrombocytosis) or II.) a decrease (thrombocytopenia) in number of platelets or III.) platelet dysfunction. Individuals with such defects usually presented with symptoms of excessive bleeding or clotting events. Since patients on ECMO are at high risk of excessive bleeding, blood products, especially platelets replacement are important to ensure haemostasis needed for patients to remain on ECMO. Children on ECMO usually receive platelet transfusion with an average of 1.3 platelet transfusions per day to maintain a platelet level of > $100x10^9$/L [201]. However, similar to the anticoagulation management strategies for patients on ECMO, current transfusion protocols are not evidence-based and are different across centres based on the experience and empirical decisions. Furthermore, platelet transfusion-related complications e.g. infections and immune-related reactions [376] have been associated with an increased in mortality and morbidity and are especially important for the ECMO patients who are already in deranged conditions [290]. Meanwhile, platelet count serves as the main parameter to determine platelet administration for ECMO patients.

Over the years, various platelet function tests have been developed and utilized in both the research and clinical settings for different purposes (Table 1.11) and platelet aggregometry has been the gold standard of platelet assays in past decades. However, each of the currently available tests is with their own limitations such as device and reagent dependence, a requirement of large volume of blood and a lack of standardization specifically for the assay protocols and reference ranges across all ECMO centres.

In recent years, whole blood flow cytometric analyses of platelet function have become the preferred method for assessing platelet activity due to its multiple advantages. These include: I.) only minimum amount of blood (as little as 5 μL) is required for test makes it especially useful in the neonatal setting (where volume of blood is often a limiting factor); II.) platelets are analysed in their physiological environment e.g. in the presence of erythrocytes and leukocytes; III.) minimal treatment which may activate or result in the loss of platelets required for sample preparation; and IV.) both the activation state and reactivity of circulating platelets (in the presence of agonists) can be determined.

Table 1.11 Summary of currently available platelet function tests.

The principles and applications for platelet function tests (adapted from [202, 203]).

Principle	Test Name	Method	Examples of applications
Platelet aggregation	Light transmission aggregometry	Change in light absorbance due to platelet aggregate formed in response to different agonists e.g. ADP, collagen, epinephrine, ristocetin, TRAP	Diagnosis of inherited/acquired bleeding disorders and monitoring of antiplatelet therapies
	Whole blood aggregometry	Change in impedance between two electrodes as platelets adhere and aggregate in response to agonists	Diagnosis of inherited/acquired bleeding disorders and monitoring of antiplatelet therapies
	VerifyNow (Accumetrics, USA)	Degree of platelet adhesion to fibrinogen-coated beads in response to agonists , TRAP, ADP and AA (integrin αIIbβ3 specific)	Monitoring antiplatelet therapy
Platelet aggregation under shear stress	PFA-100/Innovance PFA-200 (Siemens, Germany)	Measure degree of shear-induced platelet aggregation in the presence of agonists	Monitoring antiplatelet therapy
	IMPACT: Cone and Plate(Let) Analyzer (DiaMed, Switzerland)	Percentage of well surface covered by platelet aggregates and size of platelet aggregates	Monitoring of integrin αIIbβ3-fibrinogen antagonist therapy

Principle	Test Name	Method	Examples of applications
ELISA	ELISA kit for platelet factor 4 (PF-4), β-thromboglobulin (β-TG) and CD40 ligand (CD40L), P-Selectin, E-Selectin, serum thromboxane B2 etc.	Assay kits for soluble markers released upon platelet activation and activation pathway	Monitoring of soluble markers released upon platelet activation
Viscoelasticity	TEG or ROTEM	Cover platelet count, function, clotting and fibrinolytic activation	Global assessment of haemostasis and monitoring of antiplatelet therapy
Flow cytometry	Platelet activation marker/in response to agonist	Quantification of platelet-activation markers e.g. P-selectin	Diagnosis of inherited or acquired platelet dysfunctions and determination of activation state of platelet under different clinical conditions
	Heterogenous platelet aggregates	Measurement of platelet-monocyte or platelet-neutrophil aggregates	Identification of monocyte platelet or neutrophil platelet aggregates
	VASP	Measurement of VASP phosphorylation/dephosphorylation ratio	Monitoring of antiplatelet drugs

Principle	Test Name	Method	Application
Genomics	Nil	Measurement of total mRNA by microarray technology and whole genome sequencing	Identification of platelet transcriptomes
Proteomics	Nil	Measures total protein content	Characterization of platelet total protein content

[AA, arachidonic acid; ADP, adenosine diphosphate; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; TEG, thromboelastography; ROTEM, rotation thromboelastometry; TRAP, thrombin receptor activator peptide; VASP, vasodilator-stimulated phosphoprotein]

Platelet function can be assessed using flow cytometry either in the presence or absence of an exogenous platelet agonist. In the absence of an agonist, flow cytometry allows the determination of circulating platelet activation state using activation-dependant monoclonal antibody. On the other hand, the addition of agonists such as ADP, arachidonic acid and collagen allows circulating platelets reactivity to be identified in *vitro.* Specifically, the agonists induce specific physiological responses such as a change in the expression of receptors or ligands which appears as a change in the binding of monoclonal antibody target on the receptors/ligands [113].

Modifications of platelet functions associated with the changes in platelet surface markers are well-studied in patients with CPB. On the other hand, very few studies have documented the changes in platelet function in the setting of ECMO (Table 1.12 and Table 1.13) which could reflect the challenges associated with study in an ECMO setting such as the unpredictability nature of the ECMO cases and the complexity of ECMO patients. Specifically, to date, there is no existing study that has evaluated how modifications of platelet function may be associated with bleeding and clotting complications in patients on ECMO.

Table 1.12 Summary of studies for platelet function in adults on ECMO.

Adapted from [204].

Authors	Year	Summary
[205]	**2016**	**Markers of platelet function (methods):** - Soluble GPVI (ELISA) - Surface-bound GPIbα , GPVI and GPαIIb subunit (Flow cytometry) **No. of subjects:** 20 **Age group:** Range= 21 – 76 years **Blood sampling:** Peripheral blood **Main outcome:** Unspecified time point during ECMO compared with healthy individuals - Reduced GPIbα and GPVI - No change in GPαIIIb subunit - Increased soluble GPVI
[206]	**2017**	**Markers of platelet function (methods):** - β-thromboglobulin and platelet factor 4 (ELISA) **No. of subjects:** 13 **Age group:** Median (Range)= 43 (20 – 69) years **Blood sampling:** Peripheral blood **Main outcome:** 72 hours on ECMO compared with before ECMO - Reduced β-thromboglobulin - Non-significant reduction in platelet factor 4
[207]	**2016**	**Markers of platelet function (methods):** - Platelet aggregometry using AA, ADP and TRAP **No. of subjects:** 23 **Age group:** Median= 54 years **Blood sampling:** Not reported **Main outcome:** Compared with healthy individuals before and during ECMO - Reduced platelet aggregation (AA, ADP, TRAP,) (significance not tested) During ECMO compared with before ECMO - Reduced platelet aggregation (TRAP) (significance not tested) - Increased platelet aggregation (AA) (significance not tested) - No change in platelet aggregation (ADP) (significance not tested)

[208]	**2015**	**Markers of platelet function (methods):** - Platelet aggregometry using AA, ADP and TRAP **No. of subjects:** 38 **Age group:** Mean= 50 years **Blood sampling:** Peripheral blood **Main outcome:** Compared with healthy individuals for before ECMO - Reduced platelet aggregation (AA, ADP) - No change in platelet aggregation (TRAP) 24 hours on ECMO compared with before ECMO - Reduced platelet aggregation (AA, ADP, TRAP) 48 hours on ECMO compared with before ECMO - Reduced platelet aggregation (AA, ADP) - Non-significant reduction in platelet aggregation (TRAP) 24 hours after ECMO compared with before ECMO - No change in platelet aggregation (AA, ADP, TRAP)
[209]	**2015**	**Markers of platelet function (methods):** - Platelet aggregometry using AA, ADP and TRAP **No. of subjects:** 5 **Age group:** Mean ± SD = 53 ± 11 years **Blood sampling:** Peripheral blood **Main outcome:** 90 minutes on ECMO compared with before ECMO - Reduced platelet aggregation (ADP) - Non-significant reduction in platelet aggregation (AA) - No change in platelet aggregation (TRAP) 120, 150 and 180 minutes on ECMO compared with before ECMO - Reduced platelet aggregation (ADP) - Non-significant reduction in platelet aggregation (AA) - No change in platelet aggregation (TRAP)
[210]	**2015**	**Markers of platelet function (methods):** - Platelet aggregometry using ADP, collagen, TRAP and ristocetin **No. of subjects:** 10 **Age group:** Mean ± SD = 53 ± 11 years **Blood sampling:** Peripheral blood **Main outcome:** Unspecified time point during ECMO compared with healthy individuals - Reduced platelet aggregation (ADP, collagen, TRAP and ristocetin) (significance not tested)

[211]	**2018**	**Markers of platelet function (methods):** - CD62 and CD63 (Flow cytometry) - Platelet aggregometry using ADP, collagen, epinephrine and ristocetin) **No. of subjects:** 6 for flow cytometry; not reported for aggregometry **Age group:** Mean ± SD = 52 ± 16 years **Blood sampling:** Peripheral blood **Main outcome:** Unspecified time point during ECMO compared with healthy individuals - Reduced CD62 and CD63 After ECMO compared with before ECMO - Reduced platelet aggregation (ADP, ristocetin) - Non-significant reduction in platelet aggregation (collagen, epinephrine)

[AA, arachidonic acid; ADP, adenosine diphosphate; ECMO, extracorporeal membrane oxygenation; ELISA, enzyme-linked immunosorbent assay; GPαIIIb, glycoprotein αIIIb; GPIbα; glycoprotein Ibα; GPVI, glycoprotein VI; SD, standard deviation; TRAP, thrombin receptor activator peptide]

Table 1.13 Summary of studies for platelet function in neonates and children on ECMO.

Authors	Year	Summary
[212]	**1993**	**Markers of platelet function (methods):** - Platelet aggregometry using ADP, collagen and ristocetin - GPIb and IIIa (Flow cytometry) **No. of subjects:** 10 **Age group:** < 10 weeks **Blood sampling:** Peripheral blood **Main outcome:** 15 and 60 minutes on ECMO compared with before ECMO - Reduced platelet aggregation (ADP and ristocetin) - No change in GPIb and GPIIIa expressions
[213]	**2000**	**Markers of platelet function (methods):** - Platelet aggregometry using collagen - P-selectin and E-selectin (ELISA) **No. of subjects:** 10 **Age group:** 13 – 61 hours postnatal age **Blood sampling:** Peripheral blood **Main outcome:** 24 hours on ECMO compared with before ECMO - Reduced platelet aggregation (collagen) - Increased platelet but not endothelial activation as indicated by the increased plasma P-selectin concentration and no change in E-selectin
[214]	**2016**	**Markers of platelet function (methods):** - Platelet aggregometry using ADP and AA (Thromboelastography- Platelet Mapping) **No. of subjects:** 24 **Age group:** Median (Interquartile range)= 9 (1 – 70) months **Blood sampling:** Peripheral blood **Main outcome:** Patients with severe bleeding compared with patients without severe bleeding - Reduced platelet aggregation (AA) - No change in platelet aggregation (ADP)

[AA, arachidonic acid; ADP, adenosine diphosphate; ECMO, extracorporeal membrane oxygenation; ELISA, enzyme-linked immunosorbent assay; GPIb; glycoprotein Ib; GPIIIa, glycoprotein IIIa; TRAP, thrombin receptor activating peptide]

Modifications of platelet function in patients on ECMO have been associated with shear stress [74, 215] and artificial surface [120, 216] from the intact ECMO system. The majority of the existing studies had used platelet aggregometry method to assess platelet function in the ECMO population and reduced platelet response was reported for both adults and children on ECMO. For the very few studies that had utilized flow cytometry to detect platelet-specific changes and particularly for children, the results are with limited significance due to the small sample size and very few platelet-specific markers used. For the adult ECMO population, two studies that utilized flow cytometry method reported reduced GPVI and GPIb/IX/V receptor expression [205] and reduced markers of platelet activation (α-granule and lysosome release indicators) [211], respectively. The first study that examined platelet function in neonates on ECMO using flow cytometry did not detect any changes in the platelets glycoprotein receptors GPIb and GPIIIa expressions [212] (Table 1.13). However, there was a reduction in the platelet response towards ADP and ristocetin.

Recent evidence suggested that platelets are not only important to coagulation but also inflammation and immunity via LPA interactions [217]. Thus, investigating LPA in relation to thrombosis in ECMO patients is also relevant since elevated LPA is constantly observed in various thromboinflammatory conditions [218, 219]. In fact, LPA maybe a better marker for platelet activation compared to P-selectin because activated platelets lose their P-selectin rapidly after degranulation that render them undetectable in the circulation [220].

Since very limited data is available for platelet function in patients on ECMO and the existing studies especially for children are with limited significance, it is vital to evaluate platelet function using multiple markers that are important for their phenotype, activation and interactions with leukocytes. Understanding the relationships between platelet-specific changes and the development of bleeding and/or thrombosis will provide further information

and may aid in identifying the key platelet-relevant markers that could be used as the therapeutic targets in children on ECMO.

1.4 Literature review summary and objectives

ECMO is a form of life-supporting treatment (heart-lung machine) that can save the lives of critically ill children. Despite its wide and well-established application, bleeding and clotting complications remain the main challenge for patients on ECMO. Shear stress and artificial surface have been proposed to be the main cause of coagulopathy seen in the ECMO population. Particularly, management of the paediatric patients on ECMO can be further complicated by their heterogeneity in age groups, disease aetiology and duration of ECMO.

Platelets play a vital role in the coagulation system through their multiple functions such as adhesion, aggregation, activation and interactions with the other cellular components for their main *in vivo* physiological role which is to stop bleeding. Due to the unique feature of being anucleated, their physiological function is largely-dependent on the interaction between various receptors and ligands in the extracellular environment. Activation of platelets and their interaction with ligands, both of which are shear-dependent along with contact activation associated with the artificial surface of the ECMO circuitry have been proposed as the main cause of coagulopathy in the ECMO population. Furthermore, frequent platelet transfusions in the ECMO patients with excessive bleeding that display normal platelet count may indicate occurrence of platelet dysfunction. Hence, it is vital to understand the function of platelets, which has been proposed as the main cause of coagulopathy in patients on ECMO.

To date, the multifunctional role of platelets is inadequately represented by the platelet count alone and various existing platelet function tests that have multiple limitations. In recent years, whole blood flow cytometric determination of platelet function has become the preferred method of platelet function assessment for their multiple advantages. Platelets have been

proposed as the key element of the modified coagulation system that contributes to the high rate of bleeding and clotting complications in paediatric ECMO patients. However, platelet-specific changes and how they may be related to the development of bleeding or thrombosis and differ according to a patient's factors has not been assessed in this population. Furthermore, site-specific platelet-relevant changes have yet to be investigated in a paediatric ECMO system. Hence, the aim of this project is to describe the phenotype, function and cellular interactions of platelets in children on ECMO. The hypothesis of this research is that there are significant changes in platelet surface molecules that affect platelet function and contribute to the development of bleeding or thrombosis in patients on ECMO; and there are platelet-specific changes that vary at different sites in a paediatric ECMO system.

1.4.1 Aims

This study aimed to characterize the molecular indices of circulating platelets using whole blood flow cytometry approach:

1) To examine and compare the effect of pathway onto ECMO on platelet phenotype and function in paediatric ECMO population and their associations with the development of bleeding or thrombosis during ECMO.
2) To examine and compare the effect of age on platelet phenotype and function in paediatric ECMO population and their associations with the development of bleeding or thrombosis during ECMO.
3) To examine and compare the effect of duration of ECMO on platelet phenotype and function in paediatric ECMO population and their associations with the development of bleeding or thrombosis during ECMO.
4) To examine and compare the site-specific differences for platelet phenotype and function in a paediatric ECMO system.

1.4.2 Clinical outcome and significance

This study will provide the first consideration of a relationship between platelet function and measurable clinical variables in neonates and children on ECMO. Such findings may reveal the underlying reasons that contribute to the high morbidity and mortality rates associated with bleeding and clotting complications in this population. Specifically, understanding the association between the platelet-specific changes and the development of bleeding or thrombosis could provide clue for the improvement of the current clinical management with the ultimate aim to improve the overall clinical outcome of patients on ECMO. Since ECMO is one form of artificial circulation, results generated from this study may also have implications to all artificial circuit usage in neonates and children.

2 Research Design and Methods

2.1 Introduction

The whole blood flow cytometry approach that allows the assessment of platelet function with minimal amount of blood and treatment in their physiological environment was used to examine the molecular indices on the circulating platelets that are important for:

I.) Platelet phenotype

II.) Circulating platelet activation and response to thrombin receptor activator peptide 6 (TRAP-6)

III.) Interaction of platelets with monocytes and neutrophils

The above platelet-specific changes were sub-analysed according to a patient's pathway onto ECMO, age, and duration of ECMO.

2.2 Participants

2.2.1 Eligibility and selection criteria

This prospective study received ethical approval from The Royal Children's Hospital Melbourne Human Research Ethics Committee (HREC) (HREC Reference Number: HREC/15/RCHM/123) (Appendix IV). All neonates and children receiving ECMO at The Royal Children's Hospital (RCH) were eligible for this study. Written informed consent was obtained from the participant with age of eighteen and parental/guardian consent was sought for the participant under the age of eighteen. Due to the critical condition of this population, delayed consent was granted by the ethics committee for up to 48 hours following the initiation of ECMO. Such retrospective consent procedure is a novel research approach in the paediatric ECMO population. The inclusion and exclusion criteria used for the selection of participant were as follows:

Inclusion criteria: All patients in PICU receiving veno-arterial (VA) and veno-venous (VV) ECMO who are not known to have a pre-existing haematological disorder (clinically diagnosed bleeding or clotting disorder).

Exclusion criteria: Patients with previous history of haematological disorder; patients aged over 18; patients where the parent/guardian did not read, speak or understand English.

2.2.2 Data collection

Demographic details (age, gender and weight), diagnosis and duration of ECMO of each participant were recorded. Specifically, participants were categorized based on their pathway onto ECMO (Post-CPB vs. Non-CPB); age (neonatal (0 - 30 days); infants (31 days - 1 year) and children (2 – 18 years) and duration of ECMO (≤ 5 days vs. > 5 days). Mode of ECMO (VA or VV-ECMO) and details of the ECMO system were recorded.

2.2.3 Bleeding and clotting complications

The definitions used for bleeding and/or thrombosis were as follows:

Bleeding complication:

Major bleeding

I.) More than 4 mL/kg/hr for more than 4 hours, as defined by the RCH ECLS anticoagulation and blood product administration protocol
II.) Intracranial haemorrhage
III.) Gastrointestinal bleed requiring endoscopic or surgical intervention
IV.) Surgical site bleed requiring surgical exploration

Minor bleeding

I.) Bleeding or blood loss not more than 4 mL/kg/hr or persisting at that volume for more than 4 hours

Thrombosis:

I.) Objectively diagnosed clinically relevant thrombosis (including venous or arterial) that requires acute intervention or formal anticoagulation beyond the period of ECMO

II.) Radiologically proven central nervous system (CNS) embolic stroke

III.) ECMO circuit thrombosis requiring circuit change- events requiring change-out of one or more circuit components were recorded by the location of the clots and the replaced circuit components

2.3 Circuit design and set-up

Patients on ECMO were supported using a Rotaflow centrifugal pump (Jostra Medizintechnik, Germany), with either 3/8" or 1/4" diameter tubing (Medos, Germany) and an oxygenator. The decision to use the 3/8" or 1/4" diameter tubing was based on a patient's weight. The 3/8" circuit with 7000LT oxygenator was used for patient > 10.0 kg while the 1/4" circuit with 2400LT oxygenator was used for patient < 10.0 kg. Each circuit was primed with crystalloid solution, albumin and leuko-reduced packed RBCs (pRBCs).

Prior to cannulation, each patient was given a loading dose of unfractionated heparin (UFH) (50 - 100 U/kg). Following the initiation of ECMO, a continuous heparin infusion (10 – 40 units/kg/hr) was given to the patient to maintain ACT 150 – 170 seconds (hourly) (for patients < 15 kg) or APTT of 70 – 90 seconds (4 – 6 hourly) (for patients ≥ 15 kg). In addition to UFH, anti-platelet agents including epoprostenol (at 5ng/kg/min by infusion through the ECMO circuit) and nitric oxide (at 20 ppm in the sweep gas) were also given to the patients. During ECMO support, platelets were maintained at ≥ 80 – 100 x 10^9/L.

2.4 Blood collection

Whole blood was collected from each participant from the arterial line at various time points (Figure 2.1) and during clinical events as listed below:

I.) Before they were placed on ECMO

II.) Each day they were on ECMO (i.e. daily from Day 1 - 5 and every 2 days for the subsequent duration Day 7, 9, 11 etc.)

III.) 24 hours after they were weaned off ECMO

IV.) When the ECMO circuit were changed (one sample before and after the circuit change)

V.) When they experienced a bleeding or clotting complication

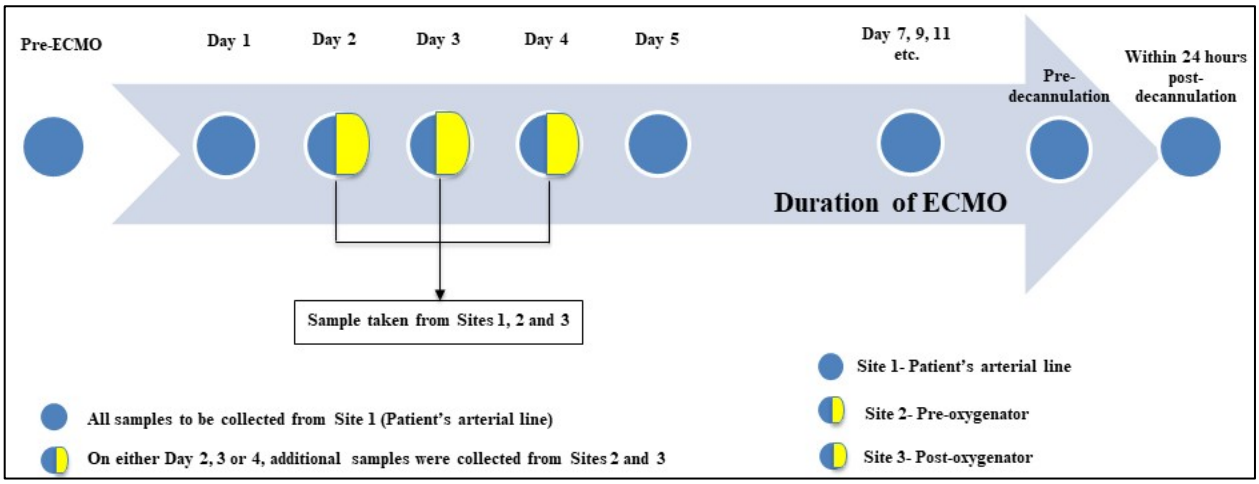

Figure 2.1 Summary of time points for blood sample collection from the ECMO patients.

To examine the platelet-specific changes at different sites in an ECMO system, in addition to the patient's arterial line, a whole blood sample was collected from the sites located at before and after the oxygenator (Figure 2.2) as a once-off event between ECMO Day 2 – Day 4.

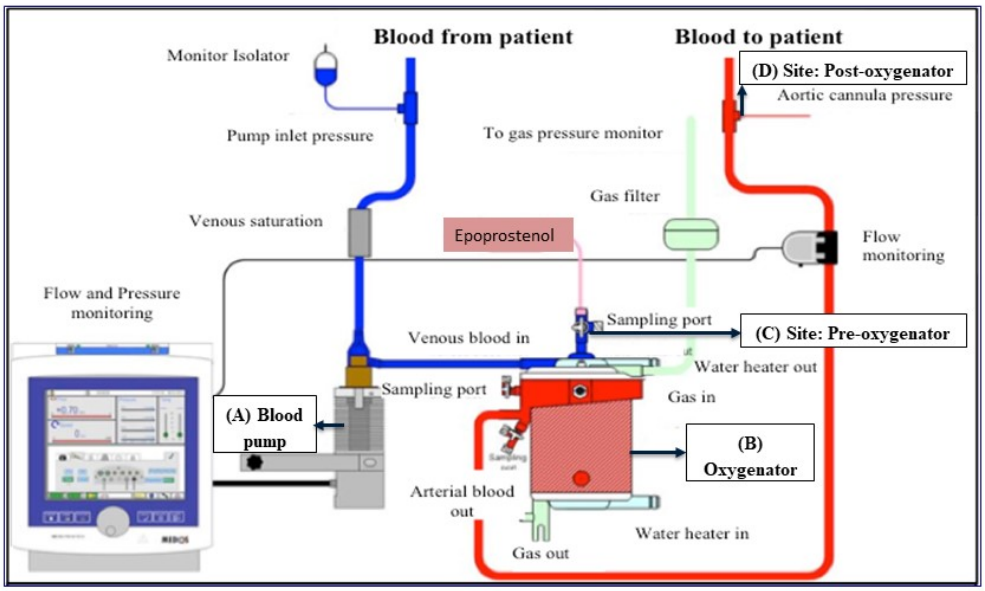

Figure 2.2 The sampling sites for platelet-specific changes at different sites in an ECMO system.

The location of sampling sites for pre-oxygenator and post-oxygenator in the intact ECMO system.

Whole blood was collected into the S-monovette® tubes (Sarstedt, Australia) containing 0.106 (3.2 %) mol/L trisodium citrate anticoagulant in a ratio of nine volumes of blood to one volume of anticoagulant. The volume of blood collected depended on the weight of the patient and was between 3 - 6 mL. Blood samples were collected at the same time as the other blood is being collected as part of the standard medical treatment to minimize line access events.

Whole blood samples were delivered to the laboratory and processed immediately.

2.5 Materials and reagents

All reagents and buffers used were listed in Appendix I.

2.6 Whole blood flow cytometry analysis of platelet function

Whole blood flow cytometry method was used to examine the expression of molecular markers on circulating platelets using three different panels as listed in the tables below:

Table 2.1 Panel 1 markers important for the platelet phenotype.

Marker	Fluorochrome	Role
CD42b	FITC	GPIb, main von Willebrand factor receptor
CD61	APC	GPIIIa, platelet identifier/ main fibrinogen receptor
GPVI	PE	GPVI, collagen receptor

Table 2.2 Panel 2 markers for circulating platelet activation and the response to thrombin receptor activator peptide 6 (TRAP-6).

Marker	Fluorochrome	Role
CD41	ECD	GPIIb, platelet identifier/ main fibrinogen receptor
CD62P	APC	α-granule release, platelet activation marker
PAC-1	FITC	Targets on activated integrin αIIbβ3
CD63	PE	Lysosome release indicator

Table 2.3 Panel 3 markers for the interaction of platelets with monocytes and neutrophils.

Marker	Fluorochrome	Role
CD14	BV711	Monocyte- and neutrophil- identifier
CD11b	BV421	Mac-1, leukocyte activation marker
CD61	APC	Platelet identifier
CD62P	PE	α-granule release, platelet activation marker

2.6.1 Full blood count

Full blood count was performed using ABX Micros 60 (Horiba Medical, USA), Cell-Dyn Emerald (Abbott, USA) or ADVIA 2120i (Siemens Healthineers, Germany) and diluted to a concentration of $5x10^7$ platelets/μL using staining buffer, HSB.

2.6.2 Instrument setup

Flow cytometry was performed on BD LSRFortessa^{TM} X-20 (BD, Germany) equipped with an ultra-violet laser (355-nm, 50-mW solid state), a violet laser (405-nm, 50-mW solid state), a blue laser (488-nm, 50-mW solid state), a yellow-green (561-nm, 50-mW solid state) and a red laser (640-nm, 50-mW solid state). The flow cytometer was calibrated daily with the cytometer setup and tracking (CS&T) beads (BD, USA) as recommended by the manufacturer, and daily adjustment to PMT settings based on the CS&T using BD application settings was performed to ensure consistent median fluorescence intensity (MFI) data for the duration of the study. Calibration beads automate the characterization of cytometer fluorescence detectors and the entire optical configuration by creating baseline performance values, which serve to standardize the cytometer performance. BD FACSDiva software (Version 6, BD, Germany) controls the connection between the flow cytometer and instrument interface being the platform to perform calibration and acquisition. Singly-stained BD^{TM} Comp Beads (BD, USA) with fluorescence-conjugated monoclonal antibodies were used for daily setting of compensation on the flow cytometer.

2.6.3 Assay for platelet phenotype (Panel 1)

2.6.3.1 Staining protocol

Within 20 minutes of blood collection, 8 µL of diluted citrated whole blood with a total number of $4x10^5$ platelets was added into 12 µL of either the monoclonal platelet (PLT Mix) or the isotype control (PLT Isotype) antibody cocktail (Appendix II) in a 1.6 mL LoBind Eppendorf tube and incubated at room temperature in the dark for 15 minutes. The PLT Mix consists of CD42b Fluorescein IsoThioCyanate (FITC) (#555472, BD, USA) (directed against platelet-specific GPIb), CD61 Allophycocyanin (APC) (#564174, BD, USA) (directed against platelet-specific GPIIIa) and GPVI PhycoErythrin (PE) (#565241, BD, USA) (directed against platelet-specific collagen receptor). The PLT Isotype consists of the CD61 Allophycocyanin (APC)

(#564174, BD, USA), IgG1κ Fluorescein IsoThioCyanate (FITC) (#555748, BD, USA) and IgG1κ PhycoErythrin (PE) (#555749, BD, USA). After incubation, samples were fixed with the addition of 300 µL of 1 % formaldehye. Fixed samples were stored on ice for a minimum of 30 minutes until acquisition.

2.6.3.2 Data acquisition

All samples were acquired with a low flow rate to minimize platelets and red blood cells coincident events. The assay was performed with a APC-threshold of channel 3000 to ensure detection of the platelet-specific anti-CD61-APC. All cytometer parameters were acquired using a logarithmic scale. For each sample, 10,000 CD61 positive events were recorded. An unstained sample was acquired with a FSC-threshold of channel 800 and 10,000 platelet specific events were recorded for the identification of platelet-specific region based on their forward and side-scatter properties.

2.6.3.3 Gating strategy and results analysis

Flow cytometric data was analysed using FlowJo_V10 software (Tree Star Incorporation, USA). Platelets were identified based on their physical parameters forward and side light scatter properties and to the expression of the platelet specific marker, CD61. CD42b and GPVI expressions were measured by setting gating threshold on the respective negative isotype matched control (Appendix III). The results were expressed as MFI of the antigen expressed on the platelets population.

2.6.4 Assay for circulating platelet activation and response to thrombin receptor activator peptide 6 (TRAP-6) (Panel 2)

2.6.4.1 Staining protocol

Within 20 minutes of blood collection, 8 µL of diluted citrated whole blood with a total number of $4x10^5$ platelets was added into 12 µL of either the monoclonal platelet (PLT-ACT Mix) or

the isotype control (PLT-ACT Isotype) antibody cocktail (Appendix II) with or without TRAP-6 (#T-1573, Sigma-Aldrich, USA) in a 1.6 mL LoBind Eppendorf tube and incubated at room temperature in the dark for 15 minutes. Fluorescence-minus-one (FMO) tubes which act as the gating controls were also prepared (Appendix II). The FMO control contains the entire panel of antibody-fluorochrome conjugates except for the one that is being measured to identify the gating boundary to ensure that any spread of the other fluorochromes into the channel of interest is properly identified. The PLT-ACT Mix consisted of CD41 PhycoErythrin-Texas Red (ECD) (#6607117, Beckman Coulter, USA) (directed against platelet-specific GPIIb), CD62P Allophycocyanin (APC) (#561920, BD, USA) (directed against P-selectin), PAC-1 Fluorescein IsoThioCyanate (FITC) (#340507, BD, USA) (directed against binding site exposed upon activation in platelet integrin αIIbβ3) and CD63 PhycoErythrin (PE) (#557305, BD, USA) (directed against CD63, a marker of lysosome release). The PLT-ACT Isotype consisted of the CD41 PhycoErythrin-Texas Red (ECD) (#6607117, Beckman Coulter, USA), IgG1κ Allophycocyanin (APC) (#554681, BD, USA), PAC-1 Fluorescein IsoThioCyanate (FITC) (#340507, BD, USA) and IgG1κ PhycoErythrin (PE) (#555749, BD, USA) and eptifibatide acetate (#SML-1042, Sigma-Aldrich, USA) (cyclic heptapeptide that inhibits integrin αIIbβ3activation). After 15 minutes of incubation, samples were fixed with the addition of 300 µL of 1 % formaldehye. Fixed samples were stored on ice for a minimum of 30 minutes until acquisition.

2.6.4.2 Data acquisition

All samples were acquired with a low flow rate to minimize platelets and red blood cells coincident events. The assay was performed with a ECD-threshold of channel 800 to ensure detection of the platelet-specific anti-CD41-ECD. All cytometer parameters were acquired using a logarithmic scale. For each sample, 10,000 CD41 positive events were recorded. An unstained sample was acquired with a FSC-threshold of channel 800 and 10,000 platelet

specific events were recorded for the identification of platelet-specific region based on their forward and side-scatter properties.

2.6.4.3 Gating strategy and results analysis

Flow cytometric data analysis was carried out using FlowJo_V10 software (Tree Star Incorporation, USA). Platelets were identified based on their physical parameters forward and side light scatter properties and to the expression of the platelet specific marker, CD41. CD62P, PAC-1 and CD63 expression was measured by setting gating threshold (less than 1% of positive platelet events) on the respective negative isotype matched control with eptifibatide inhibition of PAC-1. CD62P, PAC-1 and CD63 expressions were measured as the percentage of platelets expressing these activation markers (Appendix III). Platelet response to TRAP-6 is directly proportional to the area under the curve (AUC) derived from each dose response curve (final concentration of 0, 5, 10 and 50 μM TRAP-6) (Figure 2.3). The details for the concentrations of TRAP-6 used were included in section 3.3.2).

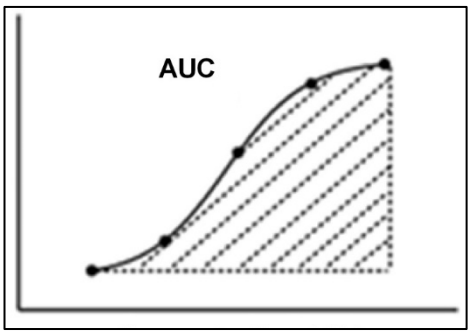

Figure 2.3 An example of dose-response curve to generate area under the curve (AUC) for platelet reactivity assay.

2.6.5 Assay for the interaction of platelets with monocytes and neutrophils (Panel 3)

2.6.5.1 Staining protocol

Within 20 minutes of blood collection, 30 µL of citrated whole blood was added into 30 µL of either the monoclonal platelet-leukocyte (PLT-LEU Mix) or the isotype control (PLT-LEU Isotype) (Appendix II) with or without TRAP-6 in a 1.6 mL LoBind Eppendorf tube and incubated at room temperature in the dark for 20 minutes. FMO tubes that acts as the gating controls were also prepared. The PLT-LEU Mix consisted of CD14 Brilliant Violet 711 (BV711) (#563373, BD, USA) (directed against leukocyte-specific CD14), CD62P PhycoErythrin (PE) (#550561, BD, USA) (directed against P-selectin), CD11b Brilliant Violet 421 (BV421) (#562632, BD, USA) (directed against CD11b, marker of activation for leukocytes) and CD61 Allophycocyanin (APC) (#564174, BD, USA) (directed against platelet-specific GPIIIa). The PLT-LEU Isotype consisted of the CD14 Brilliant Violet 711 (#563373, BD, USA) (directed against leukocyte-specific CD14), IgG1κ Allophycocyanin (APC) (#554681, BD, USA), IgG1κ Brilliant Violet 421 (#562438, BD, USA) and IgG1κ PhycoErythrin (PE) (#555749, BD, USA). The FMO control contains the entire panel of antibody-fluorochrome conjugates except for the one that is being measured to identify the gating boundary to ensure that any spread of the other fluorochromes into the channel of interest is properly identified. After 20 minutes of incubation, samples were fixed with the addition of 600 µL of 1x BD FACS Lysing solution. Fixed samples were stored on ice for a minimum of 30 minutes until acquisition.

2.6.5.2 Data acquisition

All samples were acquired with a low flow rate. The assay was performed with a forward-scatter-threshold of channel 5000 to ensure detection of the monocyte-specific event. All cytometer parameters were acquired using a logarithmic mode. For each sample, 1,000 monocyte events were recorded.

2.6.5.3 Gating strategy and results analysis

FlowJo_V10 software (Tree Star Incorporation, USA) was used for the analysis of flow cytometric data. Monocytes were identified based on their physical parameters forward and side light scatter properties and to the expression of the leukocyte specific marker, CD14 with threshold on forward light scatter (Appendix III). 1000 monocyte events were acquired for each sample. The results obtained were expressed as percentage of MPA/NPA with a total minimum of one thousand monocyte events acquired. CD62P, CD11b and CD61 on MPA/NPA were measured in MFI.

2.7 Statistical analysis

Statistical software and tests used for the analysis were included in each chapter individually. Categorical variables were recorded as the number of events and percentage. Continuous variables were expressed as the mean and standard deviation or median and range based on their distribution identified with visual inspection. A p-value of < 0.05 is statistically significant or an increasing/decreasing trend was described for p-value ≥ 0.05 - < 0.10 [221]. Power analysis was conducted using Statistical software package STATA (Release 15) (Stata Corp., College Station, Texas). Effect size was measured as Cohen's d with a value of 0.2, 0.5 and 0.8 for small, medium and large effects, respectively [404]. All statistical analysis was performed in consultation with Clinical Epidemiology Biostatistics Unit at Murdoch Children's Research Institute.

2.8 Data collection and storage

Original data collection forms were used when entering information into a secure purpose built REDCap electronic database. All information collected were re-identifiable. The participant name was removed and a specific study number was allocated to both the data collection forms and samples.

3 Methods Development and Optimisation

3.1 Methodology rationale

Since both the platelet and monocyte-platelet aggregates (MPA)/neutrophil-platelet aggregates (NPA) assays used in this study are novel, optimisation of the panels was important to ensure standardization of the methods. Platelets are known to be highly subjected to pre-analytical activation *in vitro*. Hence, the maximum time lapse between sample collection and initiation of assay and its effect on the stability of the platelet and MPA/NPA-specific markers was identified to ensure minimal pre-activation of platelets.

Platelet reactivity has been an important element for the evaluation of platelet function as it forms the basis of the most commonly used platelet function testing by aggregometry. Most assays use a single high concentration of agonist to ensure a maximum sensitivity towards potential subjects with reduced platelet function. Although useful, such high concentration of agonist will not be able to identify the subtle changes in platelet reactivity that will aid in the recognition of inter-individual variability in platelet responsiveness. To identify potential inter-individual variability in platelet reactivity, two concentrations of TRAP-6, the threshold and sub-maximal concentrations of TRAP-6 were identified to be used for the platelet reactivity assay.

Although the whole blood flow cytometric analysis of platelets and leukocytes has multiple advantages, the samples must be examined fresh [222]. Such approach is with limited practicability for studies with large sample size and/or carried out at sites remote from a flow cytometer. Currently available studies evaluating the effect of long-term fixation on whole blood for flow cytometric analysis are based on leukocytes and very limited data is available for platelets. The evaluation requirement to identify the effects of fixation on sample storage would be that it permits a certain limit of storage period of the cells post-fixation prior to flow

cytometric data acquisition without significant alteration of the light scatter and fluorescence properties of the cells [223]. At the study site of this study, flow cytometric analysis of samples either fresh or shortly after fixation is often limited by the handling of multiple samples at the same time. To allow flow cytometric analysis at a more convenient moment in the event of the handling of multiple samples, the stability of samples after fixation for a maximum storage period at 4ºC were evaluated.

3.2 Materials and Methods

Whole blood from the healthy children (HAPPI Kids Study, HREC 34183) were used for the optimization of flow cytometry Panels 1, 2 and 3 (sections 2.6.3 - 2.6.5). The details of statistical analysis were included in section 2.7. In summary, the significance of the differences for the levels of platelet-relevant markers at different time points were assessed using paired Student's t-test in Microsoft Excel.

3.3 Results for optimization of flow cytometry assays

3.3.1 Markers important for platelet phenotype (Panel 1)

3.3.1.1 The effects of time lapse between blood collection and initiation of assay on the stability of platelet-specific markers for Panel 1

A total of 5 paediatric samples (3 males and 2 females; age range: 4 – 14 years) was used in this section. The expression of fibrinogen (integrin αIIbβ3), VWF (GPIb/IX/V) and collagen (GPVI) receptors (measured in MFI) was measured over time after sample collection (Figure 3.1). There were no statistically significant changes in the expression of the above markers across all time points.

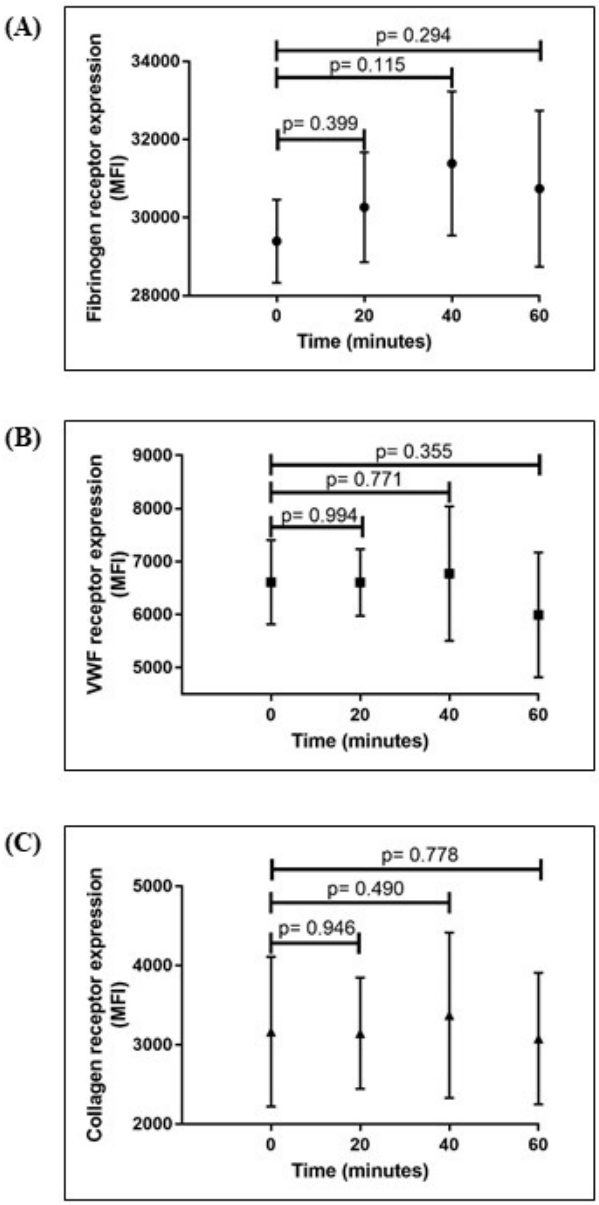

Figure 3.1 : Effect of time lapse between blood collection and initiation of assay on the stability of markers important for platelet phenotype.

The expression of (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor measured over increasing time lapse after sample collection. Data shown as mean +/- SD (n=5 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in the expressions of the above markers across all time points. [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

3.3.1.2 Post-fixation stability of Panel 1 monoclonal antibodies to paediatric platelets

A total of 3 paediatric samples (2 males and 1 female; age range: 5 – 6 years) was used in this section. There were no significant changes in the expression of GPIb/IX/V, integrin αIIbβ3 and GPVI receptors for time points 24-, 48- and 72-hours using time point 0-hour as the baseline (Figure 3.2). This showed that the expression of markers important for platelet phenotype remained stable for up to 72 hours after fixation. However, since predictable changes were observed in the expression of all three markers within the first 24 hours post-fixation, sample stained with Panel 1 monoclonal antibodies were stored at 4 ºC for 24 hours before proceeding with flow cytometric data acquisition within 72 hours.

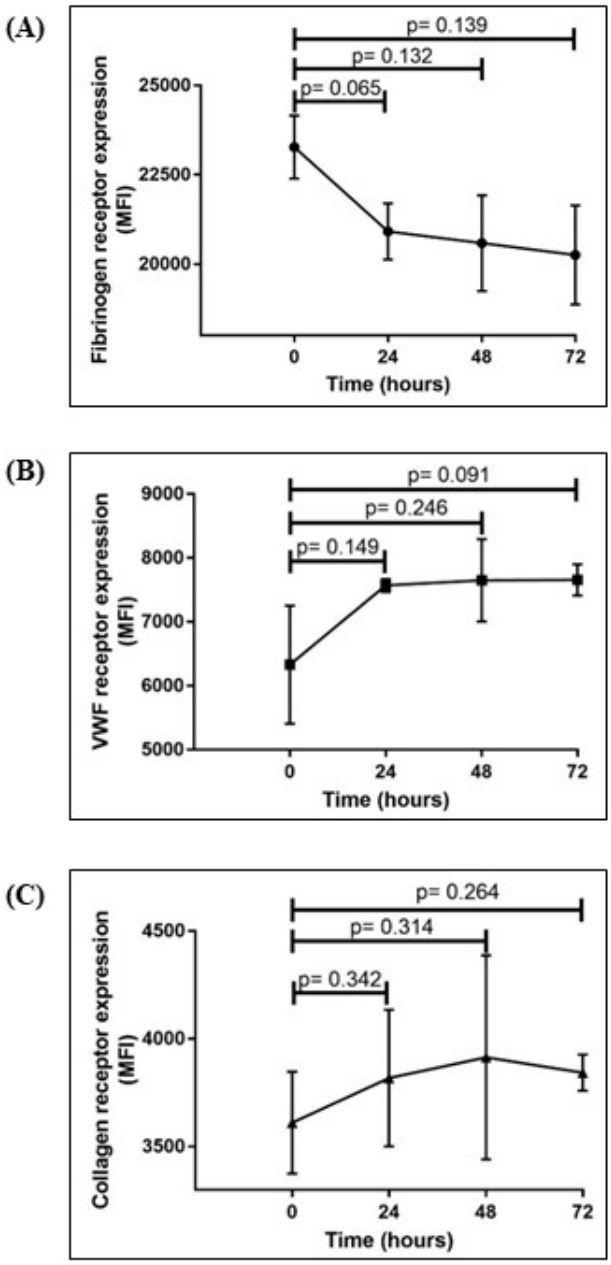

Figure 3.2 The expression (MFI) of Panel 1 markers at time points 0-, 24-, 48- and 72-hours post-fixation.

The expression of (A) fibrinogen (integrin αIIbβ3) receptor; (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor. Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in the expressions of the above markers over increasing storage period. [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

3.3.2 Markers important for platelet activation (Panel 2)

3.3.2.1 The effects of time lapse between blood collection and initiation of assay on the stability of platelet-specific markers for Panel 2

A total of 5 paediatric samples (3 males and 2 females; age range: 4 – 14 years) was used in this section. The percentage of platelets positive for α-granule release indicator (CD62P), activated integrin αIIbβ3 indicator (PAC-1) and lysosome release indicator (CD63) were measured over increasing time lapse after sample collection (Figure 3.3). Although there were no statistically significant changes in the expression of the above markers across all time points, an increasing trend was observed for percentage of platelets positive for CD62P and PAC-1 binding over time. A cut-off value of 10 % increase in PAC-1 positive platelets from the baseline was used as the maximum accepted pre-activation level that was reached 40 minutes after sample collection.

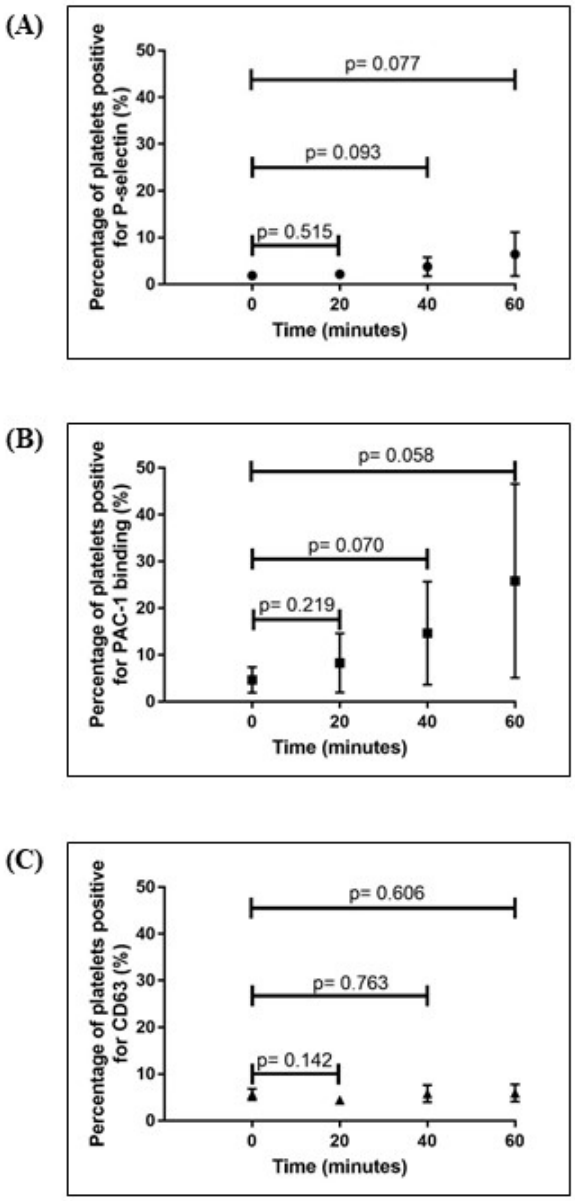

Figure 3.3 Effect of time lapse between blood collection and initiation of assay on the activation state of platelet.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 measured over increasing time lapse after sample collection. Data shown as mean +/- SD (n=5 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in the expressions of the above markers across all time points. [SD, standard deviation]

3.3.2.2 TRAP-6 concentrations for platelet reactivity assay

A total of 6 paediatric samples (1 males and 5 females; age range: 1 – 16 years) was used in this section. To identify the concentration of TRAP-6 used for the examination of the response of platelets, Panel 2 markers (CD62P, PAC-1 and CD63) were titrated against a series of TRAP-6 concentrations. A 'low' concentration is defined as stimulation of at least three-fold increase from basal activity i.e. 0 µM TRAP-6, and a 'high' concentration is defined as near to maximal (sub-maximal) level of activity measured. A concentration-response curve was used for the identification of the 'threshold' and 'sub-maximal' concentrations of TRAP-6 used for the examination of the response of platelets (Figure 3.4). The concentration-response curves were plotted for α-granule release indicator, CD62P; activated integrin αIIbβ3 indicator, PAC-1 and lysosome release indicator, CD63 against a series of TRAP-6 concentrations to identify the 'low' and 'high' TRAP-6 concentrations for each of the activation marker (Figure 3.5). It was concluded that the 'threshold' and 'sub-maximal' concentrations of TRAP-6 for all three markers of activation were at 5 µM and 10 µM respectively.

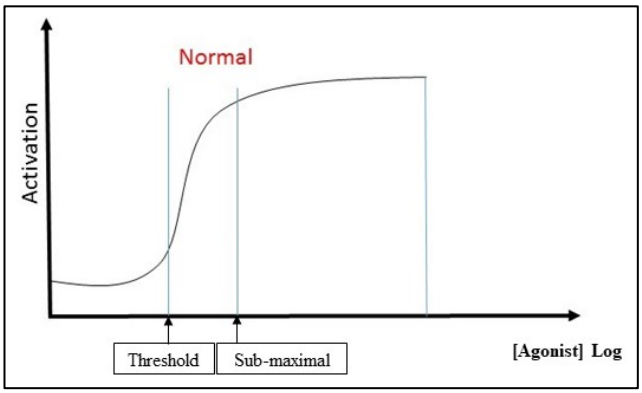

Figure 3.4 The theoretical concentration-response curve used for the identification of 'threshold' and 'sub-maximal' concentrations of TRAP-6.

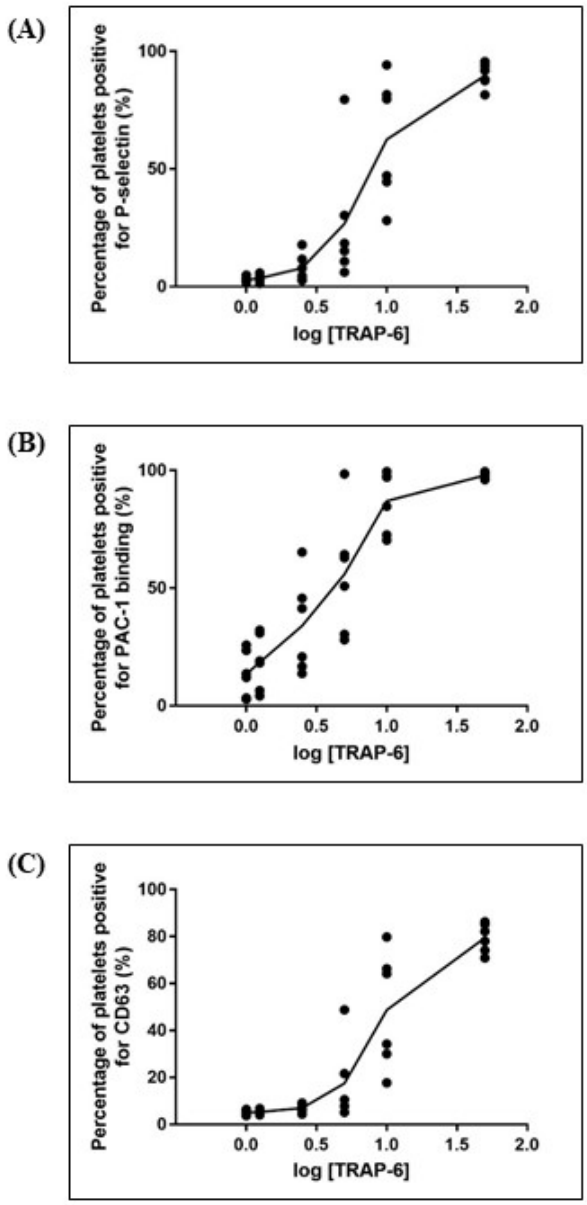

Figure 3.5 Concentration-response curves for Panel 2 markers and TRAP-6.

Percentage of platelets positive for (A) α-granule release indicator, CD62P; (B) activated integrin αIIbβ3b indicator, PAC-1 and (C) lysosome release indicator, CD63 in response to 0, 1.25, 2.5, 5, 10 and 50 μM TRAP-6 (n=6 healthy paediatric samples). A line connecting the means for the percentage of platelets positive for each of the activation marker at each TRAP-6 concentration was included in the graph.

3.3.2.3 Post-fixation stability of Panel 2 monoclonal antibodies to paediatric platelets

A total of 3 paediatric samples (2 males and 1 female; age range: 5 – 6 years) was used in this section. As Panel 2 aims to evaluate the response of platelets to TRAP-6, a potent *in vivo* thrombin mimic, it was essential to ensure that the maximum storage period for up to 72 hours is also applicable to samples activated by TRAP-6. There was no significant difference in the expression (percentage positive platelets) of the indicator of α-granule release, CD62P and activated integrin αIIbβ3, PAC-1 and the indicator of lysosome release, CD63 for time points 24-, 48- and 72-hours using time point 0-hour as the baseline across all concentrations of TRAP-6 (0, 5, 10 and 50 μM) (Figure 3.6 - Figure 3.8). These suggested that the storage procedure does not affect the expression of the indicators of platelet activation and they remain stable for up to 72 hours after fixation. Similar to Panel 1, since predictable changes were observed in the expressions of all three markers within the first 24 hours post-fixation, sample stained with Panel 2 monoclonal antibodies were stored at 4 ºC for 24 hours before proceeding with flow cytometric data acquisition within 72 hours.

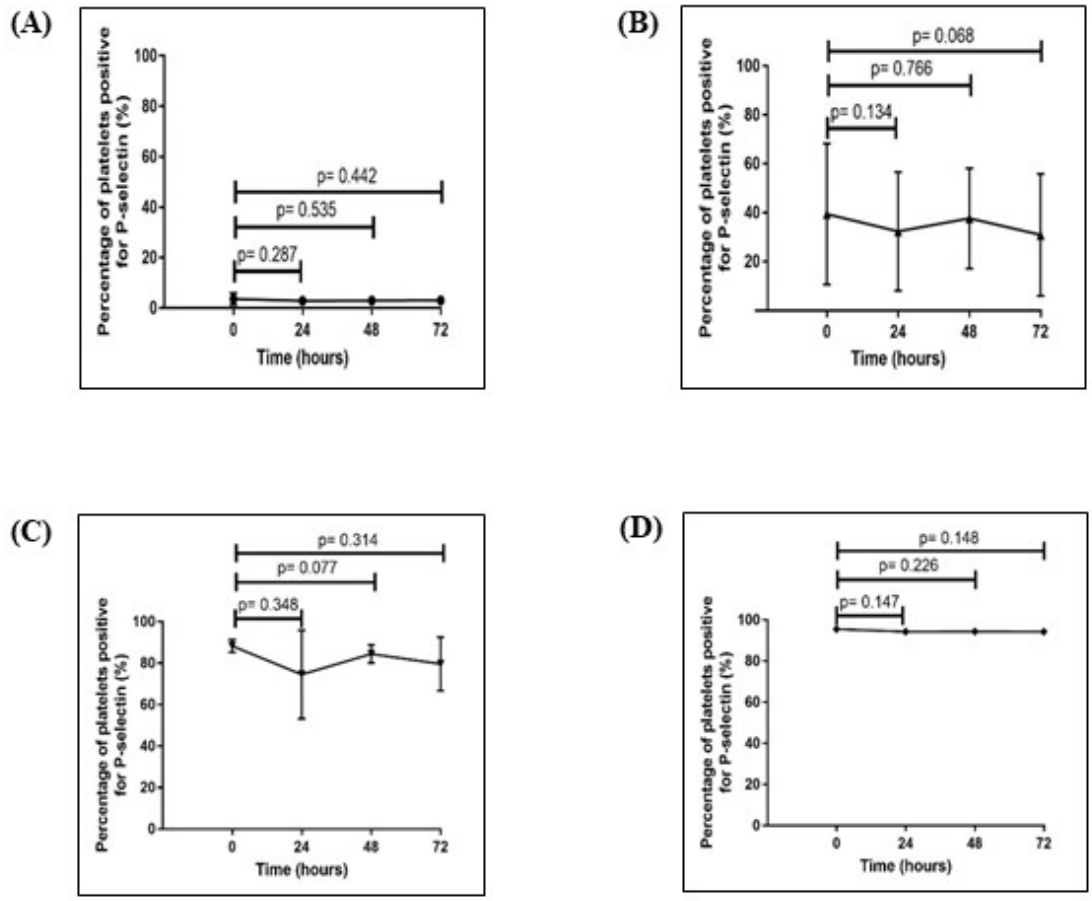

Figure 3.6 The expression (percentage positive platelets) of α-granule release indicator, P-selectin (CD62P) at time points 0-, 24-, 48- and 72-hours post-fixation.

Circulating platelets positive for α-granule release indicator, P-selectin (CD62P) at (A) 0 µM TRAP-6, (B) 5 µM TRAP-6, (C) 10 µM TRAP-6 and (D) 50 µM TRAP-6. Data shown as mean +/- SD (n=6 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in the expressions of the above markers over increasing storage period. [SD, standard deviation]

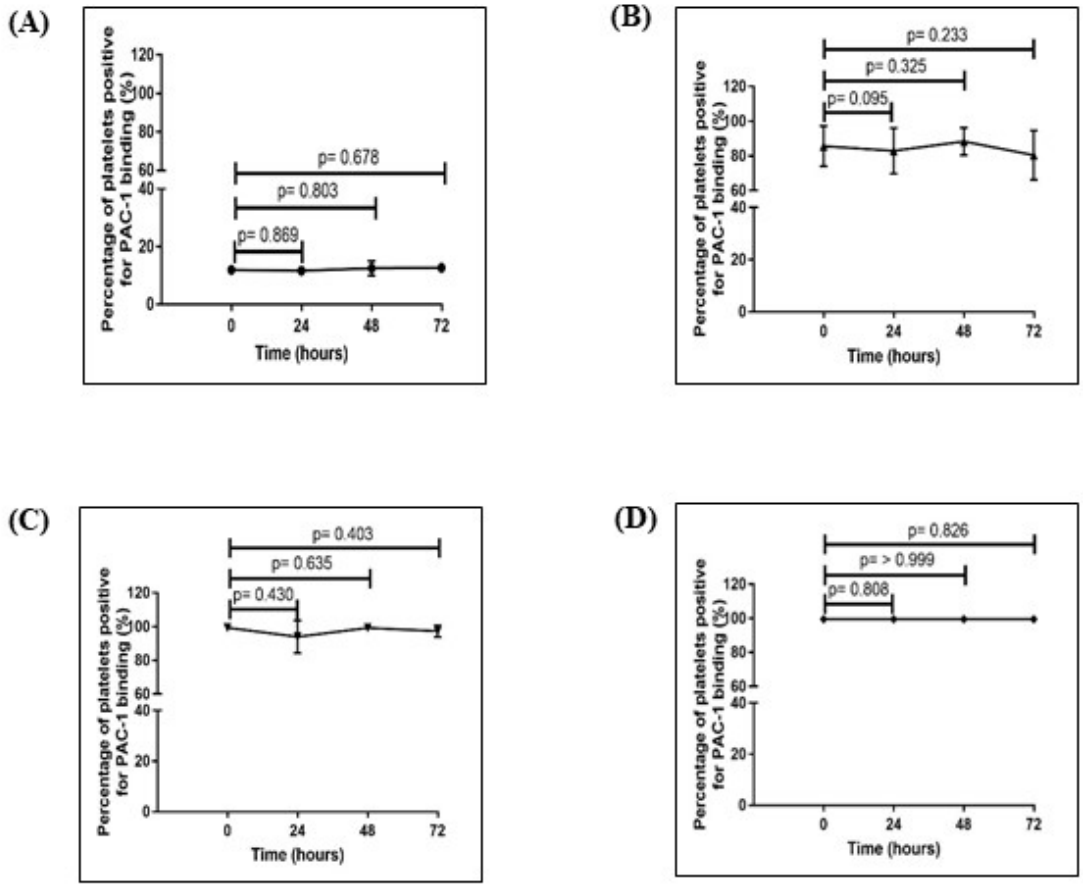

Figure 3.7 The expression (percentage positive platelets) of activated integrin αIIbβ3indicator, PAC-1 binding at time points 0-, 24-, 48- and 72-hours post-fixation.

Circulating platelets positive for activated integrin αIIbβ3 indicator, PAC-1 binding at (A) 0 µM TRAP-6, (B) 5 µM TRAP-6, (C) 10 µM TRAP-6 and (D) 50 µM TRAP-6. Data shown as mean +/- SD (n=6 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in the expressions of the above markers over increasing storage period. [SD, standard deviation]

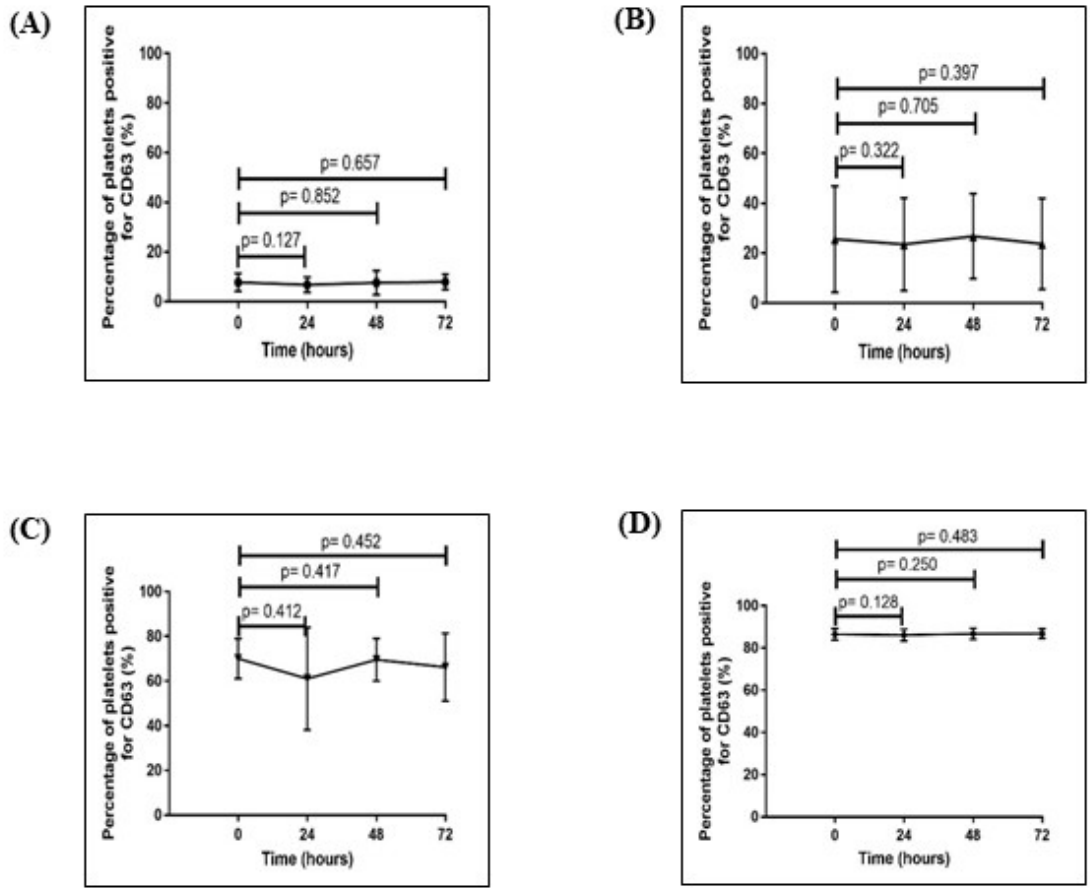

Figure 3.8 The expression (percentage positive platelets) of lysosome release indicator, CD63 at time points 0-, 24-, 48- and 72-hours post-fixation.

Circulating platelets positive for lysosome release indicator, CD63 at (A) 0 µM TRAP-6, (B) 5 µM TRAP-6, (C) 10 µM TRAP-6 and (D) 50 µM TRAP-6. Data shown as mean +/- SD (n=6 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in the expressions of the above markers over increasing storage period. [SD, standard deviation]

3.3.3 Markers important for interactions between platelets with monocytes and neutrophils (Panel 3)

3.3.3.1 The effects of time lapse between blood collection and initiation of assay on the stability of platelet- and leukocytes-specific markers for Panel 3

A total of 5 paediatric samples (3 males and 2 females; age range: 4 – 14 years) was used in this section. There was a significant increase in MPA and NPA formation across all time points up to 60 minutes after sample collection (Figure 3.9).

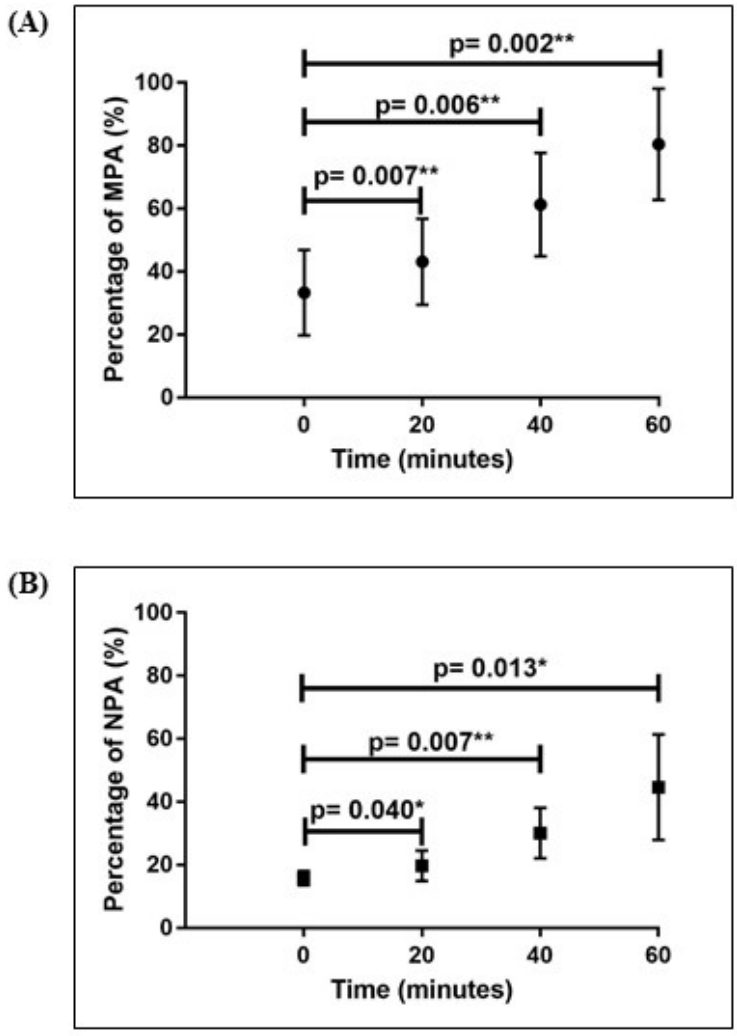

Figure 3.9 Effect of time lapse between blood collection and initiation of assay on the interaction between platelets with monocytes and neutrophils.

Circulating (A) MPA and (B) NPA levels. Data shown as mean +/- SD (n=5 healthy paediatric samples) and p-value < 0.05 is statistically significant. There was a significant increase in the interaction between platelets with monocytes and neutrophils over increasing time lapse after sample collection. [SD, standard deviation]

3.3.3.2 Post-fixation stability of Panel 3 monoclonal antibodies to paediatric platelets and leukocytes

A total of 3 paediatric samples (3 females; age range: 8 – 16 years) was used in this section. Panel 3 aims to evaluate the interaction between platelets with monocytes and neutrophils. At basal state (0 μM TRAP-6), the detection of MPA and NPA (percentage positive) (Figure 3.10 and Figure 3.11), the expression (MFI) of the platelet marker, integrin αIIbβ3 (Figure 3.12 and Figure 3.13) and CD11b (indicator of leukocyte activation) (Figure 3.14 and Figure 3.15) for both MPA and NPA remained stable after storage for up to 72 hours post-fixation. Although the expressions of the indicator of α-granule release, CD62P (marker of platelet activation) on MPA remained stable after storage for up to 72 hours post-fixation (Figure 3.16), a significant increase in CD62P expression was detected for NPA after storage for 72 hours post-fixation (Figure 3.17).

Upon stimulation by 50 μM TRAP-6, while the detection of MPA remained unchanged, the percentage positive of NPA increased significantly after storage for 72 hours post-fixation (Figure 3.10 and Figure 3.11). The expressions of the platelet marker, integrin αIIbβ3 in both MPA and NPA remained stable after storage for up to 48 hours post-fixation. A significant increase in integrin αIIbβ3 expression was observed for both MPA and NPA (Figure 3.12 and Figure 3.13). Although the expression of CD11b (indicator of leukocyte activation) on MPA remained stable for up to 72 hours post-fixation, a significant increase in CD11b was observed for NPA after storage for 72 hours post-fixation (Figure 3.14 and Figure 3.15). While the expression of the indicator of α-granule release, CD62P (marker of platelet activation) on MPA remained stable for up to 48 hours post-fixation, a significant increase was observed for NPA after storage for 48 and 72 hours post-fixation (Figure 3.16 and Figure 3.17). Due to the variability seen for the expression of the marker for platelets, integrin αIIbβ3, activation markers for platelets (CD62P) and leukocytes (CD11b) on MPA and NPA, flow cytometric

acquisition for all samples labelled with Panel 3 markers had to be completed within 24 hours after fixation.

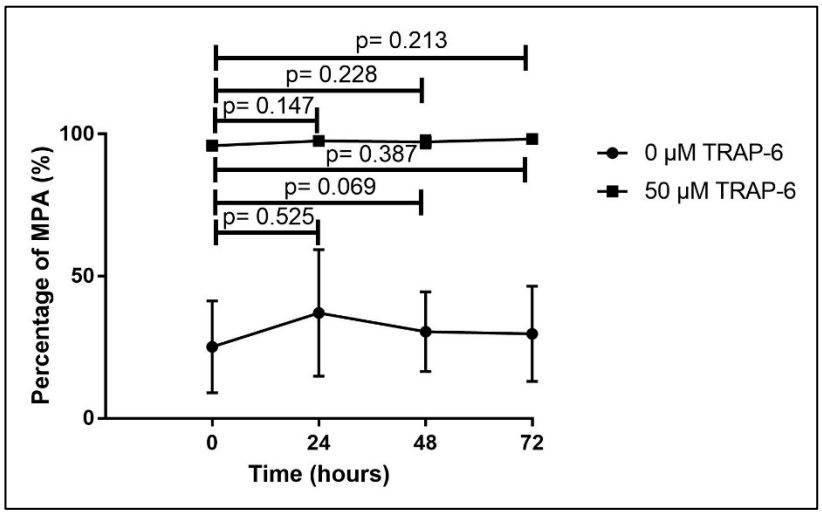

Figure 3.10 Formation of MPA (percentage positive) at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in MPA formation at both 0 and 50 µM TRAP-6 over increasing storage period. [SD, standard deviation]

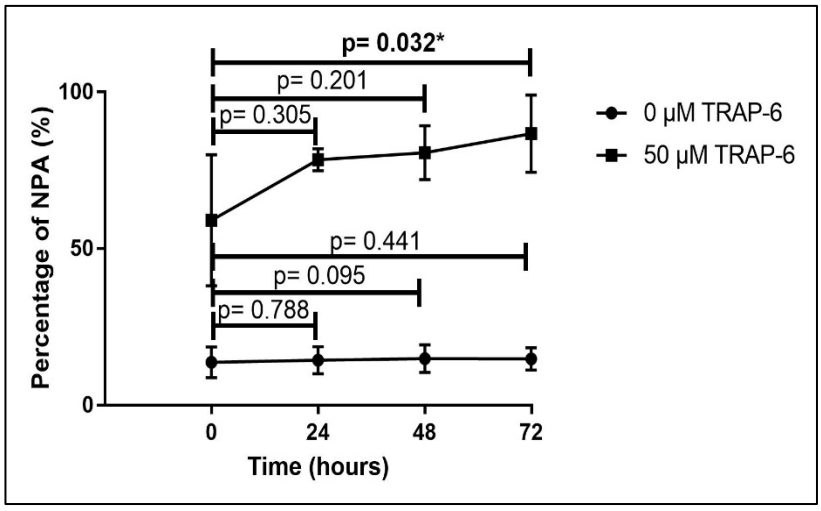

Figure 3.11 Formation of NPA (percentage positive) at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in NPA formation at 0 µM TRAP-6 over increasing storage period. At 50 µM TRAP-6, NPA formation increased significantly after storage for 72 hours. [SD, standard deviation]

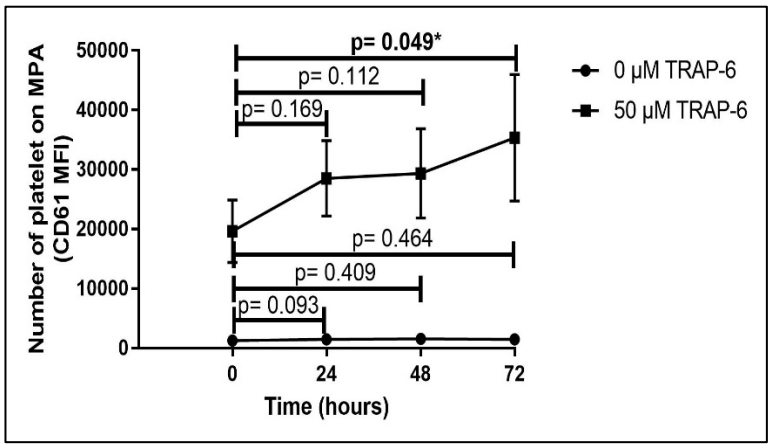

Figure 3.12 The expression of integrin αIIbβ3 on platelets in MPA at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. At 0 µM TRAP-6, there were no statistically significant changes in CD61 expressions on MPA over increasing storage period. At 50 µM TRAP-6, CD61 expressions on MPA increased significantly after storage for 72 hours. [SD, standard deviation]

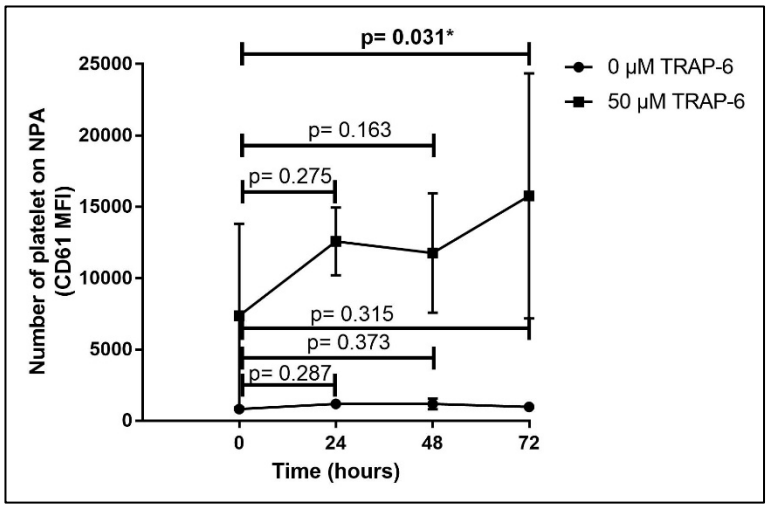

Figure 3.13 The expression of integrin αIIbβ3 on platelets in NPA at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. At 0 µM TRAP-6, there were no statistically significant changes in CD61 expressions on NPA over increasing storage period. At 50 µM TRAP-6, CD61 expressions on NPA increased significantly after storage for 72 hours. [SD, standard deviation]

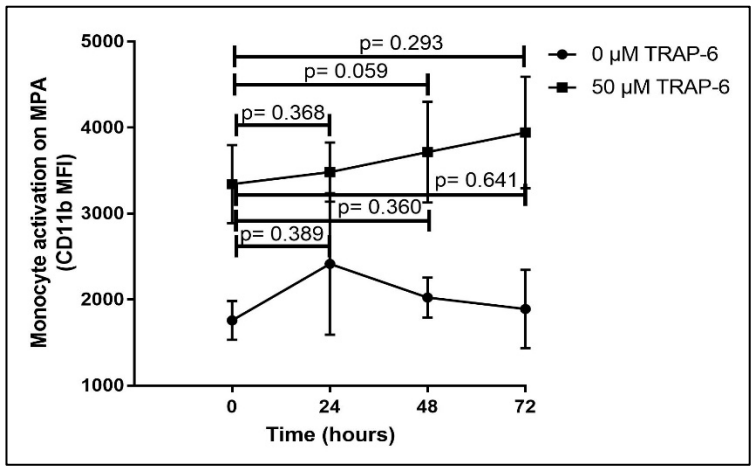

Figure 3.14 The expression of CD11b on monocytes in MPA at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. There were no statistically significant changes in CD11b expressions on MPA at both 0 and 50 µM TRAP-6 over increasing storage period. [SD, standard deviation]

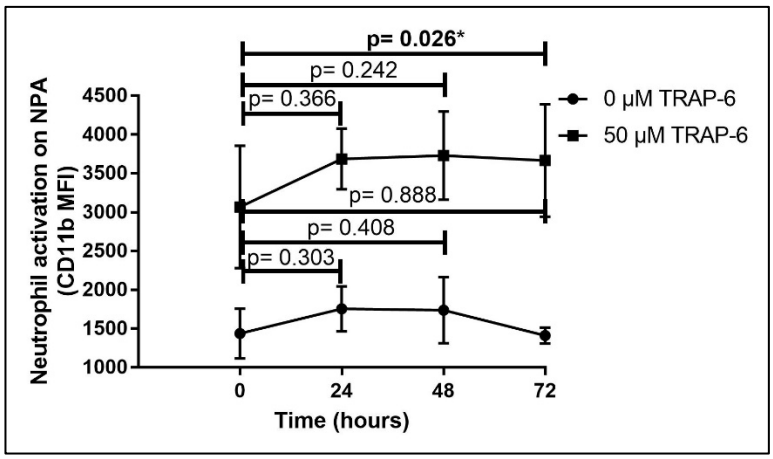

Figure 3.15 The expression of CD11b on neutrophils on NPA at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. At 0 µM TRAP-6, there were no statistically significant changes in CD11b expressions on NPA over increasing storage period. At 50 µM TRAP-6, CD11b expressions on NPA remained stable for up to 48 hours. [SD, standard deviation]

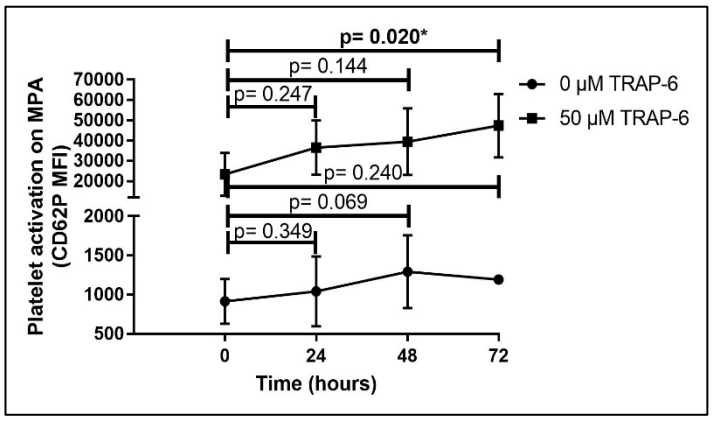

Figure 3.16 The expression of CD62P on MPA at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. At 0 µM TRAP-6, there were no statistically significant changes in CD62P expressions on MPA over increasing storage period. CD62P expressions on MPA at 50 µM TRAP-6 remained stable for up to 48 hours. [SD, standard deviation]

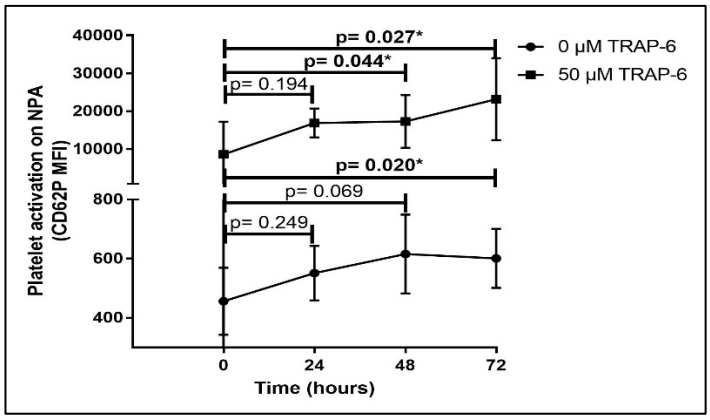

Figure 3.17 The expression of CD62P on NPA at 0 and 50 µM TRAP-6 at time points 0-, 24-, 48- and 72-hours post-fixation.

Data shown as mean +/- SD (n=3 healthy paediatric samples) and p-value < 0.05 is statistically significant. At 0 µM TRAP-6, CD62P expressions on NPA remained stable for up to 48 hours and increased significantly after storage for 72 hours. At 50 µM TRAP-6, CD62P expressions on NPA only remained stable for up to 24 hours followed by a significant increase in the expression of the marker. [SD, standard deviation]

3.4 Discussion

The main findings of this chapter were:

I.) Markers important for platelet phenotype (Panel 1) remained stable for up to 60 minutes after sample collection with an increasing trend observed for platelets activation (Panel 2) over time. In contrast, there was a significant increase in MPA and NPA formations over increasing time lapse after sample collection. For consistency and within the existing practical consideration, the staining for MPA/NPA assay (Panel 3) was initiated before the platelet assay (Panel 1 and Panel 2) and staining for Panel 1, 2 and 3 had to be completed within 40 minutes after sample collection.

II.) The 'threshold' and 'sub-maximal' concentrations of TRAP-6 to be used for the platelet reactivity assay for all three markers of activation were 5 μM and 10 μM, respectively (Panel 2).

III.) Samples stained with Panel 1 and Panel 2 monoclonal antibodies can be stored at 4 ºC for 24 hours before proceeding with flow cytometric data acquisition within 72 hours whereas flow cytometric data acquisition for samples stained with Panel 3 monoclonal antibodies had to be completed within 24 hours after fixation.

The flow cytometry assays were established to evaluate the markers important for platelet phenotype and function, the response of platelets to TRAP-6 (mimic of the most potent *in vivo* platelet activator, thrombin) and the interaction between platelets with monocytes and neutrophils. The major strength of the present assays is by combining different panels, for the first time, platelet function with regards to their phenotype, circulating activation, responsiveness and state of heteroaggregate formation could be investigated simultaneously. Together, these may contribute to the comprehensive description of the platelet function in children on ECMO. Specifically, combining measurements of markers important for platelet

phenotype and function and response to TRAP-6 will allow the identification of the cause of an enhanced or reduced platelet function that remains elusive in this population. Furthermore, combination of antibodies used for the examination of the interaction between platelets with monocytes and neutrophils allowed the investigation of the state of the MPA/NPA formation with cohesive evaluation of their activation status and mechanisms of interaction.

The sample preparation were standardized based on the few elements used to validate the sampling procedure. Using a cut-off value of 10 % as the maximum accepted pre-activation level for the platelet activation-dependent marker [224], the results showed that the platelet assays were with minimal pre-activation hence ensuring a reliable way of platelet function analysis. In contrast, increasing MPA/NPA formation seen over time was consistent with the limited processing window recommended for whole blood analysis of PLA in the currently available protocol [225]. To overcome such limitation, the staining for MPA/NPA assay was initiated before the platelet assay to ensure a minimal influence of pre-analytical variation in evaluation of the interaction between platelets with monocytes and neutrophils. Therefore, a processing window of within 40 minutes after sample collection was chosen to complete staining for all three assays.

In recent years, the utilization of multiple concentrations of different agonists in platelet function testing has gained increased interest as it allows the identification of different platelet activation pathways which is a better reflection of complex *in vivo* agonist-induced changes in platelet function. In this study, TRAP-6 which is the most potent *in vitro* activator of platelet [122, 123] was used to mimic *in vivo* thrombin effects. As thrombin is the most potent *in vivo* activator of platelets, an increased or a reduced platelet reactivity may indicate an overall platelet hyper- or hypo-responsivity. Such information will allow the identification of patients with enhanced or impaired platelet function via the thrombin PAR 1 receptor activation pathway.

To allow flow cytometric analysis at a more convenient moment for handling of multiple samples, assays that are stable with storage at 4 ºC for up to 72 hours for reliable quantification of markers important for platelet phenotype and function with simultaneous evaluation of platelet reactivity were developed in this study. On the other hand, most of the markers used to evaluate MPA/NPA displayed increasing trend in their expressions after storage for 24 hours up to 72 hours. These could be related to an overall increase in autofluorescence commonly seen for the leukocytes after long-term storage post-fixation [223].

3.5 Conclusion

In conclusion, flow cytometry assays that allow simultaneous evaluation of platelet function in relation to their phenotype, responsiveness and interactions with monocytes and neutrophils were developed. For optimal sample processing within the existing practical consideration, staining for Panel 1, 2 and 3 were completed within 40 minutes after sample collection with staining for MPA/NPA assay (Panel 3) to be initiated before the platelet assay (Panel 1 and Panel 2). To examine platelet responsiveness, the 'threshold' and 'sub-maximal' concentrations of TRAP-6 were identified to be 5 μM and 10 μM respectively for the assessment of activation-dependent platelet markers. Lastly, for flow cytometric analysis of multiple samples at a more convenient moment, samples stained with Panel 1 and Panel 2 monoclonal antibodies were stored at 4 ºC for 24 hours before proceeding with flow cytometric data acquisition within 72 hours and samples labelled with Panel 3 markers were proceed with flow cytometric data acquisition within 24 hours after fixation.

4 The effect of pathway onto ECMO on platelet phenotype, function and interactions with monocytes and neutrophils and their associations with bleeding or thrombosis

4.1 Introduction

ECMO is a modified form of cardiopulmonary bypass (CPB) which aims to provide short- to medium-term of cardiac and/or respiratory support. The ECMO population can be categorized based on the indication to be placed on ECMO, and these differ significantly in the interventions and procedures leading up to the commencement of ECMO- the "pathway onto ECMO". For respiratory indications, ECMO is used for patients with primary diagnosis of congenital diaphragmatic hernia, meconium aspiration syndrome and sepsis etc. [226]. On the other hand, cardiac patients such as those with cardiomyopathy or cardiac arrest represent 41 % of the paediatric ECMO population [227]. CPB is a heart-lung machine commonly used during cardiac surgery and more than 80 % of cardiac surgery is performed using CPB [228]. The majority of the cardiac patients (79 %) have surgery before coming onto ECMO and have higher susceptibility towards bleeding complications compared to those who had no surgery [227].

Similar to ECMO, haemostatic derangement is seen in the CPB population and has been associated with the artificial surface and shear-stress induced platelet dysfunction [229]. Furthermore, modifications of platelet function by CPB have been associated with clinical events seen in the patients during and/or after cardiac surgery. For example, more than 35 % of patients who had cardiac surgery with CPB bled more than 1 L within the 24-hour post-operative period [230]. Despite such known detrimental effects of CPB on the haemostatic function, currently, all ECMO patients receive similar management during ECMO due to the absence of evidence-based guidelines for targeted approaches.

Platelet dysfunction and activation have been associated with bleeding and thrombotic complications in patients on ECMO [231-233]. However, very few studies have documented the effects of ECMO on platelet function especially in the paediatric population due to the challenges such as the ethical approval and limited availability of blood sample volume. The limited studies previously performed, predominantly using platelet aggregometry in small number of patients have suggested that in children on ECMO there is:

I.) Unchanged expression of the receptors important for platelet adhesion and aggregation [212]
II.) Increased platelet activation [213]
III.) Reduced response to various agonists (e.g. collagen) [213, 214]

Since the effects of CPB-induced haemostatic defects on platelet function are known and have been related to the bleeding and/or clotting events seen in the patients during and/or after CPB, patients coming onto ECMO via different pathways (e.g. whether they had CPB or not) may differ in their platelet function within the first 24 hours upon ECMO initiation and may increase the risk of developing clinical event in the patient as ECMO progresses. To date, there is no study that has examined the platelet function within the paediatric ECMO population according to their pathway onto ECMO. Understanding these may provide information for whether early intervention can be tailored and given to the patients upon ECMO initiation thus minimizing the occurrence of clinical events as ECMO progresses.

Hence, the hypothesis of this chapter is that platelet phenotype, function and interactions with monocytes and neutrophils within the first 24 hours of ECMO will be different according to a patient's pathway onto ECMO and the platelet-specific changes can be associated with the development of bleeding or thrombosis during ECMO.

This chapter aims to determine the effect of a patient's pathway onto ECMO on the platelet-specific differences and their associations with the clinical events for:

1) The platelet phenotype.
2) The circulating platelet activation.
3) The response of platelets to stimulation with TRAP-6.
4) The interactions of platelets with monocytes and neutrophils.

4.2 Materials and Methods

Chapter 2 comprised the details for methods used in this chapter. Definitions in section 2.2.3 were used to identify the minor bleeding, major bleeding and thrombosis events. Flow cytometry panels and their relevant details were included in sections 2.6.3 - 2.6.5. The summary for the evaluation of platelet-specific markers are as followed:

I.) Platelet phenotype was assessed by measuring the expression of integrin αIIbβ3, GPIb/IX/V and GPVI receptors.

II.) Circulating platelet activation was measured as the percentage of platelets positive for activated integrin αIIbβ3 receptor (measured by PAC-1 binding) while platelet granule exocytosis was measured as the percentage of platelets positive for α-granule (P-selectin) and lysosome release (CD63) indicators. The response of platelet to TRAP-6 is directly proportional to the area under the curve (AUC).

III.) The interaction between platelets and monocytes/neutrophils were investigated by evaluating circulating MPA/NPA. The relative number of platelets bound on MPA/NPA and monocytes/neutrophils was measured by MPA/NPA CD61 MFI [234]. The mechanism of MPA/NPA tethering was measured by reporting the MFI of P-selectin and Mac-1 on platelet-bound and unbound monocyte/neutrophil events with/without stimulation with TRAP-6 [141].

The results of platelet-specific markers within the first 24 hours upon initiation of ECMO were used for all data analysis relevant to the platelet-specific changes in this chapter. Platelet phenotype, function and interactions with monocytes and neutrophils were compared between and within the Post-CPB and Non-CPB groups according to the clinical events except for those limited by the number of bleeding or thrombotic event (less than 3 events). Statistical analysis were described in details in section 2.7. Statistical software package STATA (Release 15) (Stata Corp., College Station, Texas) was used for all statistical analysis in this chapter. In

summary, Fisher's exact or Chi-Square test was used for the comparisons of parameters (except for the age, weight and full blood count parameters with unpaired Student's t-test) for the demographic details in Table 4.1. Unpaired Student's t-test was also used for the comparison of the platelet phenotype and function between the Post-CPB and Non-CPB group and for the evaluation of the association between platelet-related changes and clinical events (minor bleeding, major bleeding or thrombosis).

4.3 Results

4.3.1 Demographics

Patients were categorized into two groups according to whether they had ('Post-CPB' group) or had not been on CPB ('Non-CPB' group) before going onto ECMO in the same admission. Demographic information for the twenty-two patients included in this chapter was summarized in Table 4.1. Post-CPB and Non-CPB patients had comparable age, gender, weight and mode of cannulation. The Post-CPB and Non-CPB groups also had comparable rate of minor bleeding, major bleeding or thrombosis across the duration on ECMO. Also, both groups had similar full blood count parameters. In contrast, there was a trend for an increased number of patient that required at least one platelet transfusion across the duration on ECMO in the Post-CPB group compared to the Non-CPB group.

Figure 4.1 depict the longitudinal graph for patients, clinical events and platelet transfusions received by the patients according to their pathway onto ECMO. Within the first 24 hours upon ECMO initiation (Day 1), 42 % of the Post-CPB group compared to 40 % of the Non-CPB group had ≥ 1 clinical event. Similar observation was obtained for platelet transfusion with 67 % of the Post-CPB group compared to 60 % of the Non-CPB group who had ≥ 1 platelet transfusion.

The definition used to categorize between groups who had/had no clinical event for the analysis of platelet phenotype and function in relation to the clinical event was whether a patient had at least one clinical event (minor bleeding, major bleeding or thrombosis) across the duration on ECMO.

Table 4.1 Summary of demographic information and full blood count comparing the Post-CPB to Non-CPB group.

Variable			Pathway onto ECMO, n= 22 n (%)		p-value (Post-CPB vs. Non-CPB)
			Post-CPB 12 (55)	Non-CPB 10 (45)	
Age [years] median (IQR)			0.34 (0.02 - 3.11)	0.55 (0.00 - 11.40)	0.883
Gender, n (%)		Male	5 (42)	7 (70)	0.988
		Female	7 (58)	3 (30)	
Weight [kg] median (IQR)			6.20 (3.46 - 13.10)	5.55 (3.20 - 38.70)	0.231
Primary diagnosis	Cardiac	Unable to wean off CPB	6 (50)	0 (0.0)	-
		Cardiomyopathy	1 (8)	3 (30)	
		Myocarditis	0 (0)	2 (20)	
		Primary congenital cardiac abnormality	3 (25)	2 (20)	
		Other	2 (17)	1 (10)	
	Respira -tory	Meconium aspiration syndrome	0 (0)	1 (10)	-
	Other	Sepsis	0 (0)	1 (10)	-
Mode of cannulation, n (%)		Central	11 (92)	5 (50)	0.056
		Peripheral	1 (8)	5 (50)	
Clinical event, n (%)	Minor bleeding	Yes	7 (58)	5(50)	> 0.999
		No	5 (42)	5 (50)	
	Major bleeding	Yes	6 (50)	6 (60)	0.691
		No	6 (50)	4 (40)	
	Thrombosis	Yes	6 (50)	2 (20)	0.204
		No	6 (50)	8 (80)	
Platelet transfusion, n (%)		Yes	12 (100.0)	7 (70.0)	0.078
		No	0 (0.0)	3 (30.0)	
Full blood count			Pathway onto ECMO, n= 12 n (%)		p-value (Post-CPB vs. Non-CPB)
			Post-CPB 8 (67)	Non-CPB 4 (33)	
WCC (x 10^9/L)			5.24 ± 2.18	7.78 ± 6.93	0.522

RCC (x 10^{12}/L)	3.50 ± 0.71	3.30 ± 0.80	0.682
HGB (g/L)	104.88 ± 21.51	97.25 ± 22.05	0.590
HCT (L/L)	0.31 ± 0.07	0.29 ± 0.07	0.649
PLT (x 10^{9}/L)	105.38 ± 47.44	127.00 ± 58.74	0.551
MPV (fl)	8.00 ± 2.03	7.03 ± 0.57	0.239

[ECMO, extracorporeal membrane oxygenation; HCT, haematocrit; HGB, haemoglobin; IQR, interquartile range; MPV, mean platelet volume; Non-CPB, non-cardiopulmonary bypass; PLT, platelet; Post-CPB, post-cardiopulmonary bypass; RCC, red blood cell count; WCC, white blood cell count]

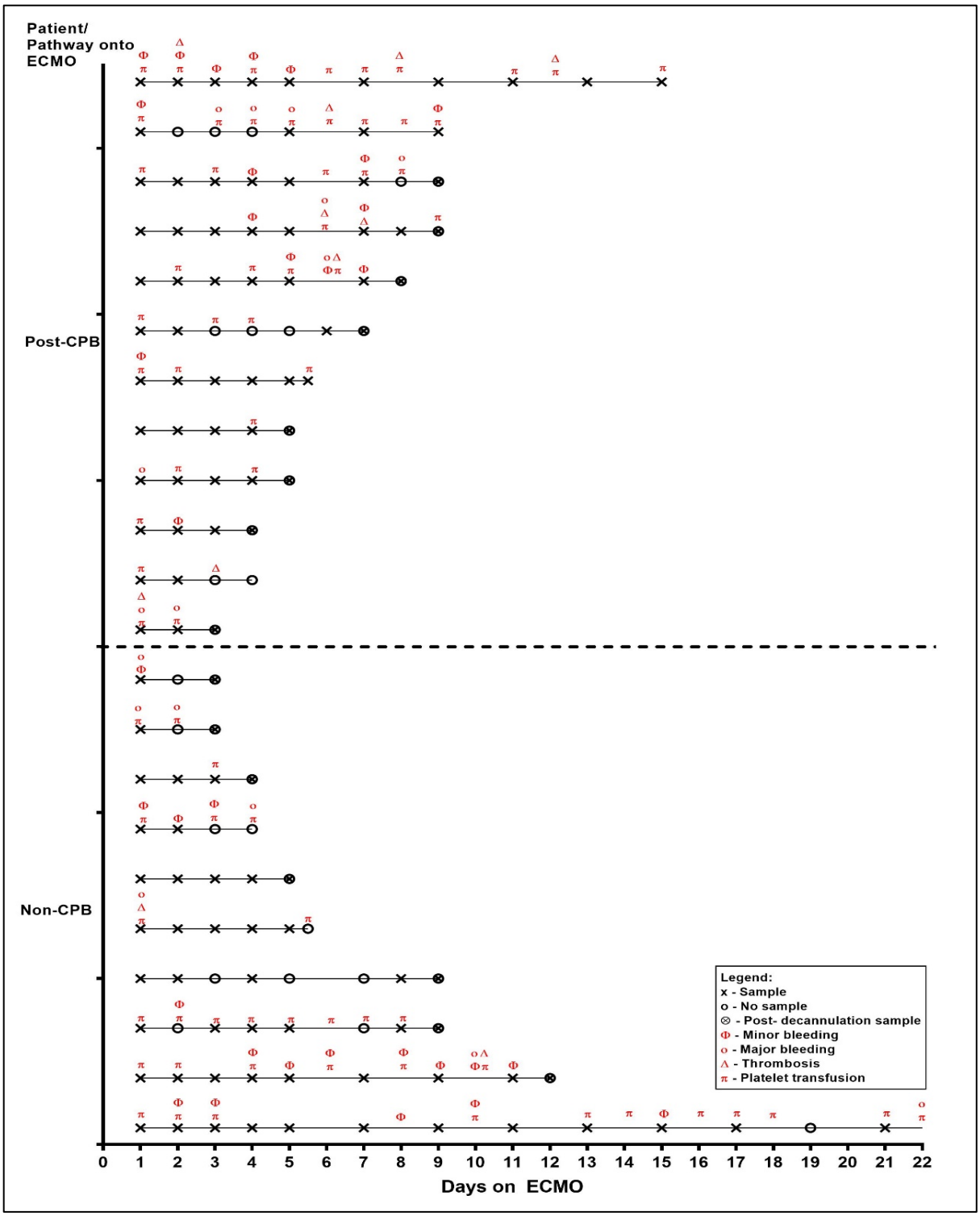

Figure 4.1 Longitudinal graph for patients, clinical events and platelet transfusions according to the pathway onto ECMO.

The dotted line depict the division for the Post-CPB and Non-CPB groups. [ECMO, extracorporeal membrane oxygenation; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass]

4.3.2 Platelet phenotype

There was no significant difference in the expression of integrin αIIbβ3, GPIb/IX/V and GPVI receptors between the Post-CPB and Non-CPB groups (Figure 4.2). However, there was a trend for an increased GPVI receptor expression in the Post-CPB group compared to the Non-CPB group (4107.00 ± 1396.38 vs. 3114.80 ± 950.55 MFI, p= 0.063; d= 0.82; power= 0.47) (Figure 4.2).

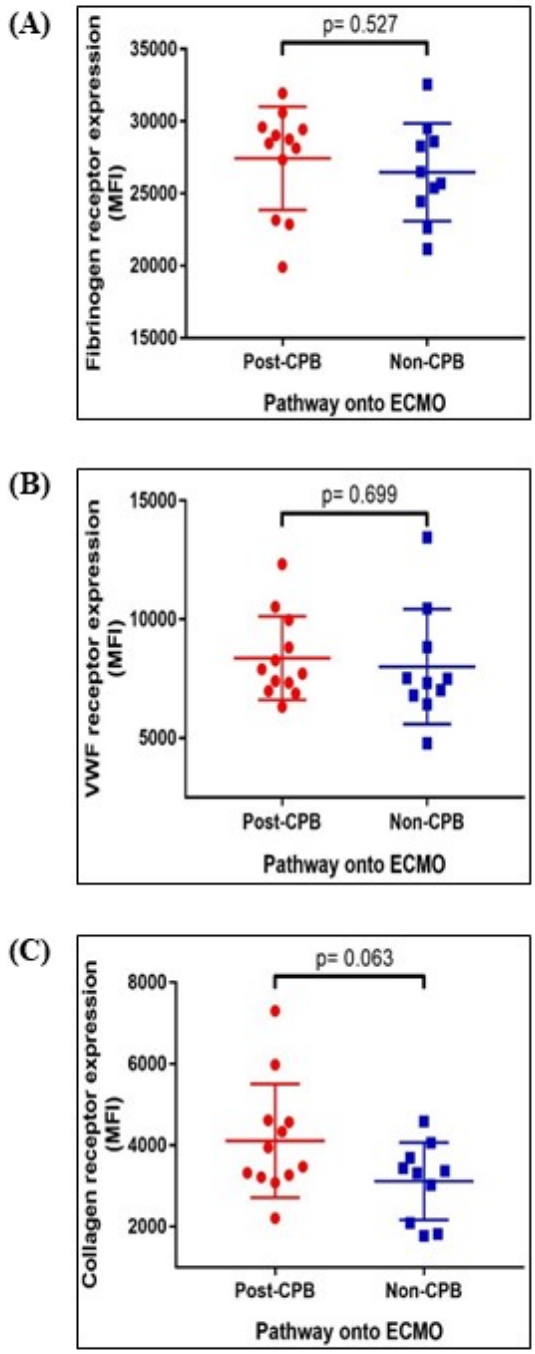

Figure 4.2 Platelet phenotype and pathway onto ECMO.

The expression of platelet (A) integrin αIIbβ3 receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor within the first 24 hours according to a patient's pathway onto ECMO. Data shown as mean MFI +/- SD (Post-CPB n=12 and Non-CPB n=10). [MFI, median fluorescence intensity; SD, standard deviation; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; VWF, von Willebrand factor]

Figure 4.3 depict the comparison of the expression for the integrin αIIbβ3, GPIb/IX/V and GPVI receptors between and within the Post-CPB and Non-CPB groups according to the clinical events. Within the Post-CPB group, patients who had major bleeding (29583 ± 1456.41 vs. 25291.5 ± 3870.48 MFI, p= 0.042; d= 1.47; power= 0.58) or thrombosis (29539.33 ± 1249.68 vs. 25335.17 ± 3998.16 MFI, p= 0.049; d= 1.42; power= 0.54) had higher integrin αIIbβ3 receptor expression than patients who had no major bleeding or thrombosis. In contrast, there was no difference for GPIb/IX/V and GPVI receptor expression between patients with or without minor bleeding, major bleeding and thrombosis within the Post-CPB group. Within the Non-CPB group, there was no difference for the integrin αIIbβ3, GPIb/IX/V and GPVI receptor expression between patients who had or had no minor /major bleeding.

For patients who had major bleeding, Post-CPB patients had higher integrin αIIbβ3 receptor expression (29583 ± 1456.41 vs. 27335.83 ± 1695.76 MFI, p= 0.034; d= 1.42; power= 0.87) and a trend for an increased GPVI receptor expression (4134.5 ± 1085.08 vs. 3023.67 ± 981.49 MFI, p= 0.094; d= 0.85; power= 0.67) compared to the Non-CPB patients.

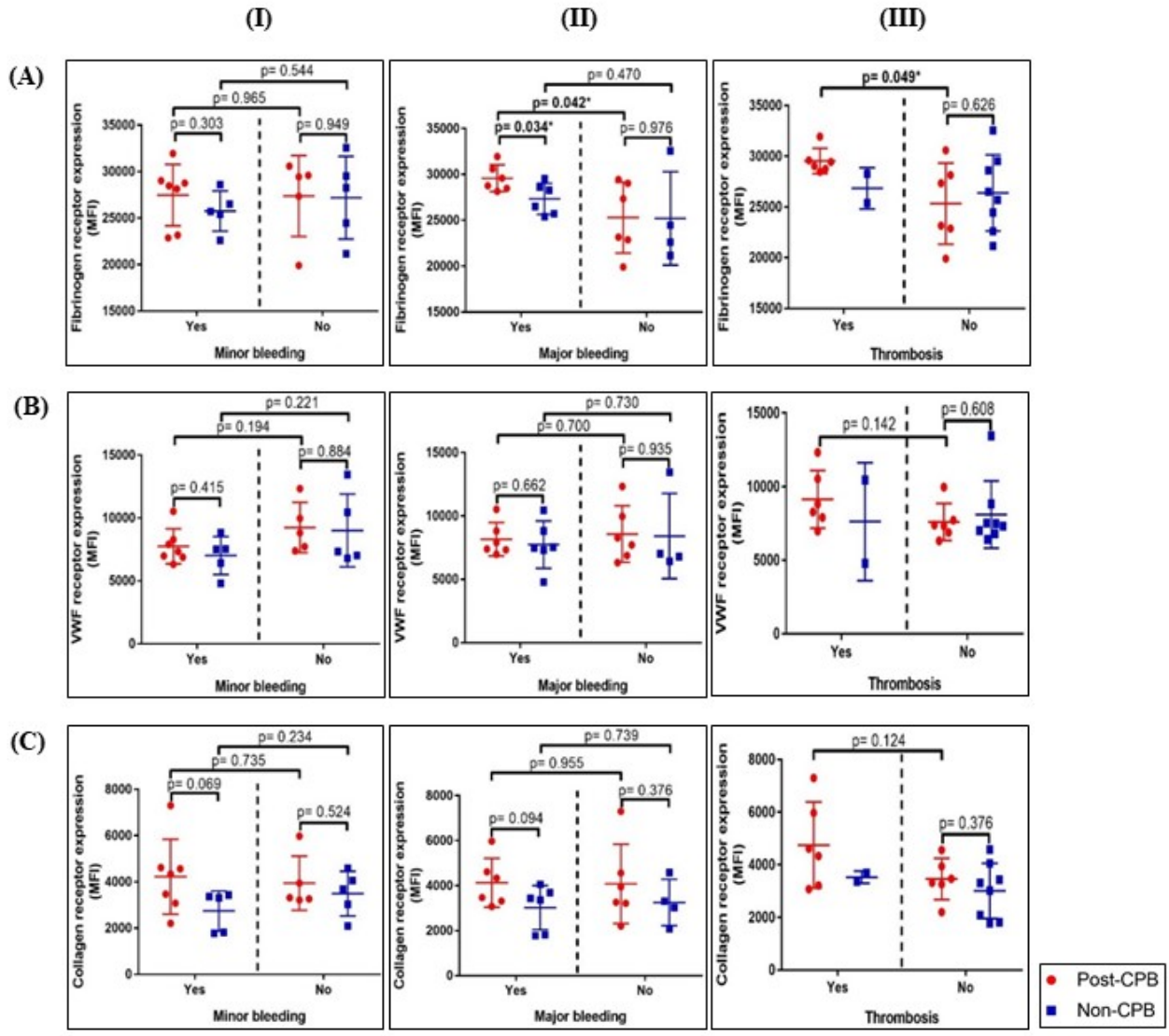

Figure 4.3 Platelet phenotype according to the pathway onto ECMO and presence or absence of clinical event.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean MFI +/- SD (Post-CPB n=12 and Non-CPB n=10). [MFI, median fluorescence intensity; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation; VWF, von Willebrand factor]

4.3.3 Circulating platelet activation

Circulating platelet activation and granule exocytosis were comparable for patients from different pathways onto ECMO (Figure 4.4). While the sample size was inadequately powered to assess differences in the coefficient of variation between groups, the inter-individual variability for the α-granule release indicator appeared to be higher in the Post-CPB group compared to the Non-CPB group (Figure 4.4).

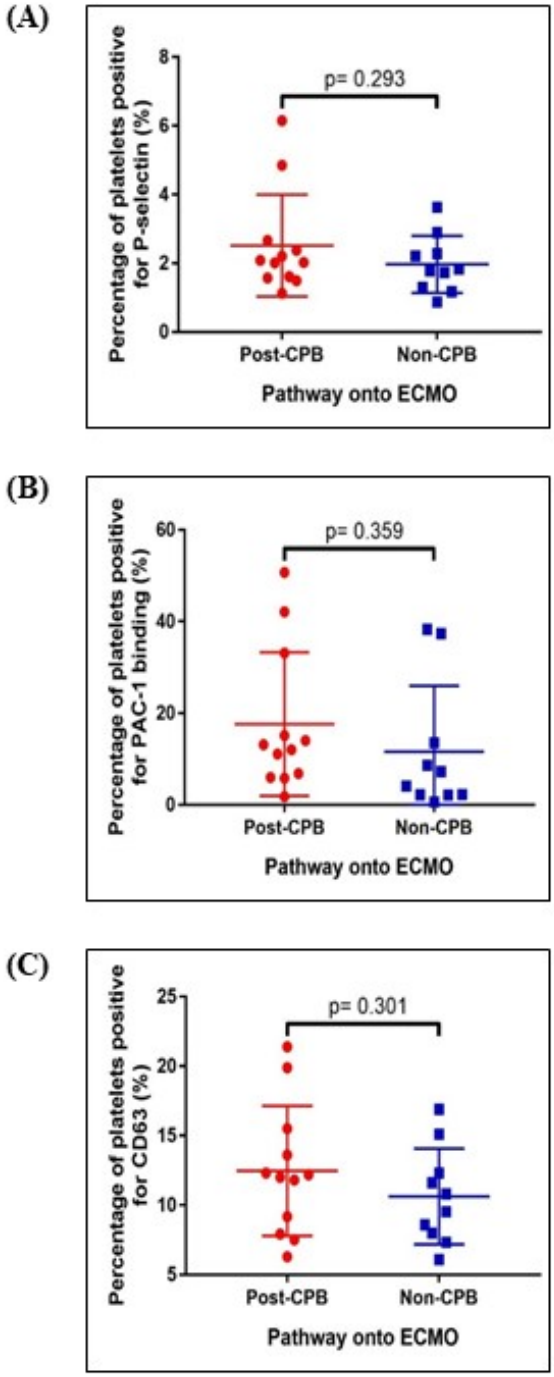

Figure 4.4 Circulating platelet activation and pathway onto ECMO.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's pathway onto ECMO. Data shown as mean % +/- SD (Post-CPB n=12 and Non-CPB n=10). [Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

Within the Non-CPB group, a trend for an increased α-granule release was observed for the patients who had major bleeding compared to those who had no major bleeding (2.33 ± 0.86 vs. 1.43 ± 0.44 %, p= 0.063, d= 1.23, power= 0.48) (Figure 4.5). On the other hand, platelet activation and granule release of the Post-CPB group were comparable between those who had minor bleeding, major bleeding or thrombosis and those who had no clinical events. Circulating platelet activation and granule release were comparable for patients between and within the Post-CPB and Non-CPB groups for minor bleeding. For patients without major bleeding, a trend for a decreased lysosome release was observed for the Non-CPB group compared to the Post-CPB group (13.32 ± 4.85 vs. 8.61 ± 2.03 %, p= 0.071; d= 1.17; power= 0.45).

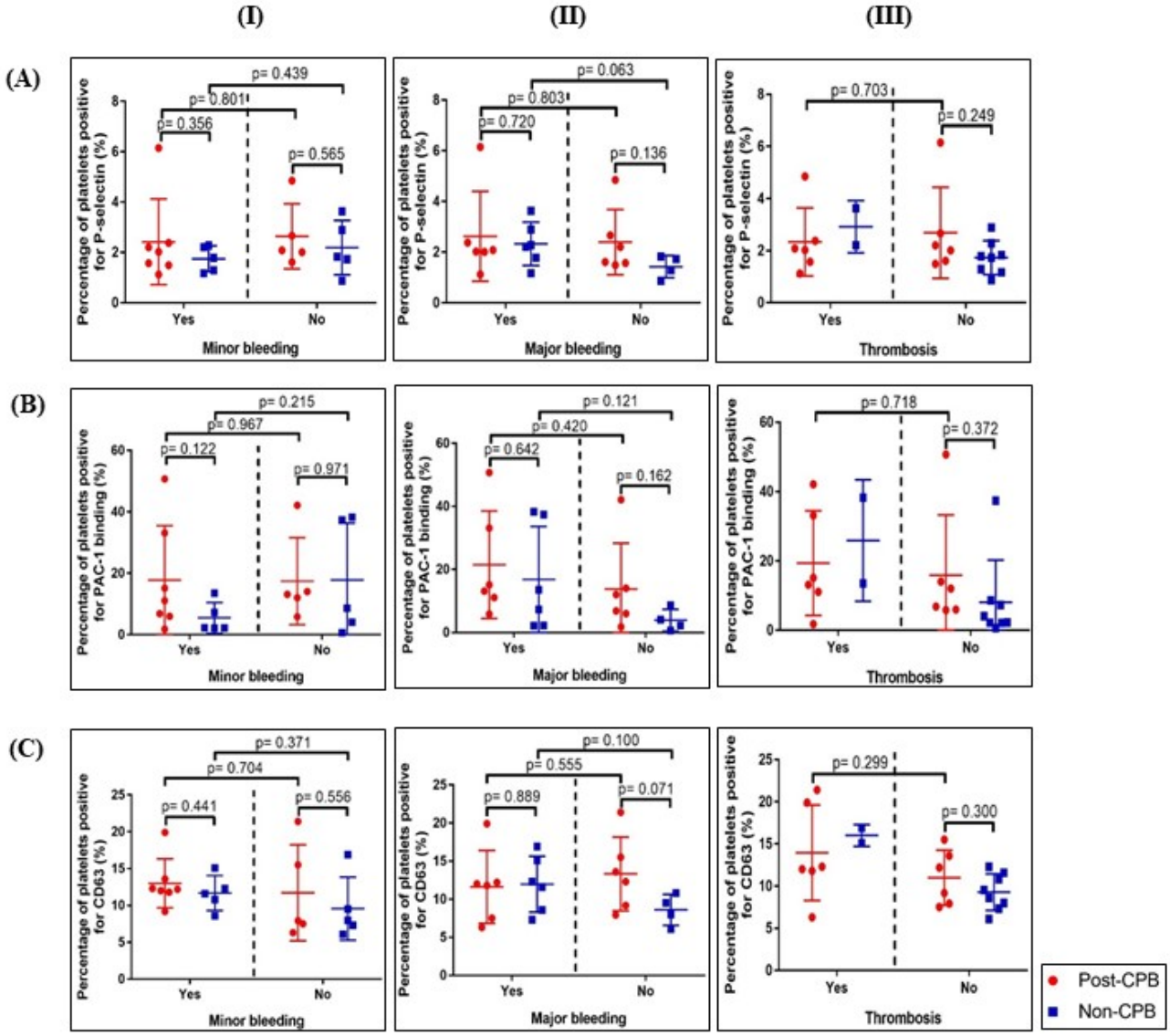

Figure 4.5 Circulating platelet activation according to the pathway onto ECMO and presence or absence of clinical event.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % +/- SD (Post-CPB n=12 and Non-CPB n=10). [Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

4.3.4 Response of platelets to stimulation with TRAP-6

The response of platelets to stimulation with TRAP-6 for activated platelet integrin αIIbβ3 receptor, and granule exocytosis (both α-granule and lysosome) was comparable for patients from different pathways onto ECMO (Figure 4.6). High inter-individual variability for the platelet response was observed within the Post-CPB and Non-CPB groups.

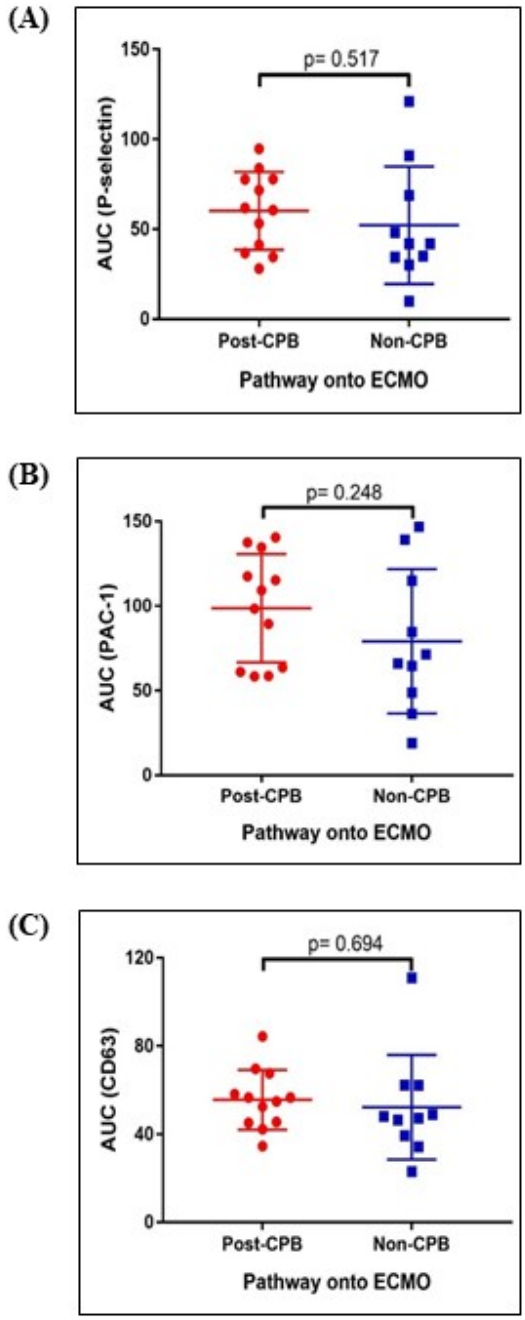

Figure 4.6 Platelet response to stimulation with TRAP-6 and pathway onto ECMO.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's pathway onto ECMO. Data shown as mean AUC +/- SD (Post-CPB n=12 and Non-CPB n=10). [AUC, area under the curve; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

Non-CPB patients who had minor bleeding had a trend for an increased platelet response [CD62P (32.87 ± 14.53 vs. 71.43 ± 35.46 AUC, p= 0.071; d= 1.42; power= 0.45) and PAC-1(55.48 ± 27.05 vs. 102.86 ± 44.16 AUC, p= 0.082; d= 1.29; power=0.42)] compared to those who had no minor bleeding (Figure 4.7). There was no difference for platelet response in patients who had or had no major bleeding within the Non-CPB group. On the other hand, Post-CPB patients who had minor bleeding, major bleeding or thrombosis had comparable platelet response to those who had no clinical events.

Non-CPB patients who had minor bleeding had lower platelet response than the Post-CPB group as indicated by the lower expression of P-selectin (57.98 ± 22.70 vs. 32.88 ± 14.53 AUC, p= 0.042; d= 1.27; power= 0.53), PAC-1 (95.95 ± 35.01 vs. 55.48 ± 27.05 AUC, p= 0.048; d= 1.26; power= 0.56) and CD63 (56.04 ± 10.31 vs. 40.28 ± 11.37 AUC, p= 0.039; d= 1.47; power= 0.58) to stimulation with TRAP-6 (Figure 4.7).

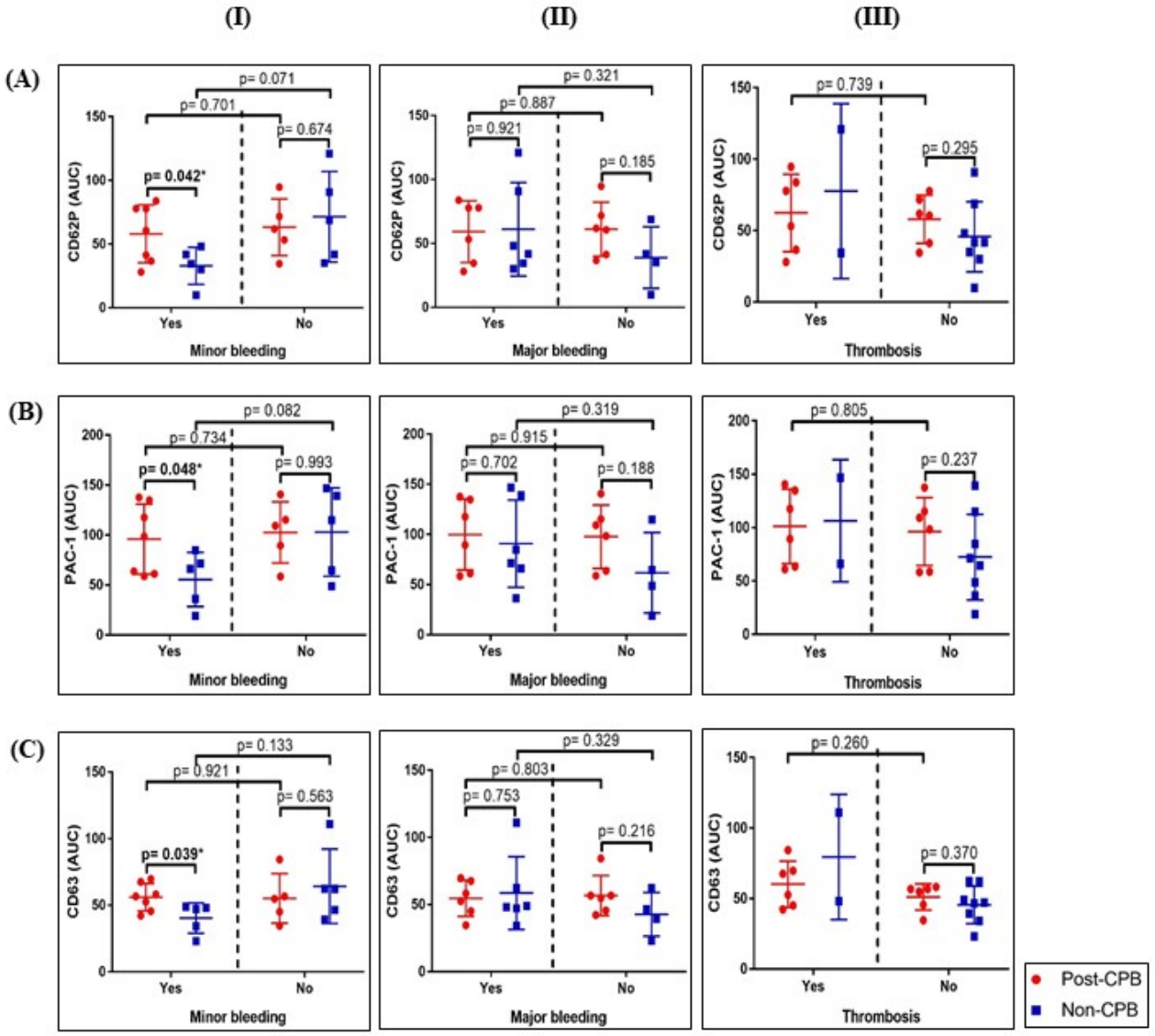

Figure 4.7 Platelet response to stimulation with TRAP-6 according to the pathway onto ECMO and presence or absence of clinical event.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean AUC +/- SD (Post-CPB n=12 and Non-CPB n=10). [AUC, area under the curve; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

4.3.5 Circulating monocyte-platelet aggregates (MPA)

The percentage of circulating MPA, the relative number of platelets bound on MPA as well as monocyte and platelet activation on MPA were comparable for patients from different pathways onto ECMO (Figure 4.8). Although the inter-individual variability for circulating MPA of the Non-CPB group appeared to be higher compared to the Post-CPB group, high inter-individual variability for monocyte and platelet activation on MPA was observed for the Post-CPB and Non-CPB groups (Figure 4.8). To assess if MPA formed via the P-selectin/PSGL-1 and Mac-1 (CD11b) adhesion mechanisms, the similar maximal expression of the P-selectin and Mac-1 upon stimulation with TRAP-6 observed for both the Post-CPB and Non-CPB groups indicated that the capacity for platelet and monocyte activation-dependent and –independent formation of MPA was equally retained in both groups (Figure 4.8).

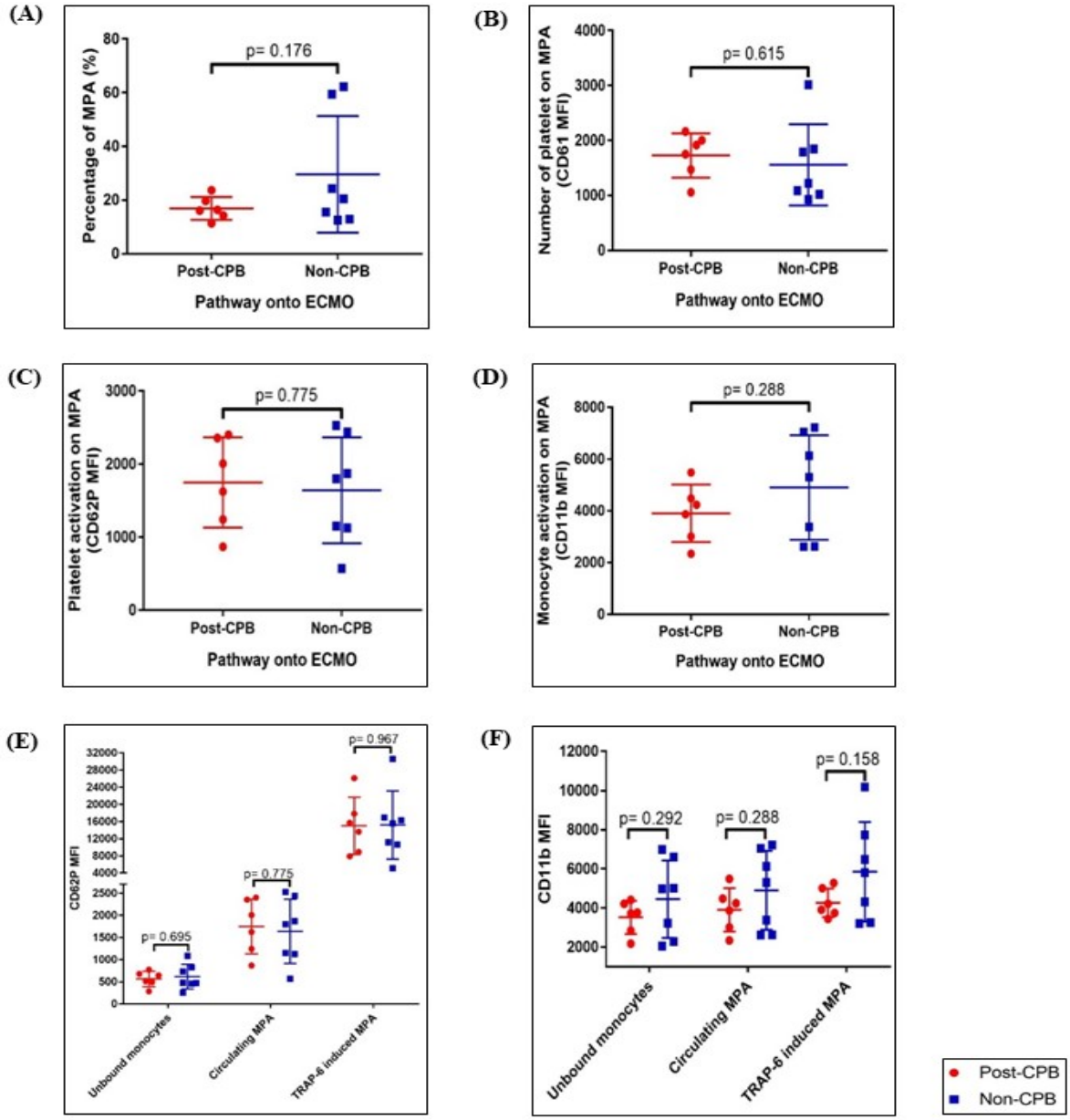

Figure 4.8 The interaction between platelets and monocytes and pathway onto ECMO.

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation, (D) monocyte activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on MPA within the first 24 hours according to a patient's pathway onto ECMO. Data shown as mean % or MFI +/- SD (Post-CPB n=6 and Non-CPB n=7). [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

For comparisons according to the clinical events, the percentage of circulating MPA, the relative number of platelets bound on MPA, platelet and monocyte activation (Figure 4.9) and mechanism of interaction (Figure 4.10) were comparable between the patients who had or had no minor bleeding or major bleeding within the Non-CPB group and also for patients with or without thrombosis within the Post-CPB group. In contrast, there was insufficient information to make comparison for MPA-related changes between patient who had or had no clinical event for minor bleeding or major bleeding within the Post-CPB group and thrombosis within the Non-CPB group.

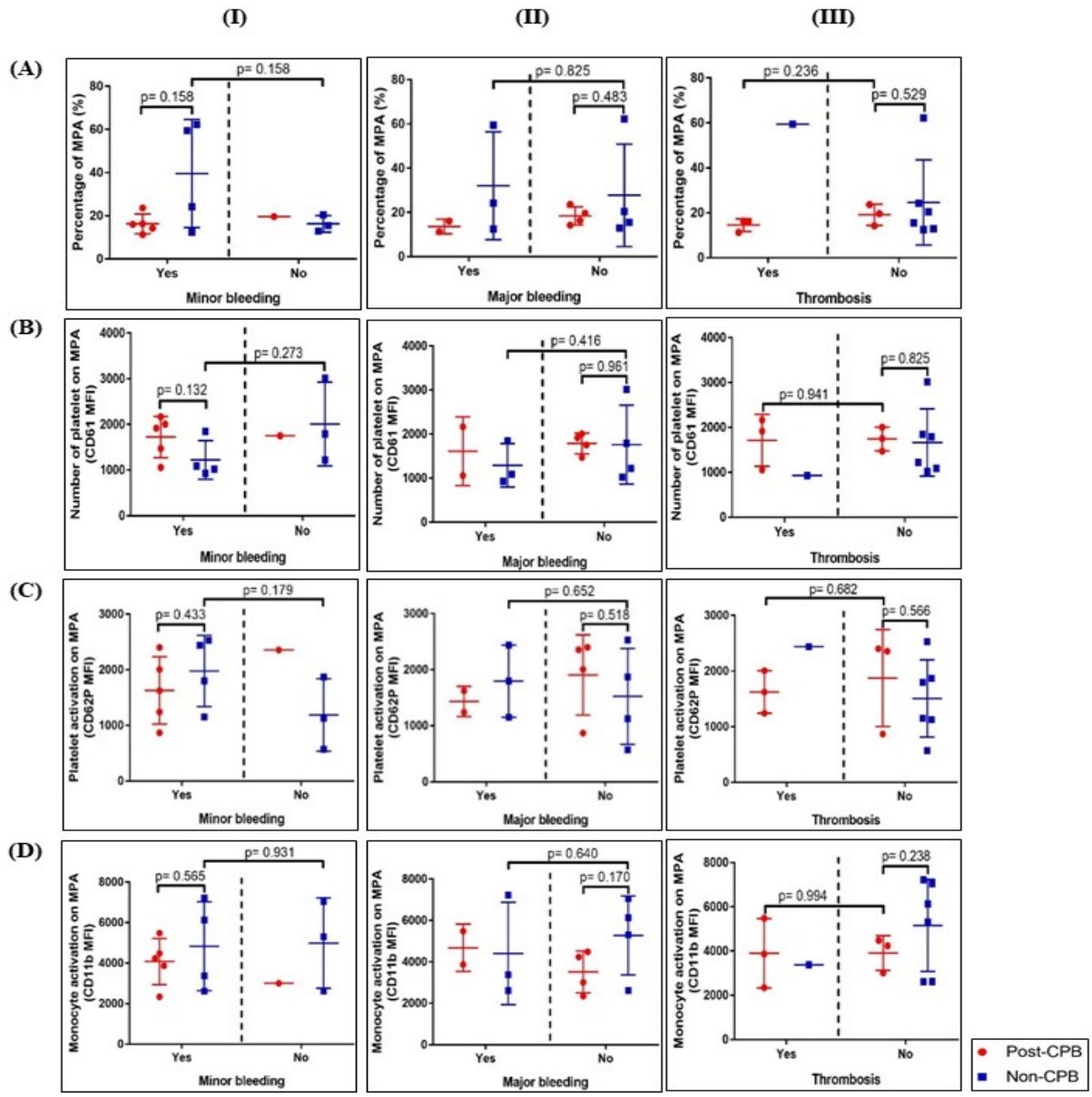

Figure 4.9 The interaction between platelets and monocytes according to the pathway onto ECMO and presence or absence of clinical event.

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation and (D) monocyte activation on MPA within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Post-CPB n=6 and Non-CPB n=7). [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass SD, standard deviation]

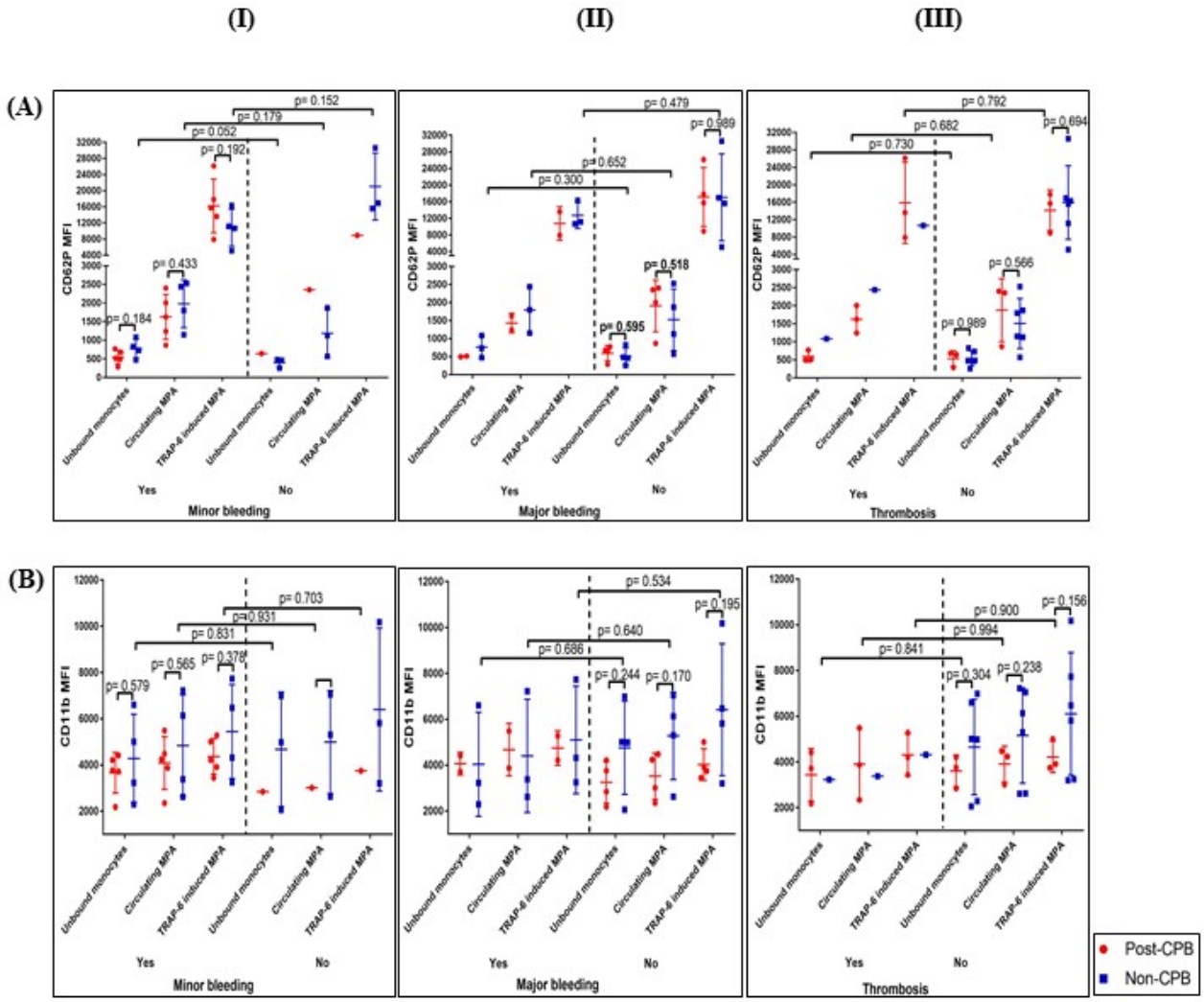

Figure 4.10 The mechanism of interaction between platelets and monocytes according to the pathway onto ECMO and presence or absence of clinical event.

The mechanism of interaction between platelets and monocytes via (A) P-selectin and (B) Mac-1 on MPA within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Post-CPB n=6 and Non-CPB n=7). [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

4.3.6 Circulating neutrophil-platelet aggregates (NPA)

There was no significant difference in the percentage of circulating NPA, the relative number of platelets bound on NPA, neutrophil and platelet activation on NPA for patients from different pathways onto ECMO (Figure 4.11). The MFI of P-selectin and Mac-1 on platelet-bound and unbound neutrophils events were examined for the mechanisms of NPA formation via P-selectin/PSGL-1 and Mac-1 adhesion mechanisms. Upon stimulation with TRAP-6, the expression of P-selectin and Mac-1 was induced to a similar maximal expression for both the Post-CPB and Non-CPB groups. This indicated that the capacity for platelet and neutrophil activation-dependent and -independent formation of NPA was retained in the Post-CPB and Non-CPB groups (Figure 4.11).

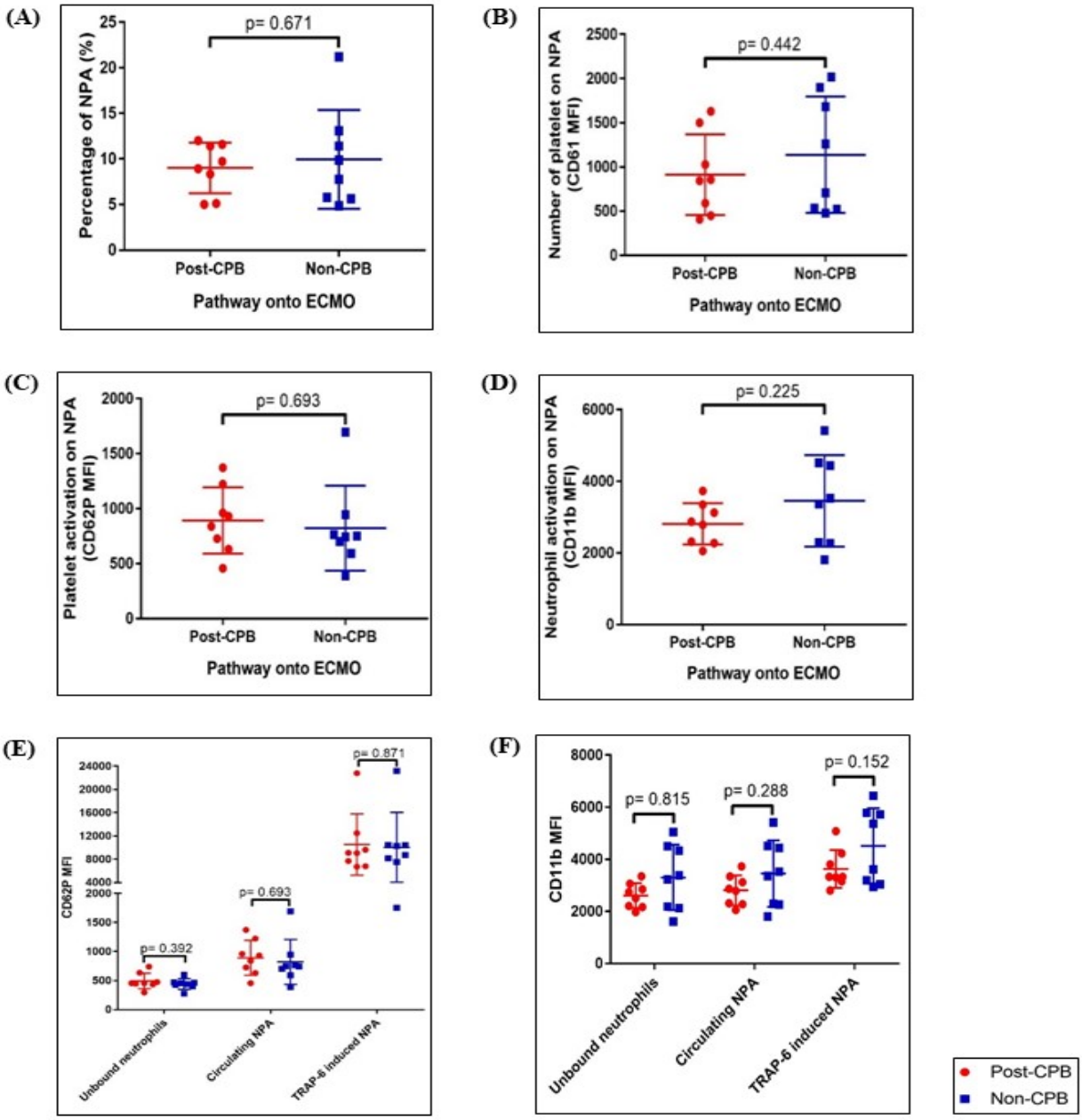

Figure 4.11 The interaction between platelets and neutrophils and pathway onto ECMO.

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation, (D) neutrophil activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on NPA within the first 24 hours according to a patient's pathway onto ECMO. Data shown as mean % or MFI +/- SD (Post-CPB n=8 and Non-CPB n=8). [MFI, median fluorescence intensity; Non-CPB, non-cardiopulmonary bypass; NPA, neutrophil platelet aggregate; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

For circulating NPA, Post-CPB patients who had major bleeding had lower circulating NPA level (6.85 ± 2.08 vs. 11.18 ± 1.01 %, p= 0.017; d= 2.64; power= 0.82) and a trend for an increased neutrophil activation (3183.75 ± 439.66 vs. 2444 ± 468.99 MFI, p= 0.061; d= 1.63; power= 0.49) compared to those who had no major bleeding (Figure 4.12). In contrast, the percentage of circulating NPA, the relative number of platelets bound on NPA, neutrophil and platelet activation on NPA and mechanism of interaction were similar between patients who had or had no minor bleeding or thrombosis within the Post-CPB group.

For patients who had no thrombosis, the Non-CPB group had a trend for an increased number of platelets (685.5 ± 305.36 vs. 1226.14 ± 658.16 MFI, p= 0.098; d= 0.96; power= 0.38) and neutrophil activation (2627 ± 405.66 vs. 3624 ± 1277.44 MFI, p= 0.094; d= 0.93; power= 0.39) on NPA than the Post-CPB group (Figure 4.12). Neutrophils on the NPA of the Non-CPB group were with higher capacity for activation in response to TRAP-6 than the Post-CPB group (3151.25 ± 244.35 vs. 4706.29 ± 1454.45 MFI, p= 0.030; d= 1.30; power= 1.00) (Figure 4.13).

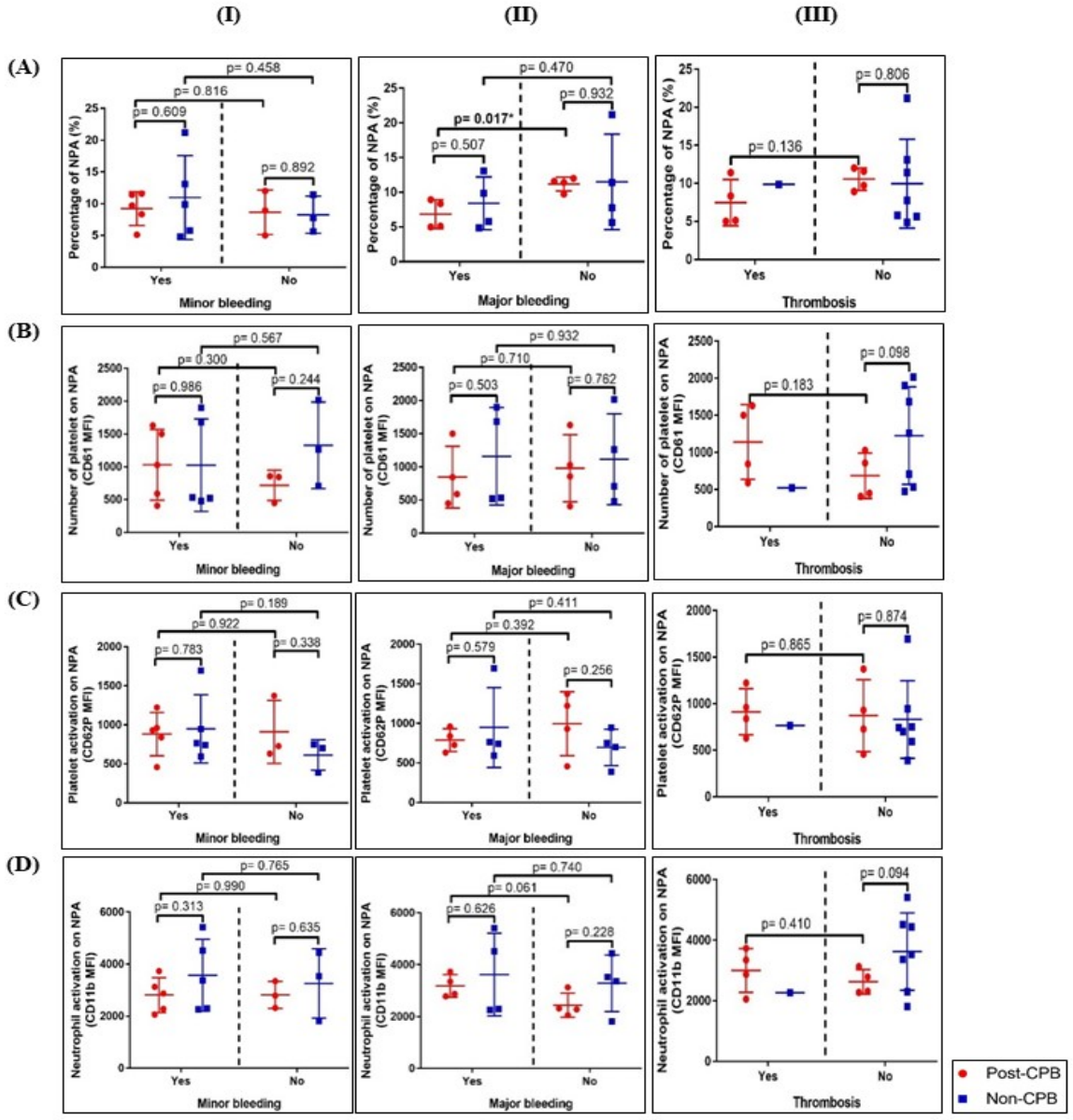

Figure 4.12 The interaction between platelets and neutrophils according to the pathway onto ECMO and presence or absence of clinical event.

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation and (D) neutrophil activation on NPA within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Post-CPB n=8 and Non-CPB n=8). [MFI, median fluorescence intensity; Non-CPB, non-cardiopulmonary bypass; NPA, neutrophil platelet aggregate; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

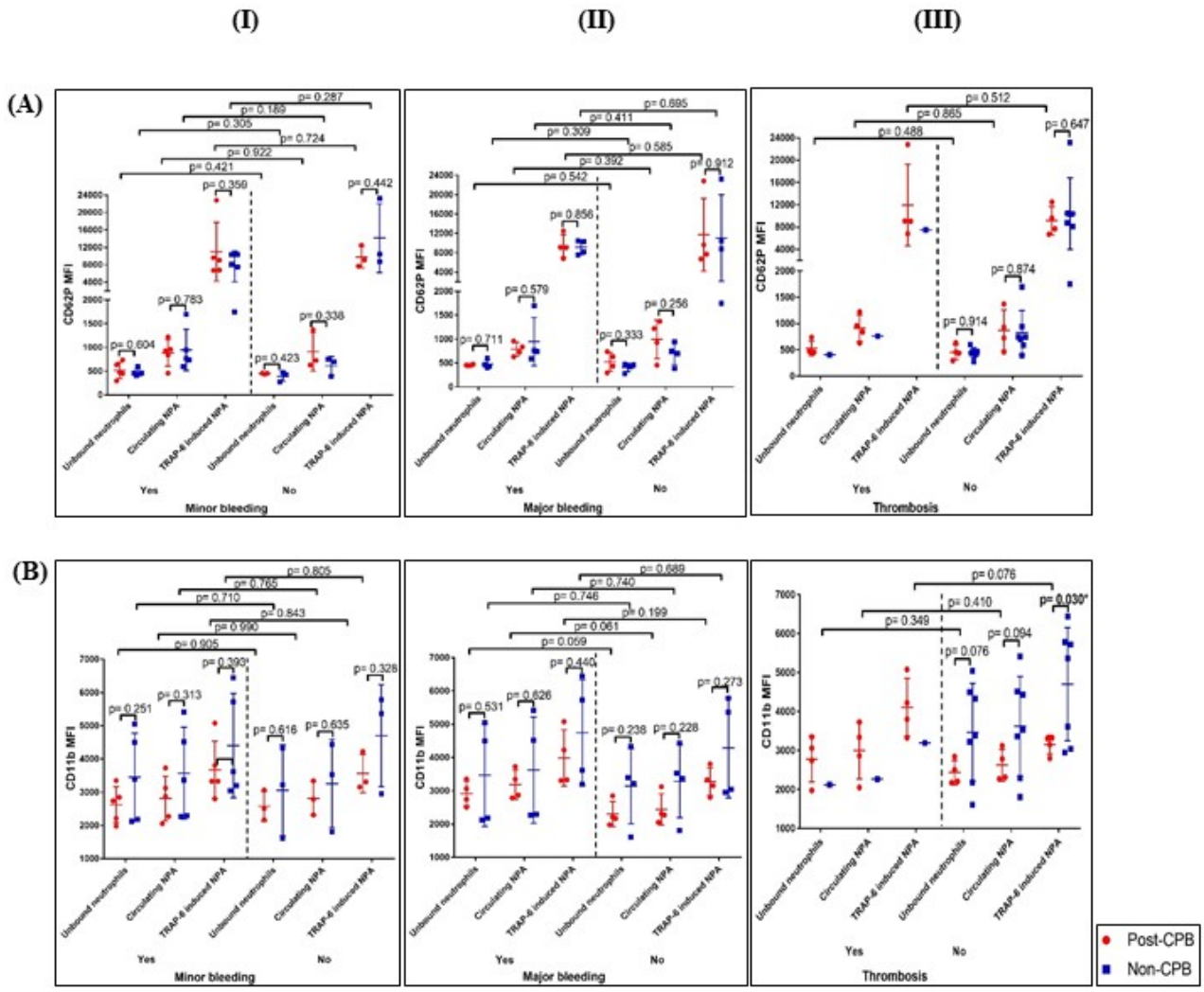

Figure 4.13 The mechanism of interactions between platelets and neutrophils according to the pathway onto ECMO and presence or absence of clinical event.

The mechanism of interaction between platelets and neutrophils via (A) P-selectin and (B) Mac-1 on NPA within the first 24 hours according to a patient's pathway onto ECMO and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Post-CPB n=8 and Non-CPB n=8). [MFI, median fluorescence intensity; Non-CPB, non-cardiopulmonary bypass; NPA, neutrophil platelet aggregate; Post-CPB, post-cardiopulmonary bypass; SD, standard deviation]

4.4 Discussion

This study demonstrated for the first time that patients coming onto ECMO via different pathways i.e. Post-CPB vs. Non-CPB had comparable platelet phenotype, function and interactions with monocytes and neutrophils within the first 24 hours on ECMO. However, when each cohort was examined according to the presence or absence of clinical events it may be that there are subtle differences in the potential mechanisms of those clinical events. For example, the changes noted in integrin αIIbβ3 receptor expression and NPA formation for the Post-CPB group and circulating platelet activation and reactivity for the Non-CPB group.

Although both the Post-CPB and Non-CPB groups had similar platelet phenotype, a trend for a decreased GPVI receptor expression was observed for the Non-CPB group compared to the Post-CPB group. This may be related to the comparable loss of receptors important for platelet adhesion (GPIb/IX/V and GPVI receptors) and aggregation (integrin αIIbβ3 receptor) from the circulating platelet surface. Adult patients with MCS i.e. ECMO and ventricular assist device (VAD) had increased loss of GPIb/IX/V and GPVI receptors from the platelet surface with concomitant increase in soluble plasma GPVI receptor due to increased platelet receptor shedding [205]. Future work should aim to investigate the correlation between the platelet surface GPVI receptor and soluble GPVI receptor in plasma to confirm such observation in paediatric patients from different pathways onto ECMO.

In addition to the platelet phenotype, both the Post-CPB and Non-CPB groups had similar circulating platelet activation and granule exocytosis. The comparable circulating platelet activation (measured by PAC-1 binding to the activated platelet integrin αIIbβ3 receptor) is consistent with the comparable integrin αIIbβ3 receptor expression hence their availability for activation in both groups. Also, both groups had similar granule exocytosis as measured by the expression of the α-granule release indicator, P-selectin and lysosome release indicator, CD63. P-selectin is known to be cleaved upon exposure on circulating platelet surface [111, 112] and

the duration of the other indicators of activation to remain expressed on the platelet surface *in vivo* remains elusive [113]. Hence, it is possible that shear originating from the intact ECMO system may have induced the loss of these activation markers and the indicators of granule release equally in both groups. Measuring the expression of these markers of platelet activation using the other methods such as ELISA may provide clarity to circulating platelet activation and granule exocytosis in patients from different pathways onto ECMO.

This research also demonstrates that patients coming onto ECMO via different pathways had comparable response of platelets to stimulation with TRAP-6. Circulating platelets in both groups may represent a mix platelet population which is hyperresponsive and/or with refractoriness to activation which could be induced via different mechanisms. The reactive oxygen species (ROS) [206, 213] and shear [235, 236] are known to induce platelet signalling pathway [237, 238] hence the enhancement of platelet responsiveness. Similarly, UFH is known to promote the response of platelets to various agonists [239] via enhancement of αIIbβ3-mediated outside-in signalling pathway [184] and platelet activation [240]. Together, these may contribute to an initial increase in platelet response followed by the exhaustion of platelets with limited capacity for further activation to a similar extent hence the comparable platelet response to stimulation with TRAP-6 as seen for both groups.

A high inter-individual variability in the circulating platelet activation and granule release was observed especially for the Post-CPB group. Such observation may be related to the CPB-induced changes in the expression of platelet surface receptor and/or platelet transfusions received by the patients during cardiac surgery. The effect of CPB on platelet phenotype and function is well-documented in existing literature with an increase in circulating platelet activation and granule release reported by multiple studies in the CPB setting [241, 242]. Also, cardiac surgery patients usually require platelet transfusions during cardiac surgery as part of the management of bleeding [243]. The circulating platelet population in the Post-CPB group

may represent a variable mix population of the patient's own platelets and transfused adult platelets which are of different phenotype and function hence the observed high inter-individual variability for this group of patients.

On the other hand, a high inter-individual variability in the platelet response to TRAP-6 was equally observed for both the Post-CPB and Non-CPB groups. Previous studies on platelet response based on the agonist-induced P-selectin and the activated integrin αIIbβ3 receptor expression in healthy adults showed that there is a high inter-individual variability in platelet response [44, 244]. Such variation may be related to polymorphism of the platelet receptor, PAR-1 (encodes for the main thrombin receptor). For example, the level of PAR-1 expression was directly correlated to the magnitude of platelet response towards SFLLRN (the synthetic thrombin mimic- TRAP-6) [245]. Also, although individuals with very high or low level of circulating platelet activation, granule release and platelet response were observed for both groups, the number of individuals observed was too small to consider these circumstances separately.

In addition to platelet adhesion and aggregation, activated platelets can interact with the monocytes and neutrophils to form MPA and NPA that have been proposed to be the key mediators for the crosstalk between the haemostatic and inflammatory systems [229]. This is important because ECMO initiation is known to induce inflammation (in addition to the underlying pathological conditions and multiple surgical wounds) regardless of the diversity of diagnoses for patients to be placed on ECMO [233, 246-248]. Two molecular mechanisms that are important for platelet-leukocyte aggregates formations are P-selectin (a receptor found on platelet surface upon activation) binding to its ligand PSGL-1 (constitutively express on leukocytes), and Mac-1 (CD11b/CD18) (an integrin constitutively expressed on leukocytes and up-regulated upon activation) binding to platelet glycoprotein GPIba [143, 249, 250].

In the setting of MCS, the interaction between platelets and leukocytes play a vital role in the haemostatic defect seen in the CPB population [229, 251]. Findings from this study showed that patients coming onto ECMO from different pathways had comparable percentage of circulating MPA and NPA. This is consistent with the comparable circulating platelet activation and the expression of α-granule release indicator, P-selectin (the main adhesion molecule responsible for the interaction between platelet with monocytes and neutrophils in adults) seen for both groups. Binding of platelets to leukocytes, particularly via P-selectin-PSGL-1 receptor-ligand pair interaction are known to modify the function of both cells within the platelet-leukocyte aggregates [252-254]. These include the up-regulation of P-selectin and Mac-1, the two main molecules involved for monocytes and neutrophils adhesion to activated platelets. The comparable P-selectin and Mac-1 expression on both MPA and NPA thus may be associated with the similar number of platelets bound to monocytes/neutrophils seen for both groups.

The higher inter-individual variability in circulating MPA of the Non-CPB group compared to the Post-CPB group may suggest that most of the circulating MPA already existed (e.g. formed during CPB) and there was only minimal MPA formation upon ECMO initiation in the Post-CPB group. CPB is known to induce MPA/NPA formation via different mechanisms such as increased platelet activation and elevated inflammatory state in the patient [251, 255]. At the same time, shear stress from the CPB could potentially induce loss of receptor such as P-selectin which is important for MPA formation from the platelet surface. This may reduce the ability of the platelets to form MPA in the Post-CPB patients upon subsequent exposure to the ECMO system. The proposed mechanisms of the MPA formation in the Post-CPB group are as follows: I.) CPB induces P-selectin release from the α-granules via different mechanisms [256]; II.) proteolytic cleavage of surface P-selectin from circulating platelets and generate soluble P-selectin [406] and III.) P-selectin negative platelets continue to circulate and function

but are unable to bind monocytes [111, 112]. Together, these may contribute to the homogeneity in the circulating MPA level seen for the Post-CPB group due to reduced capacity of platelets for MPA formation.

Platelets in the Non-CPB patients without pre-exposure to shear stress, however, with wide range of disease aetiology may have different capacity to form MPA hence the corresponding high inter-individual variability in the circulating MPA level. Measuring and comparing the circulating MPA level before ECMO initiation may provide clarity to the difference in inter-individual variability seen between groups. Contrarily, a high inter-individual variability in the platelet and monocyte/neutrophil activation within the MPA/NPA was equally observed for both groups. This may suggest that ECMO can induce platelet and/or monocyte and neutrophil activation within the MPA/NPA equally in both groups of patients.

Although patients from different pathways onto ECMO have comparable platelet phenotype, function and interactions with monocytes and neutrophils within the first 24 hours upon ECMO initiation, modification of platelet function were observed for different clinical events according to the patient's pathway onto ECMO. For patients who had minor bleeding or major bleeding, the Post-CPB group had higher platelet reactivity and integrin αIIbβ3 receptor expression, respectively than the Non-CPB group. Such observations may be relevant to pre-exposure to shear from the CPB for the Post-CPB group since shear is known to be able to increase platelet reactivity [257, 258] and expression of receptor important for platelet adhesion and aggregation [259, 260] [261, 262]. The mechanisms of how increased platelet integrin αIIbβ3 receptor expression and reactivity may be associated with the development of bleeding event will be detailed as followed.

Within the Post-CPB group, patients who had major bleeding had higher integrin αIIbβ3 receptor expression and lower circulating NPA level than those who had no major bleeding.

The same observation was obtained for the integrin αIIbβ3 receptor expression for thrombosis. Since integrin αIIbβ3 receptor is the main receptor involved in platelet aggregation, an elevated integrin αIIbβ3 receptor expression may suggest increased platelet aggregation followed by an increase in clot/thrombus formation which could eventually develop into thrombosis. Further recruitment of platelet to the clot/thrombus can in turn depletes the circulating platelets positive for integrin αIIbβ3 receptor expression. Also, since increased platelet-leukocyte aggregate level is associated with hypercoagulability, the lower circulating NPA level may indicate impaired coagulation. Together, these may increase the risk of developing major bleeding or thrombosis in the Post-CPB patient. As for the Non-CPB group, patients who had minor bleeding had reduced platelet reactivity and patients with major bleeding had a trend for an increased α-granule release compared to those who had no event. Activated platelets can bind to the circuit, existing clot/thrombus and/or subendothelial layer (e.g. from existing surgical wounds) and promote platelet adhesion and aggregation as the hyperresponsive platelets. The remaining platelets in the circulation thus may represent the population of hyporesponsive platelets with reduced function hence increasing the risk of bleeding events in the Non-CPB group.

Such differences in platelet-specific changes in relation to the clinical events for patients from different pathways onto ECMO may suggest that changes in platelet function could have a more important role in the development of bleeding in the Non-CPB group than the Post-CPB group. Other factors such as changes in coagulation may be more vital in contributing to the development of clinical event in the Post-CPB group. For example, increased plasma coagulation protein such as fibrinogen is known to promote platelet adhesion and aggregation to the CPB circuit via increased integrin αIIbβ3 and GPIb/IX/V receptor expression through positive feedback loop [263, 264]. Together, such differences in platelet-specific changes may then contribute to the different pathophysiology of clinical event in patients according to their

pathways onto ECMO. Monitoring of I.) platelet integrin αIIbβ3 receptor expression and circulating NPA level in the Post-CPB patients or II.) circulating platelet activation and reactivity to TRAP-6 in the Non-CPB patients may aid in identifying a patient's risk of developing clinical event as ECMO progresses.

This study demonstrated for the first time that paediatric patients from different pathways onto ECMO had comparable platelet phenotype, function and interactions with monocytes and neutrophils within the first 24 hours on ECMO. However, clinical event may develop as a result of modification of platelet function via different mechanism in patients according to their pathway onto ECMO. The high inter-individual variability for circulating platelet activation, granule exocytosis and MPA formation seen for the Post-CPB group may be relevant to pre-exposure to CPB. Although with the numbers of patients enrolled in this study, it is impossible to conclude with certainty the presence or absence of differences in platelet phenotype, function or cellular interactions. This study provides justification to continue this work for detailed evaluation of the platelet phenotype and function and their relationships with the development of clinical events across the duration on ECMO.

4.5 Conclusion

Clinical events may develop as a result of the modulation of platelet function via different mechanisms according to a patient's pathway onto ECMO. Modification of platelet integrin αIIbβ3 receptor expression and/or circulating neutrophil-platelet aggregates level may be associated with an increased risk for bleeding or thrombosis in the Post-CPB patients. In contrast, monitoring of circulating platelet activation and reactivity for the Non-CPB group may aid in identifying a patient's risk for bleeding during ECMO. A larger sample size is required for the detailed evaluation of the association of differences in platelet function with clinical events particularly for thrombosis. These may then provide detailed information for whether early medical intervention can be tailored and given to the patients according to their

pathway onto ECMO at the initial stage with the ultimate aim to reduce the rate of clinical events across the duration on ECMO.

5 The effect of age on platelet phenotype, function and interactions with monocytes and neutrophils and their associations with bleeding or thrombosis

5.1 Introduction

ECMO is one of the very few medical technologies that was pioneered in children and later adapted for adult usage. According to the 2017 ELSO registry report, the ECMO population is represented by 44.8 % neonates, 24.1 % paediatric and 31.1 % adult patients [265]. Although the paediatric patients represent most of the ECMO population, the majority of research has focused on adults. This may be associated with the challenges in ethical research and limited availability of blood sample volume for paediatric research. Furthermore, despite the advances in circuit-related technologies and clinical practice, the rate of clinical events remains high in the paediatric ECMO population (Table 5.1) [26, 227, 266].

Table 5.1 Summary for the rate of bleeding and thrombosis according to a patient's age and primary indication for ECMO.

Adapted from [266].

Clinical event	Number of patient with clinical event, n (%)			
	Neonatal (≤ 31 days)		Paediatric (≥ 32 days – 19 years)	
	Cardiac	Respiratory	Cardiac	Respiratory
Bleeding	71 (77.2)	91 (60.3)	91 (79.1)	63 (73.3)
Thrombosis	29 (31.5)	66 (43.7)	43 (37.4)	33 (38.4)

Platelet dysfunction has been associated with ECMO-induced coagulopathy in the adult ECMO population, however, this remains poorly studied in paediatric patients. Developmental haemostasis, especially from the aspect of platelet function has gained increased attention in recent years. Currently known platelet-related differences according to the age include:

I.) Neonates and adults have similar number and size of platelets [267-269]

II.) Platelet hyporeactivity in neonates and infants reach the level of adults by the age of one [270-273]

III.) Neonates have higher plasma VWF concentration than adults [34, 274, 275]

IV.) Platelet proteome and releasate of healthy children are different from the adults [49]

Compared to the platelet phenotype and reactivity, the interaction between platelets with monocytes and neutrophils are less well-studied in the paediatric setting. For example, the role of MPA as a marker for *in vivo* platelet activation via P-selectin-PSGL-1 receptor-ligand pair interaction followed by an increased Mac-1 (CD11b) expression to enhance their interaction are known in the adult setting [276, 277]. Contrarily, a P-selectin-independent mechanism was proposed to be more vital for MPA formation in children [137].

Age-related quantitative changes for platelet reactivity [44] and interaction between platelets with monocytes [137] and neutrophils [278] in children have important implications in the clinical management for this population [279]. To date, there is no study that has examined platelet-specific changes within the paediatric ECMO population according to their age. Understanding such age-related platelet-specific differences may provide information for whether early intervention can be tailored and given to the paediatric patients according to their age thus minimizing the incidence of clinical event as ECMO progresses.

The hypothesis of this chapter is that platelet phenotype, function and interactions with monocytes and neutrophils within the first 24 hours will be different according to a patient's age and the platelet-specific changes can be associated with the development of bleeding or thrombosis during ECMO.

This chapter aims to determine the effect of a patient's age on the platelet-specific differences and their associations with the clinical events for:

1) The platelet phenotype.
2) The circulating platelet activation.
3) The response of platelets to stimulation with TRAP-6.
4) The interactions of platelets with monocytes and neutrophils.

5.2 Materials and Methods

The methods utilized in this chapter were outlined in Chapter 2. Patients were categorized as neonates, infants or children according to their age as described in section 2.2.2. Clinical events including minor bleeding, major bleeding and thrombosis were recorded according to the definitions in section 2.2.3.

Flow cytometry panels used for this chapter were included in sections 2.6.3 - 2.6.5. Following is the summary for the evaluation of platelet-specific markers:

I.) Platelet phenotype was assessed by measuring the expression of integrin αIIbβ3, GPIb/IX/V and GPVI receptors.

II.) Circulating platelet activation was measured as the percentage of platelets positive for activated integrin αIIbβ3 receptor (measured by PAC-1 binding) while platelet granule exocytosis was measured as the percentage of platelets positive for α-granule (P-selectin) and lysosome release (CD63) indicators. The response of platelet to TRAP-6 is directly proportional to the area under the curve (AUC).

III.) The interaction between platelets and monocytes/neutrophils were investigated by evaluating circulating MPA/NPA. The relative number of platelets bound on MPA/NPA and monocytes/neutrophils was measured by MPA/NPA CD61 MFI [234]. The mechanism of MPA/NPA tethering was measured by reporting the MFI of P-selectin and Mac-1 on platelet-bound and unbound monocyte/neutrophil events with/without stimulation with TRAP-6 [141].

The results of platelet-specific markers within the first 24 hours upon initiation of ECMO were used for all data analysis relevant to the platelet-specific changes in this chapter. Platelet phenotype, function and interactions with monocytes and neutrophils were compared between and within the age group according to the clinical events except for those limited by the number

of bleeding or thrombotic event (less than 3 events). Details for the statistical analysis were described in section 2.7. Statistical software package STATA (Release 15) (Stata Corp., College Station, Texas) was used for all statistical analysis in this chapter. In summary, Fisher's exact or Chi-Square test was used for the comparisons of parameters (except for the weight and full blood count parameters with one-way ANOVA) for the demographic details in Table 5.2; the platelet phenotype and function among the three age groups were compared using one way ANOVA and Post-Hoc Tukey's test. For the evaluation of platelet-specific changes for clinical events, one way ANOVA and Post-Hoc Tukey's test was used for the comparisons among the three age groups within each patient group with/without clinical event and unpaired Student's t-test was used for the comparison between patients with/without clinical events (minor bleeding, major bleeding or thrombosis) within each age group.

5.3 Results

5.3.1 Demographics

Demographic information for the twenty-two patients included in this chapter was summarized in Table 5.2. Neonates, infants and children had comparable pathway onto ECMO, gender, mode of cannulation and full blood count parameters but differed for their weight.

Figure 5.1 depict the longitudinal graph for samples, clinical events and platelet transfusions received by the patients according to their age across duration on ECMO. Within the first 24 hours on ECMO, there were more neonates (63 %) and children (44 %) who had ≥ 1 clinical event than infants (0%). In contrast, 75 % of neonates compared to 60 % infants and 56 % children who required ≥ 1 platelet transfusion on Day 1.

The definition used to categorize between the groups who had/had no clinical event for the analysis of the platelet phenotype and function in relation to the clinical event was whether a patient had at least one clinical event (minor bleeding, major bleeding or thrombosis) across the duration on ECMO. Neonates, infants and children had comparable rate of major bleeding and thrombosis but the rate of minor bleeding differed according to the age group across the duration on ECMO. There was comparable number of patients who required at least one platelet transfusion for neonates, infants and children.

Table 5.2 Summary of demographic information comparing neonates, infants and children.

Variable			Age category (n= 22), n (%)			p-value (Neonates vs. Infants vs. Children)
			Neonates (0 - 30 days) 8 (36)	Infants (> 30 days – 1 year) 5 (23)	Children (> 1 year – 18 years) 9 (41)	
Pathway onto ECMO, n (%)		Post-CPB	4 (50)	4 (80)	4 (44)	0.482
		Non-CPB	4 (50)	1 (20)	5 (55)	
Gender, n (%)		Male	4 (50)	2 (40)	6 (66)	0.645
		Female	4 (50)	3 (60)	3 (33)	
Primary diagnosis	Cardiac	Unable to wean off CPB	2 (25)	3 (60)	1 (11)	-
		Cardiomyo -pathy	0 (0)	0 (0)	4 (44)	
		Myocarditis	1 (13)	0 (0)	1 (11)	
		Primary congenital cardiac abnormality	2 (26)	1 (20)	1 (11)	
		Other	1 (13)	1 (20)	1 (11)	
	Respira -tory	Meconium aspiration syndrome	1 (13)	0 (0)	0 (0)	-
	Other	Sepsis	0 (0)	0 (0)	1 (11)	-
Weight [kg] median (IQR)			3.10 (2.55 - 3.41)	4.27 (3.80 - 8.13)	35.00 (11.70 - 43.80)	**< 0.001*** **
Mode of cannulation, n (%)		Central	8 (100)	5 (100)	5 (67)	> 0.999
		Peripheral	0 (0)	0 (0)	3 (33)	
Clinical events, n (%)	Minor bleeding	Yes	7 (88)	3 (60)	2 (29)	**0.032***
		No	1 (12)	2 (40)	7 (71)	
	Major bleeding	Yes	4 (50)	2 (40)	6 (67)	0.645
		No	4 (50)	3 (60)	3 (33)	
	Thrombosis	Yes	2 (25)	1(20)	5 (56)	0.325
		No	6 (75)	4 (80)	4 (44)	
Platelet transfusion, n (%)		Yes	7 (88)	5 (100)	7 (71)	0.766
		No	1 (12)	0 (0)	2(29)	

Full blood count	Age category (n= 12), n (%)			p-value (Neonates vs. Infants vs. Children)
	Neonates 5 (42)	Infants 3 (25)	Children 4 (33)	
WCC (x 10^9/L)	5.04 ± 1.65	7.13 ± 2.40	6.60 ± 7.36	0.791
RCC (x 10^{12}/L)	3.64 ± 0.37	3.43 ± 0.61	3.18 ± 1.13	0.664
HGB (g/L)	110.60 ± 13.13	104.33 ± 18.50	90.50 ± 29.24	0.391
HCT (L/L)	0.32 ± 0.03	0.30 ± 0.06	0.27 ± 0.10	0.546
PLT (x 10^9/L)	118.00 ± 49.34	122.67 ± 41.20	98.25 ± 65.69	0.807
MPV (fl)	8.42 ± 2.29	6.53 ± 0.31	7.60 ± 1.22	0.352

[ECMO, extracorporeal membrane oxygenation; HCT, haematocrit; HGB, haemoglobin; IQR, interquartile range; MPV, mean platelet volume; Non-CPB, non-cardiopulmonary bypass; PLT, platelet count; Post-CPB, post-cardiopulmonary bypass; RCC, red blood cell count; WCC, white blood cell count]

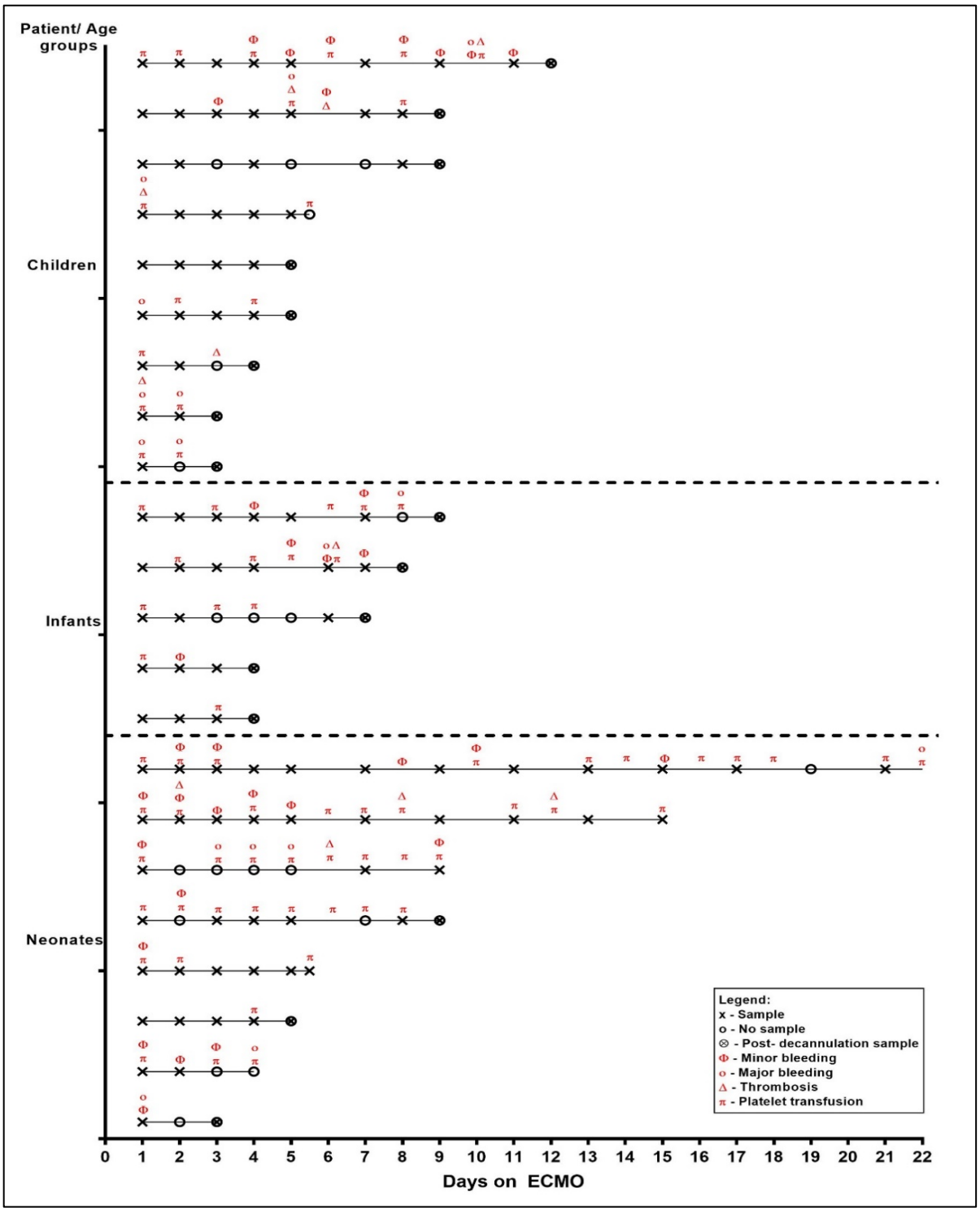

Figure 5.1 Longitudinal graph for patients, clinical events and platelet transfusions according to the age.

The dotted line depict the division for neonates, infants and children. [ECMO, extracorporeal membrane oxygenation]

5.3.2 Platelet phenotype

There was no significant difference in the expression of integrin αIIbβ3, GPIb/IX/V and GPVI receptors among neonates, infants and children (Figure 5.2). However, there was a trend for an increased integrin αIIbβ3 receptor expression in children compared to neonates (25533.0 ± 3359.17 vs. 29086.667 ± 2712.81 MFI, p= 0.072; d= 1.18; power= 0.61) (Figure 5.2). While the sample size was inadequately powered to assess differences in the coefficient of variation between groups, the inter-individual variability of GPIb/IX/V receptor expression for children appeared to be higher than neonates and infants (Figure 5.2); the reverse was observed for the GPVI receptor expression where a lower inter-individual variability was observed in children than neonates and infants (Figure 5.2).

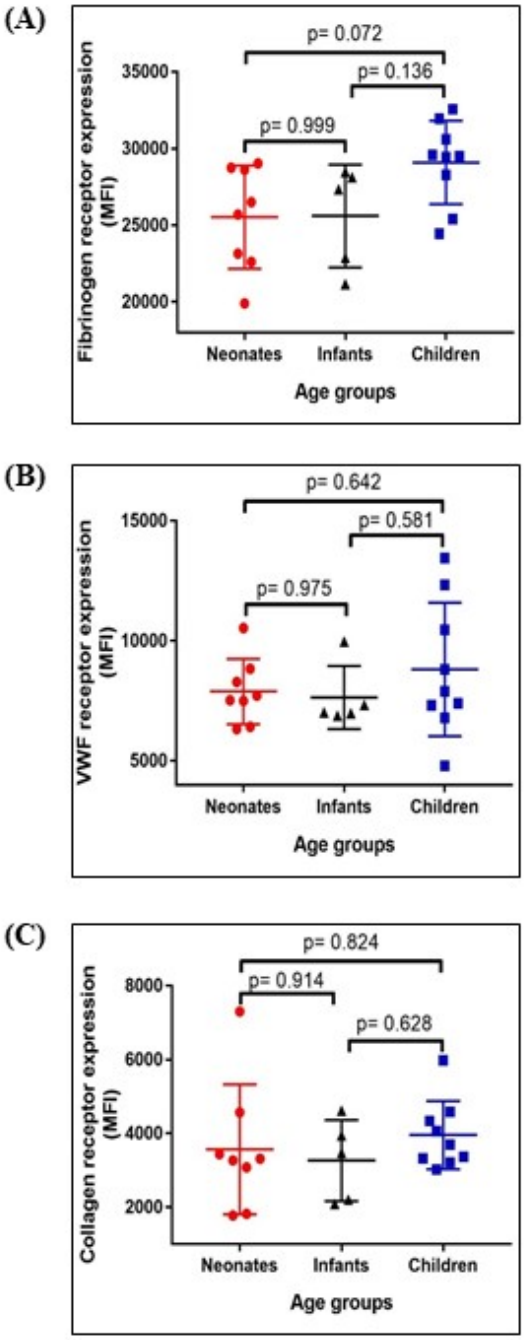

Figure 5.2 Platelet phenotype and age.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor within the first 24 hours according to patient's age. Data shown as mean MFI +/- SD (Neonates n=8, Infants n=5 and Children n=9). [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

Figure 5.3 depict the comparison of the expression for the integrin αIIbβ3, GPIb/IX/V and GPVI receptors among neonates, infants and children according to the clinical events. Neonates and children who had or had no major bleeding had comparable integrin αIIbβ3, GPIb/IX/V and GPVI receptor expressions. Similar observations were obtained for children who had or had no thrombosis.

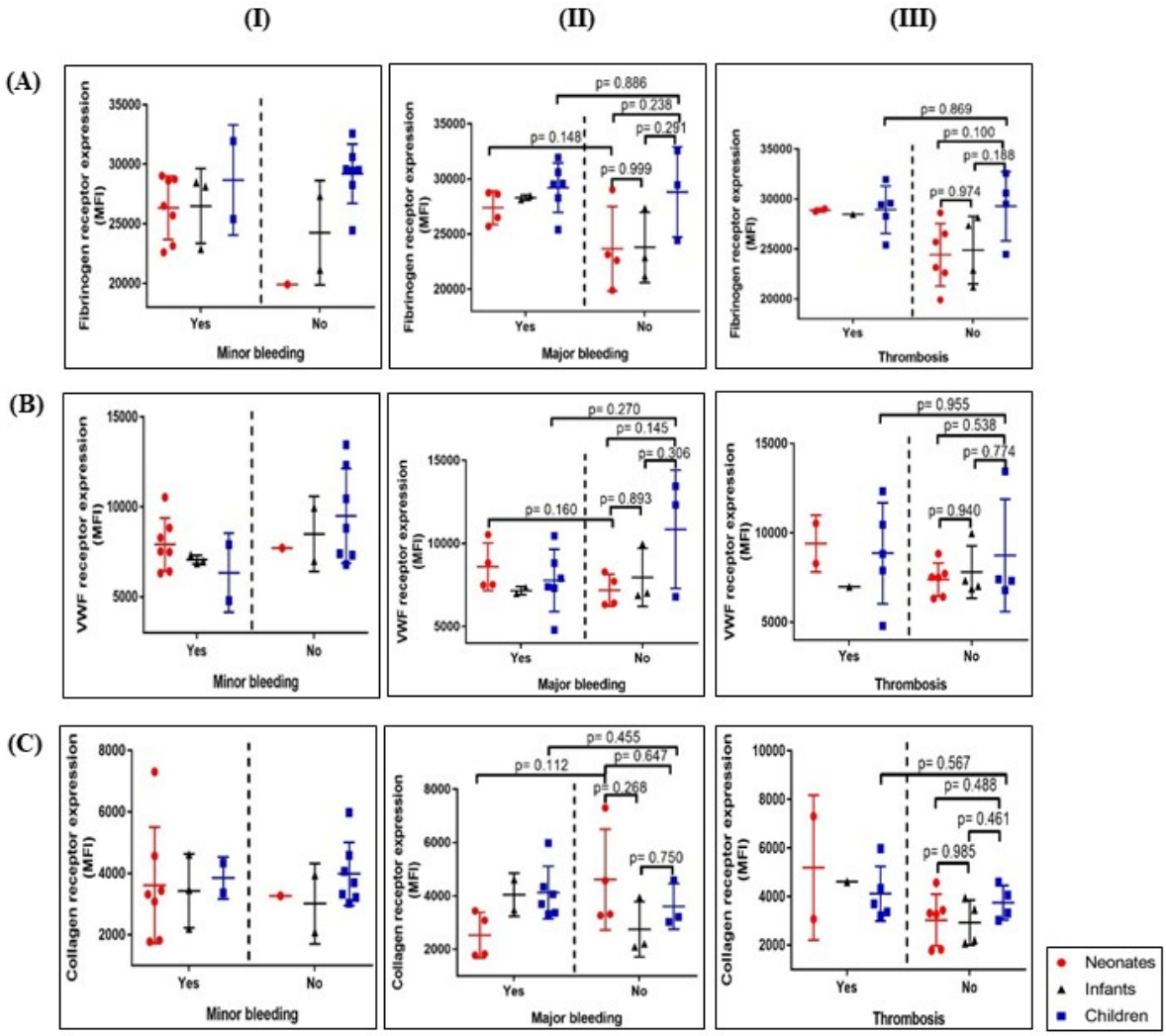

Figure 5.3 Platelet phenotype according to the age and presence or absence of clinical event.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean MFI +/- SD (Neonates n=8, Infants n=5 and Children n=9). [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

5.3.3 Circulating platelet activation

Neonates, infants and children had comparable circulating platelet activation and granule exocytosis (Figure 5.4). While the sample size was inadequately powered to assess differences in the coefficient of variation between groups, the inter-individual variability for granule (both α-granule and lysosome) exocytosis appeared to be higher in infants and children compared to neonates (Figure 5.4).

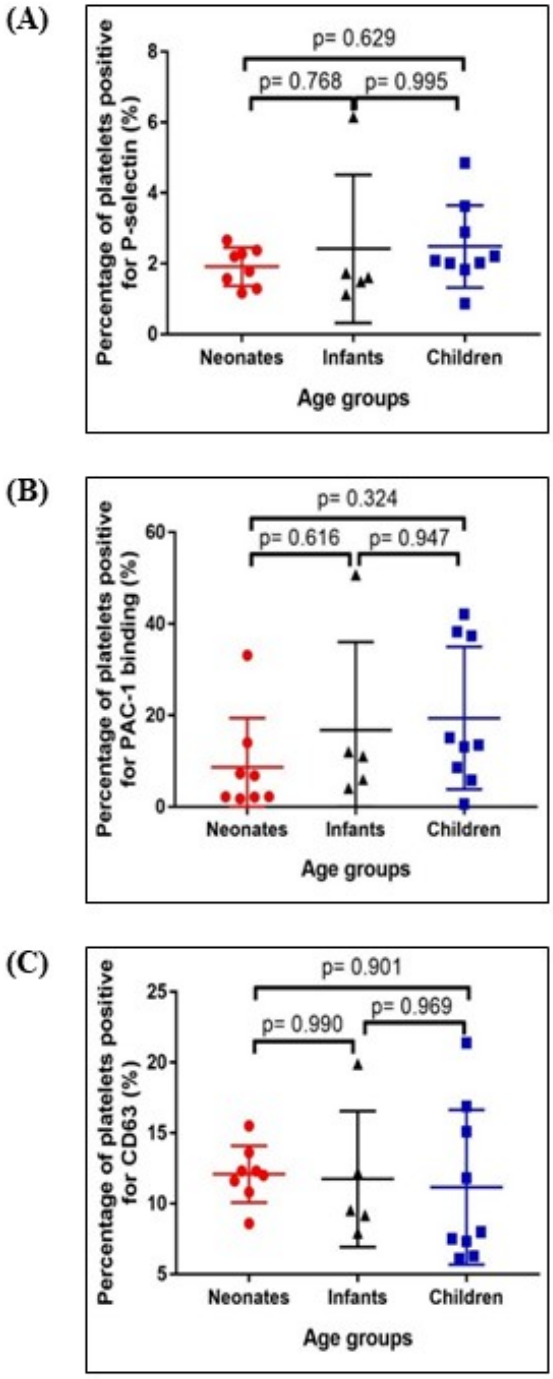

Figure 5.4 Circulating platelet activation and age.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's age. Data shown as mean % +/- SD (Neonates n=8, Infants n=5 and Children n=9). [MFI, median fluorescence intensity; SD, standard deviation]

Neonates and children who had or had no major bleeding had comparable circulating platelet activation and granule exocytosis (Figure 5.5). For thrombosis, children who had an event had higher lysosome release than those who had no event (14.30 % ± 5.66 vs 7.23 ± 0.81 %, p= 0.048; d= 1.64; power= 0.56). For individuals who had no thrombosis, children had lower lysosome release than neonates (12.07 % ± 2.37 vs 7.23 ± 0.81 %, p= 0.006; d= 2.49; power= 0.97) (Figure 5.5).

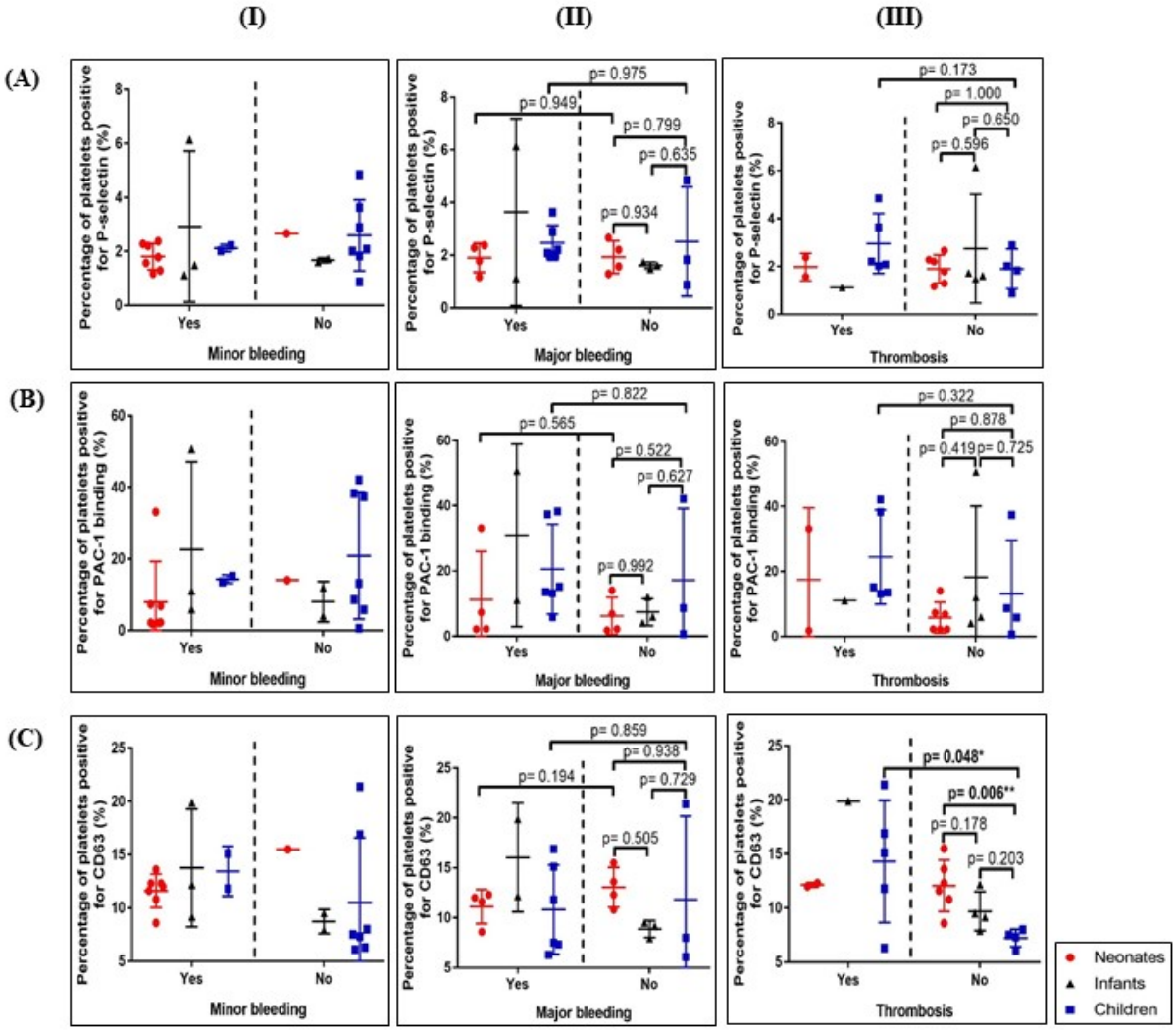

Figure 5.5 Circulating platelet activation according to the age and presence or absence of clinical event.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % +/- SD (Neonates n=8, Infants n=5 and Children n=9). [MFI, median fluorescence intensity; SD, standard deviation]

5.3.4 Response of platelets to stimulation with TRAP-6

The response of platelets to stimulation with TRAP-6 for activated platelet integrin αIIbβ3 receptor and granule exocytosis (both α-granule and lysosome) was comparable for neonates, infants and children (Figure 5.6).

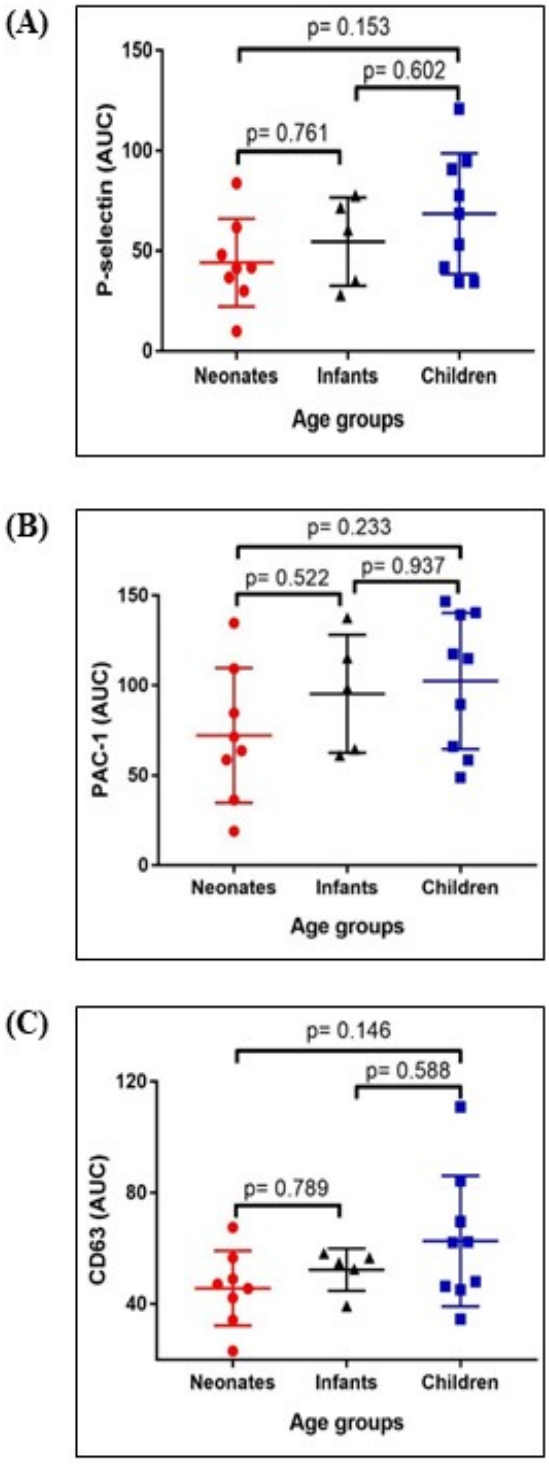

Figure 5.6 Platelet response to stimulation with TRAP-6 and age.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's age. Data shown as mean AUC +/- SD (Neonates n=8, Infants n=5 and Children n=9). [AUC, area under the curve; SD, standard deviation]

Evaluation of the association between platelet response and clinical events were limited by the number of minor bleeding event in each age group. For neonates and children, individuals who had or had no major bleeding had comparable platelet response. The same observation was obtained for children who had or had no thrombosis (Figure 5.7).

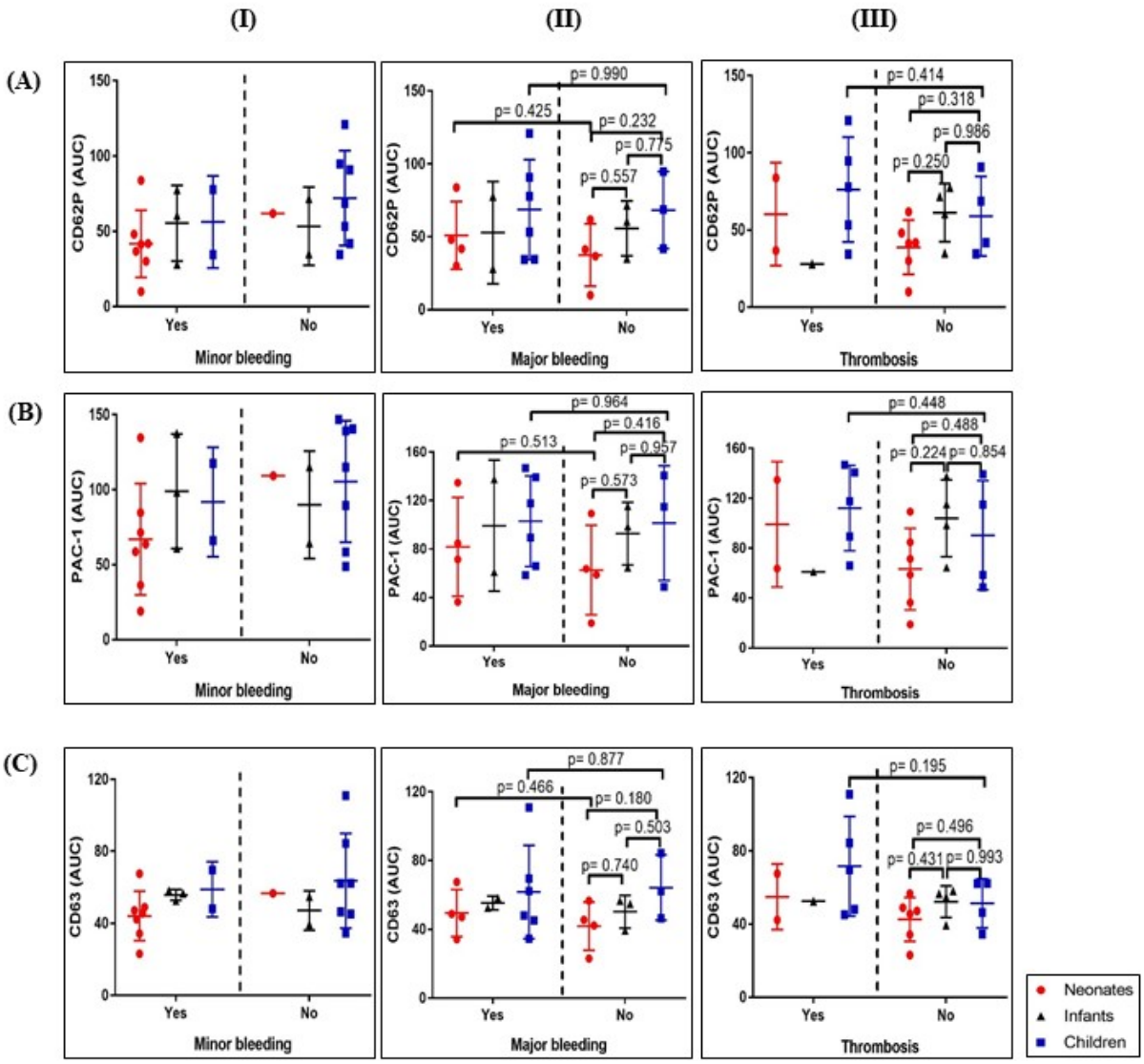

Figure 5.7 Platelet response to stimulation with TRAP-6 according to the age and presence or absence of clinical event.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean AUC +/- SD (Neonates n=8, Infants n=5 and Children n=9). [AUC, area under the curve; SD, standard deviation]

5.3.5 Circulating monocyte-platelet aggregates (MPA)

The percentage of circulating MPA, the relative number of platelets bound on MPA as well as monocyte and platelet activation on MPA were similar for neonates, infants and children (Figure 5.8). Individuals with high circulating MPA were observed for neonates and children but not infants (Figure 5.8). To determine if circulating MPA formed as a result of P-selectin/PSGL-1 and Mac-1 (CD11b) adhesion mechanisms, the MFI of P-selectin and Mac-1 on platelet-bound and unbound monocyte events was examined. Upon stimulation with TRAP-6, the expression of P-selectin and Mac-1 was induced to a similar maximal expression for neonates, infants and children. This indicated that the capacity for platelet and monocyte activation-dependent and –independent formation of MPA was retained equally in patients from all age groups (Figure 5.8).

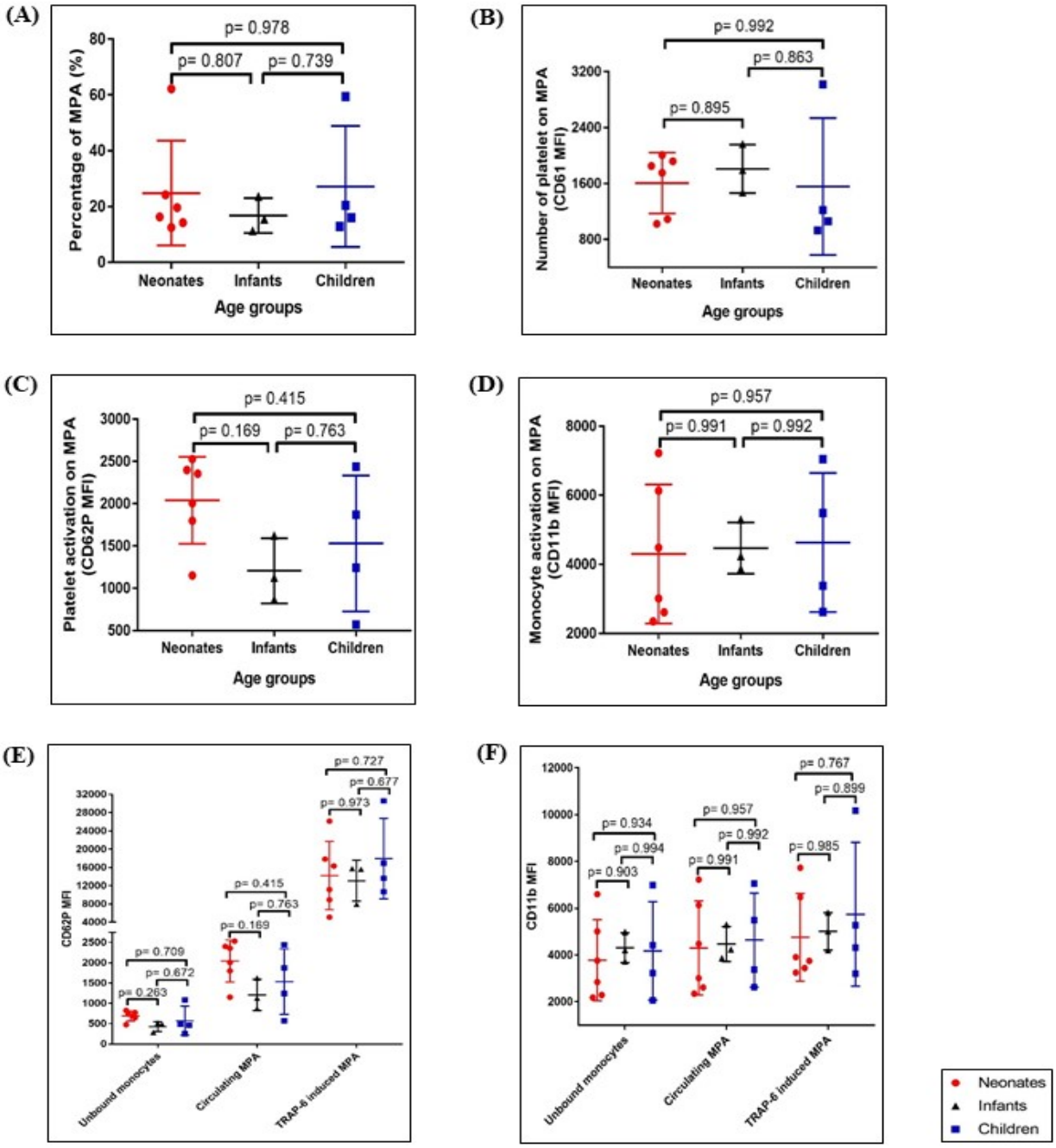

Figure 5.8 The interaction between platelets and monocytes and age.

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation, (D) monocyte activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on MPA within the first 24 hours according to a patient's age. Data shown as mean % or MFI +/- SD (Neonates n=6, Infants n=3 and Children n=4). [MFI, median fluorescence intensity; SD, standard deviation; MPA, monocyte-platelet aggregates]

Evaluation of the association between the interactions of platelets with monocytes and clinical events were limited by the number of events for minor bleeding, major bleeding and thrombosis in each age group (Figure 5.9 and Figure 5.10).

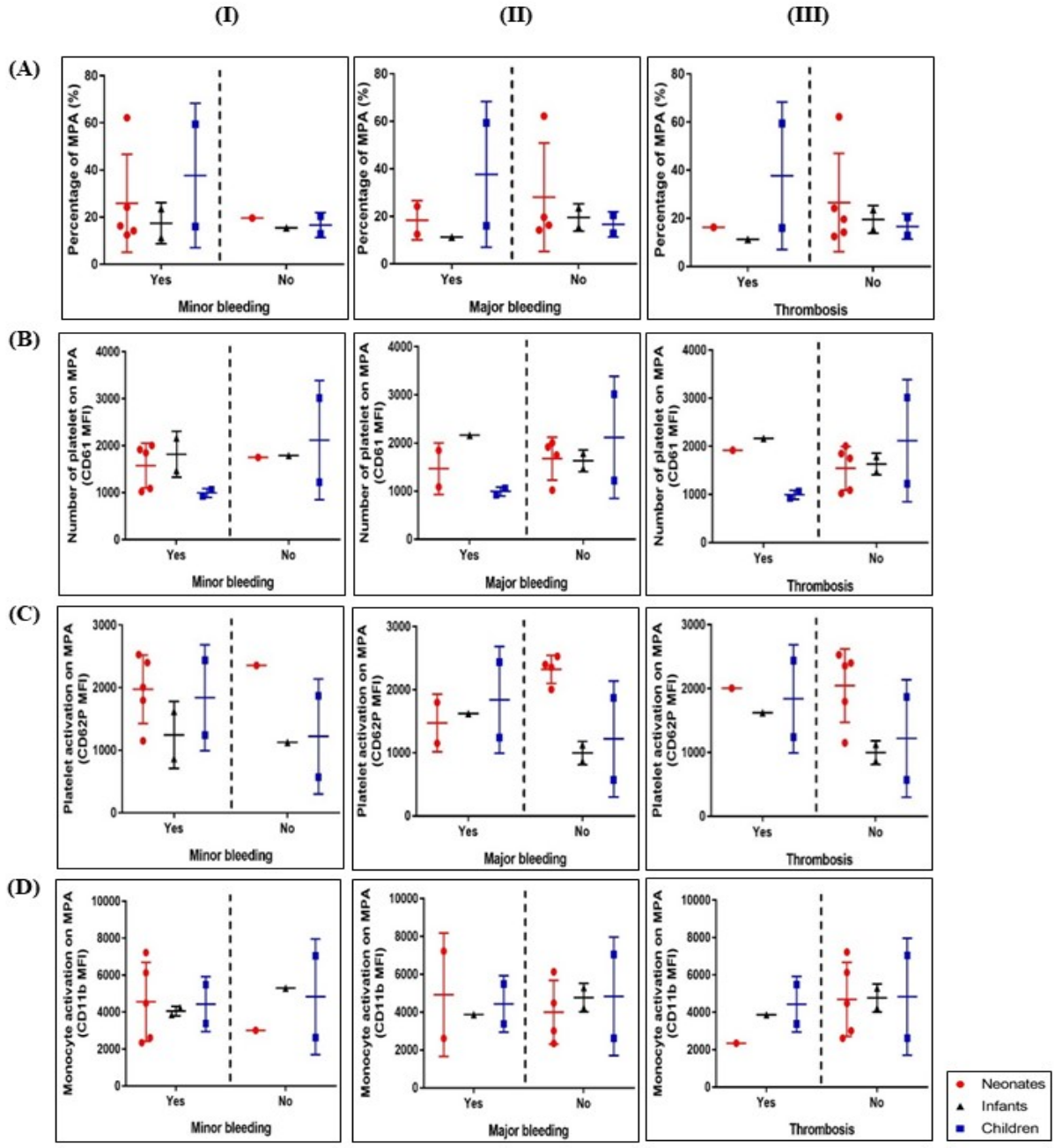

Figure 5.9 The interaction between platelets and monocytes according to the age and presence or absence of clinical event.

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation and (D) monocyte activation on MPA within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Neonates n=6, Infants n=3 and Children n=4). [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; SD, standard deviation;]

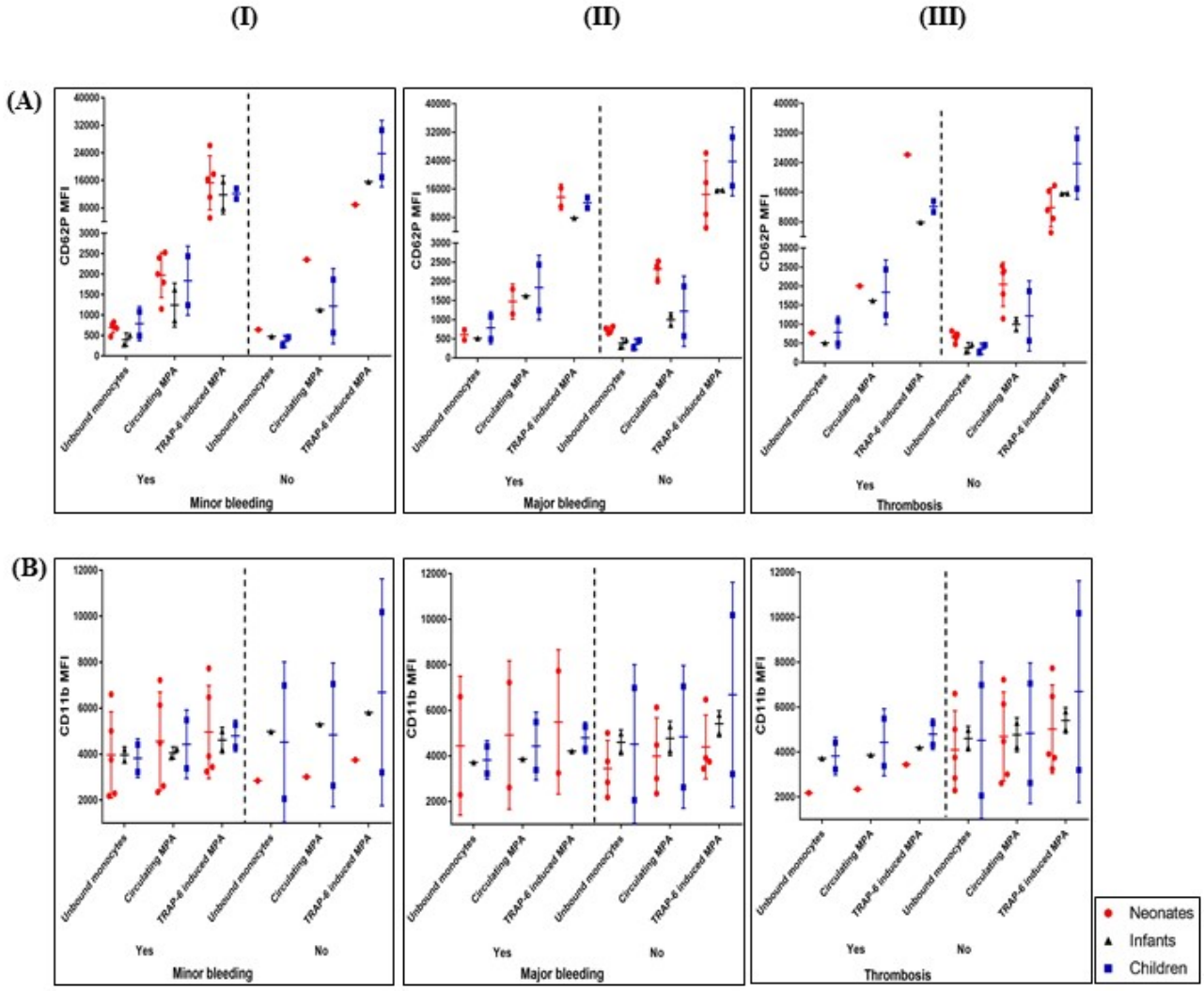

Figure 5.10 The mechanism of interaction between platelets and monocytes according to the age and presence or absence of clinical event.

The mechanism of interaction between platelets and monocytes via (A) P-selectin and (B) Mac-1 on MPA within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Neonates n=6, Infants n=3 and Children n=4). [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; SD, standard deviation]

5.3.6 Circulating neutrophil-platelet aggregates (NPA)

There was no significant difference in the percentage of circulating NPA, the relative number of platelets bound on NPA and neutrophil activation on NPA for neonates, infants and children (Figure 5.11). A trend of decreased platelet activation on NPA was observed for children compared to neonates (1072.0 ± 382.29 vs. 683.17 ± 159.10 MFI, p= 0.081; d= 1.63; power= 0.58) (Figure 5.11). To determine if circulating NPA formed as a result of P-selectin/PSGL-1 and Mac-1 adhesion mechanisms, the MFI of P-selectin and Mac-1 on platelet-bound and unbound neutrophils events was examined. Upon stimulation with TRAP-6, the expression of P-selectin and Mac-1 was induced to a similar maximal expression for neonates, infants and children. This indicated that the capacity for platelet and neutrophil activation-dependent and -independent formation of NPA was retained in all age groups (Figure 5.11).

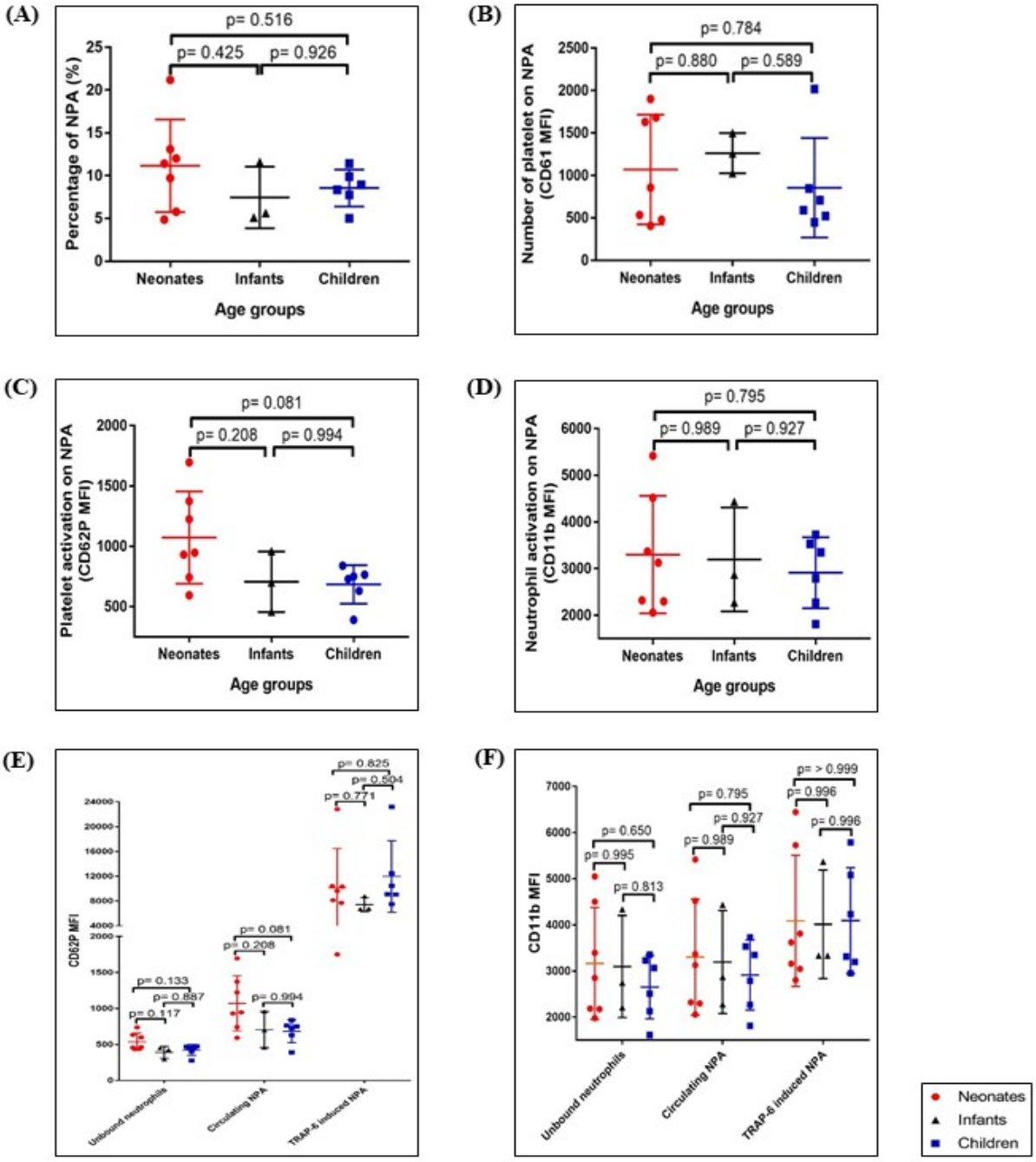

Figure 5.11 The interaction between platelets and neutrophils and age.

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation, (D) neutrophil activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on NPA within the first 24 hours according to a patient's age. Data shown as mean % or MFI +/- SD (Neonates n=7, Infants n=3 and Children n=6). [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

Evaluation of the association between the interactions of platelets with neutrophils and clinical events were limited by the number of minor bleeding event in each age group. As for major bleeding and thrombosis, comparisons were made for neonates and children who had or had no event, respectively. The percentage of circulating NPA, the relative number of platelets bound on NPA, neutrophil and platelet activation on NPA and mechanism of interaction were comparable for neonates who had or had no major bleeding. The same observations were obtained for children who had or had no thrombosis (Figure 5.12 and Figure 5.13).

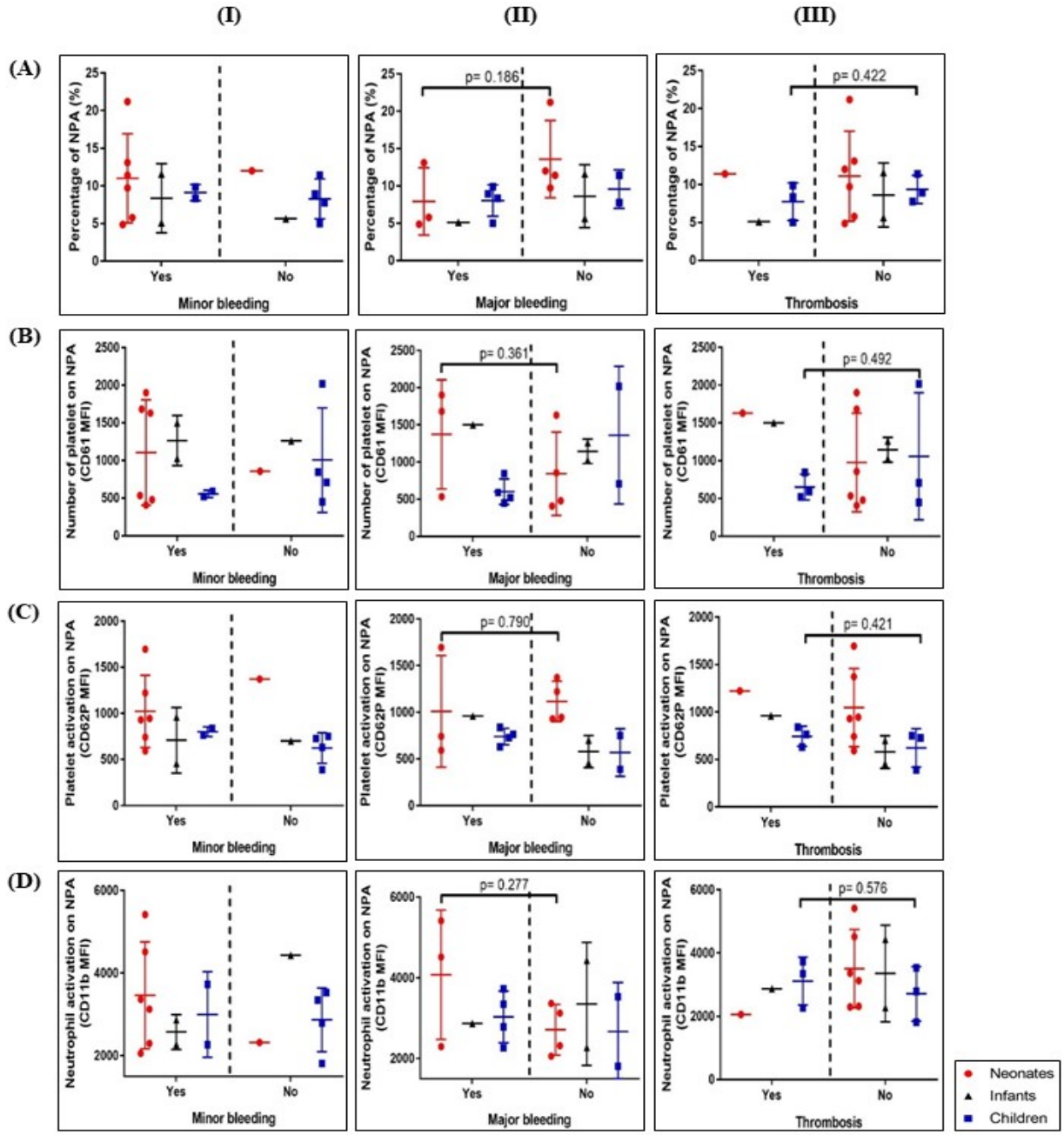

Figure 5.12 The interaction between platelets and neutrophils according to the age and presence or absence of clinical event.

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation and (D) neutrophil activation on NPA within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Neonates n=7, Infants n=3 and Children n=6). [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

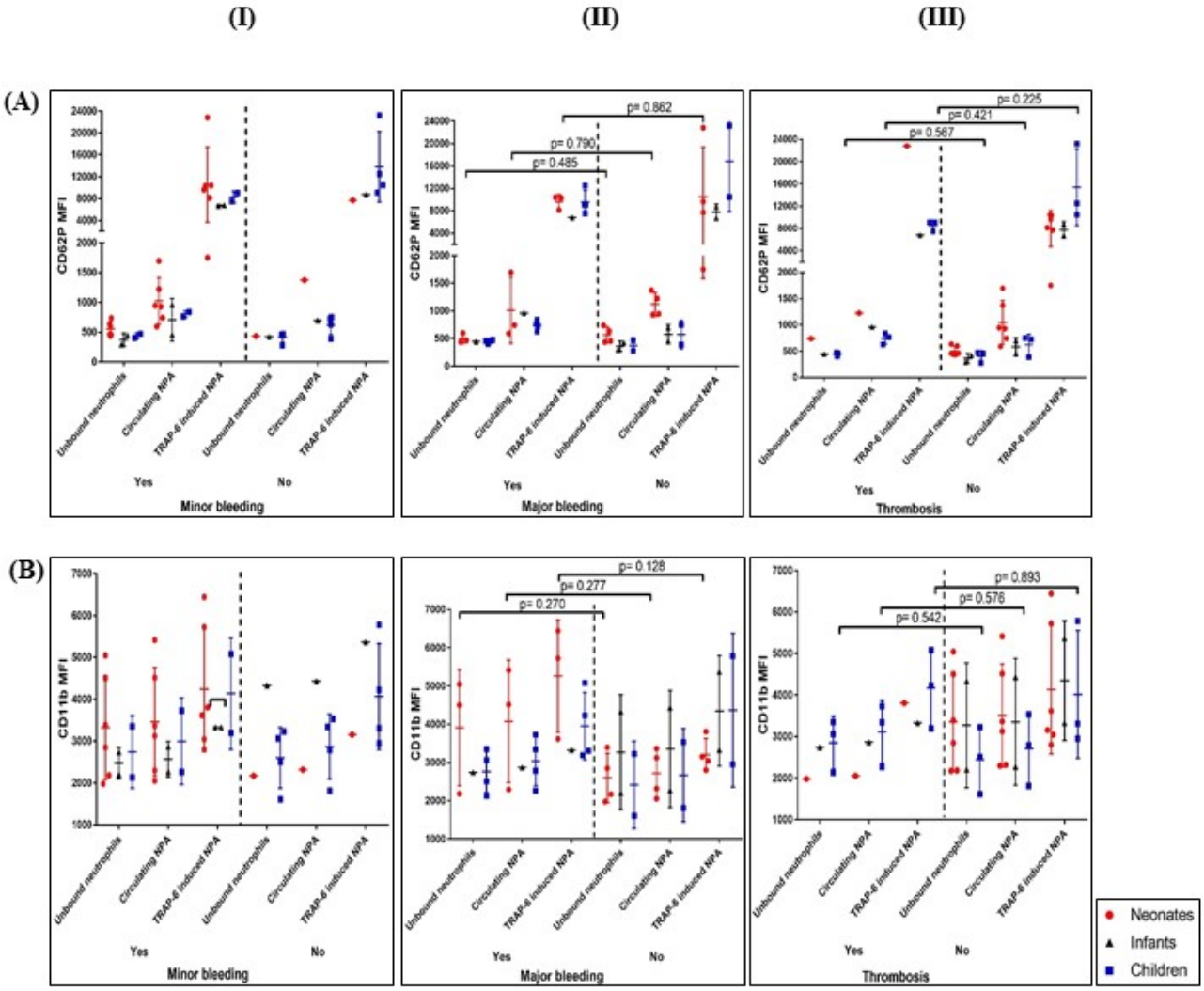

Figure 5.13 The mechanism of interaction between platelets and neutrophils according to the age and presence or absence of clinical event.

The mechanism of interaction between platelets and neutrophils via (A) P-selectin and (B) Mac-1 on NPA within the first 24 hours according to a patient's age and clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD (Neonates n=7, Infants n=3 and Children n=6). [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

5.4 Discussion

This study demonstrated for the first time that I.) neonates, infants and children had comparable platelet phenotype, function and interactions with monocytes and neutrophils within the first 24 hours on ECMO; II.) clinical events may be associated with the modification of platelet granule exocytosis as observed for the lysosome release in children with thrombosis and III.) platelet and/or leukocyte activation on MPA/NPA may be an important coagulation indicator for paediatric patients on ECMO.

Although no significant difference was observed for the expression of receptors important for platelet phenotype, children had a trend for an increased platelet integrin αIIbβ3 receptor expression compared to neonates. The elevated integrin αIIbβ3 receptor expression could be associated with an increased interaction between integrin αIIbβ3 receptor and its ligand, fibrinogen in children. As a plasma protein that gets deposited on foreign surface e.g. ECMO circuit with the highest concentration, fibrinogen undergoes shear-induced conformational change upon adhesion [263]. Hence, children with a higher weight may be subjected to a higher extent of shear-induced fibrinogen modification than neonates. Such modification of fibrinogen is known to promote platelets binding towards the foreign surface via integrin αIIbβ3 receptor followed by an increased platelet surface integrin αIIbβ3 receptor expression via positive feedback loop [280, 281] which could have detrimental effects by promoting platelet aggregation followed by thrombus formation.

In addition to the platelet phenotype, neonates, infants and children had comparable circulating platelet activation and granule release. However, children with thrombosis had higher lysosome release than those who had no thrombosis. Platelets have multiple types of storage granules that are released upon platelet activation. Compared to the α- and dense-granules, very limited information is available for the role of platelet lysosomes in haemostasis and thrombosis. Rendu and Brohard-Bohn (2001) showed that platelet lysosomes which store various enzymes

involved in carbohydrates, proteins and lipids degradation have an important role for thrombus resolution [65]. Hence, such increase in lysosome release seen for children with thrombosis may be a response to an increased demand for thrombus resolution via positive feedback loop.

The lower inter-individual variability for granule exocytosis (both α-granule and lysosome) in neonates than infants and children may be associated with lower platelet secretion in neonates due to under-developed signal transduction pathways [40, 41, 272]. On the other hand, a high inter-individual variability in platelet response to stimulation TRAP-6 was equally seen for neonates, infants and children and is consistent with the reported variation for platelet response [44, 244]. Such differences in platelet response was associated with polymorphisms of the platelet receptors. One of the receptors is thrombin receptor, PAR-1 of which the magnitude of the platelet response towards TRAP-6 is reported to be directly proportional to the level of PAR-1 expression [245].

Platelets can interact with leukocytes to form MPA and NPA, the mediators of cross-talk between haemostasis and inflammation. Specifically, an increased platelet-leukocyte aggregates level is associated with hypercoagulability and has been used as a marker of platelet activation under various pathological conditions in adults and children [148, 282-284]. Although neonates, infants and children had comparable MPA/NPA specific changes, paediatric patients from different age groups had I.) homogenised circulating MPA/NPA level; II.) high inter-individual variability for the number of platelets bound, platelet and/or leukocyte activation on MPA/NPA and III.) children had a decreased trend of platelet activation on NPA compared to neonates. Such variation seen for the MPA/NPA-related parameters may indicate that although the capacity for platelet and monocyte/neutrophil activation-dependent and -independent formation of MPA/NPA was retained in all age groups, platelet and/or monocyte/neutrophil on MPA/NPA in paediatric patients on ECMO could have an activation state that varies and are with different capacity for further activation. Hence, instead of the

circulating MPA/NPA level, platelet and/or leukocyte activation on MPA/NPA may be a more important indicator of coagulation state in paediatric patients on ECMO.

Although paediatric patients of different age groups had comparable platelet-specific changes within the first 24 hours on ECMO, platelet and/or leukocyte activation on MPA/NPA could be used to inform the coagulation state in this population. Even though the relationship between platelet-specific changes and clinical events remains elusive for paediatric patients of different age groups, modulation of lysosome release may be related to the development of thrombosis in children on ECMO. Thus, although with limited capacity to make absolute conclusion, this study provides justification to continue this work with a larger sample size for detailed evaluation for the effects of age on platelet-specific changes and their associations with the clinical events in the paediatric ECMO population.

5.5 Conclusion

Modulation of lysosome release may be associated with the development of thrombosis and the activation state of platelet and/or leukocyte on MPA/NPA could be used to inform the coagulation state for paediatric ECMO patients. A larger sample size is required to evaluate the relationship between platelet-specific changes and bleeding/thrombosis so that medical intervention can be developed accordingly to reduce the incidence of clinical events in the paediatric ECMO population.

6 The effect of duration of ECMO on platelet phenotype, function and interactions with monocytes and neutrophils and their associations with bleeding or thrombosis

6.1 Introduction

ECMO provides short to medium-length of cardiac and/or respiratory supports to the patients, ranging from a few days to a few weeks' time. Generally, the duration of ECMO is determined by the progress of recovery for the underlying cardiac and/or respiratory dysfunction. On average, a patient stays on ECMO for about 5 - 7 days [285, 286]. Various studies have shown that a patient's survival rate decreases with increasing duration of ECMO due to increased complications related to bleeding and thrombosis [226, 266]. A study in paediatric ECMO population with respiratory support showed that patients receiving support for ≥ 21 days had lower survival rate than patients supported for < 14 days (38 % vs. 61 %) [287].

Over the years, various approaches have been made attempting to improve the outcome of patients on ECMO. One approach is to review and modify the therapeutic strategies based on the specific reference parameters as ECMO progresses [286, 288]. Since platelet dysfunction and acquired von Willebrand syndrome have been associated with coagulopathy in adults on ECMO [208, 211], platelet-specific markers have been proposed to be a potential target for monitoring and guiding therapy. The currently known platelet receptor/ligand-specific changes in relation to the duration in adults on ECMO suggest there is minimal effect of ECMO on the platelet activation within the first 72 hours on ECMO and acquired von Willebrand syndrome recovers within 24 hours upon ECMO termination [206, 208, 211, 289].

Compared to the platelet phenotype and response which are well-studied in the adult ECMO population, there is no single study that has investigated the interaction between platelets and

monocytes/neutrophils in adults on ECMO, moreover, for how such platelets-monocytes/neutrophils interactions may change as ECMO progresses.

The overall effects of duration of ECMO have been suggested to be more profound in children than adults due to a lack of optimization of various ECMO-related therapeutic strategy e.g. anticoagulation in children and their increased vulnerability to complications such as bleeding [290]. However, very limited information is available for the effect of duration of ECMO in paediatric ECMO patients. Furthermore, there is no existing study which has investigated how changes in the platelet phenotype and function may be relevant to the development of clinical events as ECMO progresses in this population. Understanding the platelet-specific differences between and also within the paediatrics patients with/without clinical events may provide clarity for whether medical intervention can be tailored accordingly to their duration of ECMO to prevent the development of clinical events as ECMO progresses.

The hypothesis of this chapter is that platelet phenotype, function and interactions with monocytes and neutrophils will be different according to a patient's duration of ECMO and the platelet-specific changes can be associated with the development of bleeding or thrombosis during ECMO.

This chapter aims to determine the effect of duration of ECMO on the platelet-specific differences and the associations between these differences with the clinical events for:

1) The platelet phenotype.
2) The circulating platelet activation.
3) The response of platelets to stimulation with TRAP-6.
4) The interactions of platelets with monocytes and neutrophils.

6.2 Materials and Methods

The methods utilized in this chapter were outlined in Chapter 2. Based on the definitions in section 2.2.3, the clinical events for minor bleeding, major bleeding and thrombosis were recorded accordingly.

To assess the platelet-specific changes, comparisons at the following time points were made:

I.) Day 2 vs. Day 5 for patients on ECMO > 5 days

II.) Within 24 hours post-decannulation for patients on ECMO ≤ 5 days vs. > 5 days.

Sections 2.6.3 - 2.6.5 comprised the details for flow cytometry panels used for this chapter. Following is the summary for the evaluation of platelet-specific markers:

I.) Platelet phenotype was assessed by measuring the expression of integrin αIIbβ3, GPIb/IX/V and GPVI receptors.

II.) Circulating platelet activation was measured as the percentage of platelets positive for activated fibrinogen (integrin αIIbβ3) receptor (measured by PAC-1 binding) while platelet granule exocytosis was measured as the percentage of platelets positive for α-granule (P-selectin) and lysosome release (CD63) indicators. The response of platelet to TRAP-6 is directly proportional to the area under the curve (AUC).

III.) The interaction between platelets and monocytes/neutrophils were investigated by evaluating circulating MPA/NPA. The relative number of platelets bound on the MPA/NPA and monocytes/neutrophils was measured by MPA/NPA CD61 MFI [234]. The mechanism of MPA/NPA tethering was measured by reporting the MFI of P-selectin and Mac-1 on platelet-bound and unbound monocyte/neutrophil events with/without stimulation with TRAP-6 [141].

The results of platelet-specific markers on ECMO Day 2 (24 – 48 hours), ECMO Day 5 (96 - 120 hours) and within 24 hours post-decannulation from ECMO were used for all data analysis relevant to platelet-specific changes in this chapter. Statistical analysis were described in details in section 2.7. In summary, Fisher's exact or Chi-Square test was used for the comparisons of parameters (except for age and weight with one-way ANOVA) for the demographic details in Tables 6.1 - 6.3. Full blood count parameters and platelet phenotype and function for patients at different time points were compared using paired Student's t-test (ECMO Day 2 vs. ECMO Day 5 for patients on ECMO > 5 days) and unpaired Student's t-test (within 24 hours post-decannulation for patients on ECMO ≤ 5 days vs. > 5 days). For the evaluation of association between the platelet-related changes and clinical events (minor bleeding, major bleeding or thrombosis) in patients on ECMO > 5 days, paired Student's t-test was used for the comparison of ECMO Day 2 vs. ECMO Day 5 within patients with/without clinical events and unpaired Student's t-test was used for the comparison between patients with/without clinical events within Day 2 and Day 5.

6.3 Results

6.3.1 Demographics

Demographic information for the twenty-two patients included in this chapter was summarized in Table 6.1. All patients received VA-ECMO and had comparable age, weight, pathway onto ECMO and mode of cannulation.

Table 6.1 Summary of demographic information comparing patients on ECMO ⩽ 5 days to > 5 days.

Variable			Duration of ECMO (n= 22), n (%)		p-value
			≤ 5 days 10 (45)	> 5 days 12 (55)	
Age [years] median (IQR)			1.22 (0.04 - 10.27)	0.33 (0.01 - 1.69)	0.793
Pathway onto ECMO, n (%)		Post-CPB	5 (50)	7 (58)	> 0.999
		Non-CPB	5 (50)	5 (42)	
Primary diagnosis	Cardiac	Unable to wean off CPB	3 (30)	3(25)	-
		Cardiomyopathy	1 (10)	3 (25)	
		Myocarditis	1 (10)	1 (8)	
		Primary congenital cardiac abnormality	2 (20)	3 (25)	
		Other	2 (20)	1(8)	
	Respiratory	Meconium aspiration syndrome	0 (0)	1 (8)	-
	Other	Sepsis	1 (10)	0 (0)	-
Gender, n (%)		Male	6 (60)	6 (50)	0.691
		Female	4 (40)	6 (50)	
Weight [kg] median (IQR)			7.75 (3.44 - 35.0)	5.79 (3.35 - 9.65)	0.429
Mode of cannulation, n (%)		Central	9 (90)	7 (58)	0.162
		Peripheral	1 (10)	5 (42)	

[ECMO, extracorporeal membrane oxygenation; IQR, interquartile range; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass]

6.3.2 Clinical events

Clinical events and platelet transfusions received by the patients were illustrated in the longitudinal graph Figure 6.1 according to their duration of ECMO. Within 24 hours upon ECMO initiation (Day 1), 70 % of the ≤ 5 days group compared to 83 % of the > 5 days group had ≥ 1 clinical event. The rate of clinical events increased after 5 days on ECMO for those who were on ECMO for > 5 days. As for the platelet transfusion, 50 % of the ≤ 5 days group compared to 75 % of the > 5 days group required ≥ 1 platelet transfusion within 24 hours upon initiation of ECMO.

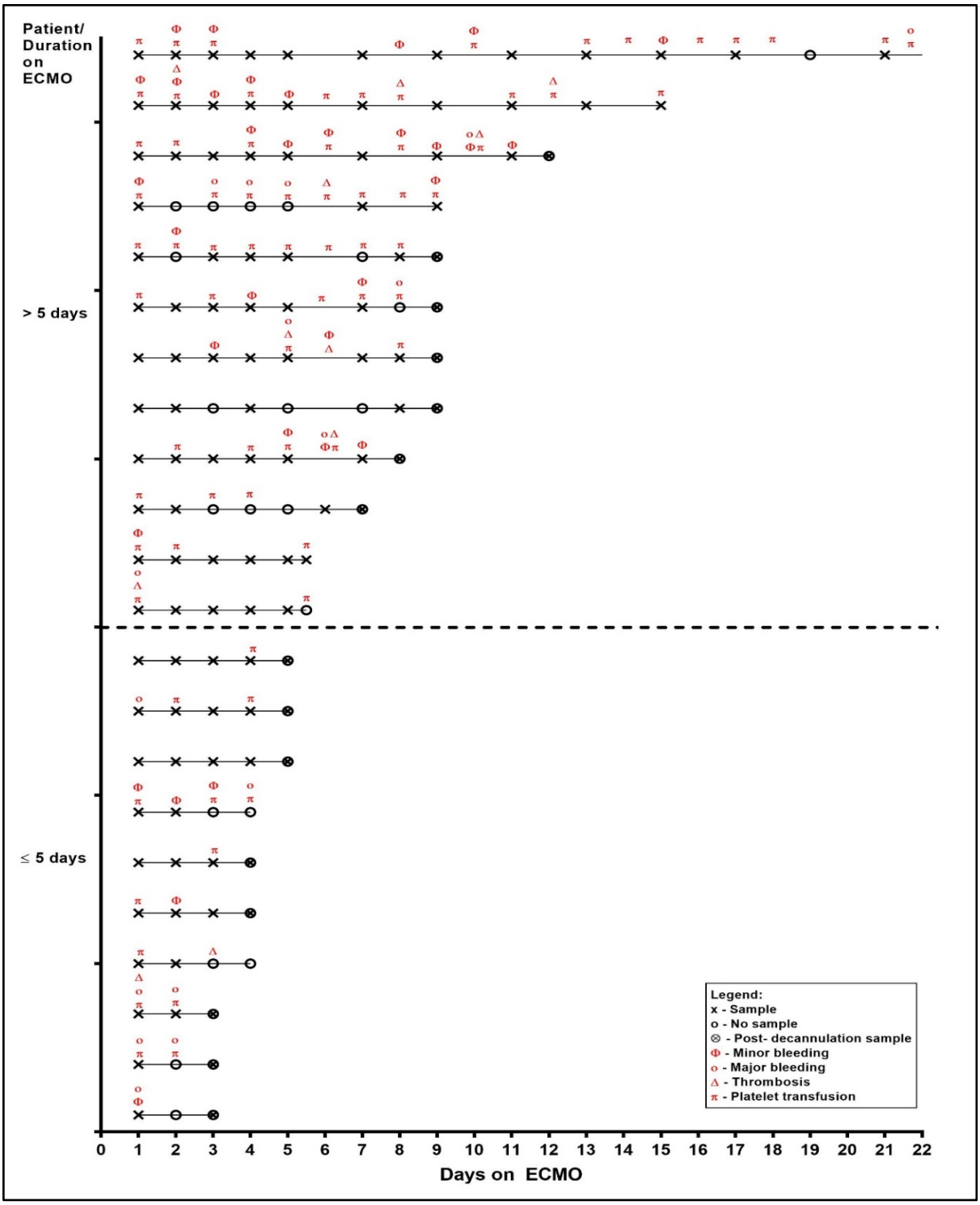

Figure 6.1 Longitudinal graph for patients, clinical events and platelet transfusions according to the duration of ECMO.

The dotted line depict the division for patients on ECMO ≤ 5 days vs. > 5 days. [ECMO, extracorporeal membrane oxygenation]

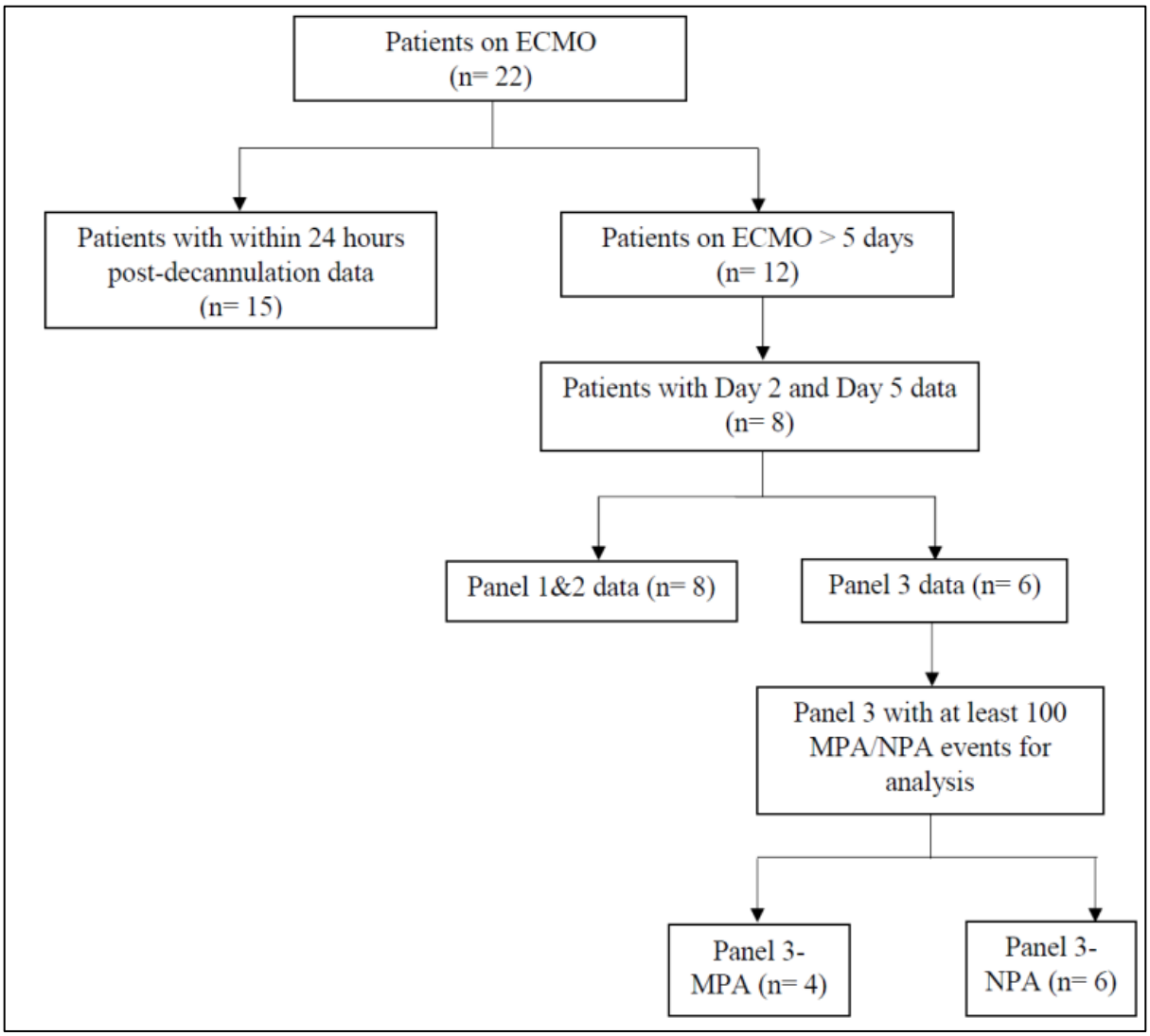

Figure 6.2 Summary for the number of sample for data analysis according to the duration of ECMO.

[ECMO, extracorporeal membrane oxygenation; MPA, monocyte-platelet aggregates; NPA, neutrophil-platelet aggregates]

To investigate the relationships between the platelet-specific changes and clinical events, patients on ECMO > 5 days were stratified into 2 groups based on whether they had bleeding/thrombosis after 5 days on ECMO. Platelet phenotype and function were analysed accordingly in relation to the clinical events with the exception for those less than 3 bleeding/thrombotic events.

Table 6.2 comprised the summary of patients with ≥ 1 bleeding/thrombotic event within the first two days and after five days on ECMO. There was no difference for the number of patients with ≥ 1 clinical event within the first two days and after five days on ECMO.

Table 6.2 Summary for the rate of clinical events comparing within the first two days to after five days on ECMO for patients on ECMO > 5 days.

Clinical event		Number and the rate of clinical event (n= 8), n (%)		p-value
		Within the first two days on ECMO	After five days on ECMO	
Minor bleeding	**Yes**	3 (37)	5 (63)	0.619
	No	5 (63)	3 (37)	
Major bleeding	**Yes**	1 (12)	4 (50)	0.282
	No	7 (88)	4 (50)	
Thrombosis	**Yes**	2 (25)	4 (50)	0.608
	No	6 (75)	4 (50)	

[ECMO, extracorporeal membrane oxygenation]

Table 6.3 recorded the summary of patients with ≥ 1 platelet transfusion within the first two days and after five days on ECMO. The number of patients who required ≥ 1 platelet transfusion within the first two days was comparable to after five days on ECMO. Also, patients had similar full blood count on both Day 2 and Day 5 (Table 6.4).

Table 6.3 Summary of platelet transfusions comparing within the first two days to after five days on ECMO for patients on ECMO > 5 days.

Transfusion product		Number and the rate of transfusion (n= 8), n (%)		p-value
		Within the first two days on ECMO	After five days on ECMO	
Platelet	Yes	7 (88)	8 (100)	> 0.999
	No	1 (12)	0 (0)	

[ECMO, extracorporeal membrane oxygenation]

Table 6.4 Summary of full blood count comparing Day 2 to Day 5 for patients on ECMO > 5 days.

Full blood count	Day on ECMO (n= 4)		p-value
	Day 2	Day 5	
WCC (x 10^9/L)	6.69 ± 3.40	7.30 ± 2.77	0.736
RCC (x 10^{12}/L)	3.31 ± 0.28	3.02 ± 0.37	0.174
HGB (x g/L)	100.00 ± 9.83	88.25 ± 17.56	0.174
HCT (x L/L)	0.29 ± 0.03	0.26 ± 0.05	0.212
PLT (x 10^9/L)	71.50 ± 44.78	74.25 ± 9.43	0.898
MPV (fl)	9.13 ± 2.03	8.03 ± 2.19	0.266

[ECMO, extracorporeal membrane oxygenation; HCT, haematocrit, HGB, haemoglobin; MPV, mean platelet volume, PLT, platelet, RCC, red blood cell count, WCC, white blood cell count]

Patients on ECMO ≤ 5 days vs. > 5 days who were compared for the platelet phenotype and function 24 hours post-decannulation were also analysed for their platelet transfusions within 24 hours before decannulation from ECMO. There was no difference for the rate of platelet transfusions and full blood count between the two groups (Table 6.5 and Table 6.6).

Table 6.5 Summary of platelet transfusions within 24 hours before decannulation from ECMO comparing patients on ECMO ⩽ 5 days to > 5 days.

Transfusion product		**Number and the rate of transfusion within 24 hours before decannulation from ECMO (n= 15), n (%)**		**p-value**
		≤ 5 days 8 (54)	**> 5 days 7 (46)**	
Platelet	**Yes**	5 (63)	4 (57)	> 0.999
	No	3 (37)	3 (43)	

[ECMO, extracorporeal membrane oxygenation]

Table 6.6 Summary of full blood count within 24 hours post-decannulation from ECMO comparing patients on ECMO ≤ 5 days to > 5 days.

Full blood count	**Duration on ECMO (n= 10)**		**p-value**
	⩽ 5 days 6 (60)	**> 5 days 4 (40)**	
WCC (x 10^9/L)	7.30 ± 4.83	12.98 ± 7.38	0.236
RCC (x 10^{12}/L)	3.05 ± 0.63	3.49 ± 0.44	0.222
HGB (x g/L)	90.83 ± 22.48	104.75 ± 14.97	0.274
HCT (x L/L)	0.27 ± 0.06	0.31 ± 0.04	0.240
PLT (x 10^9/L)	101.50 ± 28.92	152.5 ± 51.53	0.142
MPV (fl)	7.68 ± 0.54	7.63 ± 0.64	0.886

[ECMO, extracorporeal membrane oxygenation; HCT, haematocrit, HGB, haemoglobin; MPV, mean platelet volume, PLT, platelet, RCC, red blood cell count, WCC, white blood cell count]

6.3.3 Platelet phenotype

6.3.3.1 Day 2 vs. Day 5 on ECMO

Reduced integrin αIIbβIII (26086.88 ± 3835.67 vs 23019.88 ± 4105.57 MFI, p= 0.021; d= 0.77; power= 0.30) and elevated GPIb/IX/V (6093.63 ± 2132.38 vs. 8056.50 ± 1811.17 MFI, p= 0.026; d= 0.99; power= 0.45) receptor expressions were observed on Day 5 compared to Day 2 (Figure 6.3). In contrast, GPVI receptor expression was unchanged for Day 2 vs. Day 5 (Figure 6.3).

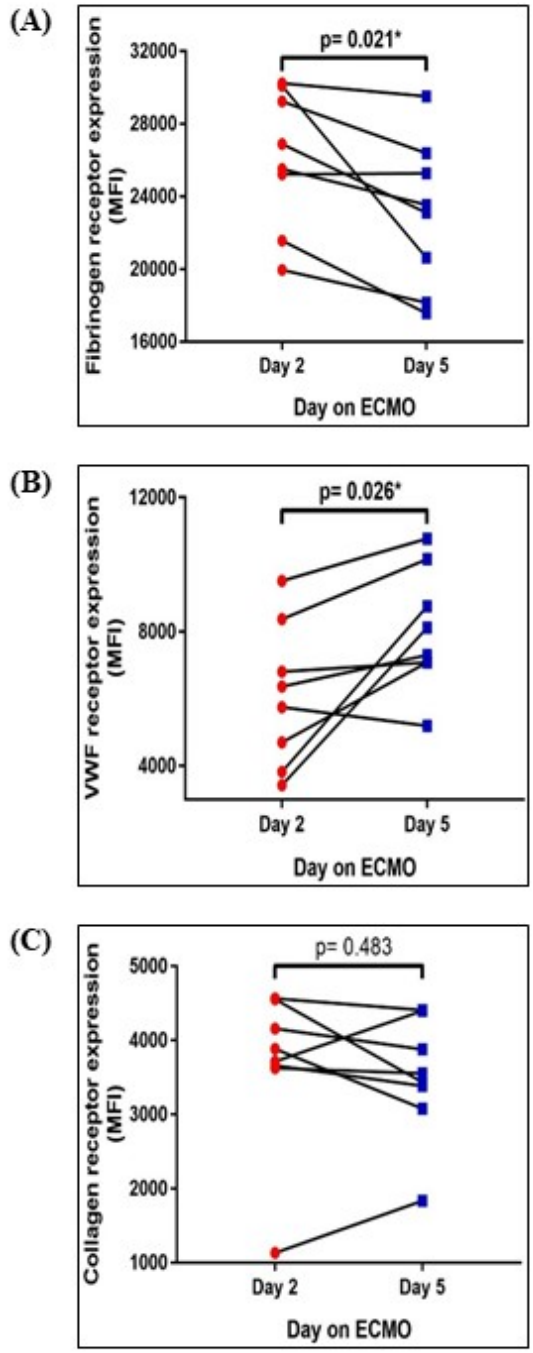

Figure 6.3 Platelet phenotype and duration of ECMO (Day 2 vs. Day 5).

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor for Day 2 vs. Day 5 on ECMO. Data shown as mean MFI +/- SD (n= 8). [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

6.3.3.2 Within 24 hours post-decannulation from ECMO

Post-decannulation integrin αIIbβ3, GPIb/IX/V and GPVI receptor expression was comparable for patients on ECMO ≤ 5 days vs. > 5 days (Figure 6.4). While the sample size was inadequately powered to assess differences in the coefficient of variation between groups, the inter-individual variability for the GPIb/IX/V receptor expression appeared to be higher for patients on ECMO > 5 days compared to ≤ 5 days (Figure 6.4).

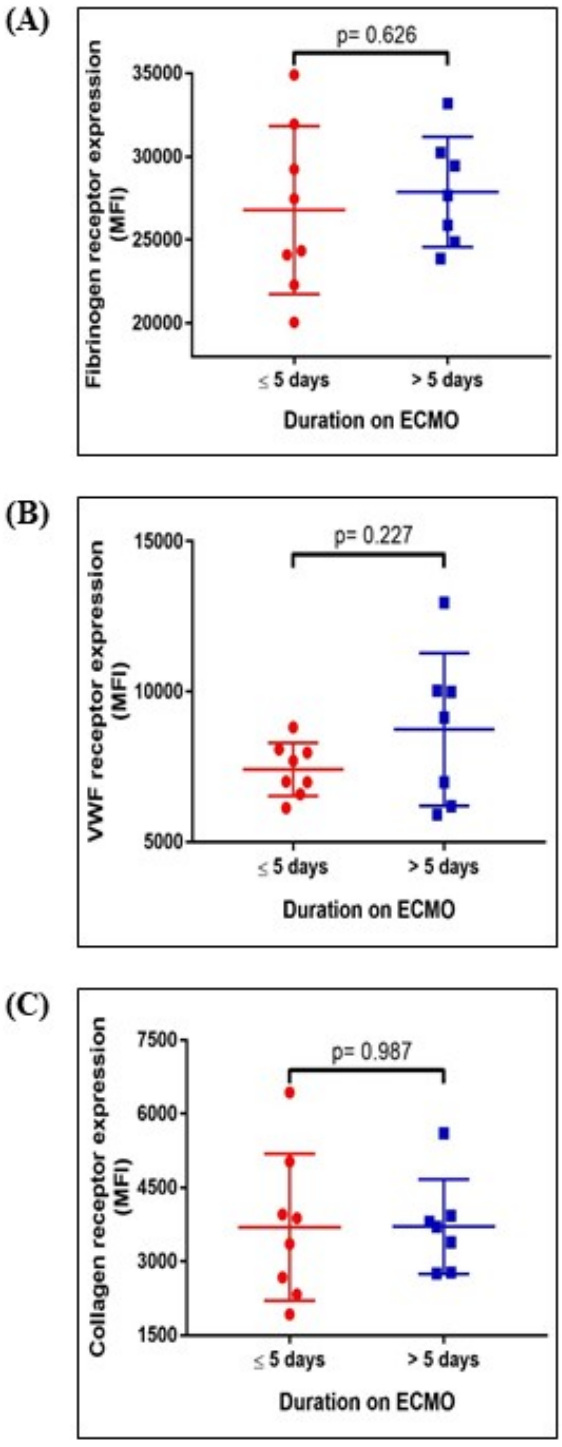

Figure 6.4 Platelet phenotype and duration of ECMO (≤ 5 days vs. > 5 days).

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor within 24 hours post-decannulation according to a patient's duration of ECMO. Data shown as mean MFI +/- SD (≤ 5 days n= 8, > 5 days n= 7). [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

6.3.3.3 The association between changes in platelet phenotype with duration of ECMO and clinical events

Patients with minor bleeding (5564.00 ± 2480.20 vs. 8405.00 ± 1481.77 MFI, p= 0.028; d= 1.39; power= 0.47) or major bleeding (4576.75 ± 1305.42 vs. 7813.25 ± 770.11 MFI, p= 0.043; d= 3.02; power= 0.92) had significantly higher GPIb/IX/V receptor expression on Day 5 than Day 2 (Figure 6.5). In contrast, a trend of decrease was observed for integrin αIIbβ3 receptor expression in patients with minor bleeding (26305.00 ± 4060.54 vs. 24486.40 ± 4228.32 MFI, p= 0.060; d= 0.44; power= 0.09) or major bleeding (25323.25 ± 3944.41 vs. 23228.75 ± 3646.05 MFI, p= 0.085; d= 0.55; power= 0.10) on Day 5 compared to Day 2 (Figure 6.5). GPVI receptor expression was comparable on both Day 2 and Day 5 in patients with minor bleeding or major bleeding (Figure 6.5). Patients with/without thrombosis or without minor bleeding/major bleeding had comparable integrin αIIbβ3, GPIb/IX/V and GPVI receptor expressions for Day 2 vs. Day 5 (Figure 6.5). Within Day 2 and Day 5, patients with/without minor bleeding or thrombosis had similar integrin αIIbβ3, GPIb/IX/V and GPVI receptor expressions (Figure 6.5). On the other hand, patients with major bleeding had lower GPIb/IX/V expression than those with no event on Day 2 (4576.75 ± 1305.42 vs. 7610.50 ± 1664.29 MFI, p= 0.030; d= 2.03; power= 0.66) but not Day 5 (Figure 6.5).

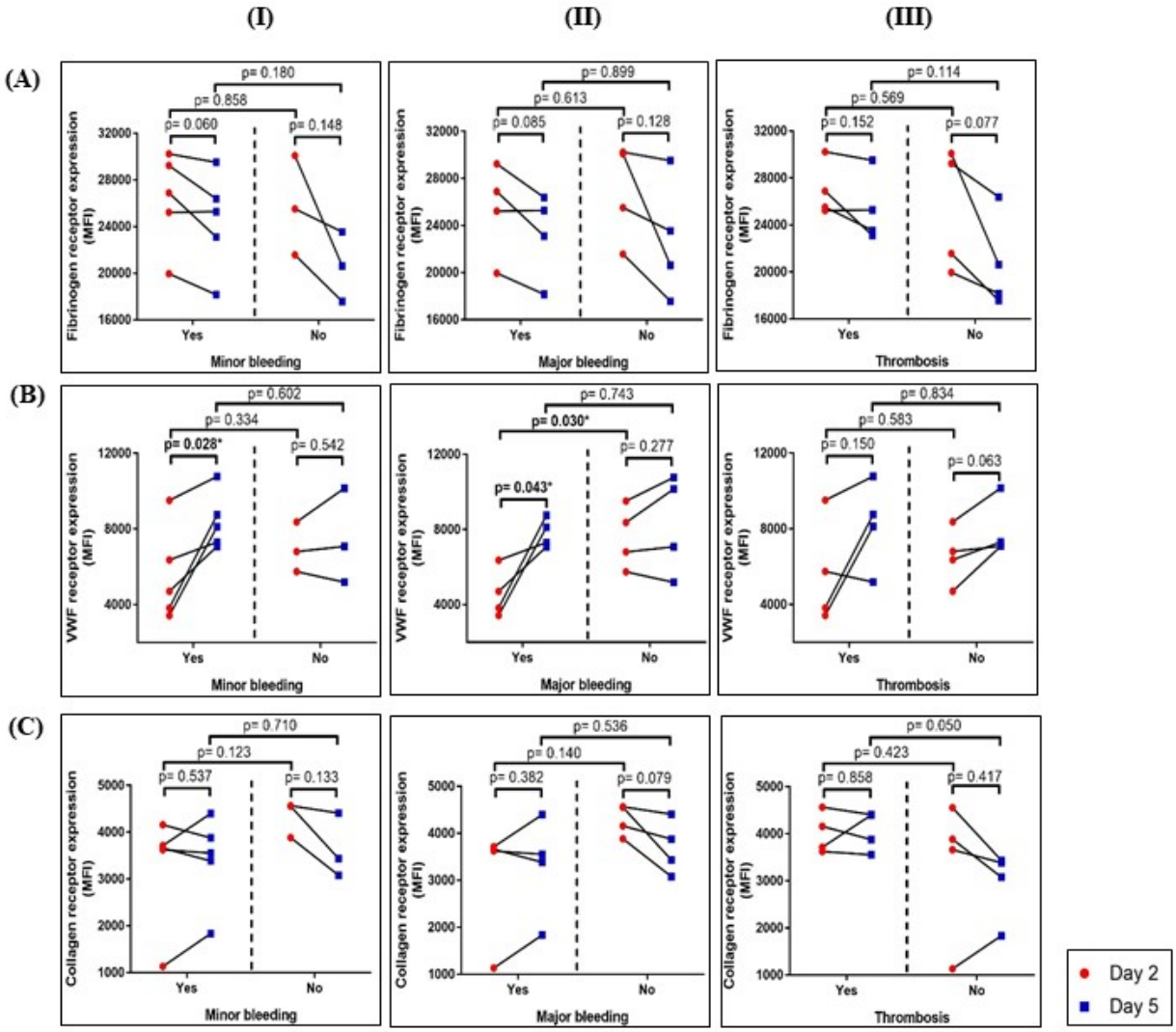

Figure 6.5 Platelet phenotype according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor for Day 2 vs. Day 5 on ECMO according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean MFI +/- SD. [MFI, median fluorescence intensity; SD, standard deviation; VWF, von Willebrand factor]

6.3.4 Circulating platelet activation

6.3.4.1 Day 2 vs. Day 5 on ECMO

Circulating platelet activation and granule exocytosis were comparable for Day 2 vs. Day 5 (Figure 6.6). The inter-individual variability for the α-granule release on Day 5 appeared to be lower than Day 2 (Figure 6.6).

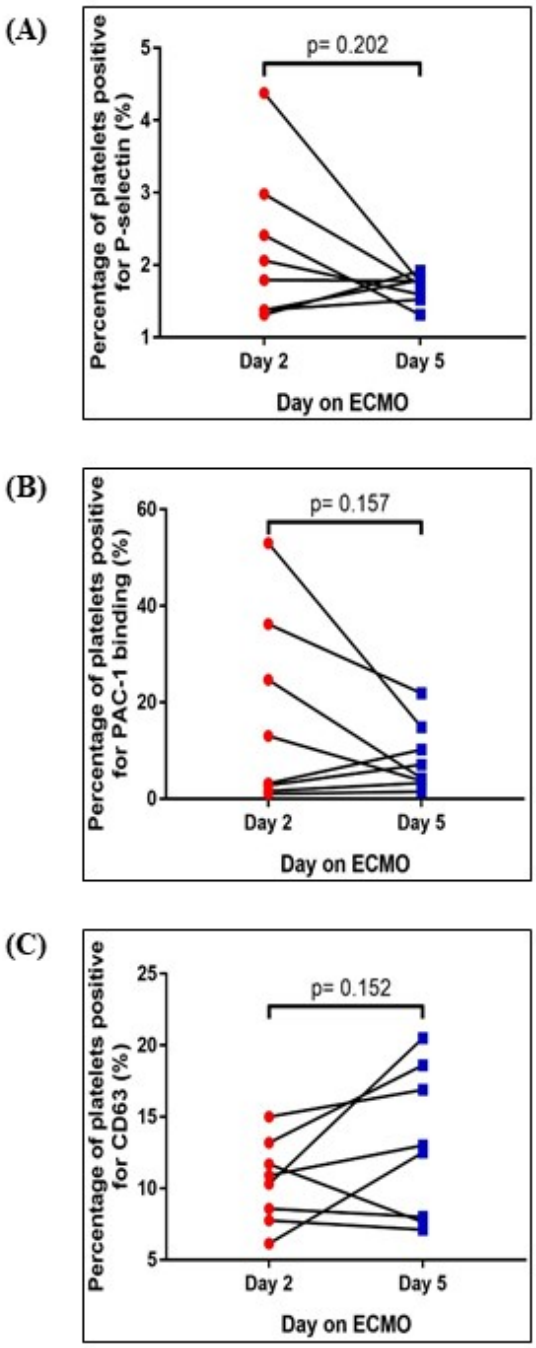

Figure 6.6 Circulating platelet activation and duration of ECMO (Day 2 vs. Day 5).

Circulating platelet activation markers for the (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 for Day 2 vs. Day 5 on ECMO. Data shown as mean % +/- SD (n= 8). [SD, standard deviation]

6.3.4.2 Within 24 hours post-decannulation from ECMO

Within 24 hours of decannulation, patients on ECMO ≤ 5 days vs. > 5 days had comparable circulating platelet activation and granule exocytosis (both α-granule and lysosome) (Figure 6.7).

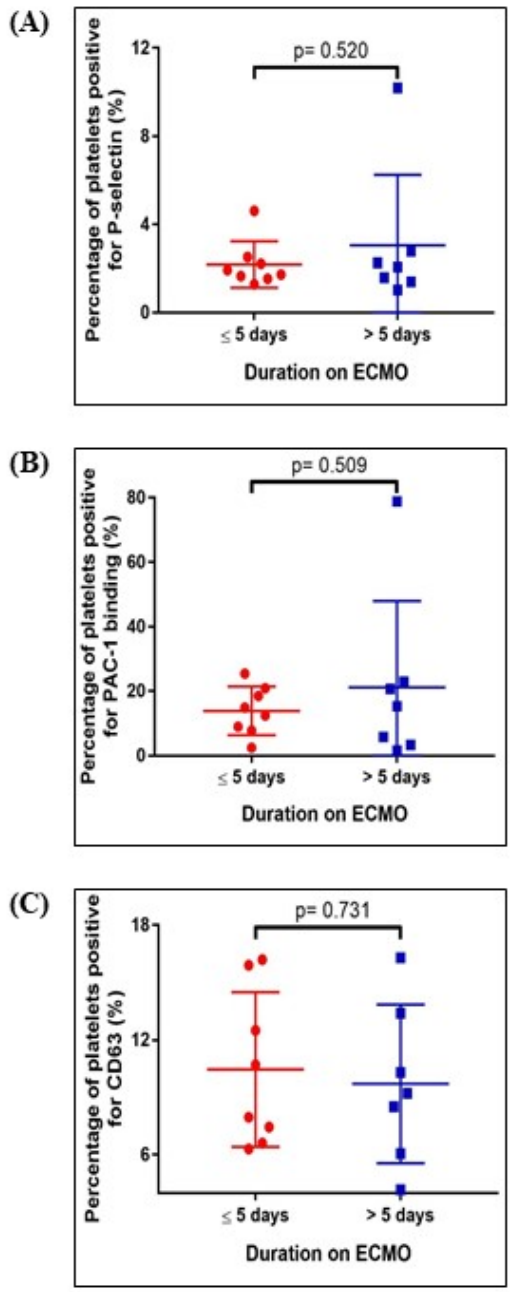

Figure 6.7 Circulating platelet activation and duration of ECMO (≤ 5 days vs. > 5 days). Circulating platelet activation markers for the (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within 24 hours post-decannulation according to a patient's duration of ECMO. Data shown as mean % +/- SD (≤ 5 days n= 8, > 5 days n= 7). [SD, standard deviation]

6.3.4.3 The association between changes in circulating platelet activation and clinical event with duration of ECMO

Patients with/without minor bleeding, major bleeding or thrombosis had similar circulating platelet activation and granule exocytosis on both Day 2 and Day 5 (Figure 6.8). Similarly, patients with/without minor bleeding or thrombosis had comparable circulating platelet activation and granule exocytosis within Day 2 and Day 5. Patients without major bleeding had higher lysosome release (8.82 ± 2.48 vs. 17.25 ± 3.19 %, p= 0.007; d= 2.88; power= 0.93) than patients with an event on Day 5 but not Day 2 (Figure 6.8).

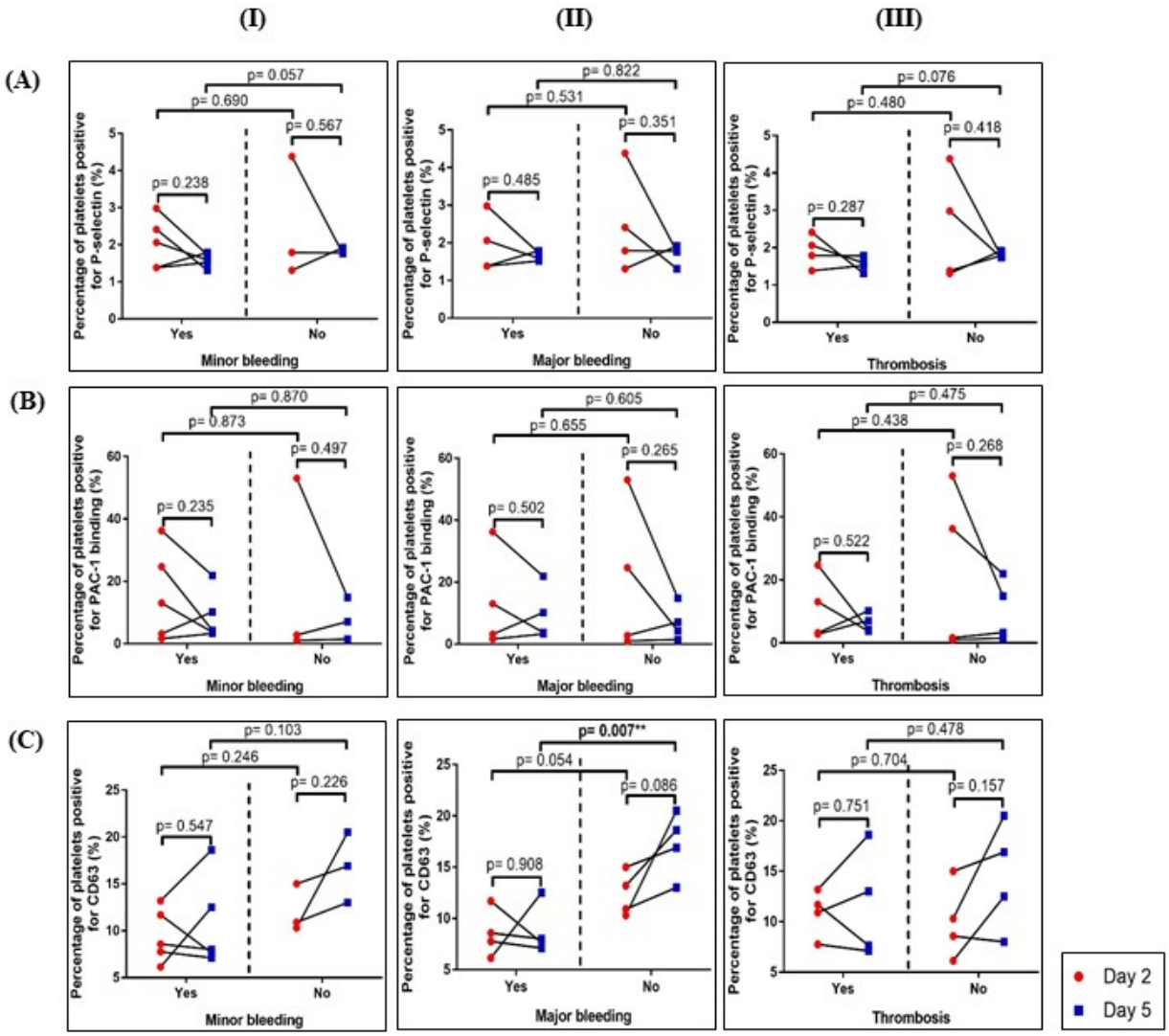

Figure 6.8 Circulating platelet activation according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

Circulating platelet activation markers for the (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 for Day 2 vs. Day 5 according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % +/- SD. [SD, standard deviation]

6.3.5 Response of platelets to stimulation with TRAP-6

6.3.5.1 Day 2 vs. Day 5 on ECMO

The platelet response to TRAP-6 is directly proportional to the area under the curve (AUC). Reduced platelet response for α-granule release (63.73 ± 30.96 vs. 40.75 ± 18.97 AUC, p= 0.026; d= 0.90; power= 0.38) (Figure 6.9) and a concomitant trend of decrease for lysosome release (57.20 ± 25.77 vs. 45.21 ± 12.07 AUC, p= 0.089; d= 0.57; power= 0.19) were observed on Day 5 compared to Day 2 (Figure 6.9). In contrast, the platelet response for activated integrin αIIbβ3 receptor on Day 2 was similar to Day 5 (Figure 6.9).

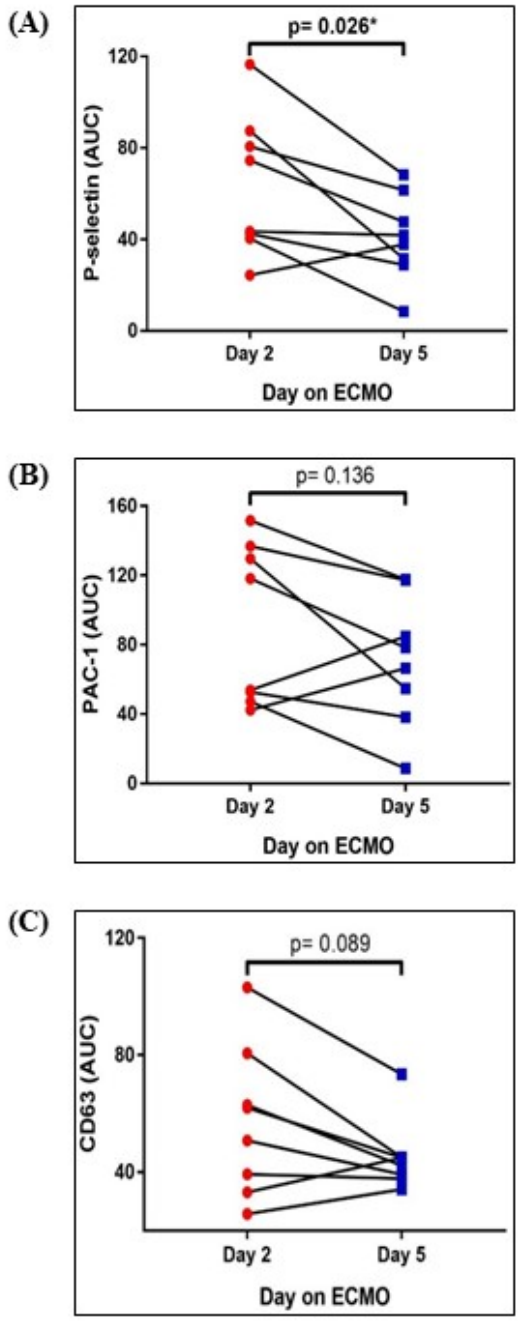

Figure 6.9 Platelet response to stimulation with TRAP-6 and duration of ECMO (Day 2 vs. Day 5).

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 for Day 2 vs. Day 5 on ECMO. Data shown as mean AUC +/- SD (n= 8). [AUC, area under the curve; SD, standard deviation]

6.3.5.2 Within 24 hours post-decannulation from ECMO

The platelet response for granule exocytosis (both α-granule and lysosome) and activated integrin αIIbβ3 receptor were comparable for patients on ECMO ≤ 5 days vs. > 5 days (Figure 6.10).

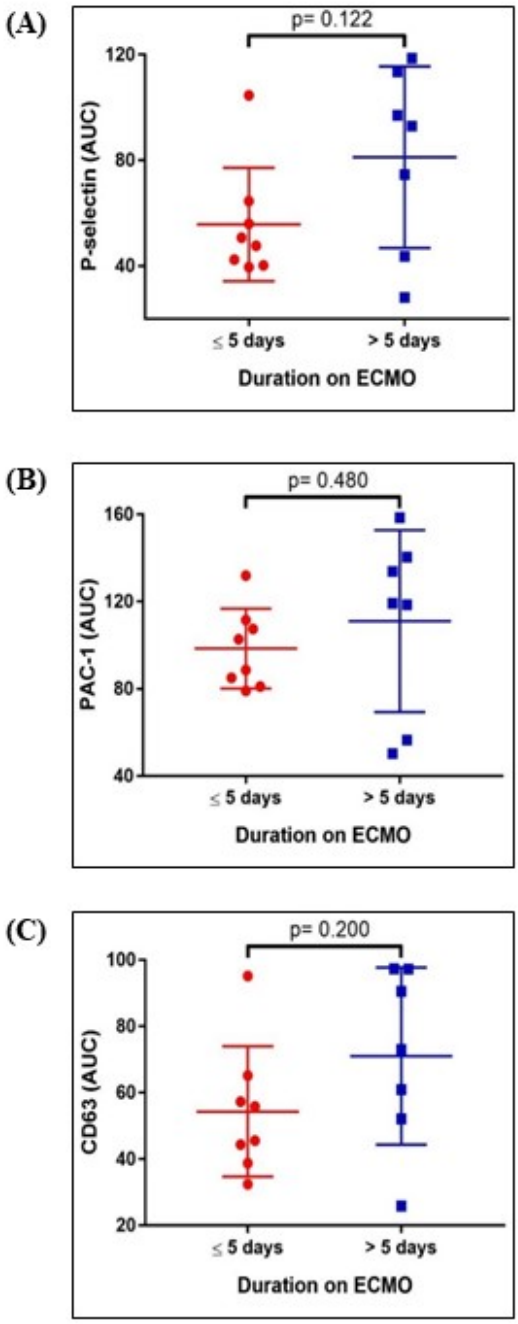

Figure 6.10 Platelet response to stimulation with TRAP-6 and duration of ECMO (≤ 5 days vs. > 5 days).

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 within 24 hours post-decannulation according to a patient's duration of ECMO. Data shown as mean AUC +/- SD (≤ 5 days n= 8, > 5 days n= 7). [AUC, area under the curve; SD, standard deviation]

6.3.5.3 The association between changes in platelet response to stimulation with TRAP-6 with duration of ECMO and clinical events

Changes in the platelet response for granule exocytosis (both α-granule and lysosome) but not activated integrin αIIbβ3 receptor was observed for patients with minor bleeding or major bleeding (Figure 6.11). Significant reduction of lysosome release (59.14 ± 15.38 vs. 41.84 ± 3.28 AUC, p= 0.037; d= 1.56; power= 0.48) and a trend of decrease in α-granule release (65.72 ± 21.28 vs. 42.30 ± 13.19 AUC, p= 0.062; d= 1.32; power= 0.43) on Day 5 compared to Day 2 were observed for patients with minor bleeding (Figure 6.11). Similar observations were obtained for patients with major bleeding (53.76 ± 11.08 vs. 41.05 ± 3.19 AUC, p= 0.056; d= 1.56; power= 0.37 for the lysosome release indicator and 60.28 ± 20.17 vs. 44.99 ± 13.56 AUC, p= 0.062; d= 0.89; power= 0.18 for the α-granule release indicator). On the other hand, platelet response was comparable on Day 2 and Day 5 for patients with thrombosis or without minor bleeding/major bleeding. Contrarily, reduced platelet response for α-granule release (70.00 ± 36.11 vs. 41.78 ± 28.12 AUC, p= 0.035; d= 0.87; power= 0.18) and activated integrin αIIbβ3 receptor (97.12 ± 54.79 vs. 70.43 ± 55.64 AUC, p= 0.019; d= 0.48; power= 0.09) but not lysosome release were observed for patients without thrombosis.

Patients with/without minor bleeding, major bleeding or thrombosis had comparable platelet response within Day 2 and Day 5 (Figure 6.11).

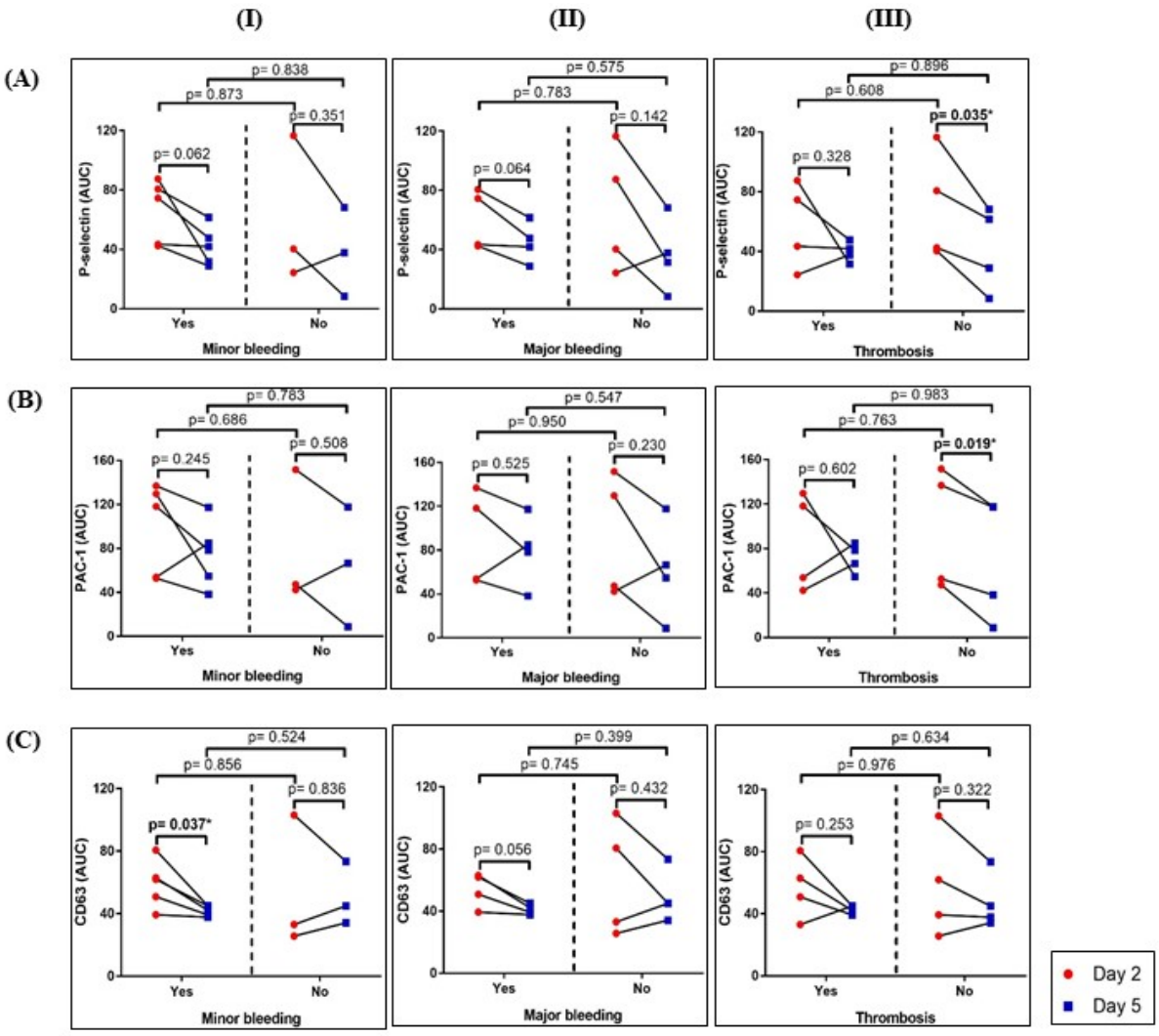

Figure 6.11 Platelet response to stimulation with TRAP-6 according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 for Day 2 vs. Day 5 according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean AUC +/- SD. [AUC, area under the curve; SD, standard deviation]

6.3.6 Circulating monocyte-platelet aggregates (MPA)

6.3.6.1 Day 2 vs. Day 5 on ECMO

Similar percentage of circulating MPA, the relative number of platelets bound on MPA as well as monocyte and platelet activation on MPA were observed for Day 2 vs. Day 5 (Figure 6.12). For mechanism of MPA formation via P-selectin/PSGL-1 and Mac-1 (CD11b), both P-selectin and Mac-1 was induced to a comparable maximal expression upon stimulation with TRAP-6. This implied that the capacity for platelet and monocyte activation-dependent and –independent MPA formation was equally preserved in patients on both Day 2 and Day 5 (Figure 6.12).

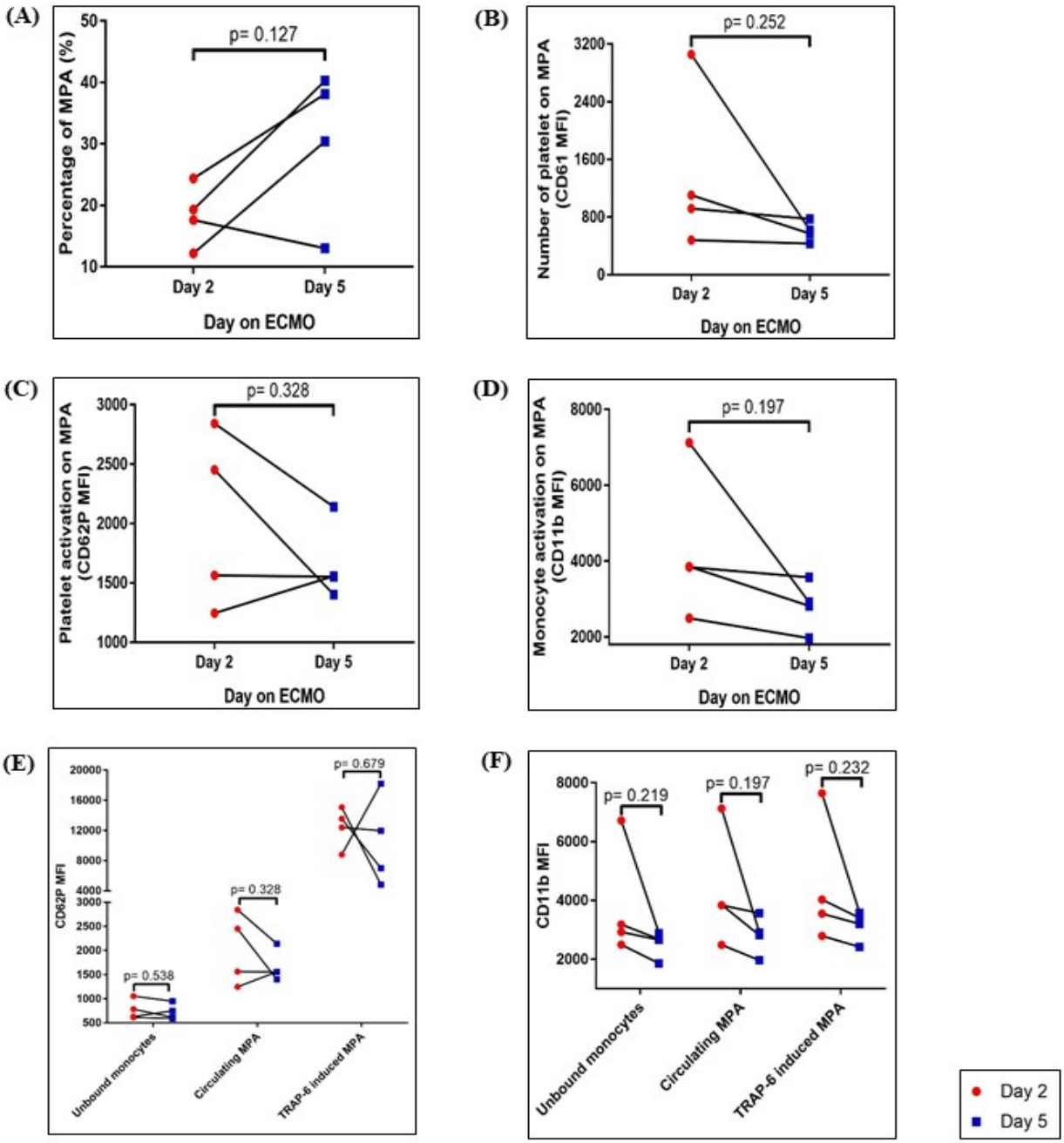

Figure 6.12 The interaction between platelets and monocytes and duration of ECMO (Day 2 vs. Day 5).

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation, (D) monocyte activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on MPA for Day 2 vs. Day 5 on ECMO. Data shown as mean % or MFI +/- SD (n= 4). [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; SD, standard deviation]

6.3.6.2 Within 24 hours post-decannulation from ECMO

Patients on ECMO ≤ 5 days vs. > 5 days had comparable circulating MPA, the relative number of platelets bound on MPA as well as monocyte and platelet activation on MPA (Figure 6.13). Also, MPA formation via both platelet and monocyte activation-dependent and –independent mechanism (P-selectin/PSGL-1 and Mac-1 (CD11b)) was equally preserved in both groups of patients (Figure 6.13).

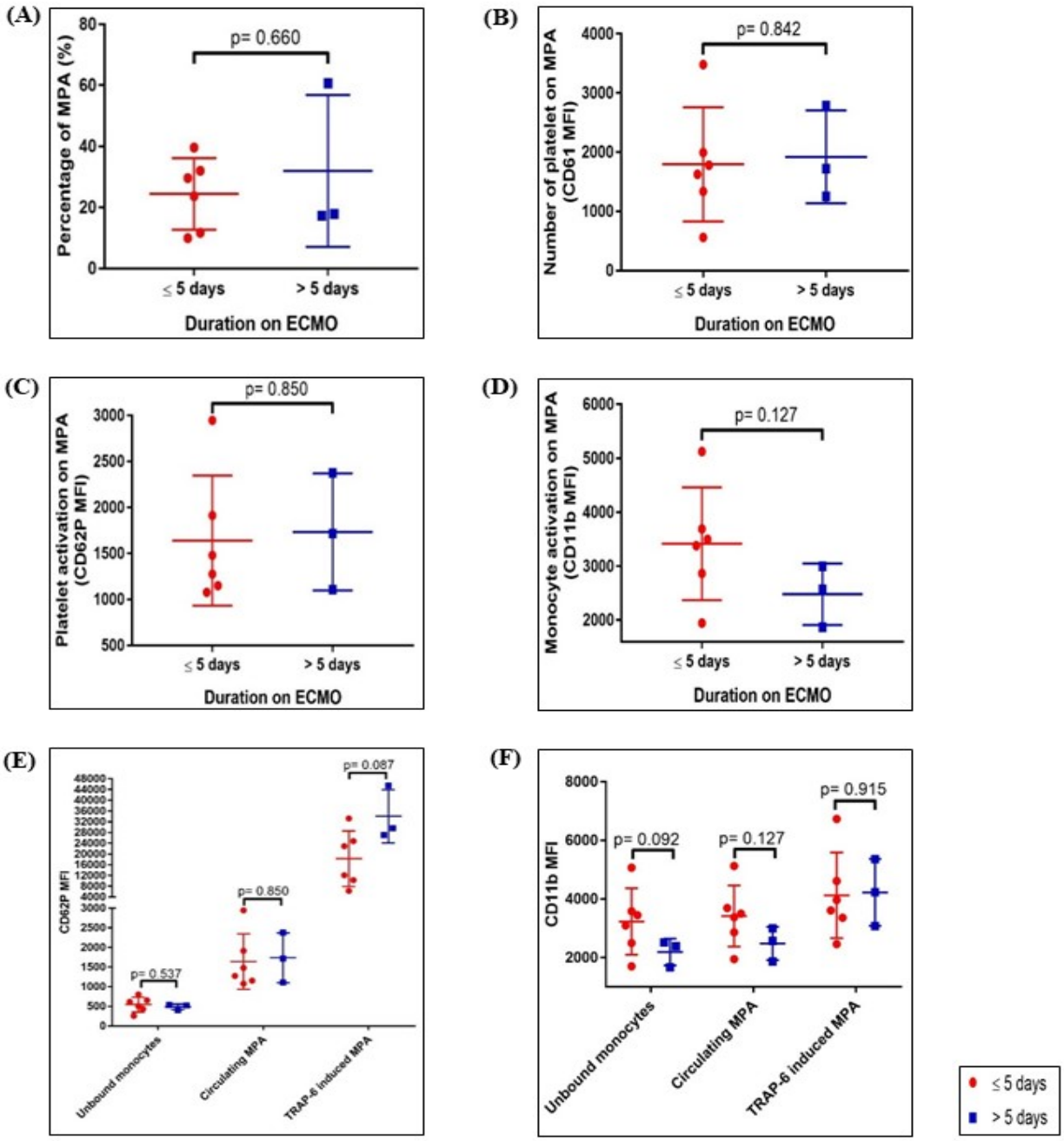

Figure 6.13 The interaction between platelets and monocytes and duration of ECMO (≤ 5 days vs. > 5 days).

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation, (D) monocyte activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on MPA within 24 hours post-decannulation according to a patient's duration of ECMO. Data shown as mean % or MFI +/- SD (≤ 5 days n= 6, > 5 days n= 3). [MFI, median fluorescence intensity; SD, standard deviation; MPA, monocyte-platelet aggregates]

6.3.6.3 The association between changes in interaction between platelets and monocytes with duration of ECMO and clinical events

The evaluation for the association between the interactions of platelets with monocytes and clinical events was limited by the number of minor bleeding and major bleeding events. For patients with thrombosis, circulating MPA level on Day 5 was higher than Day 2 (18.63 ± 6.13 vs. 36.27 ± 5.20 %, p= 0.014; d= 3.11; power= 0.80) with comparable relative number of platelets bound on MPA, monocyte and platelet activation on MPA (Figure 6.14) and also the capacity for platelet and monocyte activation-dependent and –independent MPA formation (Figure 6.15).

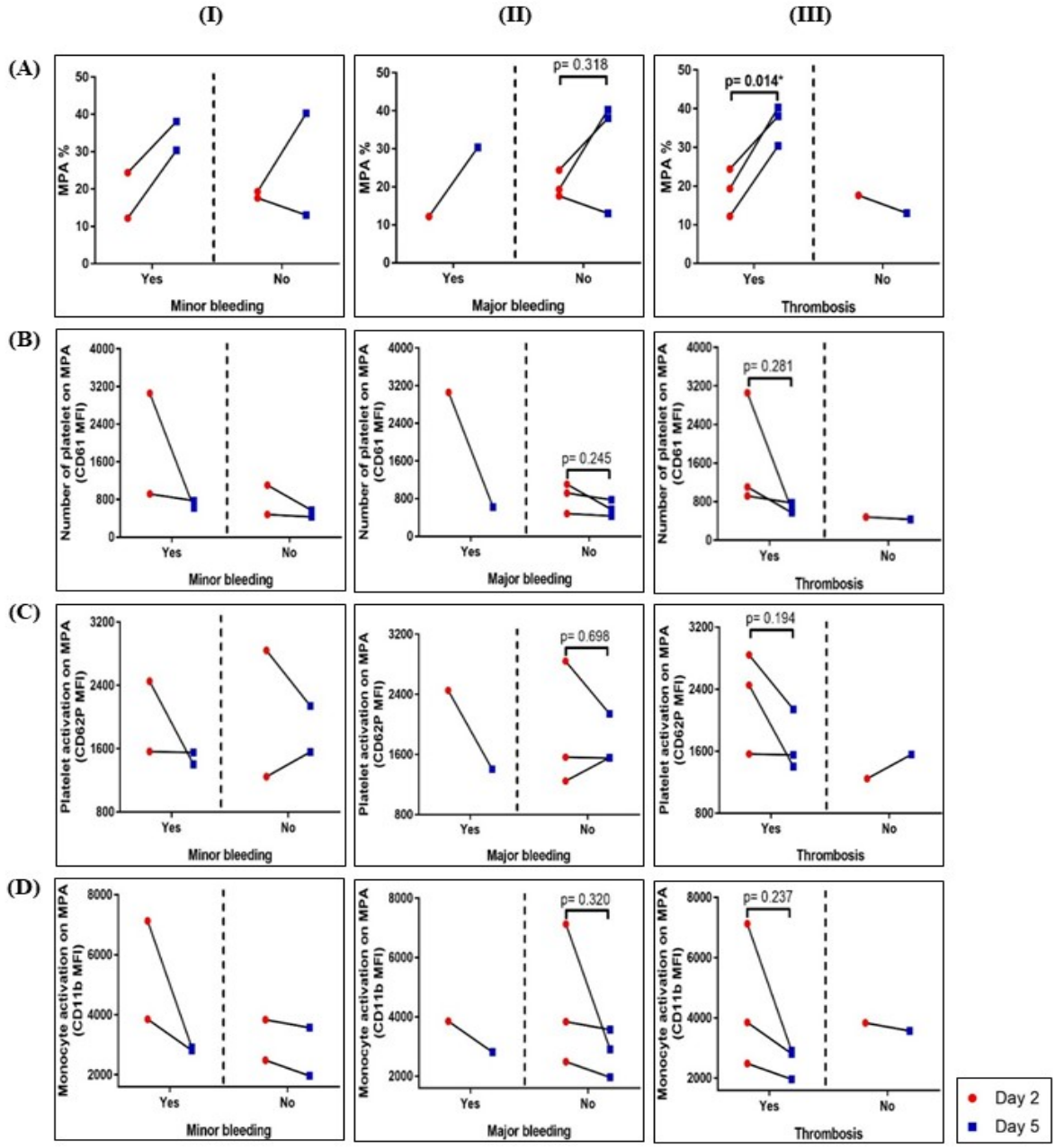

Figure 6.14 The interaction between platelets and monocytes according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

The interaction between platelets and monocytes for (A) percentage of MPA, (B) number of platelet bound on MPA, (C) platelet activation and (D) monocyte activation on MPA for Day 2 vs. Day 5 on ECMO according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD. [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; SD, standard deviation]

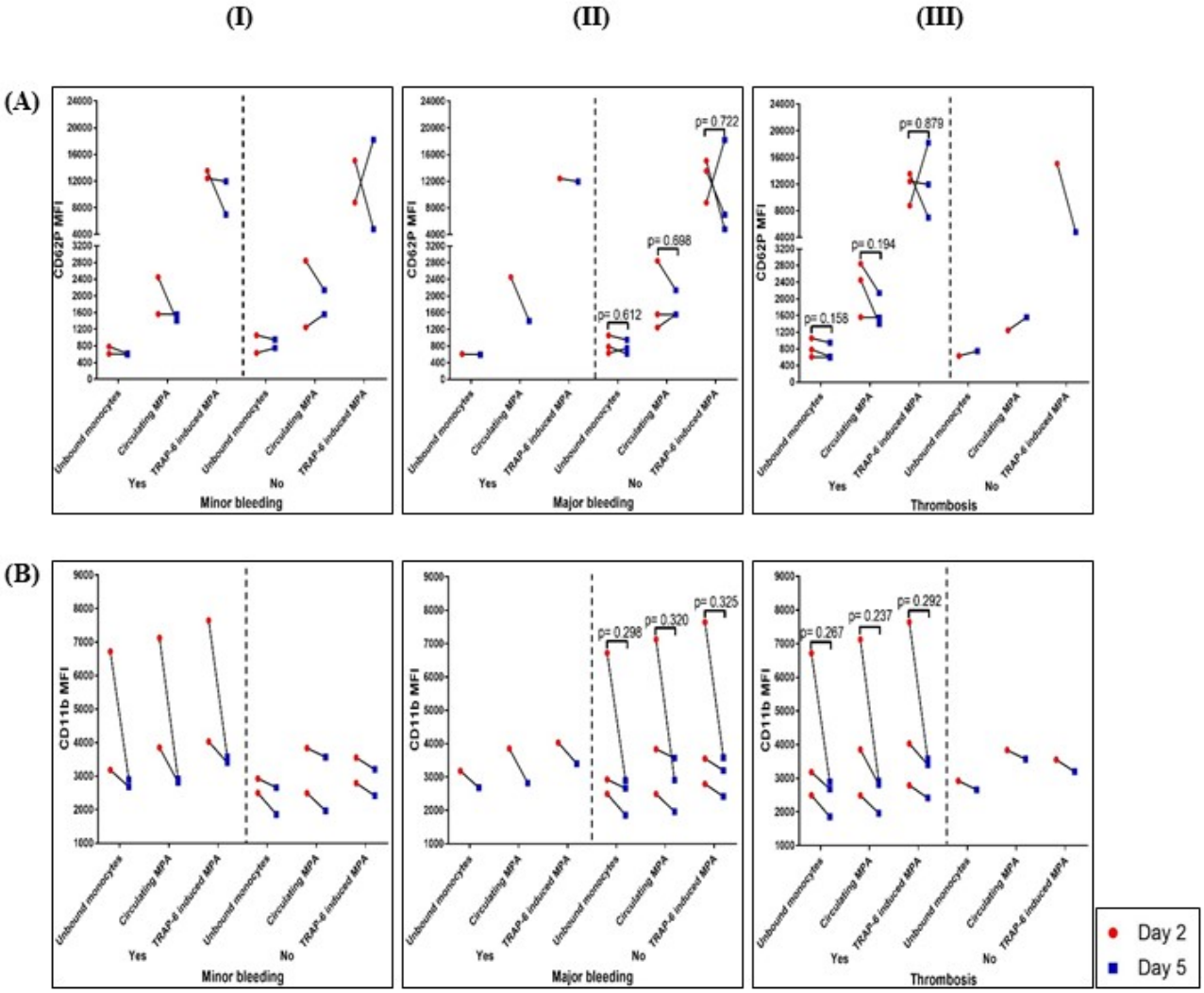

Figure 6.15 The mechanism of interaction between platelets and monocytes according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

The mechanism of interaction between platelets and monocytes via (A) P-selectin and (B) Mac-1 on MPA for Day 2 vs. Day 5 on ECMO according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD. [MFI, median fluorescence intensity; MPA, monocyte-platelet aggregates; SD, standard deviation]

6.3.7 Circulating neutrophil-platelet aggregates (NPA)

6.3.7.1 Day 2 vs. Day 5 on ECMO

A trend of decreased circulating NPA (14.89 ± 9.34 vs. 10.54 ± 7.44 %, p= 0.050; d= 0.52; power= 0.13) and the relative number of platelets bound on NPA (1234.67 ± 886.81 vs. 634.17 ± 351.27 MFI, p= 0.094; d= 0.89; power= 0.26) were observed on Day 5 compared to Day 2 while neutrophil and platelet activation on NPA were comparable on both days (Figure 6.16). Also, the capacity for NPA formation via P-selectin/PSGL-1 and Mac-1 (CD11b) adhesion mechanisms was equally retained on both Day 2 and Day 5 (Figure 6.16).

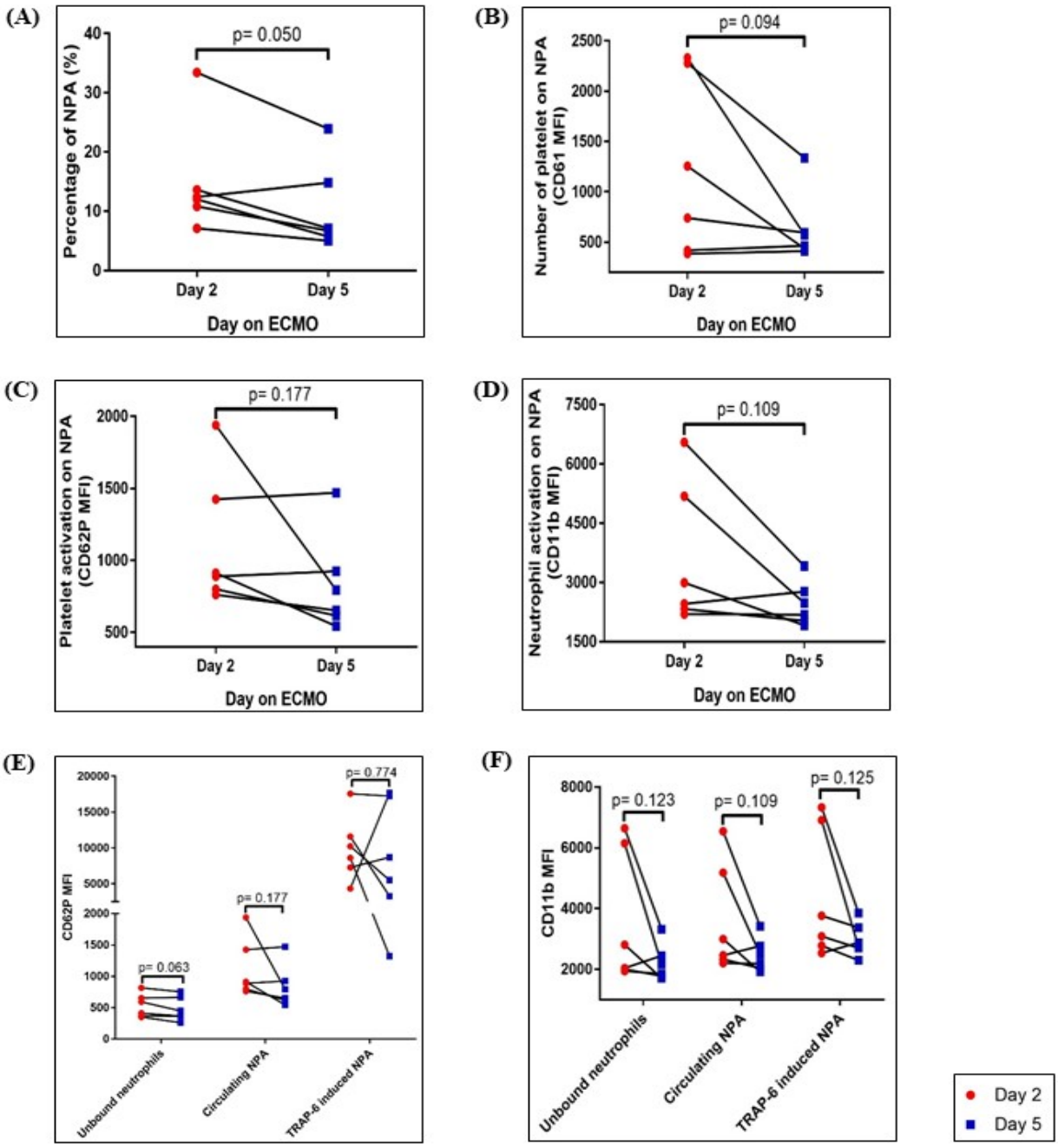

Figure 6.16 The interaction between platelets and neutrophils and duration of ECMO (Day 2 vs. Day 5).

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation, (D) neutrophil activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on NPA for Day 2 vs. Day 5 on ECMO. Data shown as mean % or MFI +/- SD (n= 6). [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

6.3.7.2 Within 24 hours post-decannulation from ECMO

Circulating NPA, the relative number of platelets bound on NPA and platelet activation on NPA were comparable for patients on ECMO ≤ 5 days vs. > 5 days (Figure 6.17). In contrast, reduced neutrophil activation was observed for patients on ECMO > 5 days compared to ≤ 5 days (2694.71 ± 789.43 vs 1766.6 ± 289.02 MFI, p= 0.021; d= 1.45; power= 0.71) (Figure 6.17). The capacity for platelet and neutrophil activation-dependent and –independent NPA formation via P-selectin/PSGL-1 and Mac-1 (CD11b) adhesion mechanisms was equally preserved in both groups of patients (Figure 6.17).

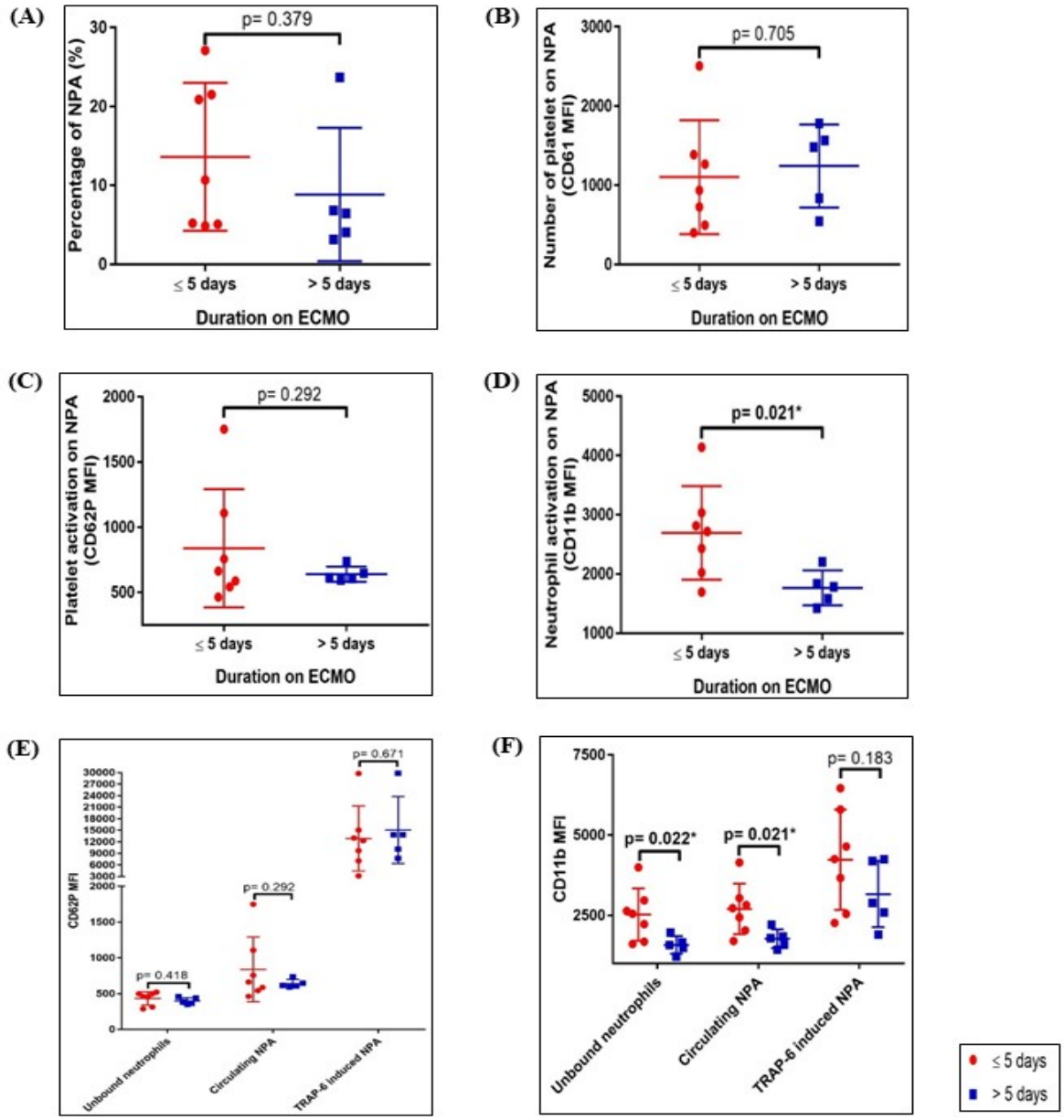

Figure 6.17 The interaction between platelets and neutrophils and duration of ECMO (≤ 5 days vs. > 5 days).

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation, (D) neutrophil activation and mechanism of interaction via (E) P-selectin and (F) Mac-1 on NPA within 24 hours post-decannulation according to a patient's duration of ECMO. Data shown as mean % or MFI +/- SD (≤ 5 days n= 7, > 5 days n= 5). [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

6.3.7.3 The association between changes in interaction between platelets and neutrophils with duration of ECMO and clinical events

Patients with minor bleeding, major bleeding or thrombosis had similar circulating NPA, the relative number of platelets bound on NPA and platelet/neutrophil activation on NPA on both Day 2 and Day 5 (Figure 6.18). Circulating NPA (19.67 ± 11.92 vs 12.24 ± 10.12 %, p= 0.019; d= 0.67; power= 0.10) in patients without major bleeding on Day 5 was lower than Day 2 (Figure 6.18). A trend of decrease in the relative number of platelets bound on NPA (1651.75 ± 784.03 vs 733.25 ± 407.31 MFI, p= 0.070; d= 1.47; power= 0.38) was observed for patients with minor bleeding on Day 5 compared to Day 2 (Figure 6.18). As for the evaluation of the mechanisms of NPA formation, patients with minor bleeding, major bleeding or thrombosis and patients without major bleeding had similar mechanisms of NPA formation via P-selectin/PSGL-1 and Mac-1 (CD11b). Similar observations were obtained for comparison between patients with and without major bleeding on Day 2 and Day 5 (Figure 6.19).

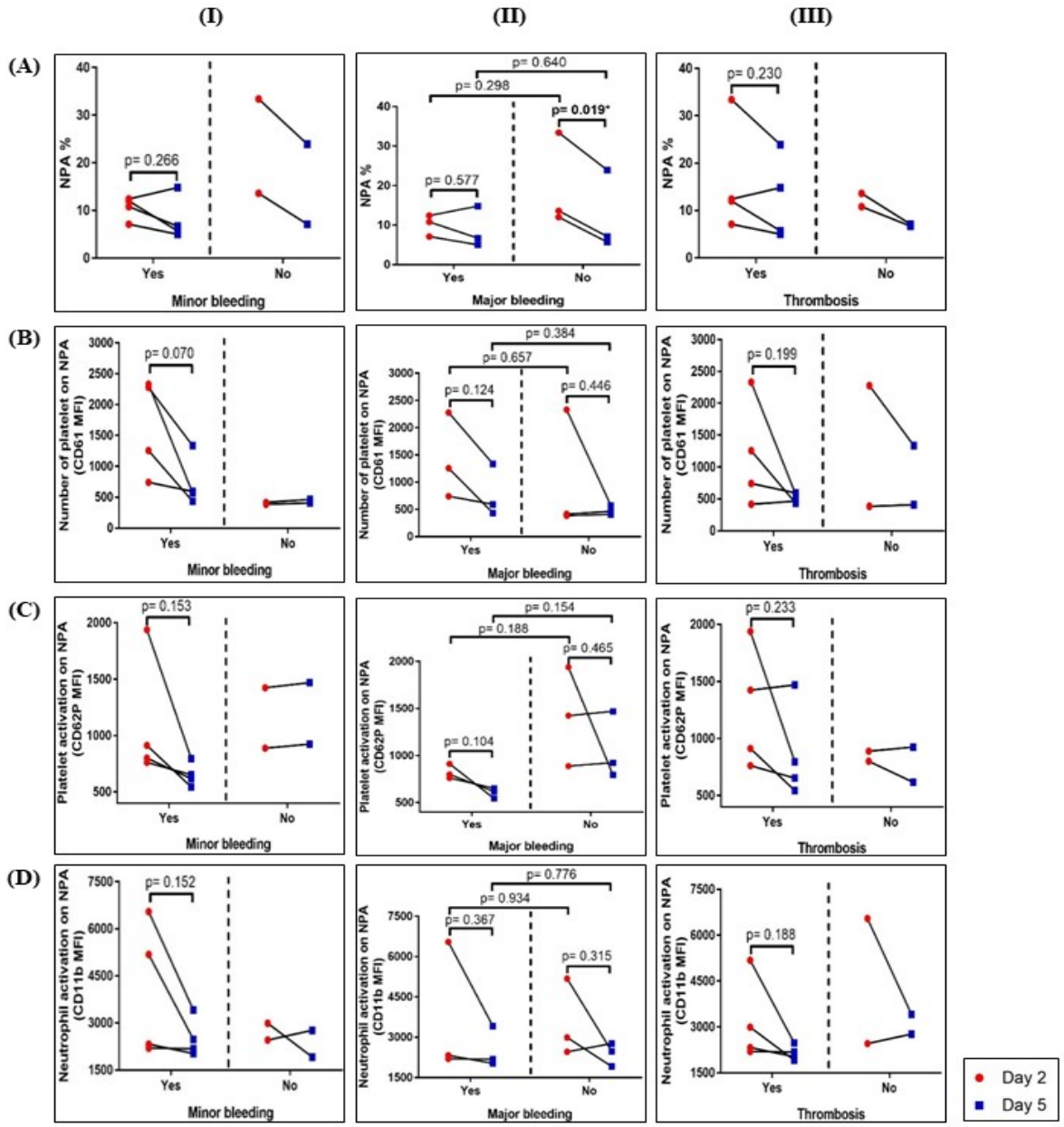

Figure 6.18 The interaction between platelets and neutrophils according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

The interaction between platelets and neutrophils for (A) percentage of NPA, (B) number of platelet bound on NPA, (C) platelet activation and (D) neutrophil activation on NPA on Day 2 vs. Day 5 on ECMO according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD. [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

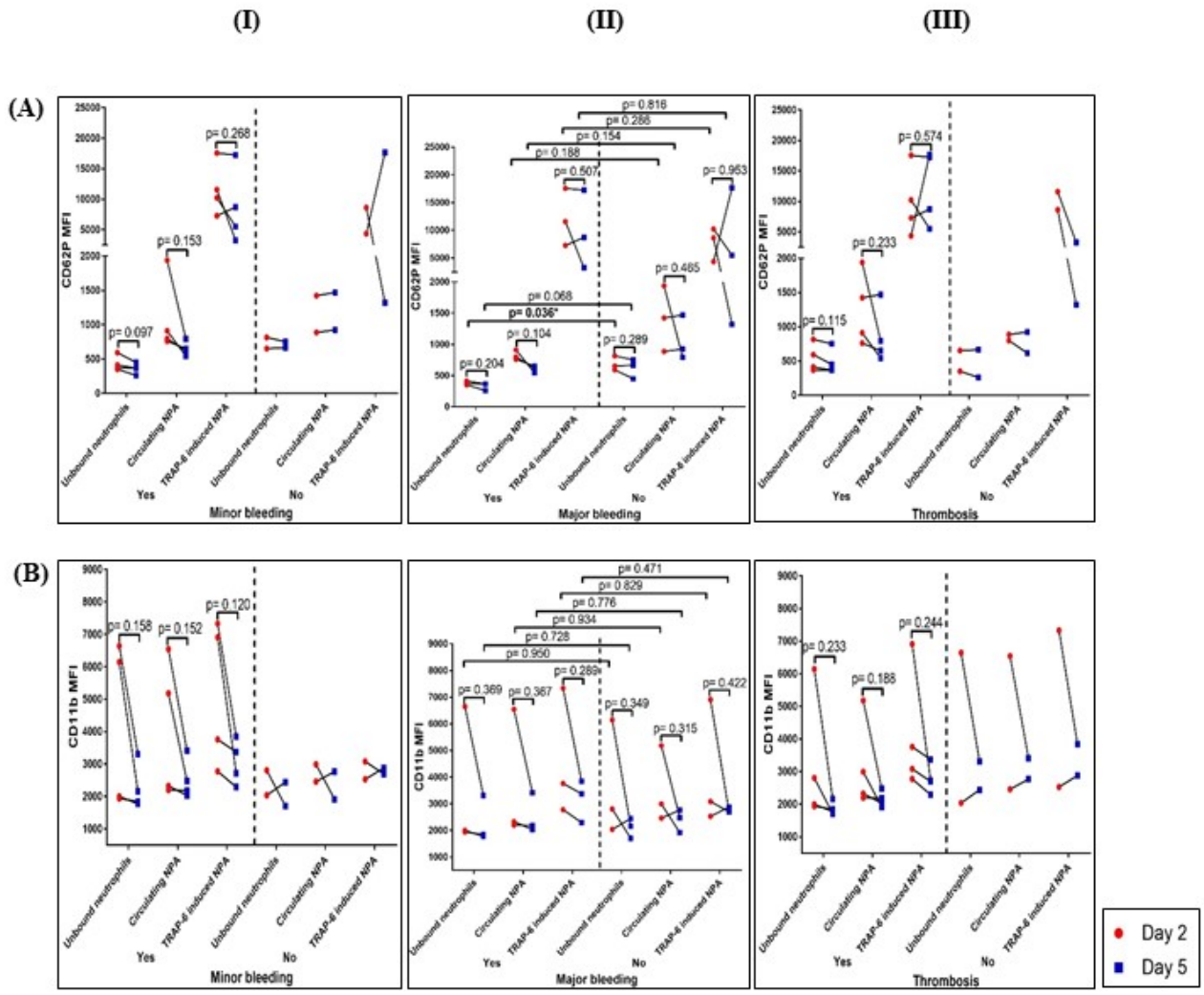

Figure 6.19 The mechanism of interaction between platelets and neutrophils according to the duration of ECMO (Day 2 vs. Day 5) and presence or absence of clinical event.

The mechanism of interaction between platelets and neutrophils via (A) P-selectin and (B) Mac-1 on NPA on Day 2 vs. Day 5 according to the clinical events (I– minor bleeding, II– major bleeding, III– thrombosis). Data shown as mean % or MFI +/- SD. [MFI, median fluorescence intensity; NPA, neutrophil-platelet aggregates; SD, standard deviation]

6.4 Discussion

This study demonstrated for the first time that the duration of ECMO may have an effect on the platelet phenotype and function in paediatric ECMO patients and could be related to the increased risk of bleeding and/or thrombosis via different pathways as ECMO progresses. The main findings for patients who were on ECMO > 5 days included: I.) lower integrin αIIbβ3 and higher GPIb/IX/V receptor expressions and II.) reduced granule exocytosis and platelet response may be associated with an increased risk of bleeding; III.) modifications of the interaction between platelets with monocytes/neutrophils may be relevant to the increased risk of bleeding or thrombosis. Also, patients with different length of stay on ECMO (≤ 5 days vs. > 5 days) recovered to comparable platelet phenotype and function upon decannulation.

Among the receptors important for platelet phenotype, this study showed that reduced integrin αIIbβ3 receptor (for platelet aggregation) expression with concomitant increase in GPIb/IX/V receptor (for platelet adhesion) expression may be associated with an increased risk of bleeding (both minor and major) as ECMO progresses. In contrast, the GPVI receptor expression was similar for patients with/without clinical event. Such observations may indicate that the modification of platelet adhesion and aggregation via the integrin αIIbβ3 and GPIb/IX/V receptors could have a more important role than the GPVI receptor for platelet phenotype that may be relevant to the development of clinical event as ECMO progresses.

Platelet dysfunction has been associated with the modification of platelet activation and/or response in the setting of ECMO [213]. This study demonstrated comparable circulating platelet activation and granule exocytosis with concomitant decreased platelet response, moreover, reduced platelet response was also seen in the patients with bleeding event as ECMO progresses. A study by Mazeffi *et al.* (2018) also showed that the adult patients on VA-ECMO had reduced platelet function on ECMO Day 5 compared to ECMO Day 2 that could not be reversed by VWF concentrate [291]. Constant exposure to the shear stress and oxidative stress

originating from the ECMO system which are both the activators of the platelet signalling pathway could promote platelet activation with concomitant depletion of the intragranular content and important mediators such as intraplatelet calcium [237, 238, 292]. The activated platelets could then I.) adhere to the ECMO circuit, existing thrombus and/or exposed subendothelial layer e.g. a patient's surgical wound and/or II.) removed preferentially by the reticuloendothelial system. Furthermore, a lower inter-individual variability was observed for circulating platelets positive for P-selectin in patients on Day 5 (compared to Day 2). Such observation may indicate the presence of a more homogenised circulating platelet population with depleted surface P-selectin and α-granule storage as ECMO progresses. This is because P-selectin is removed from the platelet surface upon release from the α-granules while the degranulated platelets can continue to circulate and function [111]. Exposure to shear from the intact ECMO system may further increase the rate of such P-selectin removal.

In addition, the reduced platelet response for granule exocytosis may also be related to the increasing number of platelet transfusions received by the patients as number of days on ECMO increases. On average, children receive 1.3 platelet transfusion per day on ECMO [201]. Studies have shown that platelet concentrates for transfusion [293-295] have minimal capacity for platelet activation due to the exhaustion of intragranule content during storage. Furthermore, Yip *et al.* (2015) showed that platelets from children have higher response to TRAP-6 than the adults [296]. Hence, the circulating platelet population in children on ECMO may represent a higher proportion of transfused adult platelets with reduced platelet response as ECMO progresses. Together, circulating exhausted platelet population with reduced capacity for platelet adhesion and aggregation originating from the effects of the ECMO system and/or platelet transfusions can thus increase the bleeding risk in patients as the duration of ECMO increases.

Upon activation, platelets can interact with monocytes or neutrophils to form hetero-aggregates. MPA/NPA have gained increased attention in recent years for their role as a stable marker of platelet activation and to inform the coagulation state in patients with cardiovascular diseases [297, 298]. Although circulating MPA and monocyte/platelet activation were comparable at both time points as ECMO progresses, an elevated MPA level was found on Day 5 (compared to Day 2) in patients with thrombosis. Even though the exact mechanisms of how modifications of the interaction between platelets and leukocytes could contribute to thrombosis remain elusive, an increased MPA level has been reported in various thromboinflammatory conditions such as cardiovascular diseases [218, 219]. Hence, such increase in MPA as observed in this study could be detrimental because existing MPA could further recruit platelets thus exacerbating the thrombus formation within the circuit and/or patients. Similar platelet activation (as indicated by P-selectin) on MPA could be associated with the comparable number of platelets bound on MPA at both time points or relevant to the P-selectin independent mechanism proposed for the MPA formation in children [137].

Different from the MPA, reduced circulating NPA and the relative number of platelet bound on NPA were observed on Day 5. Furthermore, such modification of the interaction between platelets and neutrophils could be associated with bleeding in patients as ECMO progresses and may be relevant to increased neutrophil activation for their role as the primary type of leukocyte involved in inflammation. This is important because patients on ECMO have elevated inflammatory state that could be related to their underlying disease and/or multiple surgical wounds and also exposure to the foreign surface of the ECMO circuit [233, 246-248]. Particularly, neutrophil activation increases upon the initiation of ECMO followed by the release of multiple types of cytokines and mediators from the neutrophil primary granules e.g. proteinases [299].

Gardiner *et al.* (2001) showed that binding of P-selectin to neutrophils is reduced due to increased proteolysis of PSGL-1 by proteinases after neutrophil degranulation [300]. Hence, it is expected that fewer number of platelet is able to adhere to the neutrophil for NPA formation on Day 5 compared to Day 2 due to reduction in PSGL-1 (from increased proteolysis of PSGL-1 by proteinases) as a result of increased neutrophil activation and degranulation as ECMO progresses. Furthermore, since an elevated platelet-leukocyte aggregate level is associated with hypercoagulation, a reduced circulating NPA level on Day 5 could in turn indicate an impaired coagulation state thus an increased risk of bleeding as ECMO progresses.

Platelet phenotype and function within 24 hours post-decannulation according to a patient's duration of ECMO

Upon decannulation, patients with different number of days on ECMO had comparable platelet phenotype, activation, response and interactions with platelets and leukocytes. Such observed platelet-specific changes could be expected to be with minimal effect of platelet transfusion since both groups had comparable rate of platelet transfusion within 24 hours before decannulation from ECMO. However, modification of GPIb/IX/V receptor expression and neutrophil activation on NPA were seen upon decannulation and may be relevant to a patient's duration of ECMO.

The high inter-individual variability for GPIb/IX/V receptor expression in patients with a longer duration of ECMO (> 5 days) may indicate changes in response to the modification of VWF in the plasma. High shear such as that in an ECMO system could induce unfolding of VWF and increase its adhesion to the platelet GPIb/IX/V receptor [74, 236]. Such increase in the GPIb/IX/V receptor-ligand interaction could in turn up-regulate platelet surface GPIb/IX/V receptor expression via increased release from the intraplatelet pool [301, 302]. Furthermore, this study also showed that the expression of GPIb/IX/V receptor increases as the number of days on ECMO increases (Day 5 > Day 2). Hence, patients with a longer duration of ECMO

with the corresponding longer exposure to shear thus may be subjected to a higher extent of VWF modification with a larger variation than patients with a shorter length of stay on ECMO.

In addition, patients with a longer duration of ECMO had reduced neutrophil activation on NPA that may be relevant to increased neutrophil exhaustion in this population. Mac-1 is a molecule which is ubiquitously expressed on the surface of leukocytes and is up-regulated upon leukocyte activation via increased release from the secretory granules [143]. Since neutrophil is the main type of leukocyte involved in inflammation, patients with a longer duration of ECMO may be with a higher inflammatory state thus a corresponding higher extent of neutrophil activation followed by neutrophil exhaustion. Although the platelet-leukocyte interaction is known to increase Mac-1 expression, the exhausted neutrophils in patients with a longer duration of ECMO may have limited capacity for further Mac-1 release upon interaction with platelets thus the reduced neutrophil activation on NPA as observed in this study.

In summary, platelet-specific changes in paediatric patients could be related to their duration of ECMO via modification of I.) integrin αIIbβ3 and GPIb/IX/V receptors; II.) platelet response for granule exocytosis (both α-granule and lysosome) and III.) MPA formation that these could be associated with an increased bleeding/thrombosis risk as the duration of ECMO increases.

6.5 Conclusion

This study showed that the duration of ECMO may have an effect on the platelet phenotype and function in paediatric ECMO patients. Modification of the platelet phenotype via integrin αIIbβ3 and GPIb/IX/V receptors and platelet response for granule exocytosis may be relevant to an increased bleeding risk during ECMO. In contrast, an elevated circulating MPA level could be associated with an increased risk for thrombosis as the duration of ECMO increases.

7 Platelet phenotype and response to stimulation with TRAP-6 at different sites in an ECMO system

7.1 Introduction

ECMO is a complex system composed of multiple compartments. Together with the artificial surface originating from the ECMO system, changes in fluid dynamics across an ECMO system foreign to that of a patient's circulation have been associated with ECMO-induced coagulopathy due to their effects on multiple blood components [28]. Blood is under negative pressure as it leaves the patient towards the ECMO pump. After passing through the centrifugal pump, the blood is under positive pressure as it passes towards the oxygenator, a compartment with very different surface and fluid dynamics to the rest of the ECMO system. The oxygenated blood then passes back into the patient via either the arterial or venous return catheters.

There have been no studies in children that have addressed the question of whether different parts of the ECMO circuit are more or less responsible for the coagulation changes and in particular for the changes in platelets. While the loss of cell integrity and the overall function has been described for the erythrocytes and platelets [73, 303, 304] that could eventually lead to the pathological conditions, the exact site of damage within the ECMO circuit remains unknown. Changes in platelets associated with fluid dynamics could lead to platelet dysfunction including the alteration in platelet morphology, reduction of platelet receptors and increased activation state [73]. Such platelet-specific changes associated with fluid dynamics are especially important for the expression of many of the key receptors important for platelet function and their interactions with ligands that are shear- and activation-dependent.

Of particular importance in the circuit configuration used at RCH in Melbourne is the use of epoprostenol which is infused into the top of the oxygenator at a rate of 5ng/kg/min. Also, nitric oxide (NO) is included in the sweep gas of the oxygenator at 20 ppm in all patients. Both

of these agents have known effects on the platelet function. NO is constantly released by the endothelial to inhibit platelet activity through different mechanisms which include the inhibition of I.) platelet integrin αIIbβ3 receptor, II.) release of mediators important for platelet activation and III.) the interactions between platelet receptor and their activators [189, 192]. On the other hand, epoprostenol is a synthetic prostacyclin widely used as a medication for the treatment of pulmonary arterial hypertension. Prostacyclin has been used clinically to preserve platelet count and function in CPB operations [407, 408]. To date, however, very limited information is available for the biological effect of epoprostenol on platelet function in the setting of ECLS and none is available for ECMO. The only study that is available involves an experimental model of extracorporeal perfusion which reported an inhibition of platelet activation by epoprostenol [199].

ECMO provides cardiac and/or respiratory support to patients due to multiple medical reasons from different age groups. Particularly, patients who had a cardiac surgery before coming onto ECMO had higher risk of bleeding than the other ECMO population [305]. CPB is a heart-lung machine commonly used during cardiac surgery to provide short-term cardiac and respiratory support of which its association with platelet dysfunction seen in patients during and/or after CPB are well-documented in the existing literature [306]. Developmental haematology from the aspect of platelet function has gained increased attention in recent years. Both reduced platelet adhesion and aggregation had been reported in the infants compared to the older children and adults [40, 273]. Thus, it is important to take into account of the role of developmental haematology for management of children on ECMO.

Bleeding and/or clotting are the most common complications in the ECLS population [151, 229, 307] that have been associated with the deranged haemostatic system. To ensure haemostasis for patients to remain on ECMO, platelet transfusion is an important part of the management of this population. On average, children on ECMO receive 1.3 platelet

transfusions per day to maintain platelet level of > $100x10^9$/L [201]. Since children and adults have different physiologic and haemostatic system that are known to be age-dependent and stored platelets are known to have changes in their structure and function [308], it is important to acknowledge that presence of transfused platelets in the circulation of children on ECMO may contribute to the changes in platelet phenotype and function seen in this population.

The hypothesis of this chapter is that platelet phenotype, activation and response to stimulation with TRAP-6 are different at different sites in an ECMO system and differ according to a patient's age and pathway onto ECMO. Also, the site-specific changes for platelet phenotype and function can be associated with the development of bleeding/thrombosis and platelet transfusions during ECMO.

This chapter aims to determine the site-related differences of platelet-specific changes according to a patient's pathway onto ECMO and age; and their associations with the clinical events and platelet transfusion for:

1) The platelet phenotype.
2) The circulating platelet activation.
3) The response of platelets to stimulation with TRAP-6.

7.2 Methods

The methods utilized in this chapter were outlined in Chapter 2. Whole blood was collected from the patient's arterial line, pre- and post-oxygenator sites (Figure 7.1) as a once-off event between Day 2 – Day 4 (24 – 96 hours) during ECMO. Th clinical events (minor bleeding, major bleeding and thrombosis) were recorded according to the definitions in section 2.2.3.

Details for flow cytometry Panel 1 and 2 used in this chapter were included in sections 2.6.3 and 2.6.4. Following is the summary for the assessment of platelet-specific markers:

I.) Platelet phenotype was evaluated by measuring the expression of GPIb/IX/V, integrin αIIbβ3 and GPVI receptors.

II.) Circulating platelet activation was measured as the percentage of platelets positive for activated fibrinogen (integrin αIIbβ3) receptor (measured by PAC-1 binding) while platelet granule exocytosis was measured as the percentage of platelets positive for α-granule (P-selectin) and lysosome release (CD63) indicators. The response of platelet to TRAP-6 is directly proportional to the area under the curve (AUC).

Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software Incorporation, USA). Full blood count parameters, platelet phenotype and function at different sites (arterial line vs. pre- vs. post-oxygenator sites) were compared using one way ANOVA Post-Hoc Tukey's test.

Site: Patient's arterial line

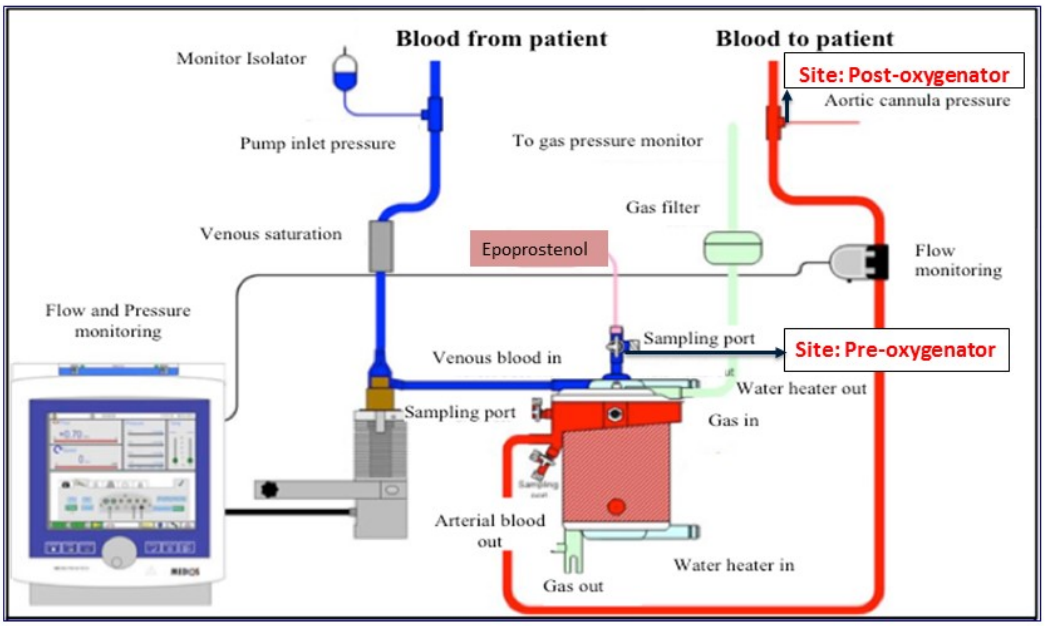

Figure 7.1 Sample collection sites for pre- and post-oxygenator and from the patient's arterial line in an ECMO system.

7.3 Results

7.3.1 Demographic information

Demographic information for the fourteen patients included in this chapter was summarized in Table 7.1. All of the patients received VA-ECMO with epoprostenol (at 5 ng/kg/min by infusion through the ECMO circuit) and nitric oxide (at 20 ppm in the sweep gas) and most of them are children. Majority of the patients had CPB before coming onto ECMO.

Table 7.1 Summary of the demographic information.

Variables	Total (n= 14)		
Age	**Neonates (0 – 30 days)**		4 (28)
	Infants (> 30 days – 1 year)		4 (28)
	Children (> 1 year – 18 years)		6 (43)
Gender, n (%)	**Male**		6 (43)
	Female		8 (57)
Pathway onto ECMO, n (%)	**Post-CPB**		8 (57)
	Non-CPB		6 (43)
Mode of cannulation, n (%)	**Central**		8 (57)
	Peripheral		6 (43)
Number of patients with ≥ 1 clinical event on the day of sample collection, n (%)	**Minor bleeding**	**Yes**	0 (0.0)
		No	14 (100)
	Major bleeding	**Yes**	2 (14)
		No	12 (86)
	Thrombosis	**Yes**	1 (7)
		No	13 (93)
Number of platelet transfusion received 24 hours before sample collection, n (%)	**0**		8 (57)
	1		5 (36)
	2		1 (7)

[ECMO, extracorporeal membrane oxygenation; IQR, interquartile range; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass]

For full blood count, all parameters except the haematocrit level are comparable at different sites of the ECMO system (Table 7.2). Comparisons were also done for patients according to their pathways onto ECMO and age. There was no difference for full blood count parameters at different sites of the ECMO system for patients who had CPB before coming onto ECMO. Neonates and children also had similar full blood count parameters at different sites in the ECMO system.

Table 7.2 Summary of full blood count comparing (A) different sites in an ECMO system and according to the patient's (B) pathway onto ECMO and (C) age.

(A)

Full blood count	Total (n= 8)			p-value
	Art line	Pre-oxy	Post-oxy	
WCC (x10^9/l)	5.64 ± 2.64	5.62 ± 2.74	5.51 ± 2.57	0.456
RCC (x10^{12}/l)	2.81 ± 0.37	2.76 ± 0.41	2.76 ± 0.39	0.058
HGB (l/l)	84.63 ± 13.14	83.63 ± 13.17	84.00 ± 12.78	0.195
HCT (g/l)	0.25 ± 0.03	0.24 ± 0.04	0.24 ± 0.04	**0.024***
PLT (x10^9/l)	97.38 ± 45.10	101.50 ± 49.46	101.50 ± 43.41	0.486
MPV (fl)	8.04 ± 1.49	7.94 ± 1.09	7.88 ± 1.48	0.760

(B)

Full blood count	Pathway onto ECMO (n= 8), n (%)							
	Post-CPB, 6 (75)				Non-CPB, 2 (25)			
	Art line	Pre-oxy	Post-oxy	p-value	Art line	Pre-oxy	Post-oxy	p-value
WCC (x 10^9/L)	5.02 ± 2.02	4.96 ± 2.08	5.00 ± 2.23	0.700	N/A (n <3)			N/A
RCC (x 10^{12}/L)	2.83 ± 0.43	2.79 ± 0.47	2.80 ± 0.45	0.283				
HGB (L/L)	86.17 ± 15.05	85.00 ± 15.13	85.67 ± 14.62	0.224				
HCT (g/L)	0.25 ± 0.04	0.25 ± 0.04	0.25 ± 0.04	0.204				
PLT (x10^9/L)	91.67 ± 43.98	99.67 ± 53.49	96.00 ± 39.14	0.287				
MPV (fl)	8.23 ± 1.64	8.05 ± 1.13	8.10 ± 1.60	0.798				

(C)

Full blood count	Age (n= 8), n (%)										
	Neonates 3 (37)			p-value	Infants 2 (25)			p-value	Children 3 (37)			p-value
	Art line	Pre-oxy	Post-oxy		Art line	Pre-oxy	Post-oxy		Art line	Pre-oxy	Post-oxy	
WCC ($x10^9$/l)	3.80 ± 0.82	3.65 ± 0.87	3.63 ± 1.07	0.342	N/A (n <3)			N/A (n <3)	6.23 ± 3.80	6.33 ± 3.88	5.97 ± 3.33	0.387
RCC ($x10^{12}$/l)	2.66 ± 0.10	2.59 ± 0.06	2.62 ± 0.08	0.210					2.62 ± 0.27	0.54 ± 0.25	2.53 ± 0.20	0.174
HGB (l/l)	84.00 ± 2.65	82.00 ± 2.00	83.00 ± 3.61	0.328					74.33 ± 10.26	74.00 ± 10.15	74.00 ± 8.89	0.794
HCT (g/l)	0.24 ± 0.00	0.24 ± 0.00	0.24 ± 0.01	0.499					0.22 ± 0.02	0.21 ± 0.02	0.21 ± 0.01	0.066
PLT ($x10^9$/l)	106.7 ± 64.38	117.3± 78.53	110.0 ± 56.67	0.521					101.7 ± 48.85	99.67 ± 39.12	108.0 ± 51.97	0.445
MPV (fl)	8.53 ± 2.15	8.30 ± 1.04	8.47 ± 2.04	0.895					8.13 ± 1.40	8.20 ± 1.38	7.90 ± 1.45	0.139

[Art line, arterial line; ECMO, extracorporeal membrane oxygenation; HCT, haematocrit; HGB, haemoglobin; IQR, interquartile range; Non-CPB, non-cardiopulmonary bypass, N/A, not available; Post-CPB, post-cardiopulmonary bypass; Post-oxy, post-oxygenator; Pre-oxy, pre-oxygenator; MPV, mean platelet volume; RCC, red blood cell count; WCC, white blood cell count]

7.3.2 Platelet phenotype at different sites in an ECMO system

In comparisons of the platelet phenotype at different sites in an ECMO system, the expression of the integrin αIIbβ3 (25012 ± 4699 vs. 26841 ± 5564 MFI, p= 0.032; d= 0.36; power= 0.15) and GPIb/IX/V (5899 ± 1373 vs. 7260 ± 1409 MFI respectively, p= < 0.001; d= 0.98; power= 0.65) receptors at the post-oxygenator site were higher than at the pre-oxygenator site (Figure 7.2). Similarly, the expression of the GPIb/IX/V receptor (5899 ± 1373 vs. 6994 ± 1584 MFI, p= 0.004; d= 0.74; power= 0.47) at the arterial line was found to be higher than the pre-oxygenator site. While not statistically significant, a similar trend was observed for the integrin αIIbβ3 receptor (25012 ± 4699 vs. 26141 ± 4869 MFI, p= 0.089; d= 0.24; power= 0.09). When comparisons were made between the post-oxygenator site and the arterial line, no difference was found for both the integrin αIIbβ3 and GPIb/IX/V receptors. There was no difference in the expression of the GPVI receptor at the pre-oxygenator site, post-oxygenator site and the arterial line in an ECMO system (Figure 7.2).

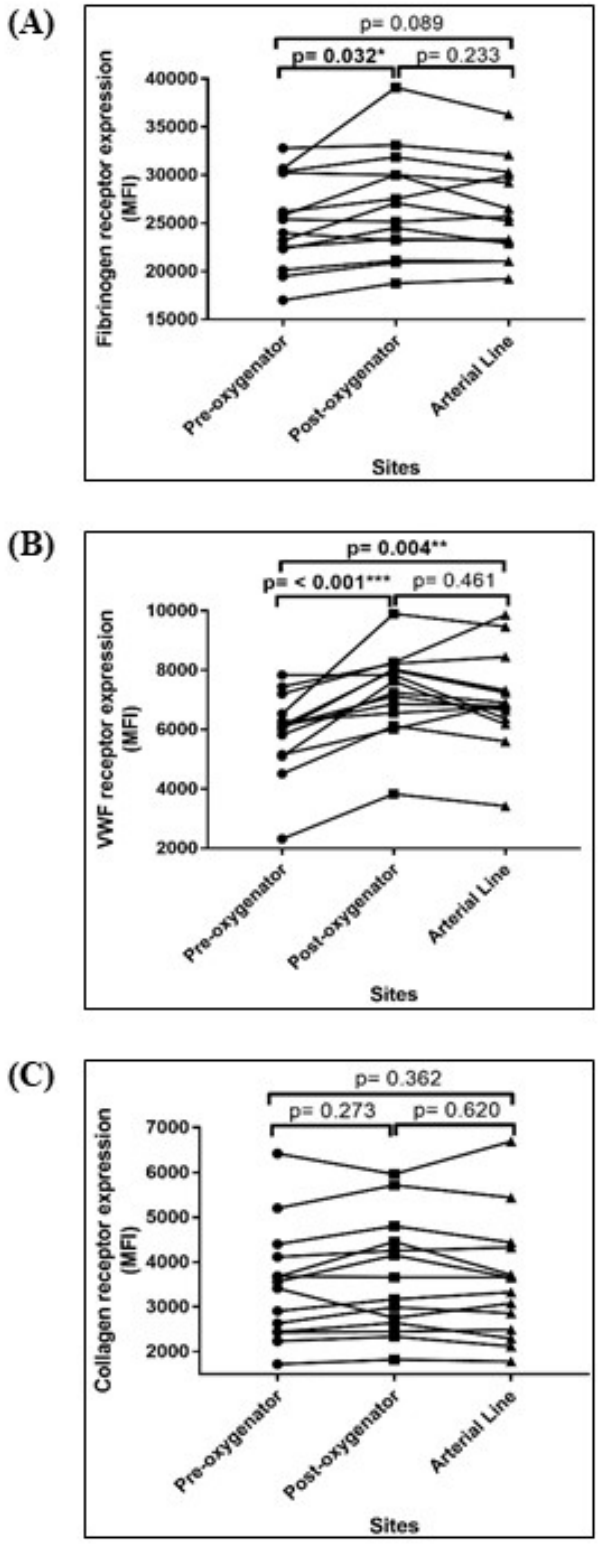

Figure 7.2 Platelet phenotype according to the sites in an ECMO system.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor at different sites in an ECMO system. [MFI, median fluorescence intensity; VWF, von Willebrand factor]

7.3.2.1 Platelet phenotype at different sites in an ECMO system according to a patient's pathway onto ECMO

When comparisons were made for different sites in an ECMO system within the Post-CPB group, only the expression of the GPIb/IX/V receptor at the post-oxygenator site was found to be higher than the pre-oxygenator site (5787 ± 1684 vs. 7437 ± 1799 MFI, p= 0.007; d= 0.95; power= 0.42) (Figure 7.3). A similar increasing trend was observed for the GPIb/IX/V receptor at the arterial line (5787 ± 1684 vs. 7092 ± 2010 MFI, p= 0.064; d= 0.70; power= 0.26) and integrin αIIbβ3 receptor at the post-oxygenator site (24939 ± 5271 vs. 27474 ± 6423 MFI, p= 0.068; d= 0.43; power= 0.13) compared to the pre-oxygenator site. The integrin αIIbβ3 receptor expression was comparable for the pre-oxygenator site vs. arterial line. On the other hand, the expression of the integrin αIIbβ3 and GPIb/IX/V receptors were comparable at sites post-oxygenator and arterial line (Figure 7.3). The expression of the GPVI receptor was comparable across all sites in the ECMO system (Figure 7.3).

When comparisons were made within the Non-CPB group, except for the GPIb/IX/V receptor, no difference was found for the expression of both the integrin αIIbβ3 and GPVI (Figure 7.3) receptors at different sites in an ECMO system. The expression of the GPIb/IX/V receptor at both the arterial line (6050 ± 941.6 vs. 6863 ± 912.2 MFI, p= 0.002; d= 0.88; power= 0.28) and the post-oxygenator site (6050 ± 941.6 vs. 7025 ± 716.4 MFI, p= 0.010; d= 1.17; power= 0.44) were found to be higher than the pre-oxygenator site (Figure 7.3). On the other hand, the expression of the GPIb/IX/V receptor was comparable at both the post-oxygenator site and the arterial line.

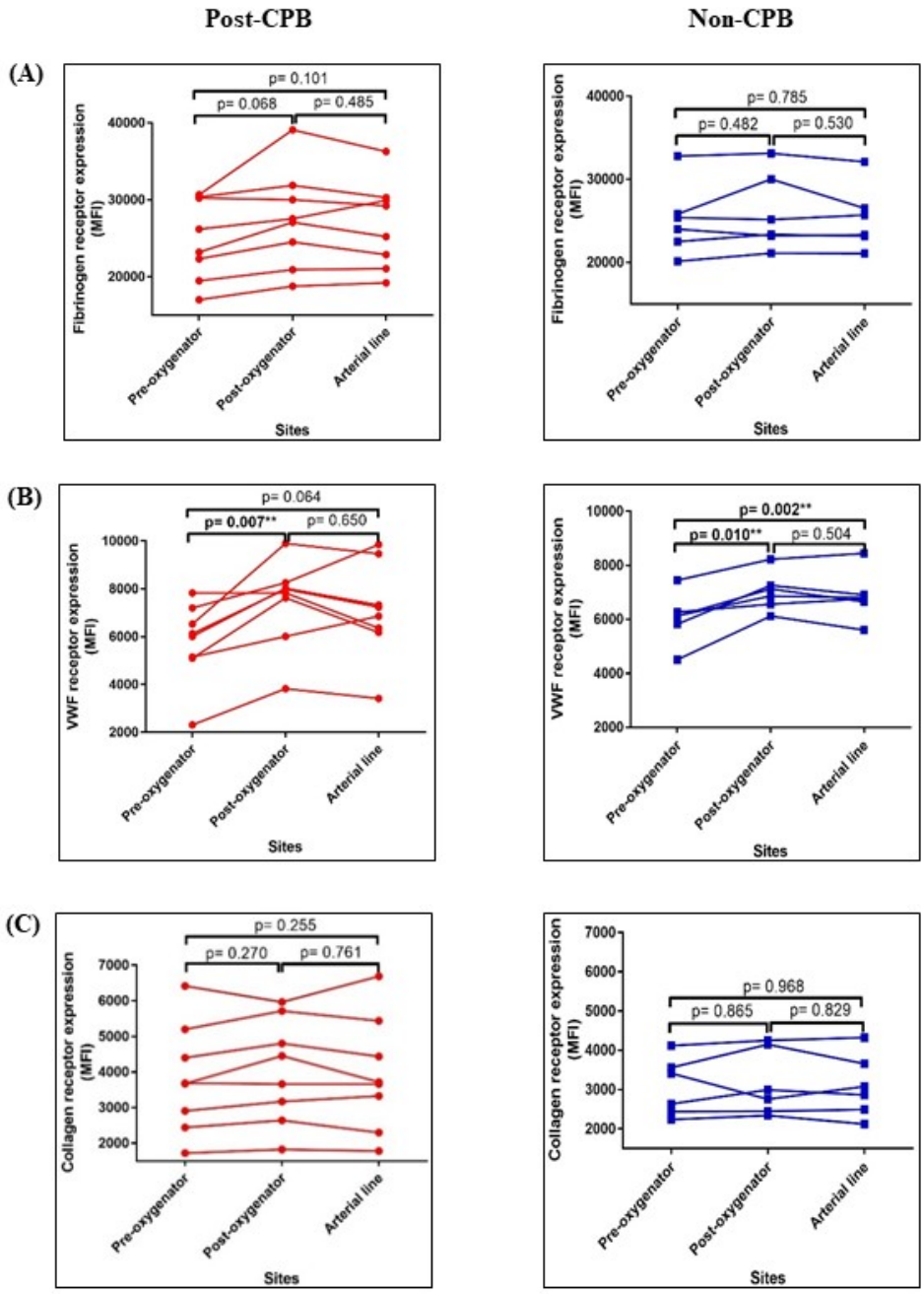

Figure 7.3 Platelet phenotype according to the sites in an ECMO system and pathway onto ECMO.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor at different sites in an ECMO system according to a patient's pathway onto ECMO. [MFI, median fluorescence intensity; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass; VWF, von Willebrand factor]

7.3.2.2 Platelet phenotype at different sites in an ECMO system according to a patient's age

The differences in platelet phenotype across different sites in an ECMO system was observed in neonates and children but not infants (Figure 7.4). In neonates, the expression of the GPIb/IX/V receptor from the arterial line was higher than at the pre-oxygenator site (5665 ± 620.3 vs. 6763 ± 435.2 MFI, p= 0.043; d= 2.05; power= 0.66) (Figure 7.4). In children, the expression of both the GPIb/IX/V (6273 ± 1063 vs. 7816 ± 1297 MFI, p= 0.024; d= 1.30; power= 0.53) and GPVI (3691 ± 1115 vs. 4041 ± 1219 MFI, p= 0.017; d= 0.30; power= 0.08) receptors at the post-oxygenator site were higher than the pre-oxygenator site respectively. The expression of the GPIb/IX/V receptor at the arterial line was also higher than at the pre-oxygenator site (6273 ± 1063 vs. 7823 ± 1697 MFI, p= 0.028; d= 1.09; power= 0.39). While not statistically significant, an increasing trend for post-oxygenator vs. arterial line GPVI receptor expression was observed (4041 ± 1219 vs. 3806 ± 1191 MFI, p= 0.070; d= 0.20; power= 0.06).

There was no difference for the expression of the integrin αIIbβ3 receptor at all sites for neonates, infants and children and for the GPVI receptor in neonates and infants (Figure 7.4).

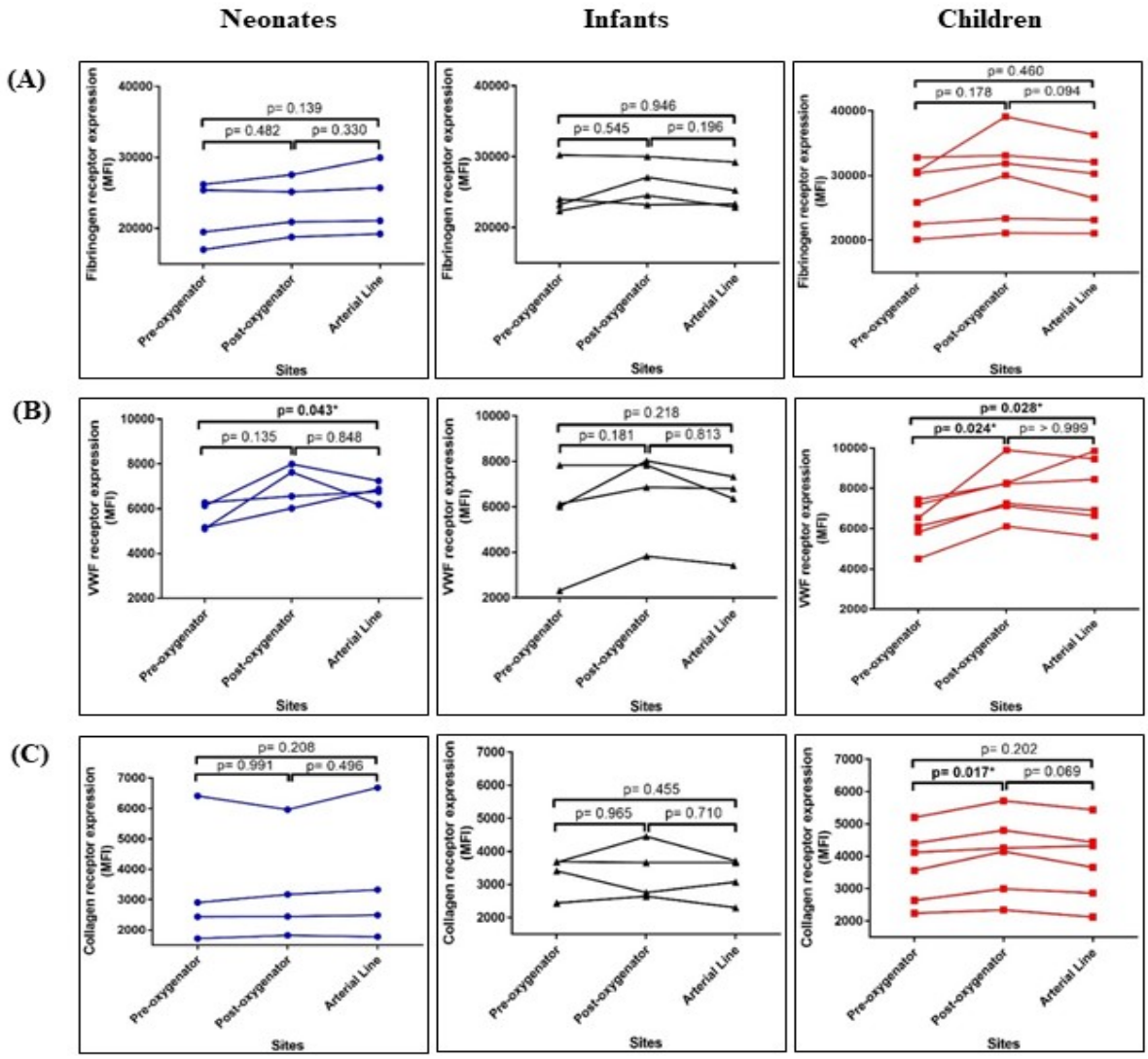

Figure 7.4 Platelet phenotype according to the sites in an ECMO system and age.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor at different sites in an ECMO system according to a patient's age. [MFI, median fluorescence intensity; VWF, von Willebrand factor]

7.3.2.3 Platelet phenotype at different sites in an ECMO system and the associations with bleeding or thrombosis

Figure 7.5 depict the comparisons of the expression for the integrin αIIbβ3, GPIb/IX/V and GPVI receptors at different sites in an ECMO system for individuals who had a bleeding or thrombosis event on the day of sample collection. Current analysis is observational because the number of patients is not adequate for statistical analysis of bleeding and thrombosis subgroups. In general, patients who had a bleeding event had lower GPIb/IX/V receptor expression across different sites in an ECMO system than majority of the patients who had no bleeding event. In contrast, no specific trend was observed for the integrin αIIbβ3 and GPVI receptors expression in the patients who had a bleeding event compared to those who had no bleeding event. More information will be required for the evaluation of changes in the expression of receptor important for platelet phenotype at different sites in an ECMO system for individual with thrombosis.

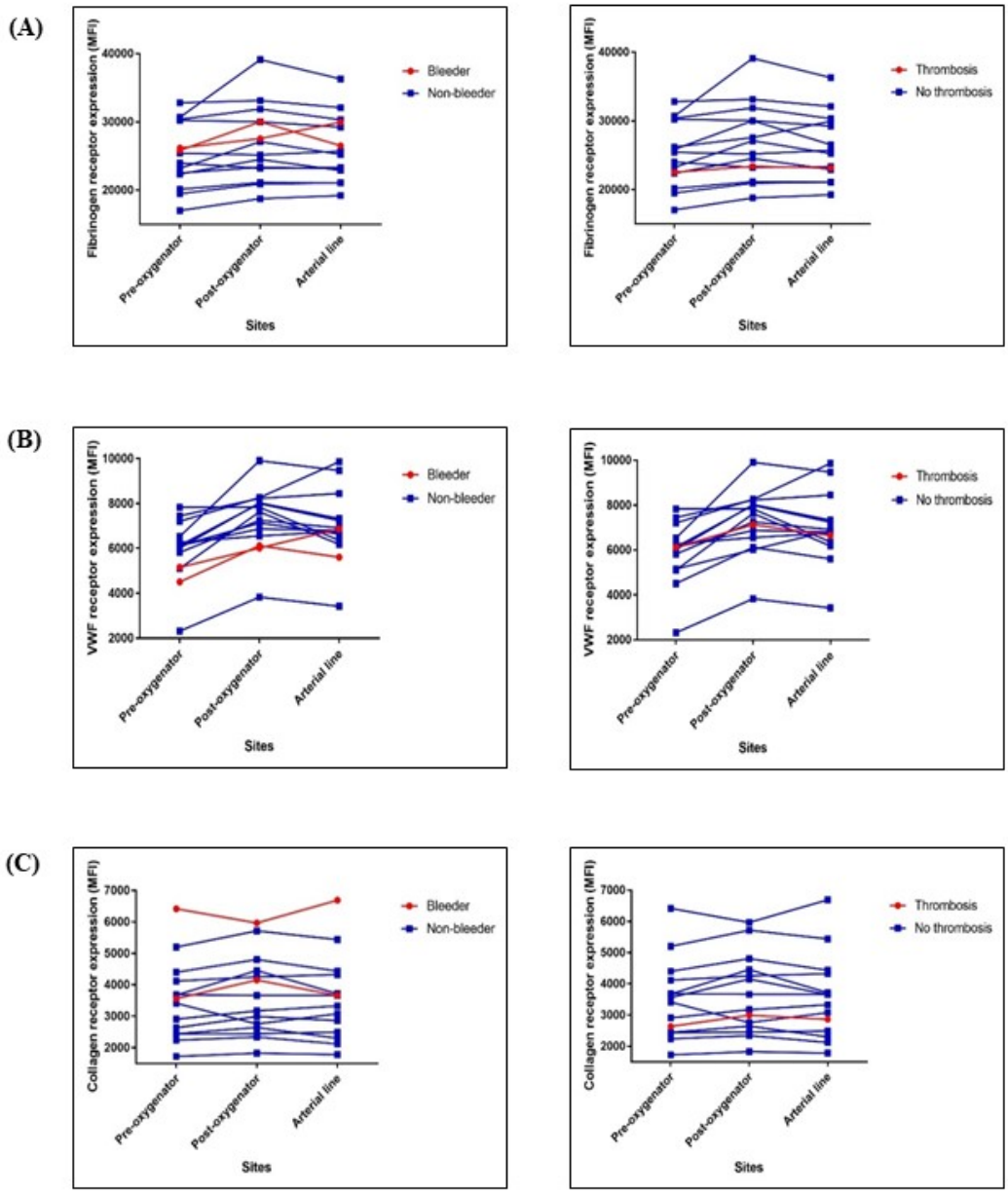

Figure 7.5 Platelet phenotype according to the sites in an ECMO system and the presence or absence of clinical event.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor at different sites in an ECMO system according to clinical events. [MFI, median fluorescence intensity; VWF, von Willebrand factor]

7.3.2.4 Platelet phenotype at different sites in an ECMO system according to the number of platelet transfusions

Figure 7.6 depict the comparisons of the expression for the integrin αIIbβ3, GPIb/IX/V and GPVI receptors at different sites in an ECMO system according to the total number of platelet transfusions received by the patient within 24 hours before sample collection. There was no difference in the platelet phenotype at different sites in an ECMO system according to the number of platelet transfusions received by the patient.

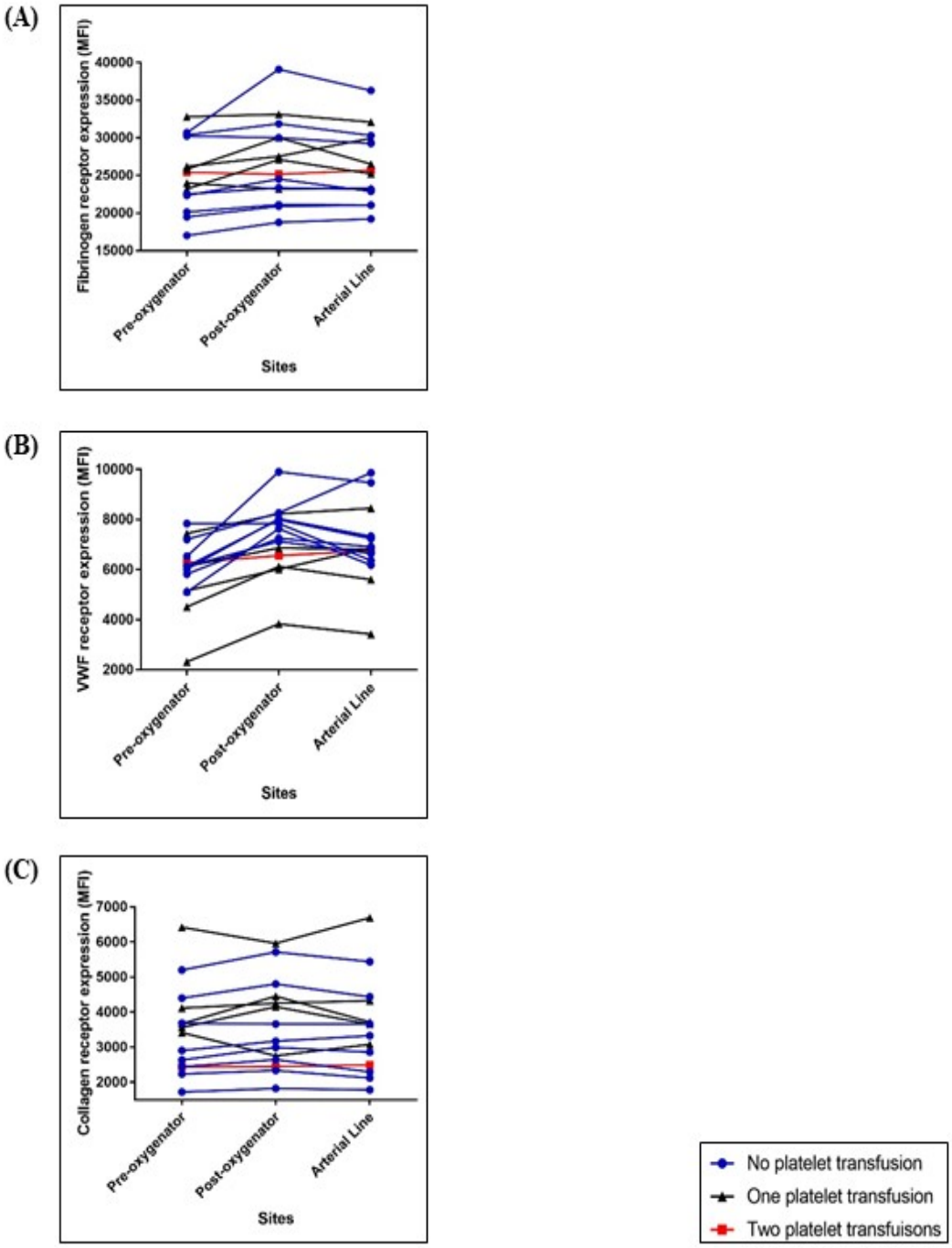

Figure 7.6 Platelet phenotype according to the sites in an ECMO system and platelet transfusions.

The expression of platelet (A) fibrinogen (integrin αIIbβ3) receptor, (B) VWF (GPIb/IX/V) receptor and (C) collagen (GPVI) receptor at different sites in an ECMO system according to the number of platelet transfusions received by the patient within 24 hours before sample collection. [MFI, median fluorescence intensity; VWF, von Willebrand factor]

7.3.3 Circulating platelet activation at different sites in an ECMO system

Circulating platelet activation was significantly increased at the post-oxygenator site compared to at the pre-oxygenator site (8.20 ± 11.62 vs. 13.78 ± 9.67 %, p= 0.040; d= 0.52; power= 0.26). However, there was no corresponding difference in granule exocytosis (both α-granule and lysosome) (Figure 7.7). There was no difference in circulating platelet activation and granule exocytosis in the arterial line compared to the pre- and post-oxygenator sites.

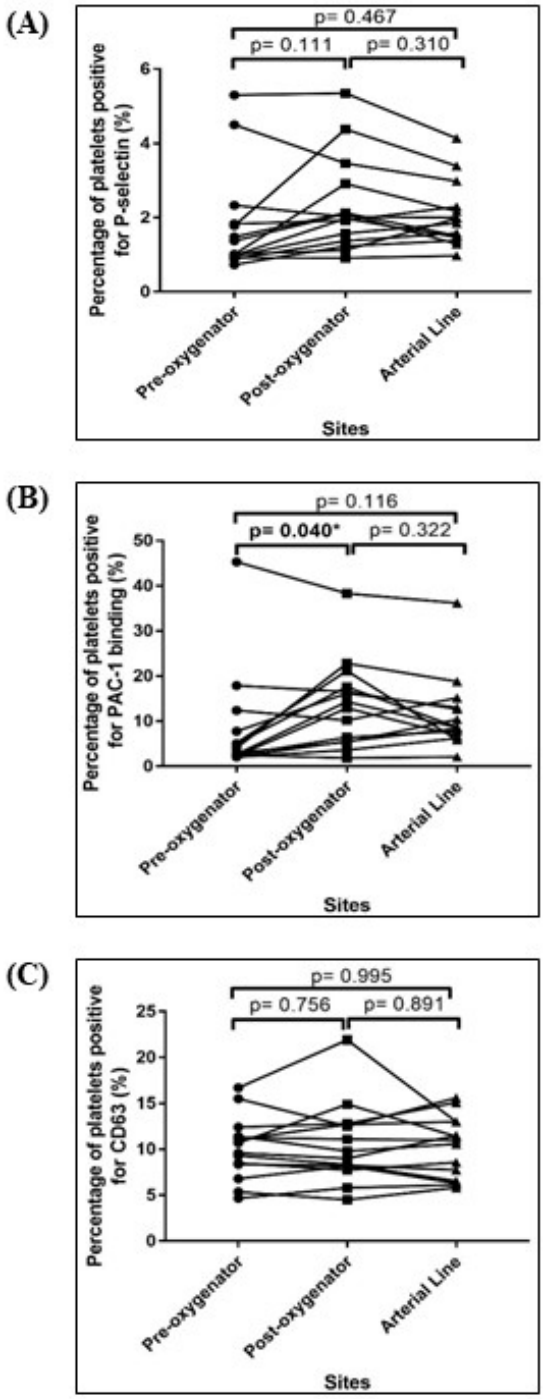

Figure 7.7 Circulating platelet activation at different sites in an ECMO system.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system.

7.3.3.1 Circulating platelet activation at different sites in an ECMO system according to a patient's pathway onto ECMO

The circulating platelet activation was comparable at different sites in an ECMO system for patients from different pathways onto ECMO (Figure 7.8).

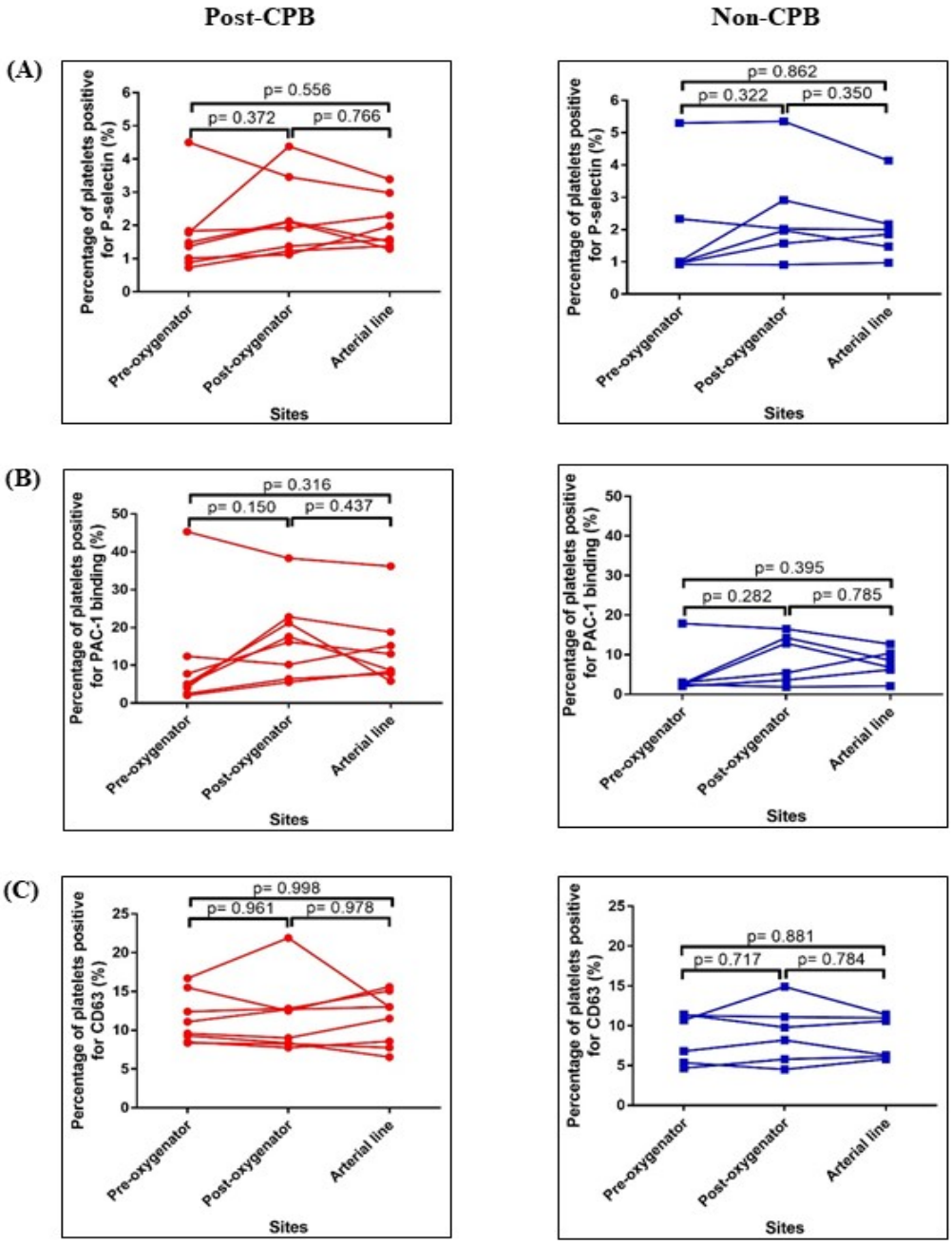

Figure 7.8 Circulating platelet activation at different sites in an ECMO system and pathway onto ECMO.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to a patient's pathway onto ECMO. [Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass]

7.3.3.2 Circulating platelet activation at different sites in an ECMO system according to a patient's age

Overall, there was no significant difference in the circulating platelet activation across different sites in an ECMO system for all age groups (Figure 7.9).

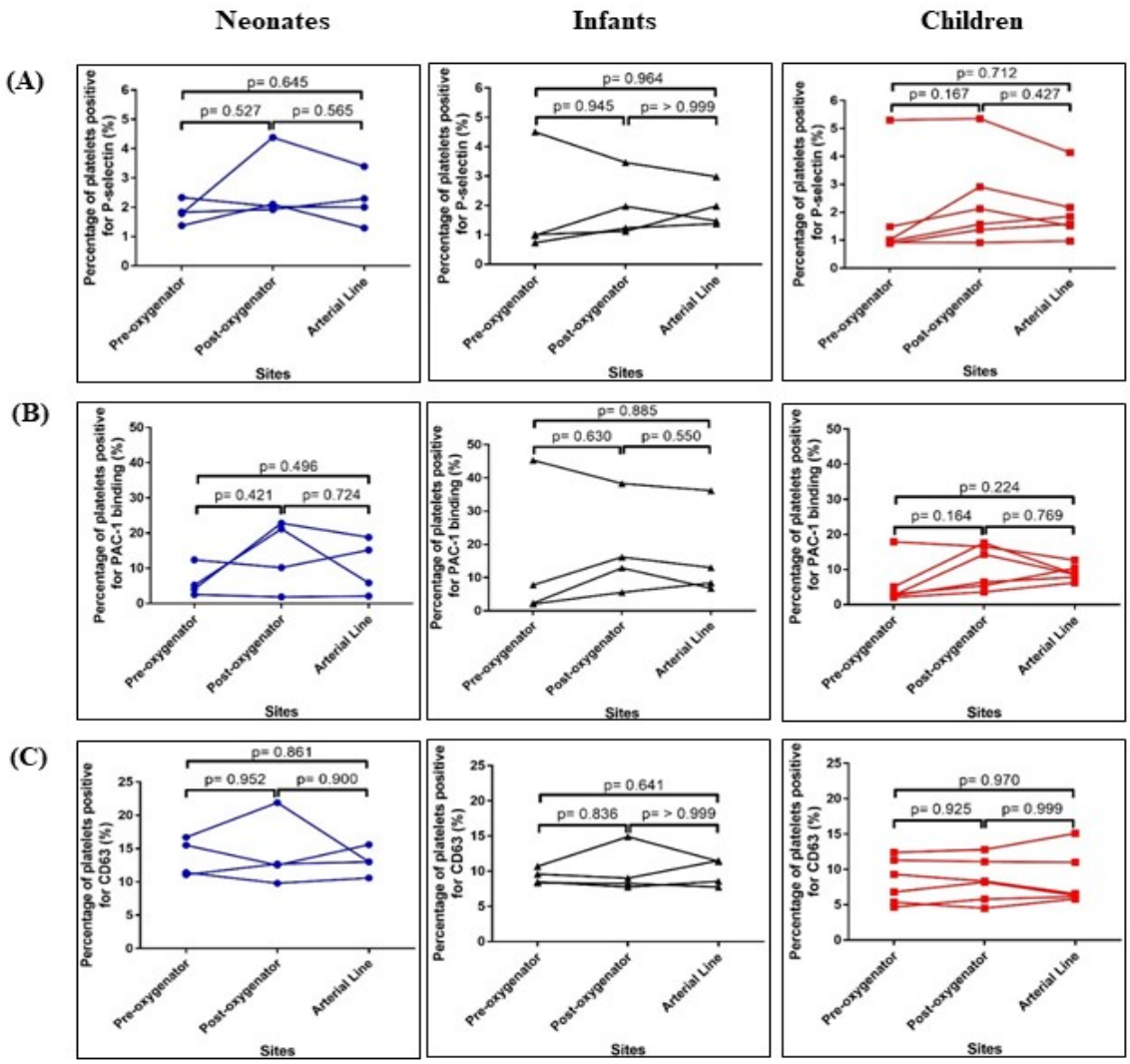

Figure 7.9 Circulating platelet activation at different sites in an ECMO system and age. Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to a patient's age.

7.3.3.3 Circulating platelet activation at different sites in an ECMO system and the associations with bleeding or thrombosis

Figure 7.10 depict the comparisons of the expression for the marker of platelet activation and the indicators of granule exocytosis for individuals who had a bleeding or thrombosis event on the day of sample collection. Patients with or without bleeding had a similar trend of changes in the expression of the activated integrin αIIbβ3 receptor and platelet lysosome release indicator across different sites in an ECMO system. However, two patients who had bleeding event had higher degree of changes in the expression for the platelet α-granule release indicator for the comparison pre- vs. post-oxygenator sites than those without bleeding event. More information will be required for the evaluation of the circulating platelet activation at different sites in an ECMO system for individual with thrombosis.

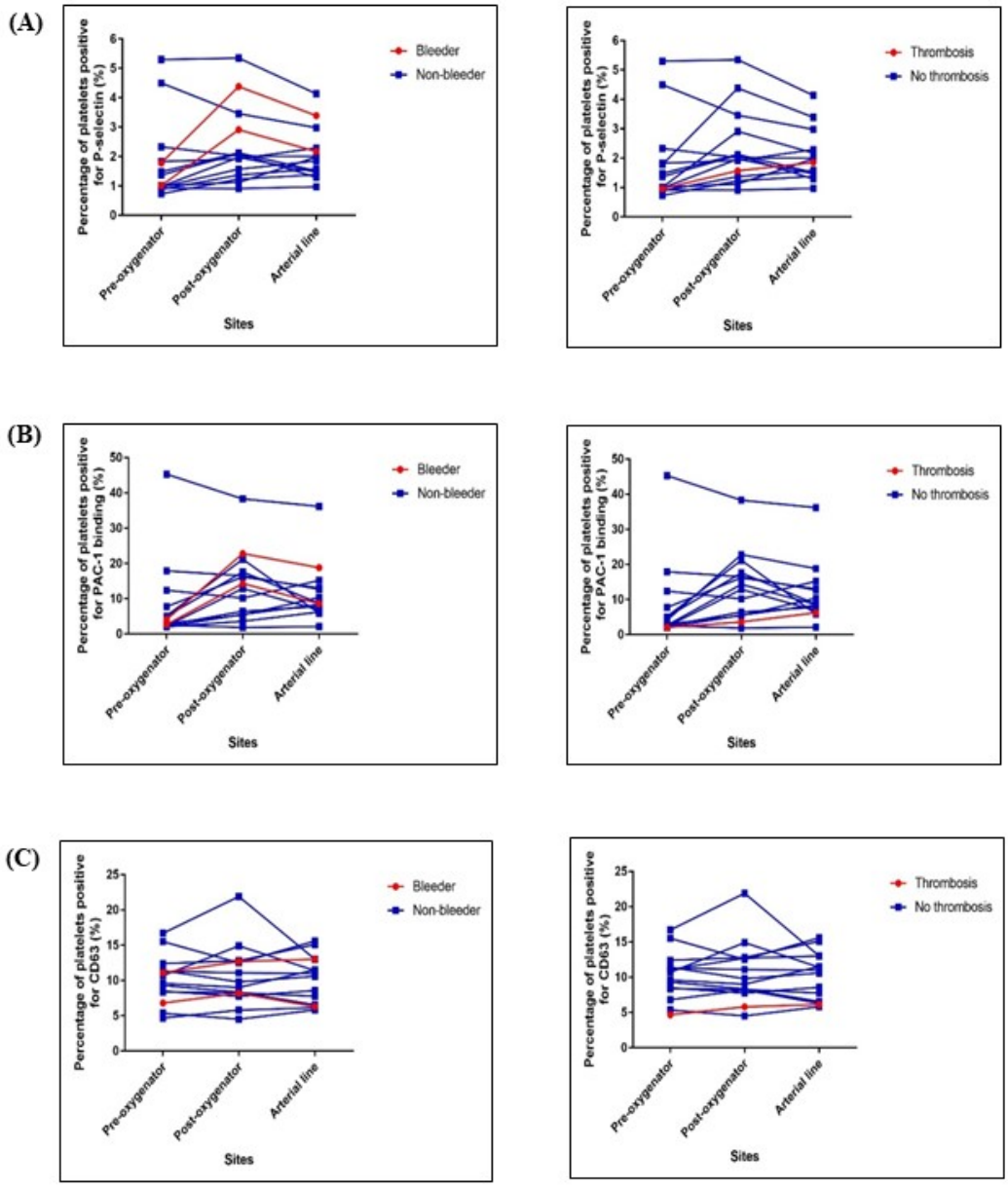

Figure 7.10 Circulating platelet activation at different sites in an ECMO system and the presence or absence of clinical event.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to clinical events.

7.3.3.4 Circulating platelet activation at different sites in an ECMO system according to the number of platelet transfusions

Figure 7.11 depict the comparisons of the circulating platelet activation at different sites in an ECMO system according to the number of platelet transfusions received by the patient within 24 hours before sample collection. There was no difference in the circulating platelet activation at different sites in an ECMO system according to the number of platelet transfusions received by the patient.

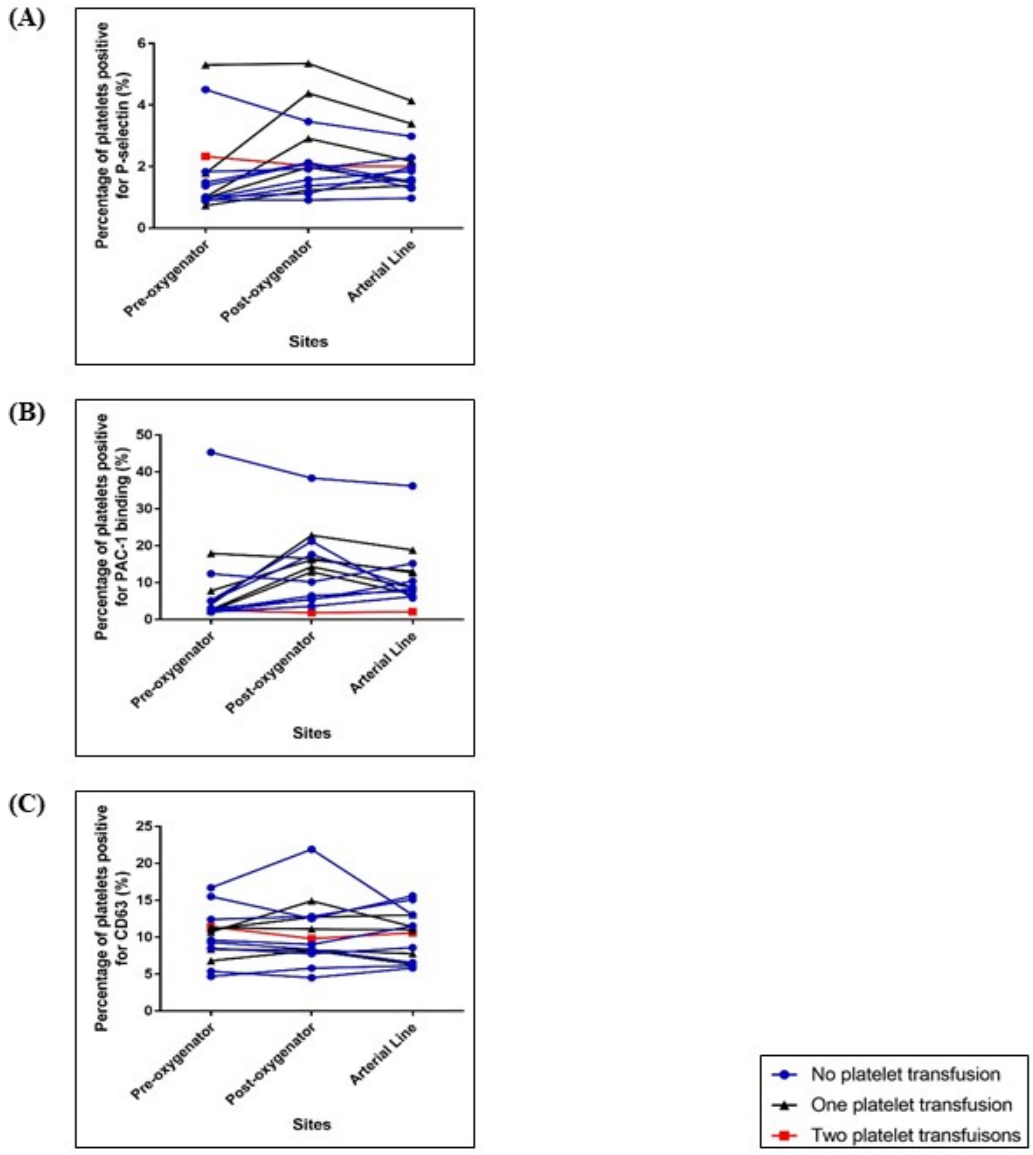

Figure 7.11 Circulating platelet activation at different sites in an ECMO system and platelet transfusions.

Circulating platelets positive for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to the number of platelet transfusions received by the patient within 24 hours before sample collection.

7.3.4 Response of platelets to stimulation with TRAP-6 at different sites in an ECMO system

The response of platelets at the post-oxygenator site was higher than at the pre-oxygenator site as indicated by the higher expression of the α-granule release indicator (22.75 ± 25.66 vs. 59.07 ± 23.0 AUC respectively, p= < 0.001; d= 1.49; power= 0.97), activated integrin αIIbβ3 receptor (39.34 ± 37.86 vs. 92.61 ± 30.43 AUC respectively, p= < 0.001; d= 1.55; power= 0.98) and lysosome release indicator (29.61 ± 14.81 vs. 55.9 ± 16.46 AUC respectively, p= < 0.001; d= 1.68; power= 0.99) in response to the increased TRAP-6 concentrations (Figure 7.12).

The platelet response was also found to be higher at the arterial line than the pre-oxygenator site for the α-granule release indicator (22.75 ± 25.66 vs. 57.97 ± 23.63 AUC respectively, p= < 0.001; d= 1.43; power= 0.95), activated integrin αIIbβ3 receptor (39.34 ± 37.86 vs. 91.67 ± 32.56 AUC respectively, p= < 0.001; d= 1.48; power= 0.96) and lysosome release indicator (29.61 ± 14.81 vs. 54.23 ± 17.27 AUC respectively, p= 0.001; d= 1.53; power= 0.97). On the other hand, the response of platelets was comparable for both the post-oxygenator site and the arterial line for the α-granule release indicator, activated integrin αIIbβ3 receptor and lysosome release indicator (Figure 7.12).

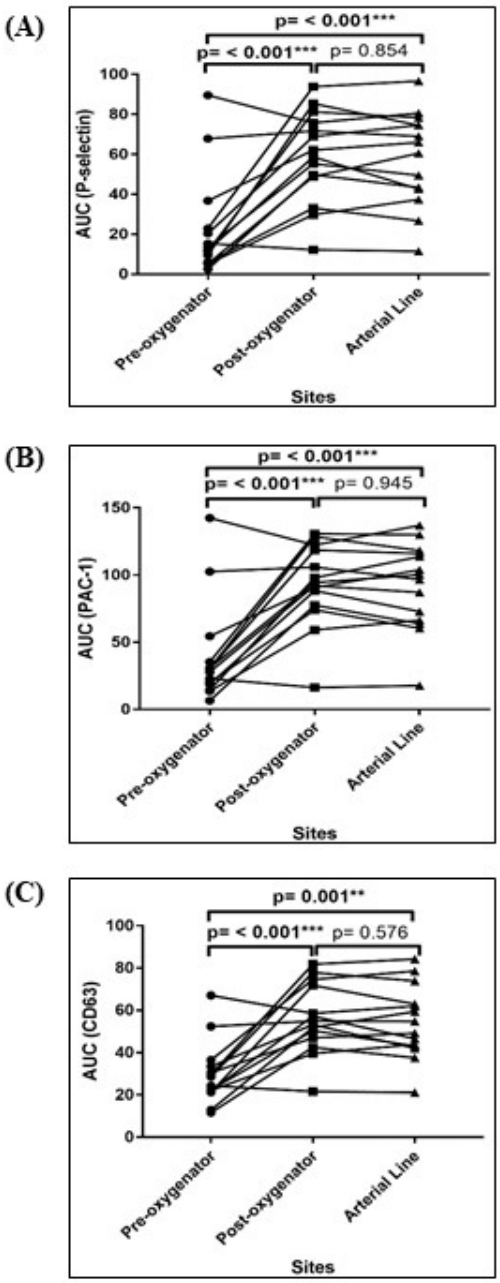

Figure 7.12 Platelet response to stimulation with TRAP-6 at different sites in an ECMO system.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system. [AUC, area under the curve]

7.3.4.1 Response of platelets to stimulation with TRAP-6 at different sites in an ECMO system according to a patient's pathway onto ECMO

For comparisons of patients who had CPB before coming onto ECMO and those who had not, the response of platelets was found to be significantly higher at the post-oxygenator site and the arterial line than the pre-oxygenator site only in the Post-CPB group for the α-granule release indicator (23.19 ± 27.39 vs. 69.05 ± 19.52 vs. 65.47 ± 23.36 AUC respectively, p= 0.008; d= 1.93; power= 0.94 (pre- vs. post-oxygenator); p= 0.009, d= 1.66; power= 0.87 (pre-oxygenator vs. arterial line)); activated integrin αIIbβ3 receptor (42.05 ± 40.93 vs. 106.1 ± 21.38 vs. vs. 102.7 ± 27.39 AUC respectively, p= 0.005; d= 2.77; power= 0.94 (pre- vs. post-oxygenator); p= 0.003; d= 1.86; power= 0.89 (pre-oxygenator vs. arterial line)) and lysosome release indicator (32.61 ± 14.97 vs. 65.67 ± 12.17 vs. 61.66 ± 16.47 AUC respectively, p= 0.006; d= 2.42; power= 0.99 (pre- vs. post-oxygenator); p= 0.012; d= 1.85; power= 0.93 (pre-oxygenator vs. arterial line)) (Figure 7.13).

Similarly, an increasing trend was observed for the pre- vs. post-oxygenator site in the Non-CPB group for the α-granule release indicator (22.18 ± 25.72 vs. 45.75 ± 21.67 AUC respectively, p= 0.079; d= 0.99; power= 0.34), activated integrin αIIbβ3 receptor (35.73 ± 36.80 vs. 74.59 ± 32.94 AUC respectively, p= 0.097; d= 1.11; power= 0.41) and lysosome release indicator (25.62 ± 14.93 vs. 42.87 ± 11.85 AUC respectively, p= 0.098; d= 1.28; power= 0.51). As for the comparison of pre-oxygenator vs. arterial line, the increasing trend was only observed for the AUC of the α-granule release indicator (22.18 ± 25.72 vs. 47.97 ± 21.87 respectively, p= 0.087; d= 1.08; power= 0.39) but not for the activated integrin αIIbβ3 receptor and lysosome release indicator. The response of platelets was comparable between the post-oxygenator site and the arterial line for both the Post-CPB and the Non-CPB groups (Figure 7.13).

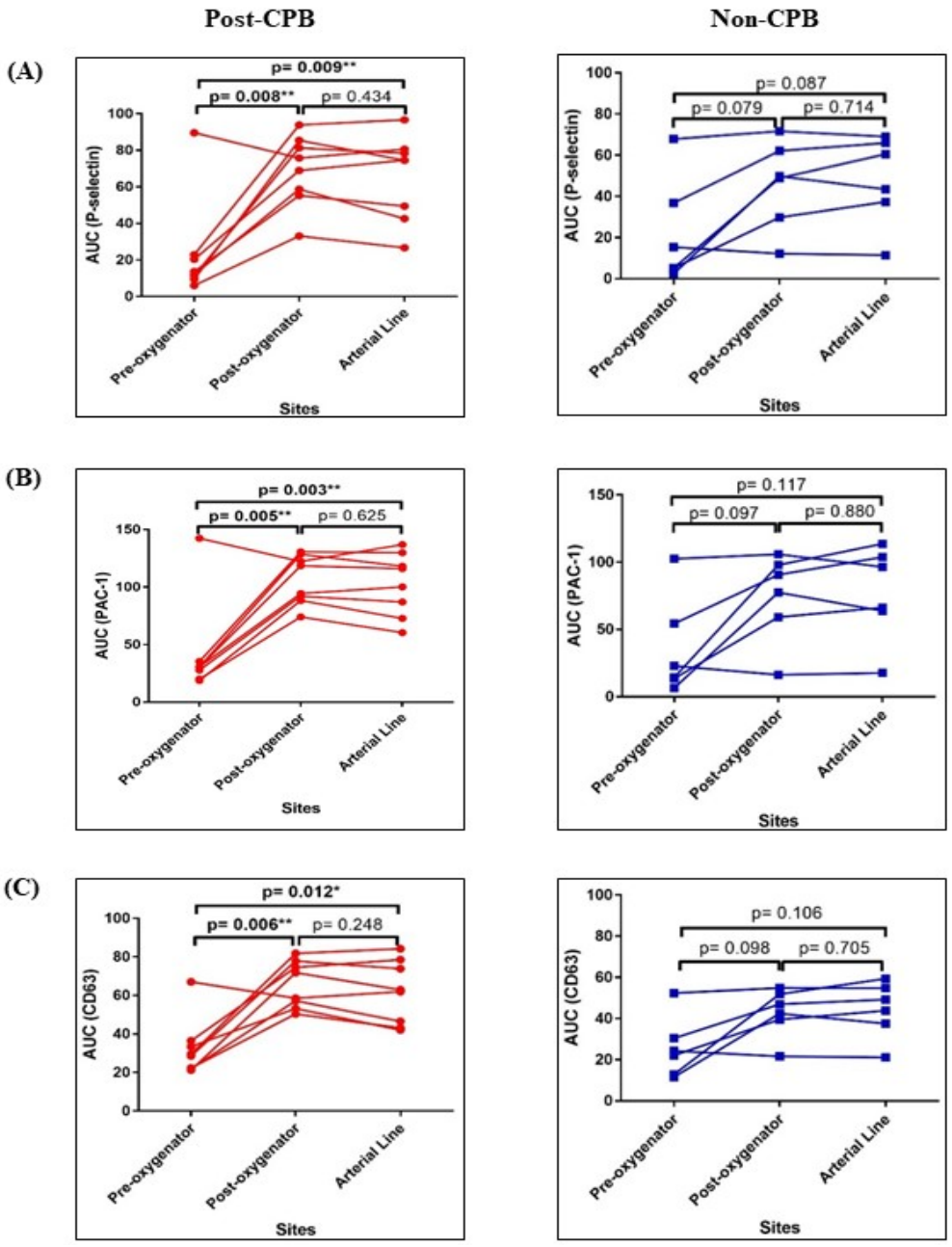

Figure 7.13 Platelet response to stimulation with TRAP-6 at different sites in an ECMO system and pathway onto ECMO.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to a patient's pathway onto ECMO. [AUC, area under the curve; Non-CPB, non-cardiopulmonary bypass; Post-CPB, post-cardiopulmonary bypass]

7.3.4.2 Response of platelets to stimulation with TRAP-6 at different sites in an ECMO system according to a patient's age

The elevated platelet response (for comparisons of sites pre- vs. post-oxygenator and pre-oxygenator vs. arterial line) as indicated by an increase in AUC for the α-granule release indicator, activated integrin αIIbβ3 receptor and lysosome release indicator were only seen for children but not in neonates and infants (Figure 7.14). For comparisons of pre- vs. post-oxygenator sites in children, the AUC measured was 24.41 ± 24.58 vs. 59.45 ± 9.71 ($p= 0.010$; $d= 1.87$; power= 0.78), 39.40 ± 35.0 vs. 93.09 ± 9.37 ($p= 0.014$; $d= 2.10$; power= 0.85) and 27.69 ± 15.5 vs. 53.49 ± 11.16 ($p= 0.015$; $d= 1.91$; power= 0.84) for the α-granule release indicator, activated integrin αIIbβ3 receptor and lysosome release indicator, respectively. For comparisons of pre-oxygenator vs. arterial line in children, the AUC measured was 24.41 ± 24.58 vs. 60.51 ± 11.94 ($p= 0.016$; $d= 1.87$; power= 0.80), 39.40 ± 35.0 vs. 94.19 ± 17.27 ($p= 0.026$; $d= 1.95$; power= 0.84) and 27.69 ± 15.5 vs. 53.79 ± 14.46 ($p= 0.025$; $d= 1.74$; power= 0.78) for the α-granule release indicator, activated integrin αIIbβ3 receptor and lysosome release indicator, respectively. As for the comparison of post-oxygenator vs. arterial line, the AUC was similar for the α-granule release indicator, activated integrin αIIbβ3 receptor and lysosome release indicator.

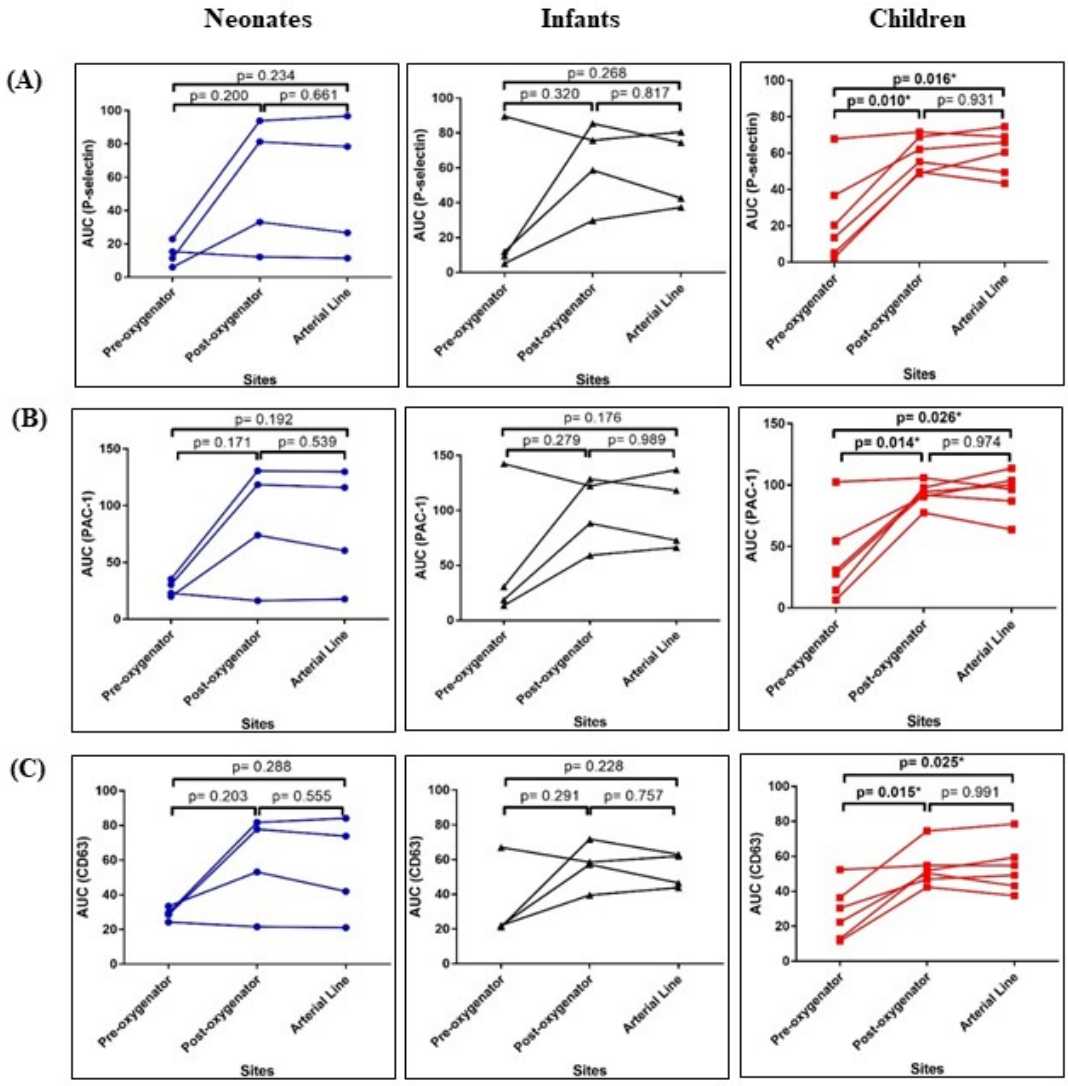

Figure 7.14 Platelet response to stimulation with TRAP-6 at different sites in an ECMO system and age.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to a patient's age. [AUC, area under the curve]

7.3.4.3 Response of platelets to stimulation with TRAP-6 at different sites in an ECMO system and the associations with bleeding or thrombosis

Figure 7.15 depict the comparisons of the AUC based on the marker of platelet activation and the indicators of granule exocytosis for individuals who had a bleeding and/or thrombosis event on the day of sample collection. Patients with or without bleeding had a similar trend of changes in the response of platelets across sites in the ECMO. More information will be required for the evaluation of the response of platelets at different sites in an ECMO system for an individual with thrombosis.

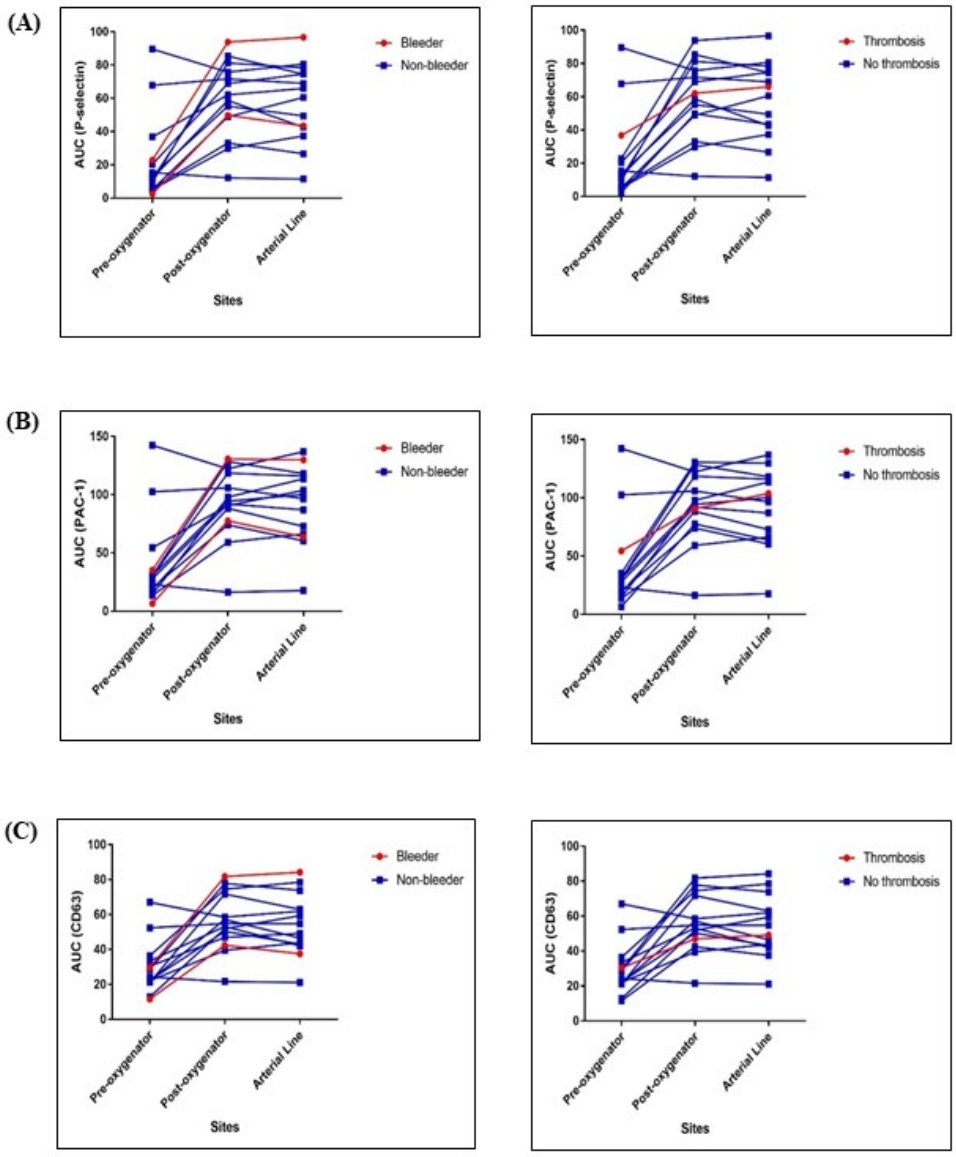

Figure 7.15 Platelet response to stimulation with TRAP-6 at different sites in an ECMO system and the presence or absence of clinical event.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to clinical events. [AUC, area under the curve]

7.3.4.4 Response of platelets to stimulation with TRAP-6 according to the number of platelet transfusions

Figure 7.16 depict the comparisons of the expression for the marker of platelet activation and the indicators of granule exocytosis according to the number of platelet transfusions received by the patient within 24 hours before sample collection. Although the number of patients is inadequate for statistical analysis of total number of platelet transfusions subgroups, the patient who received twice platelet transfusions had lower platelet response at the post-oxygenator site than the pre-oxygenator site as indicated by the lower AUC for the α-granule release indicator, activated integrin αIIbβ3 receptor and lysosome release indicator compared to the other cohorts who received none or once platelet transfusion.

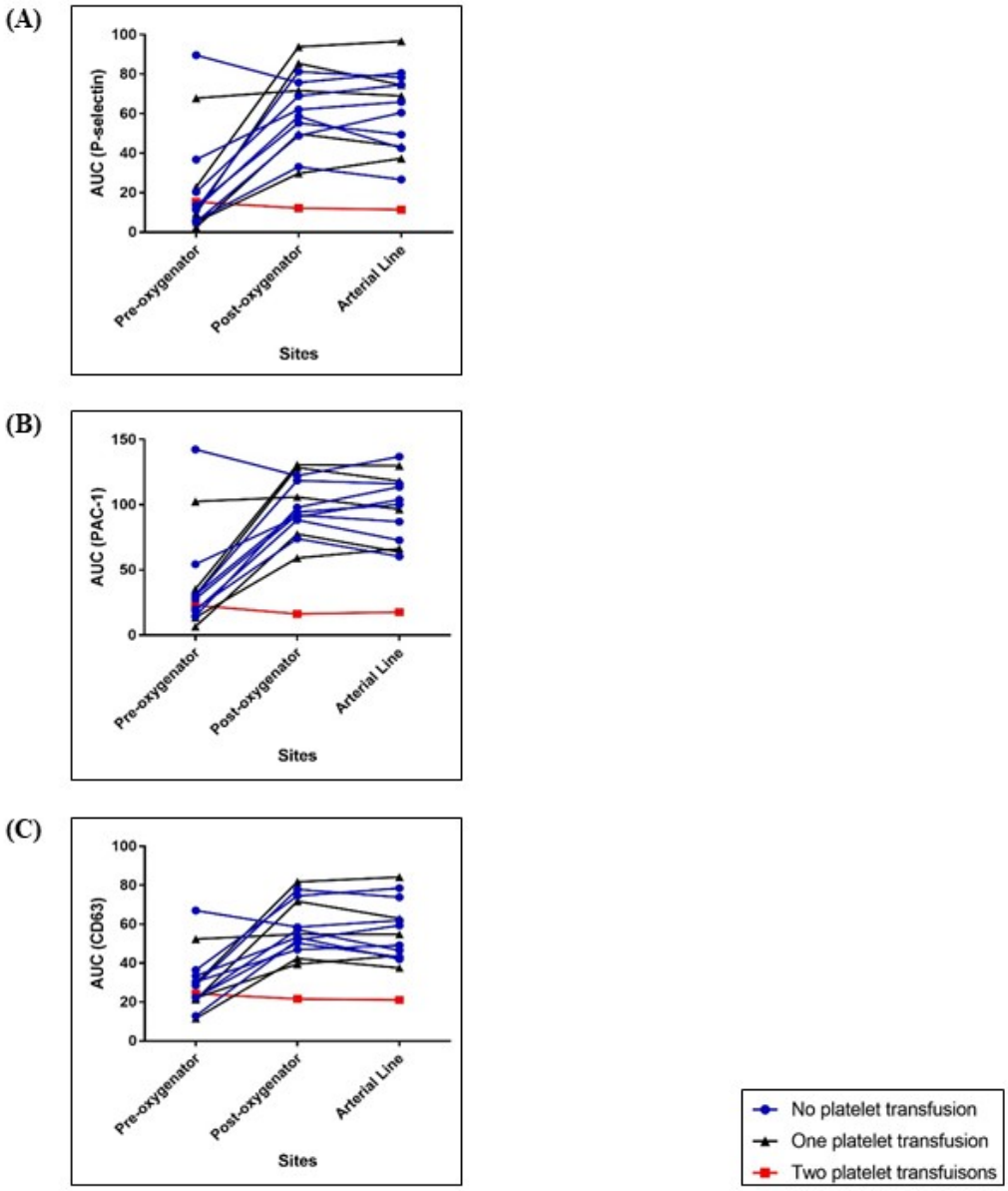

Figure 7.16 Platelet response to stimulation with TRAP-6 at different sites in an ECMO system and platelet transfusions.

The platelet response for (A) α-granule release indicator, P-selectin (CD62P) (B) activated integrin αIIbβ3 indicator, PAC-1 binding and (C) lysosome release indicator, CD63 at different sites in an ECMO system according to the number of platelet transfusion received by the patient within 24 hours before sample collection. [AUC, area under the curve]

7.4 Discussion

An ECMO system is made up of multiple compartments which ensure that the deoxygenated blood is removed from the patient (sample site- pre-oxygenator) and channeled into an oxygenator for the addition of oxygen and removal of carbon dioxide. The oxygenated blood (sample site- post-oxygenator) is then returned to the patient via the arterial circulation (sample site- arterial line) for VA-ECMO patients which represented the main cohort for this chapter of the thesis. The well-documented detrimental effects (originating from different flow and pressure) of such a complex system on multiple blood components [377-378] can eventually lead to pathological outcomes.

This study demonstrated for the first time that platelet phenotype, circulating platelet activation and response to stimulation with TRAP-6 vary across different sites in a paediatric ECMO system. The main findings are an ECMO system may affect the platelet function via I.) modulation of platelet phenotype mainly through the VWF and integrin αIIbβ3 receptors, II.) an increase in platelet activation through the activation of the integrin αIIbβ3 receptor and III.) an enhancement of platelet responsiveness.

An oxygenator is one of the important components of an ECMO system, a site where most circuit-related thromboses are found [266]. The high shear environment within the oxygenator (originating from the turbulence generated from the gas production) plays a vital role in modulating platelet function in an ECMO system. This is because the interactions between platelet receptors and ligands e.g. fibrinogen and VWF are highly-shear-dependent [235].

Among the three main receptors (integrin αIIbβ3, GPIb/IX/V and GPVI receptors) that are important for platelet adhesion and aggregation, changes in the expression across

different sites were only observed for the integrin αIIbβ3 and GPIb/IX/V receptors. Such modulations may be relevant to the changes of their ligands (e.g. structure) i.e. fibrinogen and VWF in the plasma. Specifically, VWF is the main molecule that determines platelet adhesion and aggregation under high shear stress [309]. High shear stress promotes the unfolding of VWF [310] and increases the adhesion capacity of VWF to the platelet GPIb/IX/V receptor [74, 236]. Such interaction in turns can induce the up-regulation of platelet surface GPIb/IX/V receptor via recruitment of the GPIb/IX/V receptor from the intraplatelet pool [259-262, 301, 302] .

In healthy individuals, adherence of VWF to the GPIb/IX/V receptor induces platelet activation pathway and initiates platelet aggregation and intragranule release [311]. This is accompanied by redistribution of integrin αIIbβ3 receptor from the intraplatelet pool to the platelet surface [259-261]. Fibrinogen is one of the very first plasma proteins that gets adsorbed onto the foreign biomaterial [229]. Upon deposition, fibrinogen undergoes shear-dependent conformational change [263] that can promote the binding of platelets towards the foreign surface via the integrin αIIbβ3 receptor [280, 281]. Together, such increase in interactions between the VWF and fibrinogen and their receptors may contribute to the increase in integrin αIIbβ3 and GPIb/IX/V receptor expression on platelet surface seen at the post-oxygenator site via positive feedback loop [262].

NO and epoprostenol, with their reported inhibitory effects on platelet function are part of the standard management of patients receiving ECMO at RCH. NO inhibits platelet activation via the integrin αIIbβ3 receptor in a dose-dependent manner [312, 313] and thrombin-induced up-regulation of integrin αIIbβ3 receptor, α-granule and lysosome release [312]. Although the inhibition of platelet activation by prostacyclin is also reported to be mediated through the integrin αIIbβ3 receptor, there is no existing evidence for the direct inhibitory effects of epoprostenol, a synthetic prostacyclin on platelet

function. Results from this study showed the differences in platelet phenotype and function at different sites in the ECMO system despite the presence of NO and epoprostenol. Particularly, the modification of platelet phenotype, increased circulating platelet activation and response at the post-oxygenator site may suggest that the potential inhibitory effects of the current dosage of NO and epoprostenol on platelets may either be transient that they could be overcome by the oxygenator-mediated platelet-specific changes in the current ECMO system or limited that a higher degree of platelet-specific changes is expected to be seen in the absence of NO and epoprostenol.

NO can interact rapidly and irreversibly with haemoglobin and haemoglobin-mediated NO scavenging has been associated with multiple clinical consequences such as thrombosis and platelet activation [314]. This is important because haemolysis is common in the ECMO population and is an important factor associated with outcome in paediatric ECMO patients [315]. Plasma free haemoglobin (FHb) is a marker for haemolysis with an increase of 10 – 25 fold during ECMO [316].

The oxygenator is identified as the main part of the ECMO system that contributes to ECMO-induced haemolysis [317]. Upon haemolysis, red blood cells release large amount of ADP into the circulation and induce platelet activation [391, 392]. The activated platelets can then release ADP from their intragranular storage and further potentiate platelet activation via P2Y receptors [392]. Prolonged bleeding time due to ADP infusions can be associated with platelet P2Y desensitization [393, 394].

In addition to the mechanical-stress induced haemolysis, FHb originating from the stored packed red blood cells used for priming of the ECMO circuit and transfusions may further exacerbate the haemoglobin-mediated depletion of NO [318, 319]. Together, the increase in haemoglobin-mediated NO depletion thus may reduce NO available to inhibit platelet

function. Comparing pre- vs. post-oxygenator FHb may provide further information for the potential effects of haemoglobin-mediated NO depletion on platelet-specific changes in a paediatric ECMO system.

Furthermore, currently available studies with regards to the inhibitory effects of NO and epoprostenol on platelets were based on the observations for each of the drug individually. To date, there is no existing study which has investigated the effects of coexistence of NO and epoprostenol on platelet function. Hence, the potential drug interaction between NO and epoprostenol that may have contributed to the platelet-specific changes seen at different sites in the ECMO system could not be eliminated.

ECMO-related platelet dysfunction has been associated with platelet activation [213]. This research demonstrates increased circulating (unstimulated) activation state of platelets (as demonstrated by PAC1 binding to the activated platelet integrin αIIbβ3 receptor) in the post-oxygenator circuit, which corresponds to high shear and turbulence. This is detrimental as such activation of integrin αIIbβ3 receptor may promote platelet aggregation and thrombosis. However, there was no corresponding increase in circulating platelet granule exocytosis, as measured by CD62P. This may reflect the rapid shedding of surface P-selectin from the circulating platelets [111, 112]. Furthermore, the duration of the other indicators of activation to remain expressed on the platelet surface *in vivo* are yet to be identified [113]. Investigation of the expression of these shed markers of platelet activation using other methods such as ELISA (for example, plasma soluble P-selectin, beta-thromboglobulin and platelet factor 4 have been proposed as the markers of *in vivo* platelet activation [110, 320, 321]) could clarify this issue. Specifically, both soluble P-selectin and beta-thromboglobulin had previously been found to increase in the setting of ECMO [206, 213].

The response of platelets to stimulation with TRAP-6 was higher at the post-oxygenator site than the pre-oxygenator site, as indicated by the significantly elevated platelet activation and granule exocytosis. This is consistent with the membrane oxygenator as a major stimulus for platelet hyper-activation in an ECMO system. An oxygenator serves to replace the lung function in an ECMO system. A gradient of high oxygen and low carbon dioxide is constantly created within the oxygenator to facilitate the gas exchange process. Such high oxygen concentration environment could have generated by-products such as reactive oxygen species (ROS) that includes superoxide anion, hydrogen peroxide and hydroxyl radical that are important for cellular signalling [322]. Specifically, oxidative stress has been proposed to be one of the main cause of platelet dysfunction in patients on ECMO [206, 213].

ROS can increase platelet reactivity through the enhancement of intraplatelet release of calcium [292] and attenuation of the biological activity of nitric oxide (NO) [323]. An increase in intraplatelet calcium level promotes platelet signalling pathway followed by the rearrangement of cytoskeleton, integrin activation and granule release [324, 325]. Hence, such ROS-induced increase in intraplatelet calcium level thus may lower the threshold for platelet activation pathway with the subsequent exposure to platelet activator and in this case TRAP-6. NO is a natural platelet inhibitor constantly released by the endothelia to regulate *in vivo* platelet activity. Hence, a reduction of NO production by ROS can also increase platelet reactivity.

In addition to the oxidative stress, shear stress within the oxygenator could be another potential activator of the platelet signalling pathway through the enhancement of receptor-ligand interaction and thrombin generation. High shear can promote interaction between key receptors i.e. integrin αIIbβ3 and GPIb/IX/V receptor and their ligands [235, 236] and increase platelet secretion and aggregation (via increased affinity of the integrin

αIIbβ3receptor for fibrinogen) [237, 238]. Thrombin is a protease that catalyses the final stage of clot formation by converting fibrinogen into fibrin and acts mainly via PAR1 and PAR4 in human (section 1.2.1.3.3.2). Shear-induced thrombin generation are well-studied in the setting of MCS e.g. CPB [326, 327]. Hence, it is possible that high shear within the oxygenator can promote high rate of thrombin generation and together with existing circulating thrombin in patient sensitize the platelets towards activation upon the subsequent exposure to TRAP-6, a thrombin mimic that can activate platelet via PAR1 without the presence of thrombin.

Besides, high-affinity thrombin binding site can be found on the GPIb/IX/V receptor that has a vital role in thrombin-induced platelet response [128]. This is important because GPIb/IX/V receptor was up-regulated at the post-oxygenator site and could have also play a part in potentiating the sensitizing effect of shear-induced thrombin generation on platelet response. Furthermore, monoclonal antibody against GPIb/IX/V receptor was found to be effective against shear-induced thrombin generation under high-shear [328]. Together, shear stress, high oxygen concentration and thrombin generation within the oxygenator may work synergistically and sensitize platelets towards activation via different mechanisms. These may then contribute to an increase in intragranule release and receptor activation hence the overall increase in platelet response to TRAP-6 at the post-oxygenator site.

Patients coming onto ECMO via different pathways and are of different age groups. With the existing knowledge of the effects of CPB and the role of developmental haematology and effects of CPB on platelet function, platelet phenotype, circulating platelet activation and response to stimulation with TRAP-6 at different sites in an ECMO system were also compared according to a patient's pathway onto ECMO and age. Specifically, platelet responsiveness is different according to a patient's pathway onto ECMO and age.

For the evaluation of platelet phenotype and function according to a patient's pathway onto ECMO, the increase in GPIb/IX/V receptor expression at the post-oxygenator site which may indicate changes in platelet phenotype possibly induced by shear from the oxygenator were equally seen in both groups of patients coming onto ECMO with or without CPB. On the other hand, the increase in platelet response potentially induced by shear within the oxygenator was only seen for the Post-CPB group but not the Non-CPB group. This is important because CPB is another type of MCS of which the detrimental effects on platelet function are well-documented in existing literature [329, 330]. The high shear environment in CPB may sensitize the platelets to subsequent shear condition i.e. ECMO and causes platelets to be more susceptible to shear-induced changes [257, 258]. Furthermore, CPB could have further sensitized the platelets towards ROS and shear-induced activation of the platelet signalling pathway hence further lowering the threshold for platelet activation upon exposure to TRAP-6 hence an overall increase in platelet reactivity seen for this group.

The difference in platelet phenotype and reactivity at different sites in an ECMO system constantly seen only for children but not in neonates and infants may be related to the developmental haematology relevant to platelet function. Plasma concentration of VWF was shown to increase with age in healthy individuals [331-335] and neonatal platelets were found to have decreased expression of adhesion receptors [44, 336]. Thus, the elevated GPIb/IX/V receptor expression at the post-oxygenator site seen only for children may be associated with their higher intraplatelet pool of GPIb/IX/V receptor than the other cohorts with lower age. The increase in platelet GPVI receptor expression at the post-oxygenator site that can only be seen in children may be a subsequent event following up-regulation of GPIb/IX/V receptor since binding of VWF and collagen to their receptors create synergistic effects for the adhesion-signalling pathway [337-339].

In addition to the adhesion receptors, platelet hyporeactivity was found to persist beyond the neonatal period [44] and can be related to the multiple impaired receptor-mediated signal transduction pathways [341-343]. Furthermore, platelet reactivity increases with gestational age [334, 335]. In the setting of CPB, *in vivo* platelet activation (as indicated by P-selectin expression) in infants was found to be lower than the older children [340]. Thus, the changes in platelet responsiveness at different sites in an ECMO system only seen for children may also be related to the age-related changes in platelet function.

The comparison between the post-oxygenator site and the arterial line may provide information for the platelet-specific changes in patient's body after leaving the oxygenator. The similar expression of markers important for platelet phenotype, platelet activation and response at both sites may indicate that shear stress-induced platelet-specific changes originating from the oxygenator may propagate into the patient and contribute to the clinical events commonly seen in the ECMO population. On the other hand, the higher platelet reactivity at the arterial line than the pre-oxygenator site may indicate that the hyperresponsive platelets leaving the oxygenator are with increased capacity for further platelet activation once propagate into a patient's body via the activation of the integrin αIIbβ3 receptor and intragranule release. These hyperresponsive platelets may then contribute to increased platelet activation, adhesion and aggregation that may lead to bleeding and/or clotting events.

Bleeding and/or thrombosis are the most commonly seen complications in the ECLS population [151, 229, 307] and have been associated with platelet dysfunction. Hence, platelet phenotype and function were also examined to evaluate if their differences across sites in an ECMO system could be associated with the clinical events seen in the patients. However, current analysis is observational as it is limited by the sample size. Overall, patients who had bleeding had a trend of I.) a lower GPIb/IX/V receptor expression at

different sites in an ECMO system and II.) a higher degree of change accompanied by a higher expression of α-granule release indicator at the post-oxygenator site than the pre-oxygenator site compared to those who had no bleeding event.

Platelet adhesion mediated through VWF is one of the crucial early events in haemostasis. Hence, the lower GPIb/IX/V receptor expression seen in the patients with bleeding may indicate an overall lower platelet adhesion activity than those without bleeding. Also, since it is known that the α-granule release indicator, P-selectin is rapidly loss from the platelet surface upon degranulation, the higher α-granule release indicator expression at the post-oxygenator site may indicate that majority of the platelets that leave the oxygenator and travel into the patient's body may be composed of the exhausted platelets that are with limited capacity for further activation which is vital for platelet adhesion and aggregation. Together, such changes in the expression of the GPIb/IX/V receptor and α-granule release indicator thus may contribute to the bleeding events seen in the patients. However, a larger sample size will be required for detailed evaluation of the association between changes in platelet phenotype and function across different sites in an ECMO system and the development of clinical events.

Platelet transfusion is an integrated part for the management of patients on ECMO. Although there was no difference in the platelet phenotype and circulating platelet activation at different sites in an ECMO system, platelet response was lowest at the post-oxygenator site for the single patient who received two platelet transfusions. This could be related to the patient who received more platelet transfusions may have higher population of transfuse platelets in the circulation which is with refractoriness to activation. Studies have showed that P-selectin [293, 294] and CD63 expressions [295] increase with platelet storage due to granule release. Hence, the circulating platelets in the patient may represent the transfuse platelet population with minimal capacity for

further activation due to exhaustion of intragranule content during storage. Also, the circulating platelet population may also represent the platelet remnant originating from the patient that may be with ECMO-induced refractoriness to activation because activated platelets I.) are preferably removed from the circulation by the reticuloendothelial system and/or II.) with increased binding to the exposed subendothelial layer or circuit. Together, these may contribute to the circulating platelets with reduced responsiveness seen at the post-oxygenator site. Since post-oxygenator site was observed as the main site where most changes in platelet phenotype, activation and response can be found in the current ECMO system, these may suggest that this area should be the focus of improvement to ECMO system design, or inform the use of antiplatelet agent.

Results from this study showed that all of the investigated full blood count parameters except for the haematocrit level are comparable at different sites in a paediatric ECMO system. Existing studies that have investigated the relationship between the haematocrit level and platelet activity are either based on adults or *in vitro* studies with the common finding of a liner relationship between these two parameters [402-403]. Such positive association between haematocrit and platelet reactivity may be relevant to the higher haematocrit of red blood cells that can promote margination of platelets towards the vessel wall [402] where the main platelet ligands such as VWF and collagen are commonly found. Such increased interaction between platelets and their ligands may then contribute to the differences in platelet activation and reactivity across different sites in the ECMO system observed in this study.

In summary, current analyses suggested that circulating platelets are with different phenotype, activation and response to stimulation with TRAP-6 at different sites in an ECMO system. The main receptors involved are the VWF and integrin αIIbβ3 receptors that are important for platelet adhesion and aggregation and circulating platelet activation

in an ECMO system may be mediated through the activation of the integrin αIIbβ3 receptor. Such changes in the platelet phenotype and activation may inform an increase in interaction between platelet receptors and their ligands and contribute to the subsequent increase in platelet response to stimulation with TRAP-6. Circuit at the post-oxygenator site may be a targeted area for the introduction of the modification of the ECMO system and/or anti-platelet therapy with inhibitors of the VWF and integrin αIIbβ3 receptors as the potential candidates. A larger sample size is needed for the determination of the association of differences for circulating platelet phenotype, activation and response to stimulation with TRAP-6 at different sites in an ECMO system with clinical events (bleeding and thrombosis) and the role of platelet transfusion in contributing to the differences seen.

7.5 Conclusion

Circulating platelet phenotype, activation and response to stimulation TRAP-6 are different at different sites in an ECMO system with integrin αIIbβ3 and GPIb/IX/V are the main receptors involved. A larger sample size is required for determination of the association between site-specific differences for circulating platelet phenotype, activation and response to stimulation TRAP-6 and the development of clinical events (bleeding and thrombosis) and the role of platelet transfusion for such differences seen.

8 Discussion

The feasibility of using whole blood flow cytometry as a useful platelet function test in the paediatric ECMO population was demonstrated in this study. Whole blood flow cytometry has gained increased attention in recent years due to its multiple advantages. One of such advantages is that flow cytometry only requires a minimal amount of blood for testing purpose. Study by Dalton *et al.* (2017) in the paediatric ECMO setting showed that laboratory blood sampling causes significant blood loss and is the main reason of transfusion in 42.2% of the studied population [266]. Such blood loss can in turn increase the likelihood of developing morbidity and complications related to transfusion exposure. Hence, using whole blood flow cytometry that requires minimal amount of blood as a method for platelet function test can aid in reducing the volume of sample needed for laboratory blood sampling thus reducing blood loss and the need for transfusion in children on ECMO.

In addition to the requirement for a large volume of blood, a lack of standardization for the assay protocols is another common limitation of the currently available platelet function tests. Standardization of the methods for whole blood flow cytometric evaluation of platelet function is especially important for platelets that are with high susceptibility to pre-analytical activation *in vitro*. The optimal time frame for the assessment of platelet phenotype and function was identified to ensure minimal *ex-vivo* platelet activation. In contrast, the assay for assessing MPA/NPA was consistent with the limited processing window for whole blood flow cytometry analysis of platelet-leukocyte aggregates.

Assays used in this study allowed extended storage time of samples post-fixation for up to 72 hours at 4 ºC before flow cytometric analysis for reliable quantification of markers important for platelet phenotype and function. Such flexibility in time is useful in both the clinical and research settings with a large sample size and/or allow sample

transportation across different places in multi-site studies for a site either with limited or without any accessibility to the flow cytometer. Together with the identification of the optimal time frame from sample collection to the initiation of assay and the advantage of allowing extended storage upon sample fixation ensure the feasibility and reliability of the whole blood flow cytometry method as a platelet function test.

The flow cytometry panels designated to investigate platelets for their phenotype, function and interactions with monocytes and neutrophils allowed the investigation of platelet-specific changes from different aspects in relation to their important roles in the haemostatic and inflammatory systems. Such multifaceted properties of the platelet assays are especially important for the ECMO population, a complex group of patients with different disease aetiology and whose already deranged haemostatic system could be further worsen by the medications (e.g. UFH), proinflammatory state and transfusion exposure-related complications [344]. Despite its effectiveness in saving the life of the most critically ill children, paediatric patients on ECMO are with high rate of bleeding and/or thrombosis. Platelet dysfunction, has been proposed to be the main cause of coagulopathy seen in this population [231, 233].

Platelet reactivity has been an important part for the evaluation of platelet function. Different from the other tests that utilize only a single high concentration of agonist to ensure maximum sensitivity for reduced platelet function, the platelet reactivity assay used in this study utilized two concentrations of the agonist, TRAP-6. Such approach will ensure sensitivity for the identification of subtle changes in platelet reactivity for the paediatric ECMO patients. The usage of multiple concentrations of different agonists for platelet function testing has gained increased interest in recent years [345]. This is because such approach allows simultaneous investigation of multiple platelet activation pathways which is a better representative of the complex *in vivo* agonist–induced changes

in platelet function. Although the assay utilized only a single type of agonist, TRAP-6 is a mimic of thrombin which is the most potent *in vivo* activator of platelets. Hence, an increased or reduced platelet reactivity to TRAP-6 may reflect an overall platelet hyper- or hypo-responsivity (via the thrombin PAR 1 receptor activation pathway).

This is the first study with detailed investigation of platelet physiology and function in paediatric ECMO patients. Hence, the antibodies chosen for the flow cytometry assays were targeting at platelet-relevant markers for adhesion, aggregation, activation and also their interactions with leukocytes, the fundamental and important roles of platelets in haemostasis aiming to provide an initial overview of the platelet-specific changes and their relationships with the clinical events seen in this population. However, there is increasing evidence for the extensive involvement of platelets in haemostasis beyond their well-established functions in preventing bleeding. Thus, it will also be valuable to investigate the other important haemostatic roles of platelets such as the formation of procoagulant surface as the initial step of coagulation cascade [346]. Particularly, recent evidence suggests an association between increasing platelet procoagulant activity and platelet clearance which is shear-dependent [347, 348] hence could be of importance in a high shear environment such as that of an ECMO system.

Exposure of phosphatidylserine on platelet surface is one of the features of the platelet procoagulant activity and flow cytometric measurement of Annexin V will allow the examination of such property in patients on ECMO [349]. On the other hand, tissue factor is an important component of the procoagulant activity on monocytes [341]. Since the role of PLA in the ECMO setting has gained increase attention in recent years, it will also be important to evaluate the procoagulant activity on leukocytes (e.g. by measuring tissue factor expression), particularly in relation to their interactions with platelets. Also, since phosphorylation of the intracellular proteins is vital for platelet activation, measuring the

phosphorylation state of the platelet intracellular proteins such as with the vasodilator-stimulated phosphoprotein (VASP) kit will allow the investigation of ADP-mediated platelet activation [350] in the ECMO patients. Together with the PerFix EXPOSE, which is a new kit for flow cytometric measurement of protein phosphorylation in leukocytes [342, 351], evaluating the degree of intracellular protein phosphorylation could thus provide information for the signal transduction pathways of both the platelets and leukocytes in this population.

Platelet dysfunction can develop as a result of an increased or reduced platelet function including adhesion, aggregation and interactions with leukocytes. Platelet hypofunction may develop due to deficiencies in the platelet surface receptors, depletion of the storage granules and/or defects in the signalling pathway and present as a symptom of excessive bleeding [343, 352]. The association between platelet dysfunction with coagulopathy is well-studied in the CPB setting [256, 306]. Hence, whether a patient had CPB or not before coming onto ECMO may contribute to the differences in platelet specific-changes which may develop into platelet dysfunction.

Results from this study showed increased integrin αIIbβ3 receptor expression and reduced circulating NPA level for the Post-CPB group and reduced platelet response for the Non-CPB group in patients with bleeding. Since elevated integrin αIIbβ3 receptor and reduced NPA level could indicate increased platelet aggregation and impaired coagulation while reduced platelet response signifies a reduction in platelet function, these results suggested a link between the pathway onto ECMO (Post-CPB vs. Non-CPB), bleeding and platelet dysfunction. Hence, monitoring the level of integrin αIIbβ3 receptor and circulating NPA in the Post-CPB patients and platelet response for granule release in the Non-CPB group within 24 hours on ECMO could predict an increased risk for bleeding during ECMO.

Although the duration of ECMO is determined by the progress of recovery for the underlying disease, studies have shown that the survival rate of patients on ECMO decreases with increasing duration of ECMO due to an increased risk of complications related to bleeding or thrombosis. The results showed that patients with bleeding had increased level of GPIb/IX/V receptor expression and reduced platelet response for lysosome release as the duration of ECMO increases (Day 2 vs. Day 5). Such observations may indicate increased platelet adhesion and reduced circulating platelets positive for GPIb/IX/V receptor expression.

Shear and oxidative stress in the MCS are known to induce granule release via the activation of multiple platelet signalling pathways which may be followed by subsequent depletion of granule content including platelet activators and important coagulation proteins. The remaining platelets may represent the population of exhausted platelets with reduced platelet function. These observations thus suggesting a link between the duration of ECMO, bleeding and platelet dysfunction. Hence, monitoring the marker important for platelet adhesion i.e. GPIb/IX/V receptor and platelet response for granule exocytosis could predict increased risk for bleeding during ECMO as the duration of ECMO increases.

Platelet hyperfunction mediated through an increase in platelet activation, granule release and up-regulation of platelet receptor can be associated with an increased risk for thrombosis [353]. Particularly, the platelet integrin αIIbβ3 receptor is important for aggregation and platelet-mediated thrombus formation [354]. Upon activation, platelet fibrinogen changes from a low-affinity to a high-affinity state to allow its interaction with fibrinogen. The results showed that Post-CPB patients with thrombosis had higher integrin αIIbβ3 receptor expression than those without thrombosis. This is important because shear can promote interaction between platelet integrin αIIbβ3 receptor and

fibrinogen [235] followed by up-regulation of integrin αIIbβ3 receptor via the positive feedback loop [262]. Thus, the elevated integrin αIIbβ3 receptor expression observed for the Post-CPB patients with thrombosis may suggest a link between the pathway onto ECMO, thrombosis and platelet dysfunction. Monitoring the platelet GPIIb/IIIa receptor within 24 hours upon ECMO initiation may aid in identifying the risk of Post-CPB patients for thrombosis during ECMO.

Developmental haematology for platelet function has gained increased attention in recent years. Although detailed evaluation for the association between platelet-specific changes and clinical events for different age groups was limited by the number of events especially for bleeding, children with thrombosis had significantly higher lysosome release than those with no event. Since platelet lysosome is important for thrombus resolution [65], such increase in lysosome release may imply the presence of a protective mechanism against increased thrombus formation in children with thrombosis via positive feedback loop. Such observation may thus suggest a link between the age group, thrombosis and platelet dysfunction. Thus, monitoring the lysosome release within 24 hours upon ECMO initiation in children may predict an increased risk for thrombosis during ECMO.

An increased circulating MPA level was observed in patients with thrombosis as the duration of ECMO increases. Since an elevated MPA level is associated with prothrombotic state [218, 219], such observation may suggest a link between the duration of ECMO, thrombosis and platelet-leukocyte interactions. Hence, monitoring the circulating MPA level could predict the risk for thrombosis during ECMO as the duration of ECMO increases.

Shear stress and artificial surface of an intact ECMO system have been associated with platelet dysfunction and coagulopathy in ECMO patients. However, platelet-specific

changes at different sites have not been previously investigated in a paediatric ECMO system. The first evidence of site-specific differences for platelet phenotype and function in a paediatric ECMO system was reported in this study. The results showed modified circulating platelet phenotype (via increased fibrinogen and GPIb/IX/V receptor expression), increased circulating platelet activation (via activated integrin αIIbβ3 receptor) and response to TRAP-6 at the post-oxygenator site compared to the pre-oxygenator site.

The oxygenator is an important part of an ECMO system and the site where most circuit-related thromboses can be found [266]. High shear and oxidative stress environment within the oxygenator are known to modify platelet phenotype and function [206, 213] via different mechanisms. The results may indicate increased interactions between shear-dependent platelet receptors, the integrin αIIbβ3 and GPIb/IX/V receptors and their ligands as known in the setting of MCS [235]. Such interaction can in turn induce the release of integrin αIIbβ3 and GPIb/IX/V receptors from the intraplatelet pool via positive feedback loop hence the observed elevated integrin αIIbβ3 and GPIb/IX/V receptors at the post-oxygenator site [260, 301]. On the other hand, oxidative stress can increase platelet reactivity via increased intraplatelet release of calcium [292], an important activator of platelet signalling pathway. Thus, the elevated platelet response at the post-oxygenator site may signify increased platelet activation induced by oxidative stress via increased release of intraplatelet calcium.

Another important aspect to consider for the evaluation of site-specific platelet-relevant changes in the current ECMO system configuration is the usage of NO and epoprostenol, with their reported inhibitory effects on platelet function. The platelet-specific changes observed particularly at the post-oxygenator site despite the presence of NO and epoprostenol could be related to the current dosage of the drugs used and possibly drug

interaction between the NO and epoprostenol. Since the oxygenator is an important site of haemolysis in an ECMO system, haemoglobin-mediated NO scavenging with their known effects on various haemolysis-related clinical conditions [314, 317] may have reduced the effect of NO. Also, since there is no existing study that has investigated the pharmacological effects of coexistence of NO and epoprostenol, drug interaction may have also contributed to the platelet-specific changes seen at different sites in the current ECMO system.

Comparisons were also made for the platelet phenotype and function between the arterial line and post-oxygenator site which may provide information for platelet-specific changes in the patient's body after leaving the oxygenator. The results demonstrated comparable platelet phenotype and response at both sites which may signify shear stress-induced modifications in platelet phenotype and function can propagate into the patients and contribute to the clinical events seen in patients on ECMO. In contrast, the higher platelet reactivity at the arterial line than the pre-oxygenator site may indicate platelets leaving the oxygenator are with increased capacity for further activation once propagate into the patient's circulation. Such population of hyperresponsive platelets may contribute to increased platelet activation, adhesion and aggregation and lead to bleeding and/or thrombosis.

Routine laboratory blood sampling for a variety of clinical assays including haematological parameters is an important integrated part of management of patients on ECMO. Thrombocytopenia is the main indication of platelet administration and has been identified as the predictor of haemorrhage in patients on ECMO [395-397]. However, frequent platelet transfusions in ECMO patients with excessive bleeding with normal platelet counts may indicate platelet dysfunction. Currently available studies assessing the relationship between quantitative and qualitative changes in the haematological

parameters such as platelet count vs. platelet reactivity focused on adults and yielded divergent results [398-400] that could be associated with the different types of monitoring tests used and target patient population. In a cohort of deceased paediatric patients, study by Reed and Rutledge found no correlation between platelet level and haemorrhage or thrombosis [401]. Results from this study showed that paediatric patients from different pathways onto ECMO, age groups and duration on ECMO had comparable full blood count parameters within duration on ECMO. Particularly, the similar platelet count and mean platelet volume between both groups may be related to the comparable platelet phenotype, function and interactions with monocytes and neutrophils.

Limitations

Some of the limitations relevant to the current study were discussed as followed. The platelet reactivity assay utilized a single type of agonist i.e. TRAP-6, a thrombin mimic to yield information for the overall state of platelet responsiveness. However, *in vivo* platelet function is determined by a much more complex involvement of multiple type of agonists. Hence, using the other agonists such as collagen and adenosine-diphosphate will allow identification of the other activation pathways that could have an important role in determining platelet function in the ECMO paediatric patients as shown in the other studies [206, 212]. The platelet reactivity assay provides the flexibility for such assessment of platelet response by substituting TRAP-6 with the agonist of interest.

The evaluation of the association between platelet-specific changes and bleeding/thrombosis events within some of the subgroups was limited by the number of clinical events available with the existing sample size. The recruitment of paediatric patients is dependent on the availability of patients receiving ECMO at the paediatric intensive care unit and granting of consent. While the present study sample size is comparable to the other publications [291, 355], assessing platelet-specific differences in

relation to the clinical events in a larger cohort might provide further information of the role of platelet dysfunction in coagulopathy in paediatric ECMO patients. Furthermore, an increase in sample size will allow further subcategorization of patients according to their mode of ECMO (VA vs. VV) and cannulation strategy (central vs. peripheral), another two main factors associated with the outcome of the ECMO population.

The evaluation for how site-specific differences for platelet-relevant markers in an ECMO system could be associated with coagulopathy was limited by the number of clinical events with the existing sample size. A larger cohort may allow identification of evidence for circuit at the post-oxygenator site as a targeted area for the introduction of modifications into an ECMO circuit. Also, since all paediatric ECMO patients at RCH received epoprostenol through continuous infusion via the oxygenator, the possible residual effects of a relatively higher dosage of epoprostenol at the pre-oxygenator site (one of the sampling sites which is also the infusion site for epoprostenol) than the post-oxygenator site and arterial line hence could not be eliminated.

Under normal physiological condition, the balance between coagulation and anti-coagulation activities is maintained to prevent excessive bleeding or clotting by haemostasis. Maintaining the haemostatic balance in ECMO patients is particularly challenging for their already deranged haemostatic system that can be further complicated by multiple factors such as medications or proinflammatory state associated with transfusions. Their coagulation system thus could represent the result of a complex interaction between the haemostatic and inflammatory systems. Particularly, bleeding or thrombosis observed for the ECMO patients may be due to the local instead of systemic changes. For example, bleeding or clotting on a surgical wound may be a defense mechanism for blood vessel injury from surgical procedure that requires local treatment of the surgical site instead of a direct reflection of systemic changes of the patient's

haemostatic system. Thus, monitoring systemic platelet-specific changes on its own may have limited capacity to predict bleeding or thrombosis. Furthermore, such complexity of the haemostatic system in the ECMO patients also poses challenges in identifying the cause and effect relationship between platelet-specific changes and bleeding or thrombosis. Correlating observations for platelet-specific changes and parameters for the plasma coagulation system may provide a better picture for the overall haemostatic state hence may aid in identifying more reliable indicators of bleeding or thrombosis for the ECMO patients.

Although with existing limitations, this is the first study with detailed evaluation of platelet-specific changes in relation to their phenotype, function and interactions with monocytes and neutrophils in the paediatric ECMO population. Findings from this study provide the first step in understanding the haemostatic system for especially the important role of platelets in coagulation and the development of bleeding or thrombosis in this population.

Conclusions

Whole blood flow cytometry is a reliable platelet function test for paediatric patients on ECMO. Using the multifaceted flow cytometry panels that allowed the assessments of platelet phenotype, function and interactions with monocytes and neutrophils, the first evidence for platelet-specific changes and their associations with the development of bleeding or thrombosis that differ according to a patient's pathway onto ECMO, age and duration of ECMO in children on ECMO were provided in this study. Further understanding of the platelet-specific changes and their mechanisms of causing bleeding or thrombosis may aid in identifying the key platelet-specific markers that could be used as the therapeutic targets with an overall aim to improve the outcome in this population.

9 Clinical significance and future directions

9.1 Clinical significance

The first evidence of using whole blood flow cytometry with multifaceted flow cytometry panels as a feasible and reliable platelet function test for paediatric patients on ECMO were provided in this study. This study provided new insights for the platelet-specific changes in this population and contributes to the understanding of how coagulopathy may develop via different types of platelet dysfunction according to a patient's pathway onto ECMO, age, and duration of ECMO. Together, these information will enable the development of medical interventions specific to each patient group and reduce complications related to bleeding and thrombosis with the ultimate aim to improve the outcome of the paediatric ECMO population. Since ECMO is a type of the artificial circulation, the findings from this study may also have implications on the other form of MCS such as CPB and VAD.

9.2 Future directions

Future research endeavours in continuing the effort to identify the key platelet-specific changes and their associations with bleeding and/or thrombosis, the main complications in the paediatric ECMO population.

9.2.1 Correlate results from flow cytometric detection of analysis of platelet surface markers with findings in plasma and platelet transfusion data

ECMO represents an environment of high shear and shear-induced loss of platelet receptors e.g. GPIb/IX/V and GPVI receptors have been reported in various settings of MCS [205]. Also, rapid loss of markers from the circulating platelets upon granule exocytosis is known for the marker important for platelet activation i.e. P-selectin [111, 112]. Hence, measuring the plasma level of these platelet-specific markers using

technique such as ELISA will provide further information and/or confirm the observations from flow cytometric analysis. Furthermore, acquired von Willebrand syndrome has been reported for both the adults and children in the setting of MCS [205, 356]. Measuring both the VWF level and activity and correlate with the results for flow cytometric detection of platelet surface GPIb/IX/V receptor expression might provide information for this receptor-ligand pair interaction in children on ECMO.

Platelet transfusion is an integrated part of management for patients on ECMO and this population usually receive multiple platelet transfusions per day to maintain circulating platelet level at > $100x10^9$/L. Particularly in children on ECMO, stored platelets that are of adult origin with physiology and function [40, 41, 49] that are different from patient's own circulating platelets may contribute to the high rate of bleeding/clotting events seen in this population. Correlating results for flow cytometric analysis of platelets and platelet transfusion data might provide information for the potential effects of transfused platelets on platelet phenotype and function seen in children on ECMO.

9.2.2 Platelet reactivity and MPA/NPA-related markers as the indicator of coagulation state

Point-of-care testing such as ACT, APTT, TEG and ROTEM have been used to assess the state of coagulability for both the adults and children on ECMO. However, the limitations associated with these assays including device-dependent results and a lack of standardization may reduce their usefulness as a reliable clinical tool. Modifications of platelet response have been associated with bleeding in various setting of MCS [357]. In contrast, an increased platelet-leukocyte level is associated with prothrombotic state and an elevated MPA level can be found in patients with thromboinflammatory diseases [147, 218, 358]. Hence, flow cytometric analysis for the assessment of platelet reactivity and MPA/NPA-relevant markers may be a more reliable indicator of coagulability in

paediatric patients on ECMO. Furthermore, the defect of a particular platelet activation pathway involved in the coagulopathy seen could be identified using multiple platelet-activation markers and/or agonists (in addition to TRAP-6) for the platelet reactivity assay.

9.2.3 Larger sample size for subcategorization analysis

Complications relevant to bleeding and/or clotting remain the main cause of the high mortality rate in paediatric patients on ECMO despite with increasing experience and technical improvements over the years. Multiple attempts have been made to identify the factors associated with the outcome of children on ECMO so as to develop interventions accordingly to improve their survival. In addition to the diagnosis (cardiac vs. respiratory), mode of ECMO (VA vs. VV) and cannulation strategy (central vs. peripheral) have been identified as some of these factors [152, 359]. Hence, a larger cohort of paediatric ECMO patients may allow sufficient data for further subcategory analysis (for the age, pathway onto ECMO and duration of ECMO) according to their mode of ECMO and cannulation strategy so as to identify the association between platelet dysfunction and coagulopathy in these patient groups.

9.2.4 Individualized antiplatelet therapy

The ECMO population represents a complex group of patient cohort of which their existing medical conditions can be further complicated by the proinflammatory state associated with the multiple surgical interventions, drugs and transfusions received during ECMO. Their coagulation system thus could represent the result of a complex interaction between the haemostatic and inflammatory systems. Furthermore, multiple recent evidence have highlighted the important roles of platelets in bridging haemostasis and inflammation via their interactions with leukocytes [248, 360]. The multifaceted flow cytometry panels that assess platelets from different aspects for their phenotype, function and interactions with leukocytes allow detailed evaluation of platelet-specific changes in

children on ECMO. Hence, correlating the differences seen for each of the platelet-relevant markers across panels may aid in the development of individual-specific platelets profile according to a patient's age, pathway onto ECMO and duration of ECMO. Such platelets profile could further be used to stratify the patients according to their risks for bleeding and/or thrombosis hence enable tailoring of therapeutic approach e.g. anti-platelet therapy accordingly.

NO and epoprostenol were used as the antiplatelet agents for children on ECMO in this study. However, to date, there is very limited information available for the platelet inhibitory effects of these two drugs in children, moreover, in the paediatric ECMO population. Future study that involves *in vitro* investigation of the effects of epoprostenol and NO on TRAP-6-stimulated platelets will be important to provide clue for how the platelet function may change in relation to the interplay between the stimulatory and inhibitory agonists in the paediatric ECMO population. Furthermore, since ECMO patients receive multiple medications as part of their management, it will also be important to investigate the usage of the other anti-platelet agents in addition to NO and epoprostenol and the other drugs used in patients of different disease aetiology before coming onto ECMO and during ECMO that could have an effect on the platelet phenotype and function seen in this population.

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APPENDICES

Appendix I Reagents used for flow cytometry

Table 1: List of monoclonal antibodies and manufacturers for flow cytometry experiments.

Description	Clone	Isotype	Catalogue number	Manufacturer
CD42b-FITC	HIP1	Mouse IgG1,κ	555472	Becton Dickinson Pharmingen, USA
CD61-APC	VI-PL2	Mouse IgG1, κ	564174	Becton Dickinson Pharmingen, USA
GPVI-PE	HY101	Mouse IgG1, κ	565241	Becton Dickinson Pharmingen, USA
FITC, IgG1, k	MOPC-21	Mouse (BALB/c) IgG1,κ	555748	Becton Dickinson Pharmingen, USA
PE, IgG1, k	MOPC-21	Mouse IgG1, κ	555749	Becton Dickinson Pharmingen, USA
APC, IgG1, k	MOPC-21	Mouse IgG1, κ	554681	Becton Dickinson Pharmingen, USA
CD41-ECD	P2	Mouse IgG1, chain not mentioned	6607117	Beckman Coulter, USA
CD62P-APC	AK-4	Mouse IgG1,κ	561920	Becton Dickinson Pharmingen, USA
PAC-1-FITC	SP-2	Mouse IgM, κ	340507	Becton Dickinson, USA
CD63-PE	H5C6	Mouse IgG1,κ	557305	Becton Dickinson Pharmingen, USA
CD62P-PE	AC1.2	Mouse IgG1,κ	550561	Becton Dickinson Horizon, USA
CD14-BV711	MφP9	Mouse (BALB/c) IgG2b,κ	563373	Becton Dickinson Horizon, USA
CD11b-BV421	ICRF44	Mouse IgG1,κ	562632	Becton Dickinson Pharmingen, USA
BV421, IgG1, k	X40	Mouse IgG1,κ	562438	Becton Dickinson Pharmingen, USA

Table 2: List of reagents and manufacturers for flow cytometry experiments.

Description	Catalogue Number	Manufacturer
Bovine serum albumin	A3294-50G	Sigma-Aldrich, USA
Thrombin-receptor activator peptide 6	T1573-5MG	Sigma-Aldrich, USA
Eptifibatide acetate	SML-1042	Sigma-Aldrich, USA
HEPES	H3375-100G	Sigma-Aldrich, USA
Sodium chloride	1064040500	Sigma-Aldrich, USA
Formaldehyde solution	F1635-500ML	Sigma-Aldrich, USA
BD FACS Lysing Solution	349202	Becton Dickinson, USA

Appendix II Buffer preparation for flow cytometry

a. HEPES-buffered saline (HEPES-NaCl) (10X)

HEPES	11.92 g
NaCl	43.83 g
MiliQ	500 mL

HEPES-NaCl was prepared as 10X stock concentration by diluting HEPES and NaCl in MiliQ following the amount and volume as indicated above and stored at -20°C.

b. 10 % Bovine Serum Albumin (BSA)

BSA	4.00 g
MiliQ	40 mL

10% BSA was prepared by diluting BSA in MiliQ following the amount and volume as indicated above.

c. Staining buffer, HSB

HEPES-NaCl (10X)	4 mL
10% BSA	4 mL
MiliQ	30 mL

HSB was prepared by mixing the reagents following the volume as indicated above and pH was adjusted to 7.3-7.4 with 1M NaOH.

d. 1% Formaldehyde

Formaldehyde	270 µL
HSB	10 mL

1% formaldehyde was prepared by mixing the reagents following the volume as indicated above.

e. BD FACS Lysing Solution

BD FACS Lysing solution was diluted in 1:10.

2. Monoclonal antibodies and panel antibody mixtures

2.1 Antibody panels for the assay of platelet phenotype and function (Panel 1)

a. PLT Mix

The antibodies were mixed and made up to 12 μL/test.

Description	Concentration (μg/mL)	Cat#
FITC Mouse Anti-Human CD42b	5	555472
APC Mouse Anti-Human CD61	7.5	564174
PE Mouse Anti-Human GPVI	0.5	565241

b. PLT Isotype

The antibodies were mixed and made up to 12 μL/test.

Description	Concentration (μg/mL)	Cat#
APC Mouse Anti-Human CD61	7.5	564174
FITC Mouse IgG1 isotype control	5	555748
PE Mouse IgG1 isotype control	0.5	555749

2.2 Antibody for the assay of the response of platelets to thrombin receptor activator peptide 6 (TRAP-6) (Panel 2)

a. PLT-ACT Mix

The antibodies were mixed and made up to 11 μL/test.

Description	Concentration (μg/mL)	Cat#
ECD Mouse Anti-Human CD41	0.5	6607117
APC Mouse Anti-Human CD62P	0.3	561920
FITC Mouse Anti-Human PAC-1	5	340507
PE Mouse Anti-Human CD63	2.5	557305

b. PLT-ACT Isotype

The antibodies were mixed and made up to 11 μL/test.

Description	Concentration (μg/mL)	Cat#
ECD Mouse Anti-Human CD41	0.5	6607117
APC Mouse IgG1 isotype control	0.3	554681
PE Mouse IgG1 isotype control	2.5	555749
FITC Mouse Anti-Human PAC-1	5	340507
Eptifibatide acetate	5	SML-1042

c. FMO Control Setup

Fluorochrome	FMO-APC	FMO-FITC	FMO-PE
ECD	CD41	CD41	CD41
APC	-	PAC-1	CD63
FITC	CD62P	-	CD63
PE	CD62P	PAC-1	-

2.3 Antibody for the assay of the interaction of platelets with monocytes and neutrophils (Panel 3)

a. PLT-LEU Mix

The antibodies were mixed and made up to 27.8 μL/test.

Description	Concentration (μg/mL)	Cat#
BV711 Mouse Anti-Human CD14	7.0	563373
BV421 Mouse Anti-Human CD11b	6.6	562632
APC Mouse Anti-Human CD61	7.5	555472
PE Mouse Anti-Human CD62P	0.6	550561

b. PLT-LEU Isotype

The antibodies were mixed and made up to 27.8 µL/test.

Description	Concentration (µg/mL)	Cat#
BV711 Mouse Anti-Human CD14	7.0	563373
BV421 Mouse IgG1 isotype control	6.6	562438
APC Mouse IgG1 isotype control	7.5	554681
PE Mouse IgG1 isotype control	0.6	555749

c. FMO Control Setup

Fluorochrome	FMO-BV711	FMO-APC	FMO-PE
BV711	-	CD14	CD14
BV421	CD11b	CD11b	CD11b
APC	CD61	-	CD61
PE	CD62P	CD62P	-

Appendix III Flow cytometry gating strategies

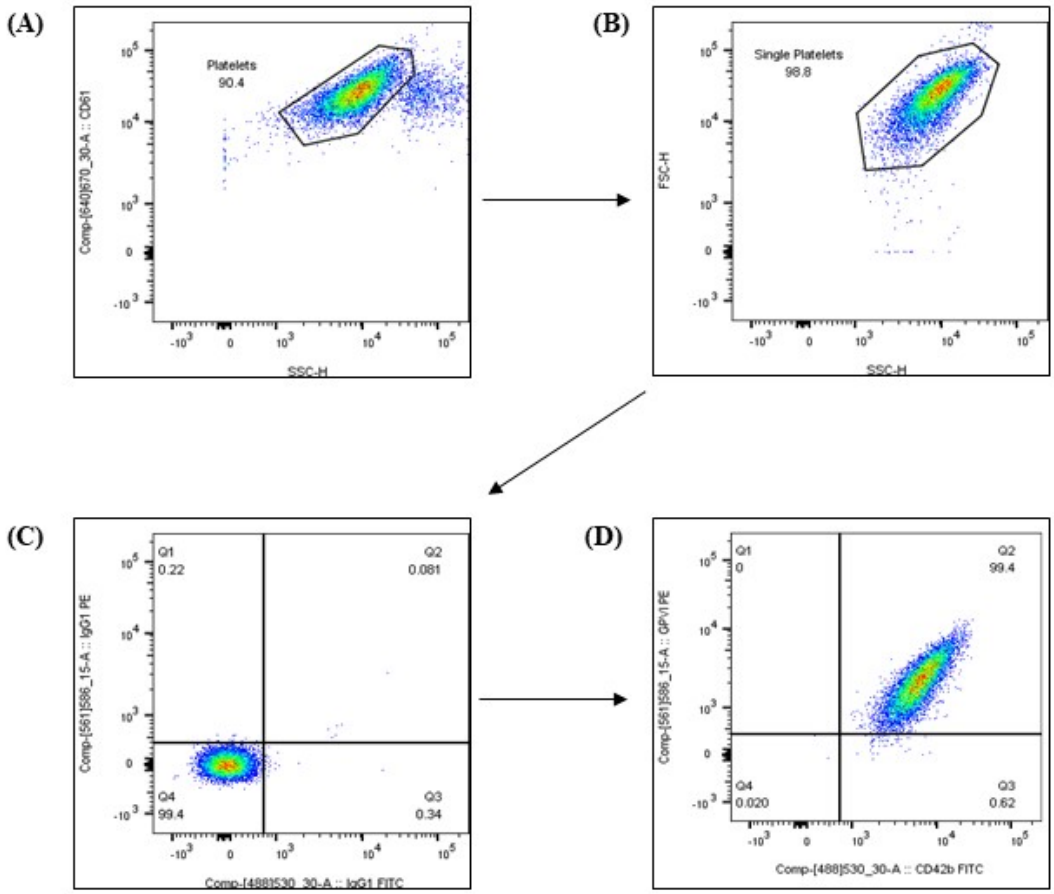


Figure 1: Gating strategy for platelet phenotype assay.

Flow cytometry detection of platelet. Platelets were identified by characteristic forward- and side-light scatter and GPIIIa (CD61) via double gating of events and threshold on CD61 (Panel A and B). Population positive for VWF (GPIb/IX/V) and collagen (GPVI) receptors (Panel D) expression was identified via gating threshold set by the respective negative isotype matched control (Panel C).

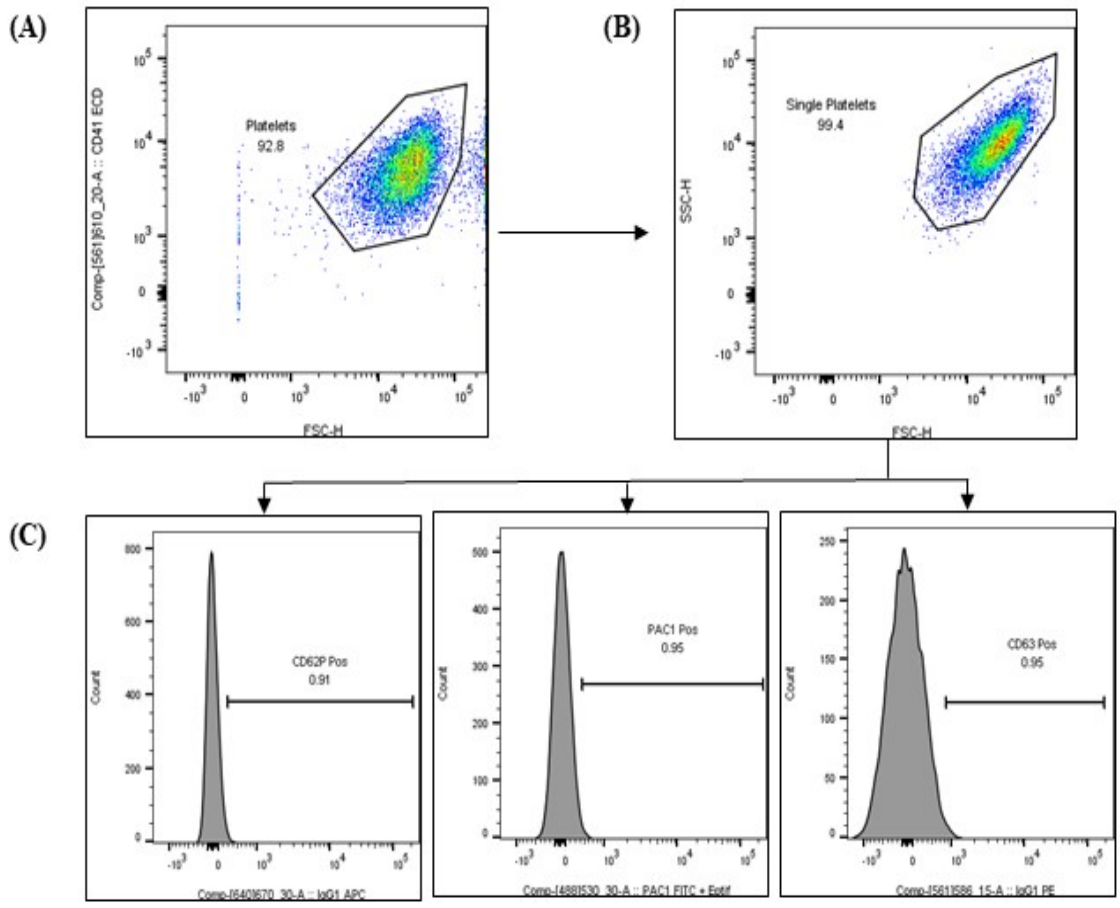


Figure 2: Gating strategy of the assay for the markers of platelet activation and response.

The detection of platelet activation markers including α-granule release indicator (P-selectin, CD62P), activated fibrinogen (integrin αIIbβ3) receptor (as measured by PAC-1 binding) and lysosome release indicator (CD63). Platelet population was identified by characteristic forward- and side-light scatter and GPIIb (CD41) via double gating of events and threshold on CD41 (Panel A and B). Population positive for P-selectin, PAC-1 and CD63 was identified via gating threshold set by the respective negative isotype matched control with eptifibatide inhibition of PAC-1 (Panel C).

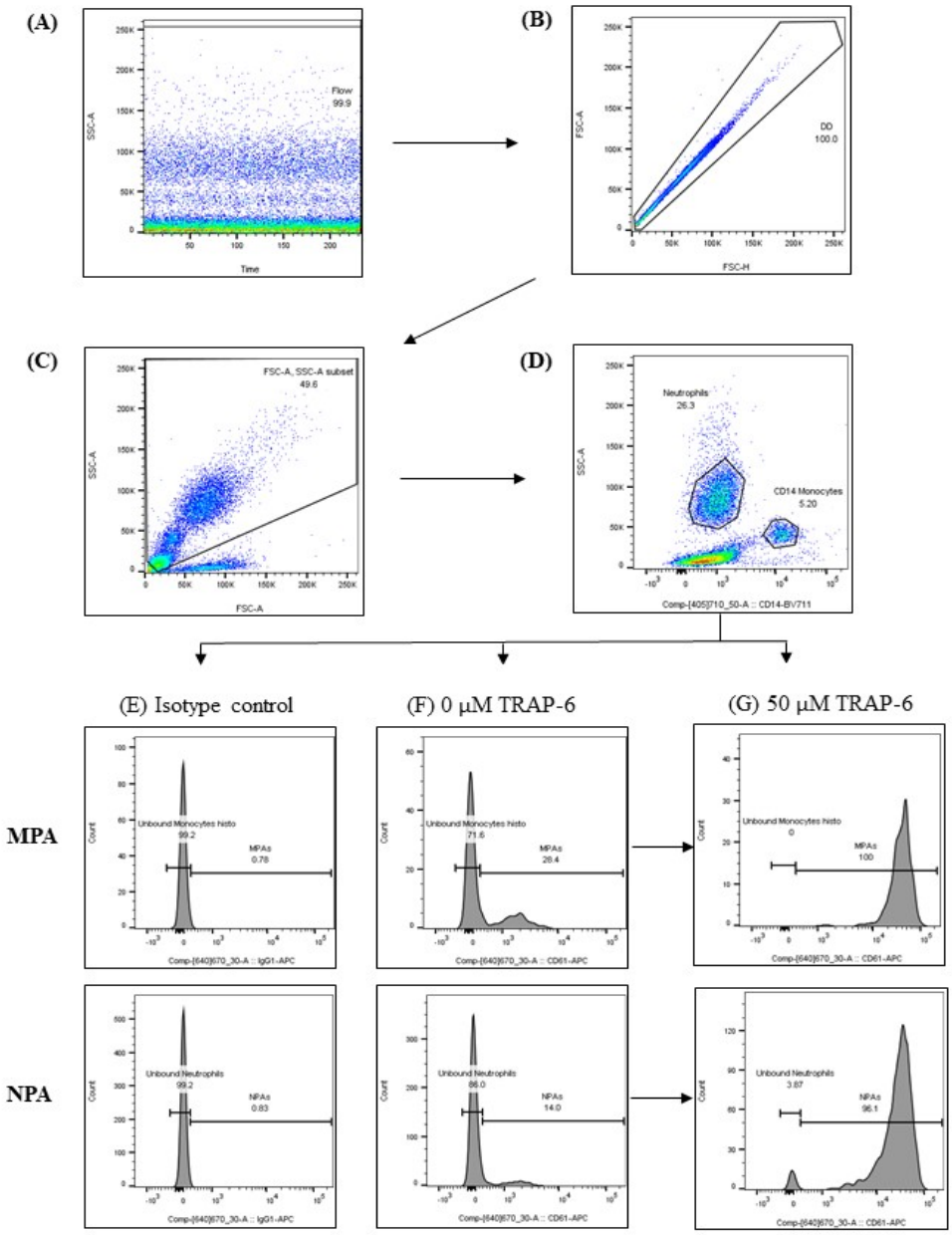


Figure 3: Gating strategy for monocyte/neutrophil-platelet aggregates (MPA/NPA) assay.

Flow cytometry detection of MPA/NPA. A gate for flow (to include region with constant flow) (Panel A) and doublet discrimination (DD) (to exclude larger leukocyte-leukocyte aggregates) (Panel B) was created, respectively. Monocytes and neutrophils were identified by the characteristics forward and side light scatter (Panel C) and low (neutrophil) and high (monocyte) expression of CD14 (Panel D). MPA/NPA population was identified by gating threshold set by the matched isotype (Panel E). Circulating MPA/NPA level at 0 µM TRAP-6 (Panel F) and increased upon stimulation with 50 µM TRAP-6 (Panel G).

Appendix IV Ethics Approval

ETHICS APPROVAL & GOVERNANCE AUTHORISATION

The Royal Children's Hospital Melbourne

16 February 2016

Prof Paul Monagle
Haematology
The Royal Children's Hospital

Dear Prof Monagle

Project Title: Coagulation Pathophysiology in Children on Extracorporeal Membrane Oxygenation (ECMO)

HREC Reference Number: HREC/15/RCHM/123
SSA Reference Number: RCH/15/RCHM/127
RCH HREC Reference Number: 35252A

I am pleased to advise that the above project has received ethical approval from The Royal Children's Hospital Melbourne Human Research Ethics Committee (HREC). The project has also received governance authorisation at the Melbourne Children's Campus (incorporating The Royal Children's Hospital, Murdoch Children's Research Institute and the University of Melbourne Department of Paediatrics).

The Royal Children's Hospital Melbourne HREC is organised and operates in accordance with the National Health and Medical Research Council's (NHRMC) National Statement on Ethical Conduct in Research Involving Humans (2007) and all subsequent updates, and in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95), the Health Privacy Principles described in the Health Records Act 2001 (Vic) and Section 95A of the Privacy Act 1988 (and subsequent Guidelines).

HREC Approval Date: 16 February 2016
Please note the HREC are no longer issuing pre-determined approval periods. Ethical approval is now ongoing, subject to the submission of an annual report on the anniversary of approval.

Participating Sites:
Ethical approval for this project applies at the following sites:

Site Name
The Royal Children's Hospital and Murdoch Childrens Research Institute

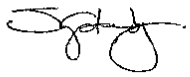
Approved Documents:
The following documents have been reviewed and approved:

Document	Version	Date
Protocol	V2	7 Jan 2016
PGIS	V2	4 Jan 2016
PGIS (delayed consent)	V2	4 Jan 2016
PIS	V1	14 Jan 2016
PIS (delayed consent)	V1	14 Jan 2016
Survey Invitation Letter	V3	6 Jan 2016

Conditions of Ethics Approval:
- You are required to submit to the HREC:

    - An Annual Progress Report (that covers all sites listed on approval) for the duration of the project. This report is due on the anniversary of HREC approval. Continuation of ethics approval is contingent on submission of an annual report, due within one month of the approval anniversary. Failure to comply with this requirement may result in suspension of the project by the HREC.
    - A comprehensive Final Report upon completion of the project.
- Submit to the reviewing HREC for approval any proposed amendments to the project including any proposed changes to the Protocol, Participant Information and Consent Form/s and the Investigator Brochure.
- Notify the reviewing HREC of any adverse events that have a material impact on the conduct of the research in accordance with the NHMRC Position Statement: *Monitoring and reporting of safety for clinical trials involving therapeutic products May 2009*.
- Notify the reviewing HREC of your inability to continue as Coordinating Principal Investigator.
- Notify the reviewing HREC of the failure to commence the study within 12 months of the HREC approval date or if a decision is taken to end the study at any of the sites prior to the expected date of completion.
- Notify the reviewing HREC of any matters which may impact the conduct of the project.
- If your project involves radiation, you are legally obliged to conduct your research in accordance with the Australian Radiation Protection and Nuclear Safety Agency Code of Practice 'Exposure of Humans to Ionizing Radiation for Research Purposes' Radiation Protection series Publication No.8 (May 2005)(ARPANSA Code).
- The HREC, authorising institution and/or their delegate/s may conduct an audit of the project at any time.

Yours sincerely



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