UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Characterisation of the Lipidome of Transfusible Platelet Components

by Sarah Green

Thesis submitted in fulfilment of the requirements for the degree of

Master of Science

under the supervision of Dr Lacey Johnson and Dr Matthew Padula

University of Technology Sydney
Faculty of Science

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Certificate of original authorship

I, Sarah Green, declare that this thesis is submitted in fulfilment of the requirements for the award of Master of Science, in the Faculty of Science, School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other institution.

This research is supported by the Australian Government Research Training Program.

30th July 2022

Sarah Green

COVID-19 Impact Statement

The COVID-19 pandemic had a dramatic impact on the progression of my studies and personally impacted me by restricting travel and access to the laboratory to complete my studies. In March 2020, I departed Australia and travelled to the United States of America to visit my husband who lives there. This was intended as a short-term holiday. However, over the next few weeks borders quickly closed and returning to Australia became near impossible. Australian borders were closed for well over 1.5 years and even once they opened, flights were prohibitively expensive and extremely limited. The situation was incredibly uncertain and I prioritised staying with my husband.

Additionally, I was working to obtain my US residency, as upon completion of my studies I was intending to move to the US, and issues arose around this process. It was suggested by our immigration lawyer that I remain in the US until the immigration proceedings could be completed or risk having my US residency application forfeited permanently. Again, I choose to prioritise my husband and my family. Given the border closures and my personal situation if I had returned to Australia, I would have been unable to see my husband for 2 years. It is my belief that being apart from my husband for 2 years would have been extremely detrimental to my own mental health, the mental health of my husband and made progression of my research project extremely difficult.

With uncertainty surrounding border reopening and flights resuming, I took an over 18 month leave of absence, hoping at some point a state of normalcy would resume and I would be able to return to Australia, the laboratory and my studies. However, no such state returned and I was unable to return to Australia. Given this, I could not perform the experiments required to complete an additional results chapter required for a PhD thesis. This study was designed to assess the functional impact for the lipid changes observed in chapters 4 and 5. As such, in May of 2022, I made the decision to complete

data analysis on the data already collected and additional data collected by my supervisors to submit a Masters thesis.

Acknowledgements

First and most importantly, I would like to thank my supervisors, Dr, Lacey Johnson and Dr. Matt Padula. This experience has been less than perfect, and without your support, understanding and encouragement it would not have been possible. Lacey, thank you for being a passionate and thorough advisor who was far too generous with your time. Matt, thank you for your much valued expertise and suggestions. Additionally, I cannot thank you enough for your assistance in performing experiments while I was unable to travel, this work literally would not have been possible without your help.

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Lastly, I would like to thank the examiners for the time they have taken to review my thesis.

Statement

This is a traditional thesis, as such it contains an introduction, materials and methods, results, and discussion chapter.

Ethics Disclosure

Ethics approval was obtained for this study from the Australian Red Cross Lifeblood Ethics Committee (Johnson 1107218), and was ratified by the University of Technology Sydney Human Research Ethics Committee (ETH18-2795).

Publications

Publications arising from this thesis:

Green SM, Padula MP, Marks DC, Johnson L: The lipid composition of platelets and the impact of storage: An overview. *Transfusion Medicine Reviews* 2020; 34: 108-16.

Peer reviewed conference abstracts arising from this thesis:

Green SM, Padula MP, Dodgen TM, Batarseh A, Marks DC, Johnson L: Alternative storage of platelets alters the abundance of bioactive lipid mediators. *Transfusion* 2020; 60: 7A-260A.

Publications alongside this thesis:

Johnson L, Waters L, Green S, Wood B, Marks DC: Freezing expired platelets does not compromise in vitro quality: An opportunity to maximize inventory potential. *Transfusion* 2019; 60: 454-9.

Johnson L, Vekariya S, Wood B, Costa M, Waters L, Green S, Marks DC: The in vitro quality of x-irradiated platelet components in pas-e is equivalent to gamma-irradiated components. Transfusion 2021; 61: 3075-80.

Table of Contents

List of Figures

List of Tables

Abbreviations

ACD	Acid Citrate Dextrose
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BioPAN	Bioinformatics Methodology For Pathway Analysis
C1P	Ceramide 1-phosphate
C1PP	Ceramide 1-phosphate phosphatase
Ca^{2+}	Calcium
CCS	Collisional cross section
CDase	Ceramidase
CE	Cholesterol ester
Cer	Ceramide
CerK	Ceramide kinase
CerS	Ceramide synthase
CID	Collision induced dissociation
CLIP-II	Cryopreserved vs Liquid Platelet-II
COX	Cyclooxygenase
CPD	Citrate-phosphate-dextrose
DAG	Diacylglyceride
DIA	Data-independent acquisition
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
eV	Electronvolts
FDA	Food and Drug Administration
GLA	Gamma-carboxyglutamic acid-rich
GP	Glycoprotein
HDLs	High density lipoproteins
HETE	Hydroxyeicosatetraenoic acid
HLA	Human leukocyte antigens

IMS	Ion Mobility Spectrometry
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LCAT	Lectin-cholesterol transferase
LDLs	Low density lipoproteins
LOX	Lipoxygenase
LPA	Lysophophatidic acid
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
m/z	Mass-to-charge ratio
MAM	Mitochondria-associated membranes
MPV	Mean platelet volume
MS	Mass spectrometry
MS^E	Mass spectrometry experiment
MTBE	Methyl tert-butyl ether
OCS	Open canalicular system
PA	Phosphatidic acid
PAF	Platelet activating factor
PC	Phosphatidylcholine
PCO	Ether linked phosphatidylcholine
PE	Phosphatidylethanolamine
PEP	Ether linked phosphatidylethanolamine
PF	Pre-freeze
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIP	Phosphoinositide
PS	Phosphatidylserine
PSL	Platelet storage lesion
PT0	Post-thaw 0
PT24	Post-thaw 24
PVC	Polyvinylchloride

PVDF	Polyvinylidene fluoride
QToF	Quadrupole Time-of-Flight
RS	Reconstitution solution
RT	Room temperature
S1P	Sphingosine 1-phosphate
S1P lyase	Sphingosine 1-phosphate lyase
S1PP	Sphingosine 1-phosphate phosphatase
SK	Sphingosine kinase
SM	Sphingomyelin
SM D	Sphingomyelin deaclyase
SMase	Sphingomyelinase
SMS	Sphingomyelin synthase
sP-selectin	Soluble P-selectin
SSP+	Platelet additive solution
TAG	Triacylglyceride
TF	Tissue factor
TRALI	Transfusion related acute lung injury
TX	Thromboxane
UPLC	Ultra-pressure liquid chromatography
v/v	Volume/volume
vWF	von Willebrand factor

Abstract

Conventional room temperature stored platelets have a short shelf life and require constant agitation. As such, there can be issues when supplying these components to remote and military locations. Alternative storage techniques, such as cold storage and cryopreservation, represent feasible options to circumvent these issues. Cold storage involves storing platelet components in the refrigerator (2-6°C); whereas cryopreservation requires the addition of DMSO and freezing at -80°C. Extensive efforts have been undertaken to understand the changes occurring in cold-stored and cryopreserved platelet components. However, the lipid profile remained incompletely understood. A few historic studies have assessed the lipidome of cold-stored platelets, however, an updated assessment was warranted due to advancements in transfusion practices and mass spectrometry technology. Further, the lipidome of cryopreserved platelets had not yet been characterised. Therefore, the aim of this thesis was to characterise the changes occurring to the lipid profile of alternatively stored platelet components.

The characterisation of the lipid profile of cold-stored and cryopreserved platelet components was conducted as two discrete studies. Apheresis derived platelet components were stored at either room temperature (20-24°C with constant agitation) or cold-stored (2-6°C without agitation) and sampled on day 1, 5, and 14 post-collection. Buffy coat derived platelet components were frozen with 5-6% DMSO and stored at -80°C. Frozen components were thawed and then stored at room temperature for 24 hours, and samples were taken before freezing, after thawing and after post-thaw storage. The platelet, microparticle and supernatant fractions were separated by differential centrifugation. Lipids were extracted using methyl tert-butyl ether (MTBE) and the lipid profile of the component fractions were assessed by LC-MS/MS. Several bioactive lipid mediators were assessed by ELISA.

The lipid profile of platelets was relatively unchanged during storage for 5 days, regardless of temperature. However, over extended storage (14 days)

changes became apparent, and these were exaggerated by cold storage. Conversely, the lipid profile of the supernatant was changed during early storage at both temperatures, but changes stabilised during extended storage. These changes may be the result of an exchange of lipids between the fractions. More specifically, the proportion of the procoagulant lipids, PS and PE, increased during extended cold storage. Further, several LPC species associated with inflammation were altered during extended room temperature storage. Most interestingly, alterations were observed in apoptosis-associated ceramide species suggesting that cold storage of platelets may delay the progression of apoptosis.

The lipidome of the cryopreserved platelets was not considerably altered immediately after thawing. However, changes became apparent during post-thaw storage. In contrast, the lipid profile of microparticles formed after thawing was significantly different to the lipid profile of the microparticles present prior to freezing. The changes present after thawing are likely the result of interactions between fractions. More specifically, externalisation of lipids associated with coagulation (PS and PE) were increased immediately after thawing. Further, lipid changes present in the post-thaw microparticles and the supernatant (LPC and LPE) may be associated with altered inflammation and signalling.

Overall, the research presented in this dissertation has expanded the knowledge of alternatively stored platelet products and thus may be valuable in expanding their utility.

Chapter 1 Overview of thesis

This chapter provides an overview of the current state of transfusion practices within Australia. It briefly outlines alternatives to conventionally stored platelets, including what is currently understood and the knowledge gaps. Consequently, an understanding of the significance of this project is provided. The aims of the project are outlined, and the structure of the thesis is also summarised.

1.1 Background and significance of project

Currently within Australia, platelets are stored at room temperature with constant agitation, and the shelf life is limited to seven days. These storage requirements are in place to minimise the risk of bacterial proliferation and lessen the effects of the storage related changes that accumulate (i.e. 'the platelet storage lesion'). However, the storage requirements of conventionally stored platelets present issues in supplying rural and austere locations. The lengthy transport times and the logistical limitations may impact the supply and quality of platelet components. Further, as there is uncertainty in component demand, the potential for wastage is high. Thus, alternative storage solutions, such as cold storage and cryopreservation, may present viable options to counter these issues.

Cold storage of platelets and platelet cryopreservation both present attractive alternatives to room temperature storage as they increase the platelet shelf life to two weeks and up to two years, respectively. Cold storage of platelets involves storing platelets between 2-6 °C without agitation, and cryopreservation involves freezing at -80 °C with dimethyl sulfoxide (DMSO). Considerable work has been done to understand the changes occurring in platelet components as a result of storage at these conditions. As such, it is known that cold-stored and cryopreserved platelets have altered metabolism, surface receptor profile, proteome, and increased haemostatic potential, compared to room temperature stored platelets. Similarly, both storage methods have been assessed *in vivo*, where they have been shown to be safe, although their efficacy is still under investigation. Despite the expanding knowledge base of cold-stored and cryopreserved platelets, little is known regarding the lipid profile of these components.

Lipids are essential for platelet structure and function. While the lipidome of room temperature stored platelets has been characterised, the lipidome of cold-stored and cryopreserved platelets is yet to be fully assessed. Lipidomic investigations are of interest as the increased haemostatic potential seen in

alternatively stored platelet components is partially attributed to the externalisation of phospholipids. Further, the transformation of phospholipids to bioactive lipid mediators is essential for platelet activation and platelet function. Additionally, certain bioactive lipid mediators have been associated with transfusion outcomes, both favourable and adverse. As such, there is a need to characterise the lipidomic changes occurring as a result of alternative storage. This would provide greater insight into how alternative storage may affect component function and safety.

1.2 Hypothesis and aims of the project

As outlined above, significant efforts have been made to understand many facets of alternatively stored platelet components. However, comprehensive lipidomic studies are yet to be completed. As such, the overall aim of this study was to characterise the lipidome of alternatively stored platelet components. It was hypothesised that cold storage and cryopreservation would affect the platelet lipidome compared to conventionally stored platelets. To assess this, two primary aims were developed.

Aim 1: To characterise the lipidome of cold-stored platelets.

Aim 2: To characterise the lipidome of cryopreserved platelets.

1.3 Thesis structure

Chapter 2: Introduction

This chapter outlines the necessary background information regarding the origins, structure, and function of platelets. Additionally, a brief background to lipids and lipids within platelets is provided. As this project assessed lipids in the context of platelets for transfusion, the collection and preparation methods for platelet components are summarised, including conventional and alternative platelet storage modes. Lastly, the body of literature regarding the lipidome of stored platelet components is critically reviewed, highlighting the clinical importance of this project.

Chapter 3: Materials and methods

This chapter describes the materials used and the methods performed in order to complete the aims of this dissertation. Contents includes platelet component production, analysis of the platelet component, lipidomic assessments and the statistical analysis performed.

Chapter 4: Results: lipidomic characterisation of cold-stored platelet components

While historic studies have been conducted assessing the lipidome of cold-stored platelets, advances in transfusion practices and improved lipidomic technologies justify a reassessment of the lipid profile of cold-stored platelets. In this chapter, the lipidome of the platelets and supernatant of cold-stored platelet components are described. Cold-stored platelet components were compared to room temperature stored platelet components for 14 days. The lipid profile of platelets was relatively unchanged at day 5 of storage at both temperatures; however, changes were evident at day 14, and these were exacerbated by cold storage. Further, the lipid profile of the supernatant was changed at day 5 of storage at both temperatures, and the changes stabilised at day 14 of storage.

Chapter 5: Results: lipidomic characterisation of cryopreserved platelet components

To date, no studies have been conducted which assess the lipidome of cryopreserved platelets. In this chapter, the lipidome of the platelets, microparticle and supernatant fractions of cryopreserved platelets are described. Thawed platelet components were compared to paired platelet components prior to freezing. While the lipidome of the platelet fraction was not extensively altered by the cryopreservation process, post-thaw storage for 24 hours at room temperature resulted in significant changes. Further, the microparticles formed after thawing had a distinct lipid profile compared to those present prior to freezing. The lipid profile of the supernatant was primarily defined by the resuspension solution.

Chapter 6: Discussion: lipidomic characterisation of alternatively stored platelet components

The results from this study outlined changes to the phospholipid and sphingolipid profile during cold storage and following cryopreservation, which are further discussed in this chapter. The changes to the cold-stored platelet component may have arisen due to exchanges of lipids between fractions and altered lipid metabolism; whereas the changes occurring following cryopreservation are likely the result of uptake from lipoproteins, microparticle formation and platelet or microparticle degradation. Functionally, the observed changes are associated with coagulation, apoptosis, inflammation and signalling. The changes that occurred in both cold-stored and cryopreserved platelet components are likely to affect the function once components are transfused; as such, further areas of study are outlined to more comprehensively understand the functional implications of these changes once components are transfused.

Chapter 2 Introduction

This chapter outlines the necessary background information regarding the origins, structure, and function of platelets. Additionally, a brief background to lipids and lipids within platelets is provided. As this project assessed lipids in the context of platelets for transfusion, the collection and preparation methods for platelet components are summarised, including conventional and alternative platelet storage modes. Lastly, the body of literature regarding the lipidome of stored platelet components is critically reviewed, highlighting the clinical importance of this project.

2.1 Platelets

2.1.1 Origins of platelets

Platelets are formed through the fragmentation of megakaryocytes [1-3]. The platelet precursor, megakaryocytes, are the largest (50-100 μm) and rarest progenitor cell within bone marrow [4]. Platelet formation can be summarised in several key steps that takes place, *in vivo,* over approximately five days (Figure 2.1). Initially, beginning from haematopoietic stem cells, megakaryocytes undergo maturation, which involves chromosomal replication, including the synthesis of organelles and granules [4-6]. The megakaryocyte then undergoes cytoplasmic changes and the formation of pseudopods [7, 8]. Lastly, platelets are released into the circulation, via the formation of pro-platelets which are formed when the cytoplasm erodes and the organelles and granules are caught within the ends of the protrusions [8, 9]. Each megakaryocyte is capable of releasing 5 000-10 000 platelets [10].

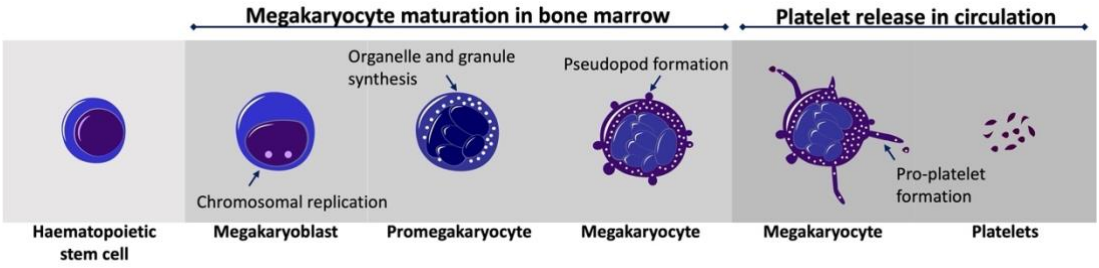

Figure 2.1. Platelet production and release from megakaryocytes

Platelets are derived from megakaryocytes. In the bone marrow, megakaryocytes undergo several key maturation steps. These steps include the replication of chromosomes, the synthesis of organelles and granules and pseudopod formation. The cytoplasm dismantles and pro-platelet formation begins. The formed organelles and granules are trapped in the ends of the pro-platelets, which are released into the circulation forming platelets. (Image by author, generated using Motifolio Biology Toolkit Suite).

2.1.2 Resting platelets

Platelets are small anucleate discoid cells, approximately 2-4 μm in diameter, that circulate within the vascular system and have a lifespan of 7-10 days [10, 11]. The normal platelet count of whole blood is 150-400 x 10^3/μL [10]. *In vivo*, platelets have an essential role in the maintenance of haemostasis, control of thrombosis and in mediating immune responses [10, 11].

Regardless of their small size, platelets contain a complex array of organelles and express a number of surface receptors which allow for their diverse and dynamic function (Figure 2.2) [12, 13]. The platelet membrane is a lipid bilayer primarily composed of phospholipids interspersed with other lipids, including cholesterol, sphingolipids, and glycoproteins (GP), including GPIb-IX-V, GPVI, integrin αIIbβ3 and integrin α2β1 [10, 14]. Glycoproteins and integrins are important for platelet signalling during activation and facilitate the haemostatic response [13, 14]. Throughout the membrane surface are invaginations, referred to as the open canalicular system (OCS), which provides a channel for the transport of contents to and from the platelet, and provides the membrane surface area needed for platelet spreading following platelet activation [14, 15]. Within the platelet are alpha granules and dense granules which contain a variety of soluble mediators, and a dense tubular system which stores calcium [12, 14]. Platelets also contain mitochondria, necessary for meeting the energy requirements of the cell, and a well-purposed cytoskeleton [12, 14].

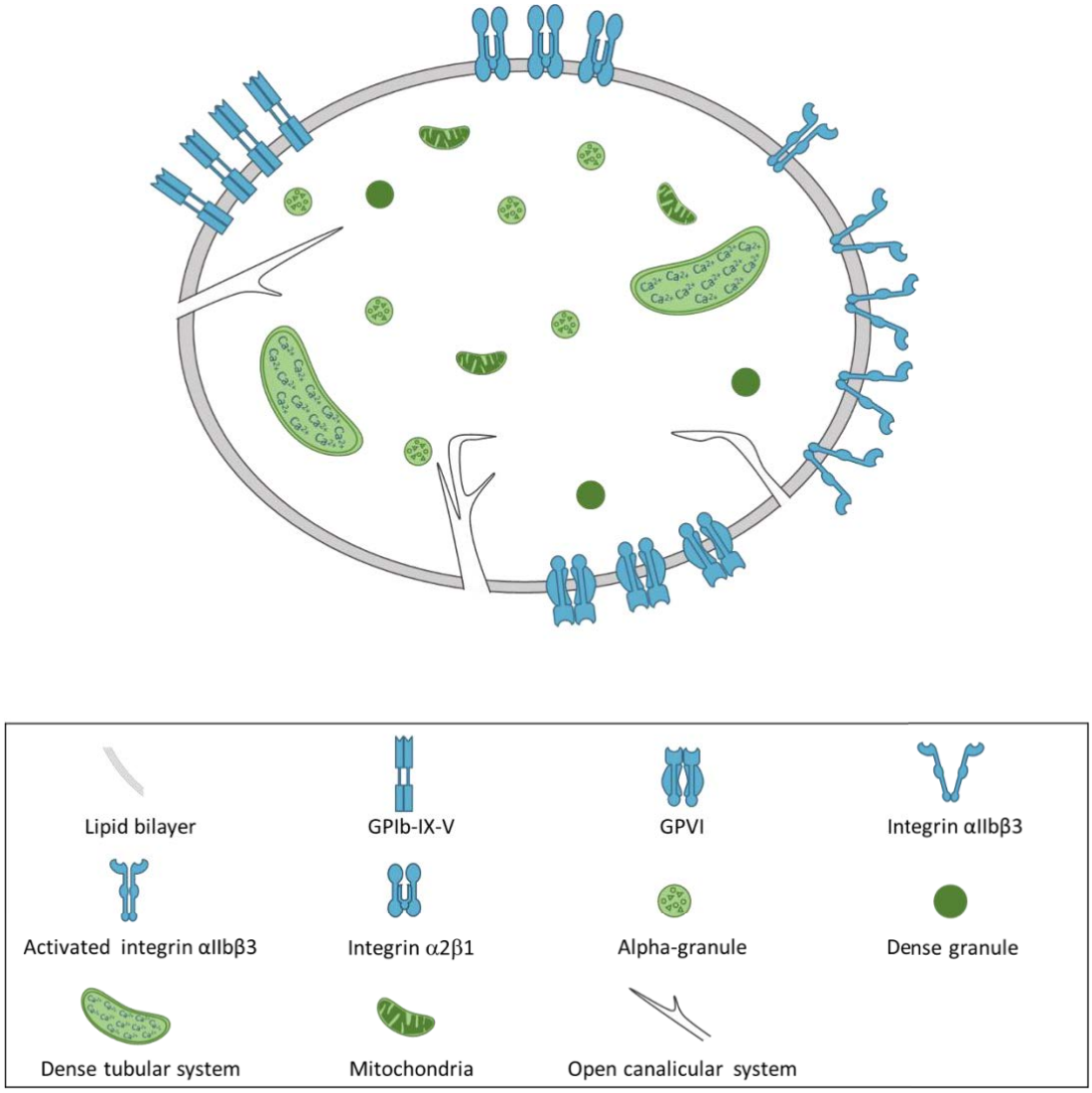

Figure 2.2. Schematic of platelet structure

Platelets contain an assortment of organelles. Within resting platelets are mitochondria, alpha and dense granules which contain a number of soluble mediators, and a dense tubular system that houses calcium (Ca^{2+}) stores. The platelet membrane is a lipid bilayer composed mostly of phospholipids and dispersed throughout are glycoproteins (GP) required for platelet signalling. Platelets also contain membrane invaginations that allow for transport of vesicles and increased surface area during activation, referred to as the open canalicular system. (Image by author, generated using Motifolio Biology Toolkit Suite).

2.1.3 Platelet activation and haemostatic function

One of the primary functions of platelets is to maintain haemostasis through the formation of a platelet plug at the site of vascular injury [16, 17]. The key steps of this process are tethering, adhesion, activation, aggregation, coagulation, and stabilisation through fibrin formation (Figure 2.3) [16, 17].

Haemostasis is initiated when vascular injury exposes collagen and other platelet activators. This process leads to the adhesion of platelets to the site of injury [16, 17]. Exposed collagen captures von Willebrand factor (vWF) circulating within the bloodstream [18]. Platelets are able to form an impermanent interaction (tethering) with the site of injury through the binding of GPIbα (a subunit of the GPIb-IX-V complex) to vWF [18]. Firm adhesion is then formed through the binding of GPVI and integrin $\alpha 2\beta 1$ to collagen [16, 18].

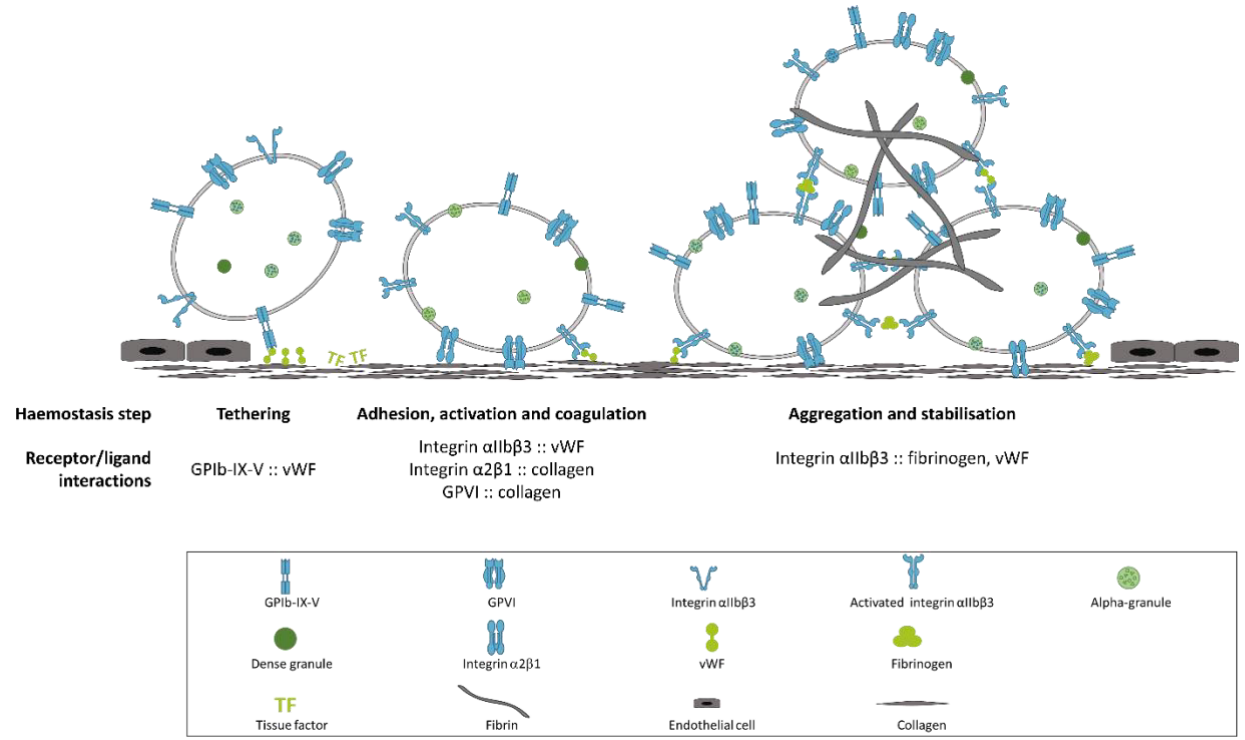

Figure 2.3. Haemostatic function of platelets

The primary role of platelets is the restoration of haemostasis, achieved through the formation of a platelet plug and initiation of coagulation. Collagen exposed at the site of vascular injury captures platelets through interaction of GPIb-IX-V with von Willebrand factor (vWF) (tethering). At the same time, tissue factor (TF) released at the site of injury initiates the coagulation cascade. This initial capture activates platelets allowing the release of granule contents and further receptor interaction, including GPVI and integrin $\alpha 2\beta 1$ with collagen (adhesion). Integrin αIIbβ3 becomes activated, which facilitates platelet-platelet interactions (aggregation) through fibrinogen and vWF. The coagulation cascade culminates in the formation of thrombin and fibrin and a stable platelet plug (Image by author, generated using Motifolio Biology Toolkit Suite).

Following surface receptor binding to agonists, platelet activation occurs [16, 18]. Initially, downstream signalling, which is mediated by tyrosine kinase and G protein-coupled receptor signalling, leads to the mobilisation of calcium [19, 20]. Several of these pathways involve the transformation of phospholipids to bioactive lipid mediators [19, 21]. Cytosolic calcium is increased due to the release from the dense tubular network and the influx of calcium via activated calcium channels in the plasma membrane [19, 21]. These signals culminate in dramatic changes to the platelet including a morphological shape change, cytoskeleton reorganisation, externalisation of phosphatidylserine (PS), microparticle formation, the release of intracellular granules, and conversion of surface receptors to a more activated state [19, 20, 22-25].

Activated platelets are distinct from resting platelets. Platelet shape change involves formation of filopodia and platelet spreading that increases the platelet surface area, necessary for establishing connections during aggregation [26]. Simultaneously, during the platelet activation process, membrane asymmetry is lost, resulting in the release of microparticles and the externalisation of PS [27, 28]. These processes will be discussed in greater depth in section 2.1.3.1 and 2.1.4.2. Platelets release alpha and dense granules during activation, which contain potent mediators of platelet adhesion and activation, including adenosine diphosphate (ADP), calcium, thromboxane A_2 and P-selectin [17, 18, 21, 29, 30]. In addition, platelet activation changes the conformation of integrin αIIbβ3 receptor to allow high affinity binding to fibrinogen, thereby allowing the platelet-platelet interactions which are necessary for aggregation [19, 31].

In parallel to platelet adhesion and activation, the tissue factor released from the site of injury by cells, such as smooth muscle and fibroblasts, initiates the coagulation cascade. Both the intrinsic and extrinsic pathway involve a series of proteolytic reactions of coagulation proteins, which culminate in the formation of the tenase complex (Factor IXa and Factor VIIIa). The formation of tenase is necessary to form the prothrombinase complex (Factor Xa and Factor Va) [18, 32, 33]. The action of the prothrombinase complex leads to the

formation of a large amount of thrombin, which is responsible for the conversion of fibrinogen to fibrin (stabilisation) [18, 32, 34]. Importantly, several steps in the coagulation cascade are dependent on platelet membrane phospholipids, which will be discussed further in section 2.1.4.2.

2.1.3.1 Platelet microparticles and platelet microparticles formed during activation

Microparticles arise from many different cell types, however, those derived from platelets are the most abundant in the bloodstream [35-37]. Microparticles can be classified based on size and the expression of markers on the surface [38]. Microparticles are defined as being between 0.1 – 1 μm in diameter and typically have exposed PS, while other particles, such as exosomes, are smaller in size (less than 0.1 μm) and express CD9 or CD63 [38-40]. Additionally, platelet microparticles can be further discriminated from other microparticles due to their expression of receptors typically found on platelets, including integrin αIIb, integrin β3 and GPIbα [41]. Additionally, microparticles are known to contain protein, RNA, and lipids [28, 42-44].

The formation of microparticles in the circulation arise due to processes such as apoptosis, shear forces and activation [35, 37]. The biological process leading to the formation of microparticles remains relatively unknown [41]. However, it is evident there is a rise in intracellular calcium, a loss in lipid membrane asymmetry of the parent cell and a degradation of cytoskeletal proteins [45, 46]. Increasingly, it is becoming apparent that microparticles play an important role *in vivo,* as they to transport molecules, participate in cell to cell communication and the inflammatory process, contribute to the restoration of haemostasis and play a part in disease processes [36, 47]. The function of microparticles is believed to be dependent on the mechanism of generation, which may influence the number, size, lipid profile, protein expression and molecules packaged within the microparticles [37, 48]. Further, the number of microparticles produced and the composition of the microparticles is also affected by the source of platelet activation [28, 49].

2.1.4 Lipid classes and their role in platelets

Platelets are composed of lipids from several categories, including phospholipids, sterols, sphingolipids, free fatty acyls and glycerolipids (Table 2.1) [27, 50-52]. These categories are distinguished from one another by a functional group or structural motif, and are further classified by fatty acyl chain length and double bond number and position (Figure 2.4) [53]. In addition, platelets contain an extensive number of bioactive lipid mediators, which are produced from parent lipids via specific pathways and are important signalling molecules [54]. Platelet lipids have distinct functional roles, which are briefly outlined below.

Table 2.1. Distribution of lipids within resting platelets and an indication of changes occurring in response to platelet activation

	Percentage distribution of lipids in resting platelets (%) *	Changes following thrombin activation†
Cholesterol	27.3 (24.9-30.0)	ND
Phosphatidylcholine (PC)	25.8 (24.6-28.0)	Unchanged
Phosphatidylethanolamine (PE)	13.8 (13.0-15.0)	Unchanged
Sphingomyelin (SM)	11.7 (11.3-12.0)	Unchanged
Phosphatidylserine (PS)	10.3 (10.0-11.0)	Unchanged
Phosphatidylinositol (PI)	2.4 (1.0-3.2)	Decreased
Cholesterol Ester (CE)	1.96 (1.9-2.0)	ND
Lysophosphatidylcholine (LPC)	1.2 (0.8-2.0)	Increased
Ceramide	0.76 (0.3-1.0)	ND
Phosphatidylglycerol (PG)	0.75 (0.5-1.0)	Unchanged
Phosphatidic Acid (PA)	ND	Increased
Lysophosphatidylethanolamine (LPE)	ND	Increased
Lysophosphatidic Acid (LPA)	ND	ND

*Values reported as mean (range); adapted from references [55-57]
†The described changes in response to thrombin activation (decreased, increased, unchanged) are compared to resting platelets; adapted from references [27, 50]
Not determined (ND)

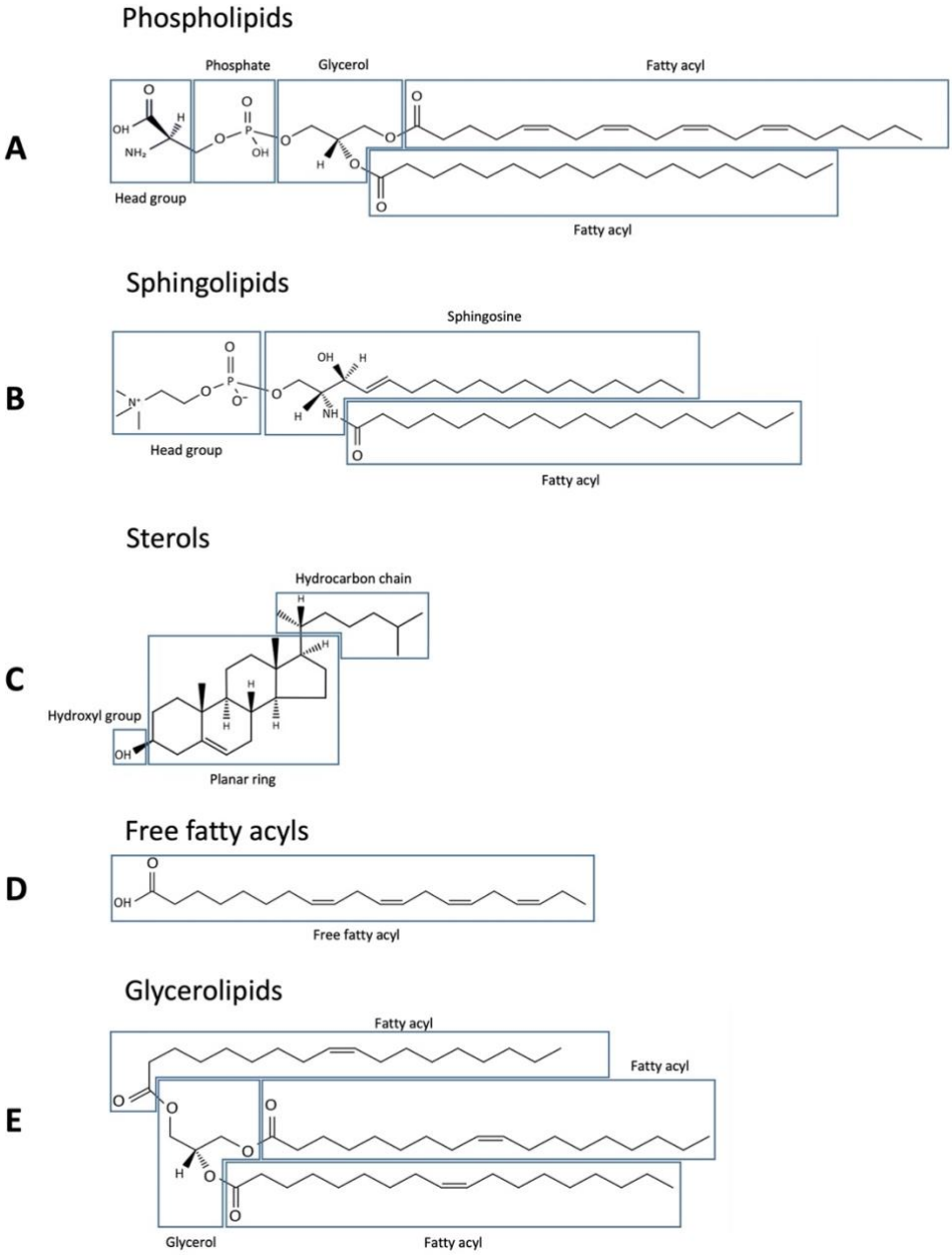

Figure 2.4. General structure of lipids within platelets

(A) Phospholipids are comprised of a head group attached to two fatty acyl tails, shown phosphatidylserine. (B) Sphingolipids are formed from a sphingosine motif, to which the attachment of a fatty acyl tail forms the ceramide class and the addition of head groups forms more complex sphingolipids, such as sphingomyelin; shown sphingomyelin. (C) Sterol lipids; shown cholesterol. Cholesterol is constructed of four fused, planar rings attached to a hydroxyl group with a hydrocarbon chain at the opposing end. (D) Free fatty acyls form the most basic lipids; shown arachidonic acid. (E) Glycerolipids are formed from the attachment of one, two or three fatty acyls to a glycerol; shown triacylglyceride.

Phospholipids are the dominant category of lipids in platelets (Table 2.1), and include phosphatidylcholine (PC), phosphatidylethanolamine (PE), PS, phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidic acid (PA) classes [27, 43, 50, 51, 55, 58, 59]. These classes are defined by their polar-phosphate containing head group attached to two fatty acyls containing chains (Figure 2.4A) [53]. Phospholipids are the major structural lipid of the platelet membrane and provide the substrates for the formation of many bioactive lipid mediators [53, 60]. For example, phospholipids can be cleaved to produce biologically active lysophospholipids and free fatty acyls through the activity of phospholipases [61, 62]. Further, phospholipids can undergo a biochemical change which imparts a distinct structure and function [63], and as such have been assessed in this study. Ether linked phospholipids are a subclass in which an alkyl chain is attached by an ether bond to the glycerol backbone [63]. The moiety is most commonly found on PC and PE [63]. Platelet activating factor (PAF) is an ether linked PC species, in which the sn-2 fatty acyl chain is very short (2:0) [64]. It is a potent bioactive lipid mediator, capable of inducing proinflammatory functions [64].

The main classes of sphingolipids present in platelets are ceramide and sphingomyelin (SM; Table 2.1). Ceramides are formed by the addition of a fatty acyl chain to a sphingosine base chain [65]. To this, more complex sphingolipids, such as SM, are formed by the addition of a head group at the C_1 position of the sphingosine (Figure 2.4B) [65]. Sphingolipids are a significant constituent of lipid membranes [65-67], where they function as key signalling molecules. Sphingosine 1-phosphate (S1P) and ceramide are well characterised signalling molecules, mediating an array of cellular functions via G protein-coupled signalling pathways and by acting as second messengers, respectively [65-69]. The importance of sphingolipids in cellular signalling is primarily due to their ability to modulate intracellular calcium stores [70, 71]. Sphingolipids are also enriched in lipid rafts [65, 66, 72], which provides a platform for mediating signal transduction, particularly for ceramide [65].

The sterol lipids within platelets include cholesterol and cholesterol esters (CE) [73], which make up a significant proportion of the platelet lipidome (~30 %; Table 2.1) [55-57]. Sterols are composed of a hydrophobic, fused, planar ring structure with a polar hydroxyl group at one end and a short hydrocarbon chain at the opposing end (Figure 2.4C) [74]. The presence of cholesterol within the platelet membrane provides structure and fluidity [74, 75]. Functionally, an alteration in the cholesterol content of platelets results in a proportional alteration to agonist induced aggregation [76]. Further, cholesterol is a necessary component of lipid rafts, which are specialised micro-domains of lipids and proteins within the platelet membrane. The assembly of lipid rafts is thought to localise and/or compartmentalise components required for signalling through certain pathways, including G protein-coupled receptors and tyrosine kinase receptors [72, 77]. As such, the biological consequences are far reaching, but include immune cell signalling via T-cell and B-cell receptors, platelet activation via the thromboxane A_2 receptor and GPVI, and they may also play a role in microparticle formation [78-81].

The free fatty acyls present within platelets include arachidonic acid (Figure 2.4D), linoleic acid, eicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid, dihomo-γ-linolenic acid, palmitic acid, stearic acid and oleic acid [82, 83]. Free fatty acyls can be formed by enzymatic cleavage from phospholipids [82, 84, 85]. Fatty acyls are for the most part inactive, however, they are converted to important lipid-derived signalling molecules, eicosanoids, via the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 pathways [82, 84, 85]. The term eicosanoids collectively refers to the fatty acyl derived bioactive lipid mediators such as prostaglandins, leukotrienes, and the lesser investigated lipoxins, resolvins and protectins [82, 84, 85]. These bioactive lipid mediators are potent regulators of platelet function, mainly through the binding of G protein-coupled receptors [82, 84, 85]. As an example, the oxidation of arachidonic acid by the COX and LOX pathways yields biologically important lipid mediators, such as thromboxane, 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE and 15-HETE [84].

Glycerolipids are formed by the condensation of one, two, or three fatty acyl molecules with glycerol (Figure 2.4E). Triacylglyceride (TAG) and diacylglyceride (DAG) constitute only a small proportion of the platelet lipidome [73]. However, DAGs represent an important and potent group of bioactive lipid mediators [86]. DAGs are generated by several enzymatic pathways, the most important being from phospholipids by phospholipase enzyme activity. This is due to the concurrent formation of other bioactive lipid mediators and subsequent activation of downstream signalling pathways [86, 87].

2.1.4.1 Lipid profile of resting platelets

In line with other circulating blood cells, the platelet membrane is composed mainly of PC, PE, PS, SM and cholesterol (Table 2.1). Compared to other hematopoietic cells, the platelet membrane contains more cholesterol, which assists in the maintenance of the membrane in a more rigid state, thought to prevent early clot formation [55]. Under resting conditions, the choline containing phospholipids, SM and PC, are contained on the outer leaflet of the membrane, and the aminophospholipids, PS and PE, are present on the inner leaflet (Figure 2.5A) [88]. This conformation is thought to keep the surface of the platelet in an anti-coagulant state, as the bulky configurations and tight packing ability of SM and PC block hydrophobic interactions of coagulation factors [89, 90]. The choline head group of PC and SM sterically hinders the binding of the gamma-carboxyglutamic acid-rich (GLA) domains of coagulation proteins to the phosphate group of these phospholipids [90]. The other minor lipid species, such as PI and PA, are predominantly contained on the inner leaflet, which positions them to readily form bioactive lipid mediators involved in downstream signalling [91, 92].

The integrity of the platelet lipid membrane is maintained in this conformation by adenosine triphosphate (ATP)-dependent translocase, which transports PE and PS from the outer leaflet to the inner leaflet, and the activity of ATP-dependent floppase, which supports the movement of PC and SM from the inner leaflet to the outer leaflet [89, 93, 94]. The action of these transporters is to correct disruptions in membrane asymmetry that may occur during membrane fusion, such as exocytosis and endocytosis, or during the transport of phospholipids synthesised *de novo* (Figure 2.5A) [89, 94]. However, this conformation of the platelet lipid membrane is highly dynamic and very receptive to activation, allowing platelets to respond quickly to activation signals.

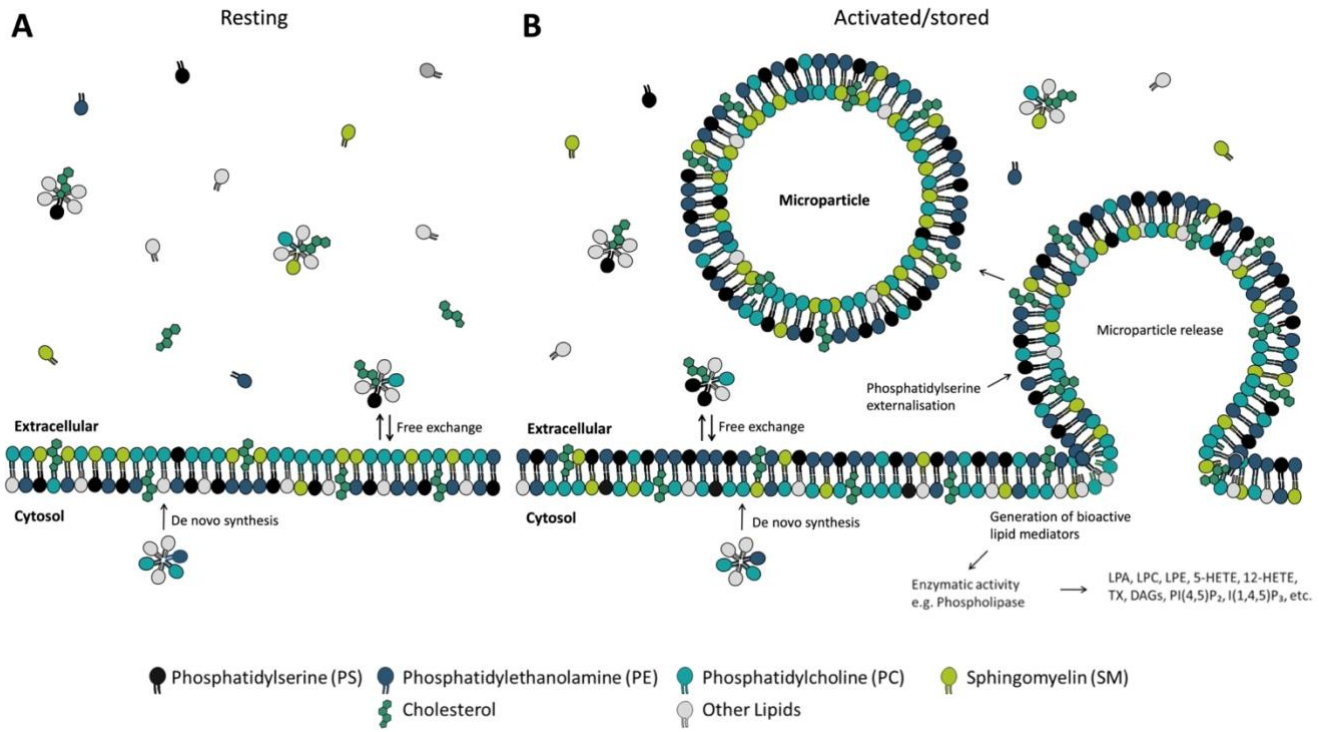

Figure 2.5. Lipid dynamics of resting, activated and stored platelets

The platelet lipidome is highly dynamic. This is exemplified by the ability of platelets to synthesise phospholipids and fatty acyls *de novo*, and freely exchange lipids with the plasma. (A) Under resting conditions, the choline containing phospholipids, SM and PC, are contained on the outer leaflet, and the aminophospholipids (PS and PE) are present on the inner leaflet of the platelet membrane. (B) During activation and storage, the lipidome of the platelets undergo greater change, characterised by externalisation of aminophospholipids as well as microparticle release, and the formation of bioactive lipid mediators, such as lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and thromboxane (TX) (Image by author, generated using Motifolio Biology Toolkit Suite)

2.1.4.2 Changes to platelet lipids as a result of activation

Platelet activation results in significant alterations to the platelet membrane lipids (Figure 2.5B) [27, 50], which are essential to support coagulation and thrombus growth [33, 95]. Calcium-dependent scramblase activity rapidly moves PE and PS to the outer leaflet and PC and SM to the inner leaflet (Figure 2.5B), negating the activity of the ATP-dependent translocase and the ATP-dependent floppase [89, 93]. Once externalised, PS localises the tenase (Factor IXa and Factor VIIIa) and prothrombinase (Factor Xa and Factor Va) complexes of the coagulation cascade to the platelet membrane through electrostatic and hydrophobic interactions, which is necessary for the formation of thrombin [33, 95]. PE also modulates pro-coagulant activity, where the fatty acyl chain length impacts the ability for PE to support coagulation [96]. For example, long chain fatty acyls, specifically arachidonic acid, provide better support for tissue factor dependent thrombin generation compared to short chain species [96]. Despite the extensive rearrangement of the platelet lipid membrane, stimulation of platelets by thrombin does not significantly alter the proportion of the majority of the lipids in the platelet membrane (PC, SM, PE, PS; Table 2.1) [27, 50].

Following thrombin activation, phospholipids are also converted to lysophospholipids through the action of phospholipases. The formation of lysophospholipid species is primarily through the action of phospholipase A_2 [61, 62], which acts upon the sn-2 position of phospholipids releasing fatty acyls, such as arachidonic acid [61, 62]. Platelet activation has been shown to result in an increase in the content of lysophospholipid species, including lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE; Table 2.1) [50] and lysophosphatidic acid (LPA) [97]. Despite increases in LPE and LPC, no decrease in the phospholipids PE or PC has been observed, suggesting the involvement of aspects other than simple enzymatic conversion. The content of PC and PE in platelets may be maintained through exchange with plasma lipoproteins, as it has been shown that platelets are capable of incorporating PC and PE from low density lipoproteins (LDLs) and

high density lipoproteins (HDLs), and this process is increased during activation of platelets with thrombin [98, 99].

2.1.4.3 Changes to the lipid profile of platelet microparticles as a result of activation

During activation platelets release microparticles (Figure 2.5B). The lipid composition of platelet microparticles varies depending on the source of platelet activation [28, 43]. Further, the lipid composition of the parent platelets may be affected by the release of microparticles. Biro *et al.* have shown the percentage of SM, PC and PE present in microparticles is dependent on the mechanism of generation [43]. The lipid composition of platelet microparticles most closely resembles platelet plasma membranes and platelet granule membranes, and it has been suggested platelet microparticles may be an amalgamation of plasma and granule membranes [43]. Despite an initial investigation into the membrane phospholipid content of platelet microparticles [43], little is known regarding the packaging of other lipid species or bioactive lipid mediators into microparticles.

2.2 Platelet components for transfusion

2.2.1 Platelet collection and preparation

Within a clinical setting, platelet components are collected and stored for transfusion purposes. Patients with platelet function disorders or low platelet counts may receive platelet transfusions prophylactically [100, 101]. Alternatively, patients who are actively bleeding, such as during surgery or massive trauma, may receive platelet transfusions to stop bleeding [102-105]. The transfusion of platelets for prophylactic reasons requires platelets to remain in circulation for an appropriate amount of time; however for patients who are actively bleeding, transfused platelets are likely consumed almost immediately in the haemostatic process. Therefore, the requirement for an extended circulation time differs depending on the clinical indication.

Australian Red Cross Lifeblood collects and manufactures platelet components from whole blood donations and apheresis donations (Figure 2.6). Whole blood (470 mL) from a healthy donor is collected into bags containing citrate-phosphate-dextrose (CPD) to prevent coagulation (Figure 2.6A) [106-109]. The whole blood component is centrifuged and separated by an automated press (MacoPress Smart, Macopharma, Tourcoing, France) into individual components: red blood cells, buffy coat (containing white cells and platelets) and plasma [109]. To produce buffy coat derived platelet components (Figure 2.6B), four buffy coats from blood type matched donors are pooled with platelet additive solution (SSP+), centrifuged and the platelet rich upper layer is collected using a MacoPress Smart [108, 109]. The platelet component is filtered through an in-line filter to remove white cells (leukoreduction) and is composed of approximately 70 % SSP+ and 30 % plasma [108-110]. Collecting platelets by apheresis involves removing blood from a donor into a Trima apheresis machine (Figure 2.6C). The donor is connected to the Trima machine, and blood is drawn and centrifuged, where the red blood cells, and a portion of the plasma are returned to the donor. Additionally, the white blood cells are removed by a filter within the collection

consumable [108, 109]. Apheresis platelets are collected into bags containing an anticoagulant (Acid Citrate Dextrose (ACD-A)) [111]. Once collected, a low volume of plasma and platelet additive solution is added to achieve a final ratio of 60 % SSP+ and 40 % plasma [111]. The collection of platelets by apheresis yields sufficient platelets for multiple transfusion doses from a single donation.

As transfusion practices have evolved, there has been interest in supplementing plasma with platelet additive solution as the storage medium. Platelet additive solutions are saline based, with the addition of other constituents (acetate, phosphate, magnesium and potassium) to optimise aerobic metabolism and/or decrease platelet activation. Much of the benefit of reducing the plasma content in the storage medium has been associated with the reduced incidence of adverse transfusion reactions such as transfusion-related acute lung injury (TRALI), the reduced risk of prion protein disease transmission and the increased availability of plasma for fractionation [112-115]. While the composition varies depending on institutional practices, most agree a plasma composition of at least 30 % results in acceptable *in vitro* quality parameters, including a comparable metabolic rate and level of platelet activation to platelets stored in 100 % plasma over 7 days of storage [116-121].

The platelet component is comprised of several “fractions”: the platelets themselves, microparticles and the storage solution. Microparticles are shed from platelets during the preparation and storage of the platelet component [122, 123]. The platelet storage solution generally contains between 30-100 % plasma together with 0-70 % platelet additive solution, depending on the manufacturing institute [110, 124]. Regardless, over the storage period the composition of the storage solution is altered as platelets release their granule contents, release microparticles and are degraded [110, 122, 125].

Buffy coat derived and apheresis platelet components have advantages and disadvantages [106, 126]. Following the preparation of red blood cell and plasma components from a whole blood donation the remaining donation can

be maximised through the preparation of buffy coat derived platelet components [127]. While buffy coat derived platelet components are derived from multiple donors, apheresis platelets are derived from a single donor, which is advantageous for the safety of the recipient [106, 108]. For apheresis components the risk of exposure to the causative agents of transfusion transmitted infection and transfusion associated sepsis is reduced [128]. Further, the use of apheresis platelet components allows for human leukocyte antigens (HLA) cross matching when recipients may be at risk of developing platelet refractoriness (a platelet count following transfusion that is lower than expected due to alloimmunisation to HLA and/or platelet-specific antigens as a result of prior exposure) [129]. Importantly, buffy coat derived platelet components and apheresis platelet components appear to be comparable in terms of quality [106, 109, 126]. As such, in our organisation, despite the difference in plasma carryover, buffy coat derived and apheresis platelet components are treated interchangeably. For this reason and based on component availability both component types were examined in this study.

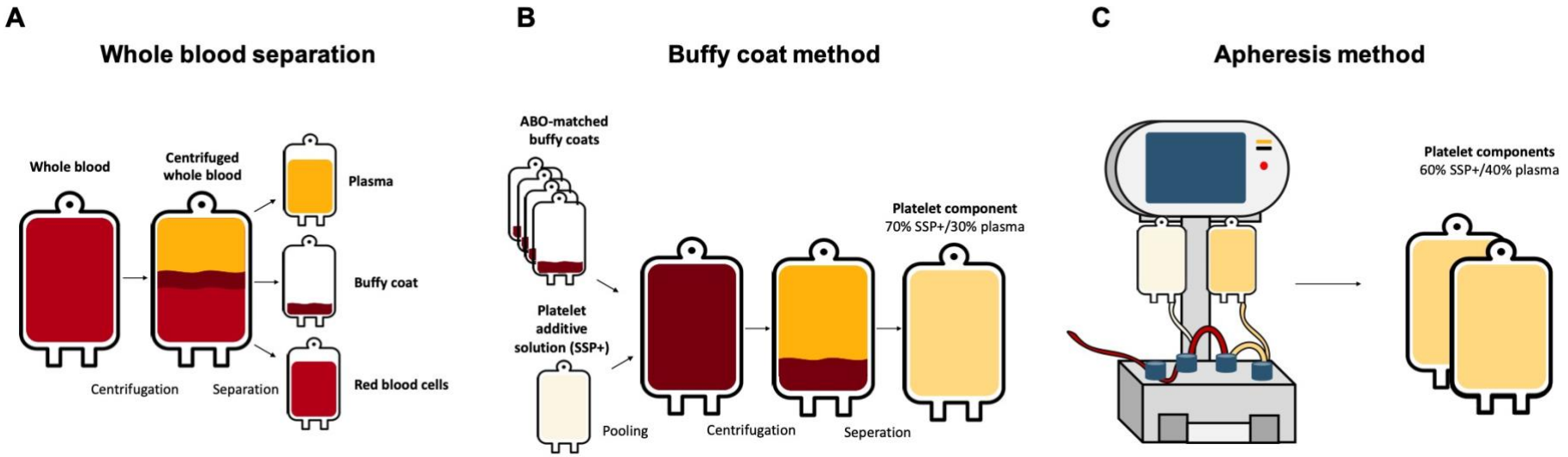

Figure 2.6. Methods of platelet component preparation

(A) Whole blood donations are centrifuged and separated into three individual components: plasma, buffy coat and red blood cells. Plasma and red blood cells are pressed into new storage bags for storage as components. Plasma is frozen and stored at -30°C. Red blood cells are stored between 2-6°C. Buffy coats are further processed into platelet components. (B) Platelet components are prepared by the buffy coat method by pooling four ABO-matched buffy coats with platelet additive solution (SSP+). The pooled component is centrifuged, separated and leukodepleted by filtration. The final component has a composition of 70 % SSP+/30 % plasma. (C) Platelet components can be collected by the apheresis method. This method uses an apheresis machine which separates cells, collecting the plasma and platelets and returning the red blood cells to the donor. A donation from a single donor can yield sufficient platelets for up to three transfusion doses. During the donation process, the component is leukoreduced by filtration and the component is suspended in plasma and platelet additive solution at a final composition of 60 % SSP+/40 % plasma. (Image by author)

2.2.2 Conventional platelet storage

Conventionally, platelets are stored at room temperature with constant agitation. The shelf life of platelets in Australia is currently seven days [108, 130]. Constant agitation and use of gas permeable storage bags facilitate the exchange of oxygen and carbon dioxide, which is required to support platelet metabolism [131]. Further, constant agitation prevents aggregation of platelets while in storage bags [131]. The shelf life limitation has been implemented to minimise the risk of bacterial proliferation, which could potentially be introduced into the platelet component during the donation process [132]. Any potential contamination can be exacerbated by the conditions at which platelets are stored [132]. Further the short shelf life and strict storage requirements aim to lessen the effects of the compounding storage related changes, known as the platelet storage lesion (PSL) [133, 134].

The PSL is best defined as the progressive decline in quality of stored platelet components [134]. The storage related defects are similar to the changes associated with platelet activation, characterised by shape change, degranulation, alteration to surface glycoproteins, release of microparticles, a reduced aggregation response and weaker clot formation *in vitro* (Figure 2.7A) [133-135]. While the process is not entirely understood, all facets of the donation process and *ex vivo* storage of platelets play a role in the accumulation of declining platelet quality [134, 136]. The decline in *in vitro* quality indicators seen over storage is associated with faster clearance *in vivo* [137], suggesting platelet components become less effective in maintaining haemostasis the longer they are stored [134, 137, 138]. Further, the changes that occur as a result of the PSL are associated with adverse transfusion reactions [135, 139, 140].

2.2.2.1 Challenges to conventional platelet storage

The storage requirements of conventional platelets limit their availability. Lengthy transport times are required when supplying rural and remote locations [141]. As such, the supply of platelets in these locations is limited

and the quality of platelet components may be impacted [131, 141]. In addition to the short shelf life, the demand for platelets in these locations can be unpredictable resulting in a high potential for wastage [141]. These issues are further exacerbated when supplying remote military locations [142-144]. An inability to readily provide platelets in these environments results in a failure to meet current standards of resuscitative care [141, 144]. The issues surrounding conventionally stored platelets could be resolved through the use of alternative storage techniques, such as cold storage and cryopreservation.

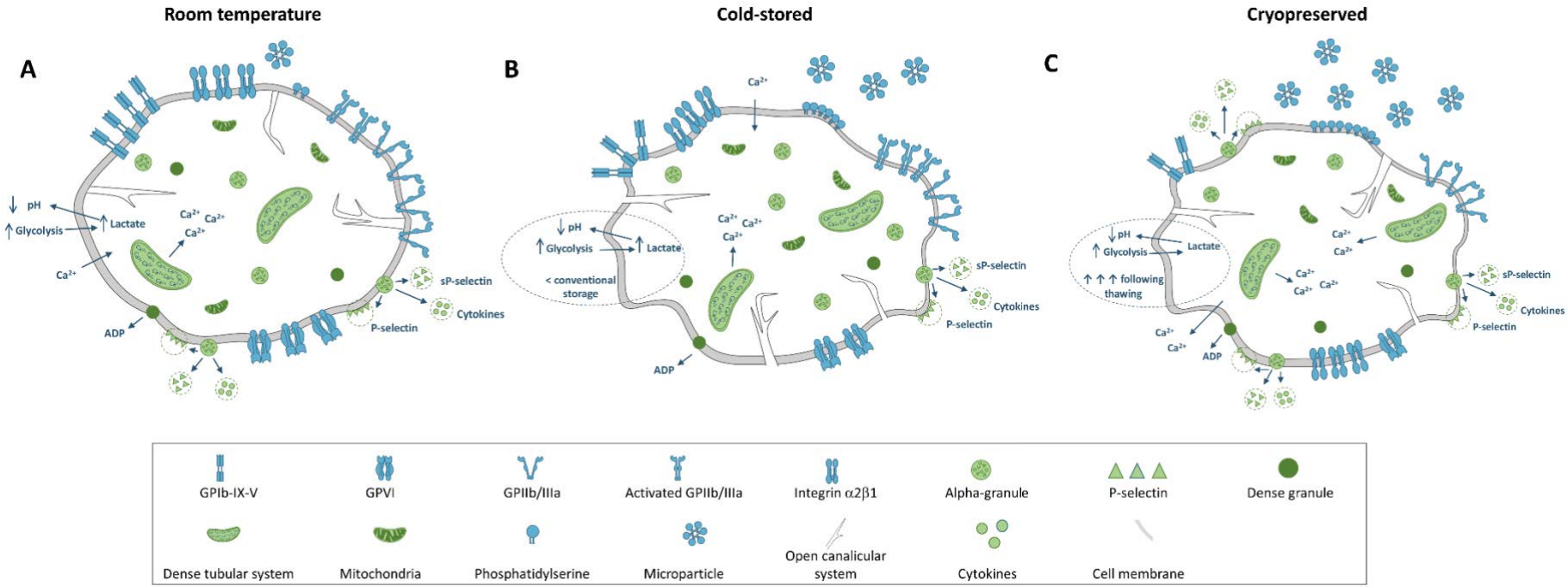
Room temperature
Cold-stored
Cryopreserved
A
B
C
pH
Glycolysis
Lactate
Ca2+
ADP
sP-selectin
Cytokines
P-selectin
< conventional storage
following thawing
GPIb-IX-V
GPVI
GPIIb/IIIa
Activated GPIIb/IIIa
Integrin α2β1
Alpha-granule
P-selectin
Dense granule
Dense tubular system
Mitochondria
Phosphatidylserine
Microparticle
Open canalicular system
Cytokines
Cell membrane

Figure 2.7. Conventional and alternate storage results in changes to the platelet structure

(A) Conventionally stored platelets, undergo a number of changes, including shape change and membrane rearrangement, through the externalisation of phosphatidylserine. Throughout the storage period, platelets respire, consuming glucose and producing lactate which can decrease the pH of the storage medium. There is release of microparticles and alpha granule secretion, which exposes P-selectin on the surface of the platelet, and release soluble P-selectin (sP-selectin) into the surrounding environment. Glycoprotein expression is altered, including increased expression of glycoproteins associated with activation. (B) Cold storage of platelets results in a number of changes, which differ to those seen during conventional storage. Cold storage results in shape change, loss of membrane asymmetry and release of microparticles. While to a lesser extent than conventional storage, cold-stored platelets still respire, consuming glucose and producing lactate, which can decrease the pH of the storage medium during extended storage. Compared to conventional storage, alpha granules are retained, while several glycoproteins are lost, including GPVI and GPIb-IX-V. Further, glycoproteins, such as integrin αIIbβ3, are altered to their more active conformation. (C) Platelet cryopreservation results in a number of changes compared to conventionally stored and cold-stored platelets. Cryopreservation results in shape change, a significant loss of membrane asymmetry resulting in PS externalisation and a significant increase in microparticles released, much of this is driven by the significant increase in cytosolic calcium. Upon thawing, platelets have significantly increased metabolism. In comparison to conventionally stored platelets, alpha granule secretion is greater, while several surface glycoproteins, including GPIb-IX-V and GPVI are lost. Further, the activated conformation of integrin αIIbβ3 is not present. (Image by author, generated using Motifolio Biology Toolkit Suite).

2.2.3 Cold-stored platelets

Cold storage of platelets is a viable alternative to conventional platelet storage. The cold storage of platelets involves storing platelets in a refrigerator between 2-6 °C, and does not require agitation. This method is purported to extend the platelet shelf life up to two weeks [145-148]. Historically platelet components were stored in the cold, but in the 1970s it was found that cold-stored platelets were cleared more quickly from circulation than platelets stored at room temperature [146, 149]. However, cold-stored platelets provide other benefits that may compensate for the reduced circulation time.

Interest in cold-stored platelets has resurfaced due to the extended shelf life, less burdensome storage and transport logistics, lower risk of bacterial proliferation and enhanced haemostatic capabilities. The *in vivo* efficacy of cold-stored platelets has been assessed [146, 149-152]. The results from these studies suggest the use of cold-stored platelets is feasible for the treatment of active bleeding [150]. Further, cold-stored platelets stored for 14 days remain functionally active once transfused [150].

Extensive *in vitro* studies have been conducted to examine metabolism, surface receptor expression and the proteome [122, 148, 153-156]. Cold-stored platelets possess altered platelet quality parameters compared to room temperature stored platelets [122, 146, 147]. Cold-stored platelets have a higher abundance of activation markers, including activated integrin αIIbβ3 and P-selectin compared to conventionally stored platelets (Figure 2.7B) [153, 154, 157, 158]. Further, storage of platelets at cold temperatures results in increased PS externalisation, increased release of microparticles and a less discoid appearance than conventionally stored platelets [145, 147, 154, 158, 159]. However, compared to conventionally stored platelets the release of granule contents is reduced [122, 147]. Cold storage also reduces the rate of glycolysis, such that the pH is maintained and the glucose in the storage solution remains for at least 2 weeks, thus allowing for the 14 day shelf life [122, 145, 147, 154-156, 159, 160]. Further, the ability for platelets to

aggregate is retained during cold storage, and it has been suggested that cold-stored platelets may be able to form clots more quickly than conventionally stored platelets [145, 147, 154, 158, 159]. The increased haemostatic capability of cold-stored platelets has been partly attributed to the increased externalisation of PS and increased formation of microparticles with externalised PS [154, 161]. However, these changes vary based on storage duration and the composition of the storage solution [153, 154, 162].

The re-introduction of cold-stored platelets for transfusion has begun. In the United States, the Food and Drug Administration (FDA) has authorised limited use of cold-stored platelets in civilian hospitals and the component has been used within military settings [163-165].

2.2.4 Cryopreserved platelets

Platelet cryopreservation presents an attractive alternative to room temperature storage for supplying austere environments, as it can extend the shelf life to at least two years [141, 142, 166]. The cryopreservation of platelet components requires the addition of the cryoprotectant, DMSO, to a final concentration of 5-6 % volume/volume (v/v) and freezing at -80 °C [142]. For transfusion, cryopreserved platelet components are rapidly thawed and reconstituted in an appropriate solution [142, 167-169]. The thawed platelet component can be stored at room temperature for up to 6 hours [166, 169, 170]. Cryopreservation is a more labour intensive and expensive technique than cold storage [167], and has not been routinely used in a civilian context.

Cryopreservation has been used in a military setting for 20 years [142, 171], and several clinical trials have been performed to assess the feasibility of use in a civilian setting [170, 172, 173]. The results from military usage and clinical trials have concluded that cryopreserved platelets are at least as comparable to conventionally stored platelets in their haemostatic effectiveness [141, 142, 170-173]. Further, there is no evidence of serious adverse reactions arising from the transfusion of cryopreserved platelets [142, 170-173]. However, cryopreservation of platelets is not routine and clinical trials are still underway,

including the Cryopreserved vs Liquid Platelet-II (CLIP-II) study being conducted in Australia. CLIP-II is a Phase III randomised clinical trial, aimed at demonstrating the non-inferiority of cryopreserved platelets in comparison to conventionally stored platelets to treat bleeding in cardiac patients [174].

It has been well documented that cryopreservation results in platelet loss and considerably alters the platelet surface receptor phenotype [167, 175-177]. Specifically, cryopreserved platelets appear more activated, expressing higher levels of P-selectin, increased granule secretion, externalisation of PS and increased formation of PS-expressing microparticles (Figure 2.7C). Cryopreserved platelets lose surface receptors that are important for adhesion and aggregation, including GPVI, GPIbα and integrin αIIb [167, 168, 175, 176, 178, 179]. Further, following cryopreservation platelets exhibit increased metabolism, as shown through a significantly higher glucose consumption and lactate production compared to conventionally stored platelets [145, 167]. Despite these changes which are traditionally viewed as being characteristic of poor *in vitro* quality, cryopreserved platelets have been shown to be more haemostatically active [145, 170, 175, 178]. It has been suggested that this is due to the significant PS externalisation and release of PS-expressing microparticles that contribute to clot formation [178].

2.2.4.1 Microparticle formation in cryopreserved platelets

Cryopreserved platelet components contain up to 100-fold more PS-expressing microparticles compared to conventionally stored platelets [122, 180]. Further, the microparticles formed following cryopreservation have an altered surface receptor phenotype compared to the microparticles present prior to freezing [180]. Microparticles formed following cryopreservation have a higher abundance of platelet specific markers compared to microparticles in freshly collected platelet components [180], thus eluding to the potential for differential packaging of contents taking place during the formation of microparticles occurring as a result of the cryopreservation process.

2.3 Changes to the lipidome during platelet storage

2.3.1 Lipidome of conventionally stored platelets

Ex vivo storage alters the lipidome of platelet components. Overall, when stored in 100 % plasma, the platelets undergo a reduction in total lipid content, which is suggested to be due to the selective loss of lipids to the plasma [51, 52, 57]. Specifically, following five days of storage, cholesterol in platelets decreases, which is proposed to be due to the transfer of cholesterol to microparticles shed during storage [43, 56, 161]. Additionally, PC decreases and LPC increases in the plasma of platelet components over storage [56, 57]. SM and ceramide have been shown to increase in platelets, microparticles and plasma after five days of storage [56, 57]. The increases in SM and ceramide within stored platelets is thought to be the result of the incorporation of SM from LDLs and HDLs [98], as platelets lack the enzymes required for *de novo* sphingolipid synthesis [181]. However, other phospholipids, including PE, LPE, PG, PI, PA and LPA, remain unchanged or have not been detected in global lipidomic studies assessing platelet components stored at room temperature [51, 56, 57].

The body of research that has been undertaken on the lipidome of room temperature stored platelet components provides a valuable understanding of the changes occurring over the five day shelf life. However, scope exists to achieve a greater understanding of the lipidome of stored platelet components. Platelet components can be stored at room temperature for seven days, and are increasingly being prepared with platelet additive solution rather than plasma as the storage medium. To date, no lipidomic investigations have been undertaken on platelets stored in platelet additive solution.

2.3.2 Lipidome of cold-stored platelets

A limited number of studies have assessed the lipidome of cold-stored platelets. Hamid *et al.*, reported that the total lipid content of platelets stored at 4 °C did not significantly change after three days [51]. However, Okuma *et*

al., reported significant decreases in the total lipid content of platelets stored for 3 and 6 days at cold temperatures [52]. Differences in the method of sample collection from the platelet component are highlighted as a possible explanation for these discrepancies. The early time points examined also limit the applicability of the data to the current situation, as it has been proposed that the feasible shelf life of cold-stored platelet components could be at least 14 days [164]. Further, recent evidence demonstrates that cold storage results in increased PS externalisation, but the timing is variable [153, 154, 162]. From this literature, it is difficult to interpret the overall impact that cold storage may have on the platelet lipidome. However, given that PS externalisation is increased, it is hypothesised that additional changes in specific lipid classes could be expected.

Historic studies of the lipidome of cold-stored platelet components were focused on the platelet fraction of the component, thus overlooking their interaction with the storage solution and its contribution to the overall lipid profile of the platelet component. Due to the dynamics of the lipid profile, it is important to fully characterise the lipidome of the entire platelet component over the shelf life of the component, as has been undertaken in room temperature stored platelet components by Pienimaeki-Roemer and colleagues [56]. The recent renewed interest in cold-stored platelet components and advances in lipidomic technologies present an opportunity to expand our knowledge of the lipidomic changes occurring within these platelet components.

2.3.3 Lipidome of cryopreserved platelets

To date the lipidome of cryopreserved platelets has not been assessed. It is known that the cryopreservation process increases the proportion of platelets with externalised PS to approximately 70 %, as well as generating a large number of PS-expressing microparticles [178]. Despite this extensive remodelling of the platelet membrane, no studies have been conducted to determine the impact of the cryopreservation process on the global lipidome

of platelets, platelet microparticles, storage solution or downstream lipid-mediated signalling pathways. As the use of cryopreserved platelets has expanded, the impact of the cryopreservation process on the lipidome should be determined.

2.3.4 Clinical importance of understanding the lipid profile of platelet components

Storage related changes decrease *in vitro* quality and lead to faster clearance *in vivo* and reduce the efficacy of the component once it is transfused [137]. Changes in the global lipidome of platelets have been observed during storage at room temperature [56, 57, 182], and these changes, although small and subtle, may affect the clinical outcomes of the platelet components once transfused. Specifically, bioactive lipids, including 5-HETE, 12-HETE, 15-HETE and LPCs, have been shown to accumulate in stored red blood cell and platelet components which have been implicated in TRALI [183-185]. While the role of 5-HETE, 12-HETE and 15-HETE in mediating TRALI remains unclear [186], it is hypothesised that LPC facilitates TRALI through the activation of pro-inflammatory pathways in endothelial cells and the priming of neutrophils, which allows for them to be readily activated following an additional insult [187, 188]. This is particularly interesting as LPC has been shown to increase in platelets, microparticles and plasma during conventional storage of platelet components [51, 56, 57].

2.4 Definition of research question

The platelet component is comprised of three parts: the platelets, microparticles and the storage solution. The lipid profile of these parts are in constant flux, through *de novo* synthesis, free exchange, and biochemical processes, resulting in changes that could affect component function or safety, favourably or adversely, once the platelet component is transfused. Given the essential role of lipids and bioactive lipid mediators in platelet function and the potential clinical impact of these molecules, and the growing interest in alternatively stored platelet components, there is an impetus to more fully understand the impact of storage on the lipid profile of platelets. While significant efforts have been made to advance the understanding of the lipidome of platelet components in a transfusion setting, an understanding of the impact of developing transfusion practices is still required.

As such the aim of this study was to comprehensively characterise the lipidome of alternatively stored platelet components.

Aim 1: To characterise the lipidome of cold-stored platelets and compare it to the lipidome of room temperature stored platelets.

Cold storage may extend the platelet shelf life to 14 days and is known to alter some *in vitro* characteristics of the platelet component, particularly at later time points. It was hypothesised that cold storage would alter the lipidome of the platelets and the storage solution.

Aim 2: To characterise the lipidome of cryopreserved platelets and compare it to the lipidome of platelets prior to cryopreservation.

Cryopreservation is known to alter many *in vitro* characteristics of the platelet component. However these changes may be beneficial for treating active bleeding. It was hypothesised cryopreservation would alter the lipidome of the platelets, microparticles and storage solution.

Chapter 3 Materials and Methods

This chapter describes the materials used and the methods performed in order to complete the aims of this dissertation. It includes platelet component production, analysis of the platelet component, lipidomic assessments and the statistical analysis performed.

3.1 Ethics

Ethics approval was obtained for this study from Australian Red Cross Lifeblood Ethics Committee and was ratified by the University of Technology Sydney Human Research Ethics Committee (ETH18-2795).

All donations were collected from eligible, voluntary, non-remunerated donors in accordance with the guidelines established by Australian Red Cross Lifeblood.

3.2 Materials

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) except where otherwise stated.

3.3 Platelet collection and preparation

3.3.1 Apheresis platelet components

Apheresis platelet components were collected using an apheresis system (Trima Accel, TerumoBCT, Lakewood, CO, USA) with the anticoagulant Acid Citrate Dextrose (ACD-A), according to standard Australian Red Cross Lifeblood procedures. The platelet component was leukoreduced and stored in 40 % plasma and 60 % SSP+ (Macopharma, Tourcoing, France) The platelet components were stored at 20-24 °C with agitation (Helmer Inc., Noblesville, IN, USA) for 24 hours until day 1 of the study.

3.3.2 Buffy coat derived platelet components

Whole blood was collected in accordance with Australian Red Cross Lifeblood guidelines. Buffy coat derived platelet components were prepared by standard methodologies [189]. Briefly, whole blood donations were collected into bags containing CPD. The buffy coat was separated from red bloods cells and plasma by centrifugation (Beckman J6-MI, Beckman Coulter, Brea, CA, USA) at 5 000 x g for 10 minutes and an automated blood press (MacoPress Smart, Macopharma). ABO-matched buffy coats from four donors were pooled with

300 mL of SSP+ (Macopharma). Platelets were further separated by centrifugation at 500 x g for 6 minutes and platelets were extracted with an automated blood press. The platelet component leukoreduced by in-line filtration and stored in a polyvinylchloride (PVC) bag (ELX, Haemonetics Corporation, Braintree, MA, USA) at 20-24 °C with agitation (Helmer Inc.) for 24 hours until frozen.

3.4 Experimental design

The lipidomic characterisation of transfusible platelet components was conducted as two discrete studies.

3.4.1 Lipidomic characterisation of cold-stored platelets

The cold storage study was performed using double-dose apheresis platelet components from 8 donors. The component was split into single dose equivalents and the matched components were randomly assigned and stored at either room temperature (RT; 20-24°C) with constant agitation (Helmer Inc.) or under refrigerated conditions (2-6°C) without agitation. Cold-stored platelet components were directly compared to conventionally stored platelet components from the same donor (Figure 3.1).

Double-dose apheresis platelet components were sampled (10 mL) on day 1 post collection. Double-dose apheresis platelet components are routinely pooled for quality control testing and bacterial contamination sampling, and from this pooled component a sample (10 mL) was taken on day 1 before splitting into matched pairs. Platelet components were sampled on day 5 and 14 of storage. The cold-stored component was placed on an agitator at room temperature for 10 minutes prior to sampling to ensure homogeneous sampling [190]. Days 5 and 14 were selected as time points as they represented the maximum shelf life of room temperature stored platelets (at the time of conducting the study) and the current feasible shelf life of cold-stored platelets, respectively [164].

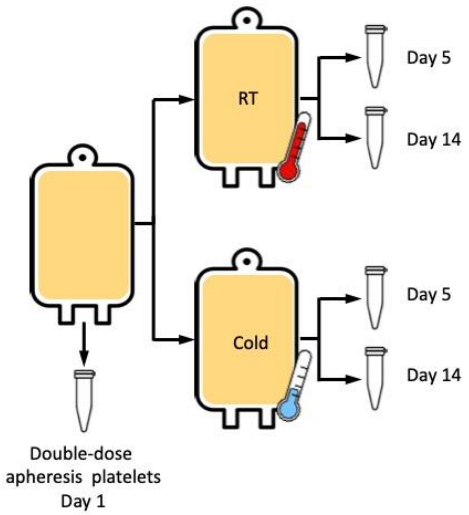

Figure 3.1. Study design for the lipidomic characterisation of cold-stored platelet components

Double-dose apheresis platelet components (n=8 donors) were sampled (10 mL) on day 1 post collection. The pooled component was split into two equal components (n=8 in each group) which were stored at either room temperature (RT; 20-24°C with constant agitation) or under refrigerated conditions (2-6°C without agitation). Platelet components were sampled on days 5 and 14 of storage. (Image by author)

3.4.2 Lipidomic characterisation of cryopreserved platelets

The cryopreservation study was conducted using buffy coat derived platelet components from six donors. The lipidomic characterisation of cryopreserved platelets was conducted as a paired study (Figure 3.2); whereby the same platelet component was sampled before freezing, after thawing and after 24 hours of post-thaw storage at room temperature with agitation.

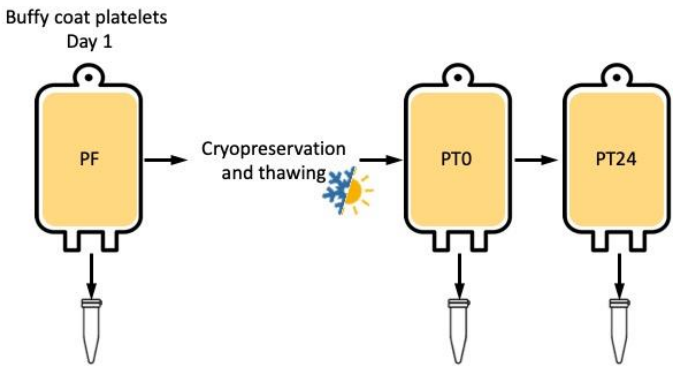

Figure 3.2. Study design for the lipidomic characterisation of cryopreserved platelet components

Buffy coat derived platelet components (n=6; derived from 24 donors) were sampled on day 1 after collection (pre-freeze; PF). Components were cryopreserved and thawed using well established methodologies. Thawed and reconstituted components were sampled immediately (post-thaw 0; PT0) and after 24 hours of post-thaw storage at room temperature (20-24 °C) with agitation (post-thaw 24; PT24). (Image by author)

3.4.2.1 Cryopreservation process

On day 1 following collection, buffy coat derived platelet components (n=6) were cryopreserved using previously published methodologies [189], as depicted in Figure 3.3. Prior to cryopreservation, a pre-freeze (PF) sample was taken (10 mL). A fistula set with a 17G needle (Asahi Kasei Kurraray Medical Co., Ltd, Tokyo, Japan) was sterile welded (TSCD-II, Terumo BCT, Lakewood, CO, USA) to the platelet component. Within a biosafety cabinet, approximately 100 mL 27 % (w/v) DMSO/0.9 % saline (Sypharma Pty. Ltd., Danenong, VIC, Australia) was added to achieve a final concentration of 5-6 % DMSO. The DMSO was added by gravity at a constant flow by spiking the bottle with the needle of the fistula set, while the platelet component was under constant agitation (rocking platform mixer; Ratek Instruments, Boronia, VIC, Australia). The DMSO containing platelet component was transferred to a 450 mL PVC freezing bag (Macopharma) and the platelets were pelleted by centrifugation (Beckman J6-MI, Beckman Coulter, Brea, CA, USA) at 1350 x g for 10 minutes with no brake. The supernatant was removed using a manual plasma press (Baxter Healthcare, Fenwal Division, Deerfield, IL, USA), resulting in a final volume of 20-30 mL. The pelleted platelets were resuspended in the residual supernatant by gentle rubbing. The platelet hyperconcentrate was vacuum sealed (Magic Vac, Brescia, Italy) and placed in a rigid box. The platelet component was frozen and stored in a -80°C freezer for an average of 23 days (range 20-34 days).

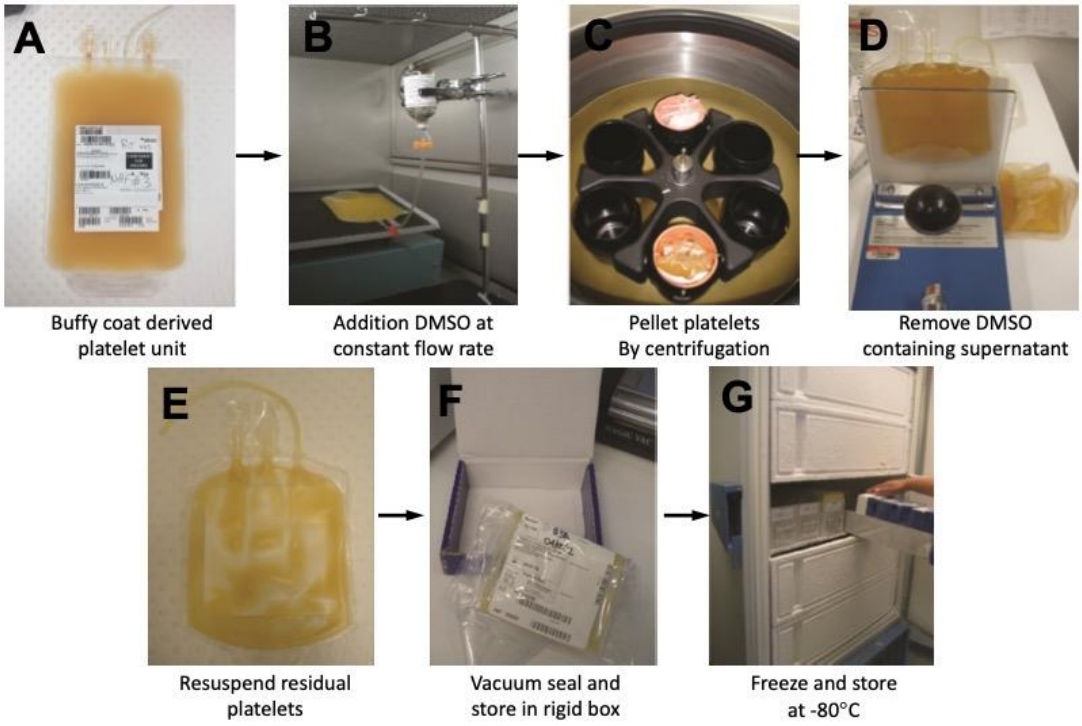

Figure 3.3. Overview of the platelet cryopreservation process

(A) Buffy coat derived platelet components were cryopreserved by (B) adding dimethyl sulfoxide (DMSO) to the component to achieve a final concentration of 5-6 %. The (C) platelets were pelleted by centrifugation and (D) the DMSO containing supernatant was removed using a manual press. The platelets were (E) resuspended in the residual supernatant by gentle rubbing, (F) vacuum sealed and placed in a rigid box. The (G) hyperconcentrated platelet component was frozen and stored at -80°C.

3.4.2.2 Thawing process

Following thawing, the platelet hyperconcentrate must be reconstituted in a solution appropriate for platelet storage and transfusion [167]. For this study, 30 % plasma/70 % SSP+ was chosen as it is the standard storage solution for buffy coat derived platelets, thus matching the PF and PT composition.

In order to reduce donor variation in the reconstitution solution and allow any differences to be attributed to the cryopreservation process, the same plasma was used to reconstitute all platelets. To facilitate this, fresh frozen plasma (plasma frozen and stored at -30 °C) from six donors was rapidly thawed in a 37 °C water bath (Thermoline Scientific, Sydney, NSW, Australia). Thawed plasma components were pooled into a single bag and split into aliquots (containing approximately 80 mL) in PVC freezing bags (Macopharma) by sterile welding. Pooled plasma aliquots were frozen and stored at -30 °C until the platelet components were thawed for analysis.

The platelet components were thawed using previously published methods (Figure 3.4) [189]. An aliquot of fresh frozen plasma was rapidly thawed in a 37 °C water bath (Thermoline Scientific) until the component reached approximately 30 °C. The thawed plasma component was combined with SSP+ (Macopharma) to reach a final volume of 250 mL, producing a reconstitution solution of 30 % plasma/70 % SSP+. The reconstitution solution (reconstitution solution; RS) was sampled as a control. The cryopreserved platelet hyperconcentrate was rapidly thawed in a 37 °C water bath (Thermoline Scientific) until the component reached approximately 30 °C. The thawed platelet component was rested on a platelet agitator for 10 minutes before reconstitution. The reconstitution solution and the platelet component were sterilely docked (TSCD-II) together, and the reconstitution solution was added to the platelet component by gravity and reconstituted by gentle mixing. The component was sampled immediately (post-thaw 0; PT0). The component was stored on a platelet agitator at 20-24 °C (Helmer Inc.) for 24 hours before sampling (post-thaw 24; PT24).

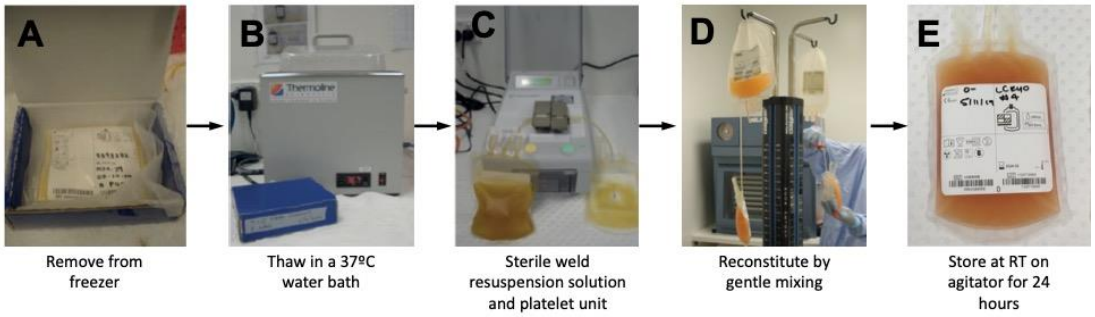

Figure 3.4. Overview of the platelet thawing process.

An aliquot of fresh frozen plasma was thawed in a 37°C water bath, until the component reached a temperature of 30°C (not shown). The plasma was combined with platelet additive solution to produce the reconstitution solution (not shown). The cryopreserved platelet component was (A) removed from the freezer and (B) thawed in a 37°C water bath, until the component reached a temperature of 30°C. The (C) reconstitution solution and platelet component were sterile welded together and the (D) reconstitution solution was added to the platelet by gravity. The (E) final reconstituted platelet component was stored on an agitator at room temperature for 24 hours.

3.5 Analysis of platelet component

The platelet count and mean platelet volume (MPV) were determined using a haematology analyser (CELL DYN Ruby, Abbott Laboratories, Chicago, IL, USA). All platelet components were assessed for presence of swirl and macroaggregates by visual inspection [122, 191].

3.5.1 Platelet recovery of cryopreserved platelets

The platelet count was used to determine platelet recovery after cryopreservation, according to the calculation below:

$$\text{Percentage recovery (\%)} = \frac{\text{Post-thaw platelet count (x10}^9\text{/unit)}}{\text{Pre-freeze platelet count (x10}^9\text{/unit)}} \text{ X } 100$$

3.6 Flow cytometric analysis

The number of microparticles released was assessed by flow cytometry (FACSCanto II, Becton Dickinson, Franklin Lakes, NJ, USA). The absolute number of microparticles was determined using TruCount tubes (BD Biosciences, San Jose, CA, USA). TruCount tubes contain a lyophilised pellet with a known number of beads, which can be used to calculate the absolute number (cells/ μL) of platelets or microparticles in a sample. A sample of the platelet concentrate (5 μL) was diluted in 95 μL of annexin V Binding Buffer (BioLegend, San Diego, CA, USA) in a TruCount tube. For all assays, annexin binding buffer was filtered through a 0.1 μm polyvinylidene fluoride (PVDF) membrane syringe filter (Merck Millipore, Merck KGaA, Darmstadt, Germany). The sample was stained with 5 μL annexin V-FITC (BioLegend) and 5 μL CD61-APC (Dako, Glostrup, Denmark) for 15 minutes, protected from the light at room temperature. The stained samples were diluted in 1 000 μL of filtered annexin V Binding Buffer and measured by flow cytometry, by collecting 10 000 bead events. The microparticle population was distinguished from the platelet population using forward and side scatter parameters, determined

using beads of known sizes (0.6 μm, 1.0 μm and 3.0 μm; Sigma-Aldrich), with microparticles defined as being less than 1.0 μm (Figure 3.5). The absolute number of platelets or microparticles was determined according to the calculation below:

$$\text{Absolute number/ } \mu\text{L} = \frac{\text{Positive events}}{\text{Bead events (10000)}} \times \frac{\text{Bead count per tube (lot specific)}}{\text{Sample volume (5 } \mu\text{L)}}$$

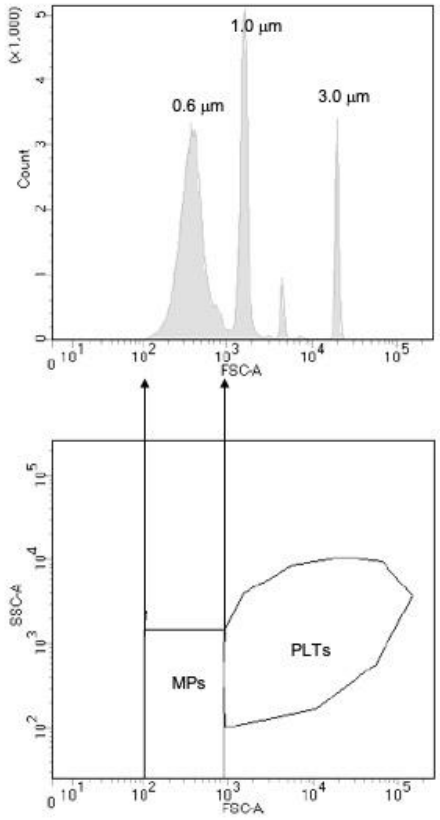

Figure 3.5. Gating strategy for discrimination of microparticles and platelets by flow cytometry

Platelets (PLTs) and microparticles (MPs) were discerned from one another using forward (FSC-A) and side scatter (SSC-A). Beads of known size (0.6 μm, 1.0 μm and 3.0 μm), were used to set gates. Microparticles were defined as less than 1.0 μm.

The externalisation of PS was assessed by flow cytometry. Platelets (5 μL) were diluted in 95 μL of annexin V Binding Buffer in a FACS tube. The sample was stained with 5 μL annexin V-FITC (BioLegend) for 15 minutes, protected from the light, at room temperature. The stained samples were diluted in 1 000 μL of filtered annexin V Binding Buffer and measured by flow cytometry, by collecting 10 000 events.

The externalisation of PE was assessed by flow cytometry. Platelets (5 μL) were diluted in 95 μL of 1 x Tyrode's Buffer (Table 3.1) in a FACS tube. For all assays, Tyrode's buffer was filtered through a 0.1 μm PVDF membrane syringe filter (Merck Millipore, Merck KGaA). The sample was stained with 500 nM Duramycin-Cy5 (Molecular Targeting Technologies, Inc., West Chester, PA, USA) for 15 minutes, protected from the light at room temperature. The stained samples were diluted in 1 000 μL of filtered Tyrode's Buffer and measured by flow cytometry, by collecting 10 000 events.

Table 3.1. Solutions used in flow cytometric analysis

Solution	Preparation
10 x Tyrode's Buffer	10.08 g Sodium Bicarbonate, 100 mL HEPES (1M), 80.06 g Sodium Chloride, 2.013 g Potassium Chloride, 9.008 g D-glucose. Made up to 1 L of ddH_2O and adjusted to pH 7.2-7.3 10 x Tyrode's buffer diluted to 1 x Tyrode's in ddH2O

3.7 Isolation of component fractions

Platelets, microparticles and the supernatant were separated by differential centrifugation (Figure 3.6). A standard number of platelets (500 x 10^6 platelets) were transferred to a 1.5 mL tube and centrifuged (Eppendorf 5415D; Eppendorf, Germany) at 1 500 x g for 15 minutes at room temperature to pellet platelets. The supernatant was transferred to a new tube and the platelet pellet was stored at -80°C until analysis. The supernatant was then cleared of residual platelets and microparticles by multiple rounds of centrifugation at 1 500 x g for 15 minutes at room temperature, followed by 14 000 x g for 2 minutes at 4°C. The supernatant was then transferred to a new 1.5 mL tube between spins. Lastly to isolate microparticles, the supernatant was centrifuged at 16 000 x g for 30 minutes at 4°C. The cleared supernatant was transferred to a new 1.5 mL tube and both the supernatant and microparticle pellet were frozen and stored at - 80°C until analysis.

The cleared supernatant was assessed by flow cytometry to determine if platelet and microparticle removal was successful (Figure 3.7). Flow cytometry was conducted as outlined in section 3.6.

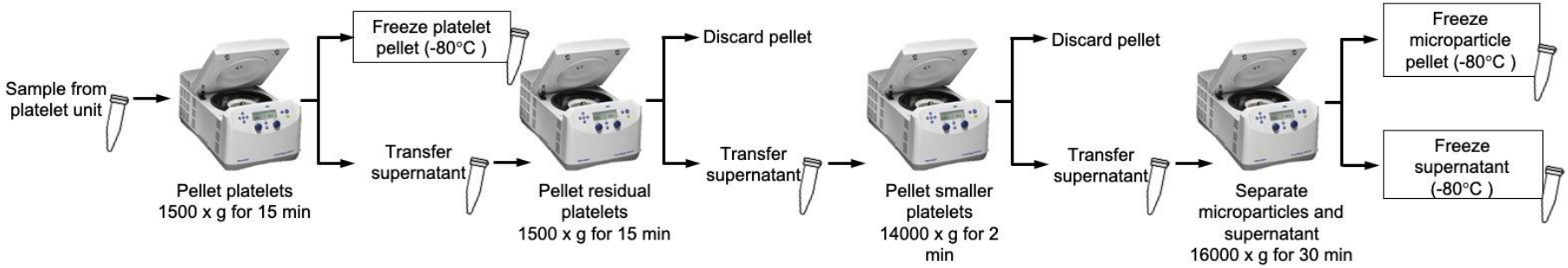

Figure 3.6. Summary of sample preparation be differential centrifugation

Samples were taken from platelet components at each testing point. The volume of the sample was normalised to $500x10^6$ platelets and transferred to a 1.5 mL tube. Platelets were pelleted by multiple rounds of centrifugation and stored at -80°C. The supernatant was cleared of residual platelets by further centrifugation and pellets discarded. The microparticle pellet and cleared supernatant were isolated by multiple rounds of centrifugation and stored at -80°C.

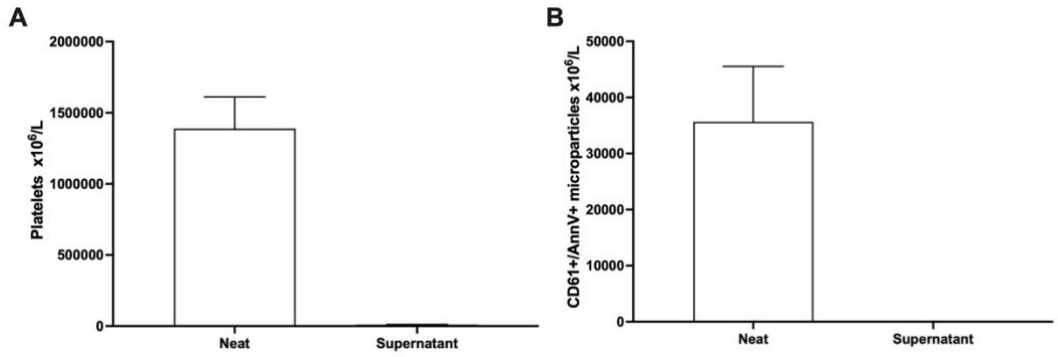

Figure 3.7. Differential centrifugation clears supernatant of most platelets and microparticles

Samples were subjected to multiple rounds of centrifugation. The absolute number of (A) platelets and (B) annexin-V positive and CD61 positive microparticles were determined in the sample prior to centrifugation (neat) and in the supernatant following centrifugation by flow cytometry. The data represents mean + SD (error bars); n=3 from post-thaw 0 samples.

3.8 Lipidomic analysis

3.8.1 Lipid extraction

Lipidomic investigations were performed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). Lipids were extracted by methods adapted from Matyash *et al.* [192]. Lipids were extracted from platelets (500 x 10^6), microparticles (100 x 10^6) and 50 μL supernatant by resuspension in 300 μL of methanol. The samples were transferred to a clean glass auto sampler vial and 5 μL of EquiSPLASH LIPIDOMIX Quantitative Mass Spectrometry Internal Standard (Avanti Polar Lipids, Alabaster, AL, USA) was added. Samples were vortexed and incubated on ice for 10 minutes. Following incubation, 1 000 μL of the organic solvent, methyl tert-butyl ether (MTBE) was added. Samples were vortexed and incubated on a rotisserie shaker at 4 °C for 1 hour. Phase separation was induced by the addition of 250 μL of ddH_2O. Samples were incubated for 10 minutes, before centrifugation at 1 000 x g for 10 minutes at room temperature. The upper organic phase, containing lipids, was transferred to a clean glass auto sampler vial and dried by nitrogen evaporation, before being resuspended in 100 μL of 2:1 isopropanol: methanol and transferred to a clean glass auto sampler vial fitted with a bottom-spring insert.

3.8.2 Lipid mass spectrometry

Global lipidomic analysis was conducted using an ACQUITY™ ultra-pressure liquid chromatography™(UPLC) I-Class system (Waters Corporation, Milford, MA, USA) coupled to a Vion Ion Mobility Spectrometry (IMS) Quadrupole Time-of-Flight (QToF) Mass Spectrometry (MS) (Waters Corporation, Milford, MA, USA) in an untargeted data-independent acquisition (DIA) manner. Data was collected in DIA method as only a limited understanding of the lipidome of alternatively stored platelet products exists, and thus could be collected in an unbiased manner. Samples were run as biological replicates (n=8 for cold

storage study and n=6 for cryopreservation study) and as technical replicates (n=3).

Each sample (1 μL) was loaded at 400 μL/min onto a C18 Acquity UPLC column (2.1 x 100 mm), packed with 1.7 μm particles (Waters Corporation). Lipids were then eluted from the column and into the source of a Vion IMS Q-ToF using the following gradient formed by mixing solvent A (60 % acetonitrile/40 % water (v/v) buffered with 0.01 % formic acid and 10 mM ammonium formate) and solvent B (90 % isopropanol/10 % acetonitrile (v/v) buffered with 0.01 % formic acid and 10 mM ammonium formate): 40 % solvent B for 2.0 min, 43 % solvent B for 0.1 min, 50 % solvent B for 9.9 min, 70 % solvent B for 0.1 min, 99 % solvent B for 5.9 min, 40 % solvent B for 0.1 min, 40 % solvent B for 1.9 min. The eluted lipids were ionised at 2 000 Volts for positive mode and 1 000 Volts for negative mode. A MS^E acquisition was performed in both positive and negative mode, with alternating low and high energy scans performed and a precursor mass-to-charge ratio (m/z) range of 100-2 000 m/z continuously scanned for lipids with an intensity of more than 30 counts/second. Ions were separated by ion mobility and then subjected to sequential low collision energy (6 electron volts (eV); containing all precursor ions) and high collision energy (20-50 eV sliding ramp) scans, with ions fragmented through Collision Induced Dissociation (CID) with argon gas. As mobility separation occurs before CID, Collisional Cross Section (CCS) was measured for all precursor ions and assigned to resulting product ions, using standard t-wave IMS settings and nitrogen gas. An example of mass chromatograph generated by LC-MS/MS is depicted in Figure 3.8. Additional representative chromatograms from other fractions and time points are presented as supplemental data, S.1-S.6.

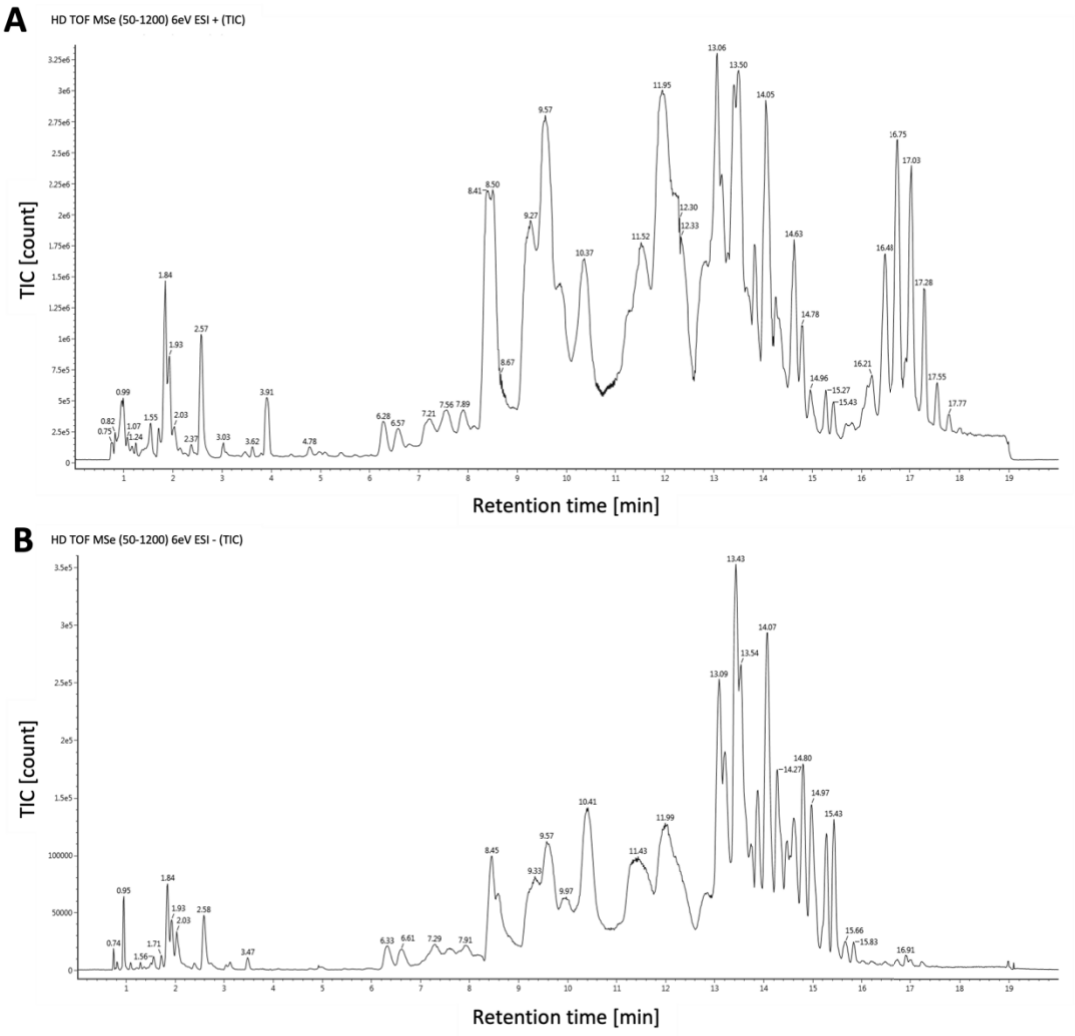

Figure 3.8. Representative total ion chromatograms from platelet samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from pre freeze platelets are shown.

3.8.3 Mass spectral data processing

Mass chromatograms were deconvoluted and data was normalised using Progenesis QI software version 2.3 (Nonlinear Dynamics, a Waters Company, Newcastle upon Tyne, UK), according to manufacturer's workflow. Sample ion alignment was performed automatically. Standard peak picking settings were used, with sensitivity set to automatic, default. Chromatographic peak width was not activated and the entire chromatogram was processed. High energy limits were set to 1 % of the base precursor peak. Adducts selected for deconvolution included M+H, M+Na, M+K and 2M+H for positive mode and M-H and M+Cl for negative mode. Lipids were searched for within the software against the LipidMaps database with search parameters of 8 parts per million (ppm) for precursor mass error tolerance and 10 ppm for fragmentation mass error tolerance [193]. Lipids were reported as normalised relative abundance, as determined by the comparison of ion intensities of the internal standard with a known concentration of lipid (μg/mL), or normalised to sum of ion content within a respective class (percentage %).

3.8.4 Nomenclature

Data is presented at the lipid species level as described by Liebisch *et al* [194]. Lipid species level represents the glycerophospholipid class followed by the total number of carbon atoms and total number of double bonds in the fatty acyl moieties e.g. PS(38:4). For sphingolipids, it has been assumed that 18:1 was the major sphingoid base. Data visualisation is representative of the lipid species that exceeded a value of 1 % across all biological replicates [195].

3.8.5 Bioinformatics methodology for pathway analysis

Lipid pathway analysis was performed using the open access web-based tool, Bioinformatics Methodology For Pathway Analysis (BioPAN) [196]. LC-MS/MS data was loaded into the platform and analysed according to the developer's workflow. BioPAN calculates a Z-score, and determines if a given reaction is significant ($p<0.05$). Further, a reaction is determined to be activated

(Z>1.645) or suppressed (Z<-1.645) depending on the direction of the change. The following options selected; type: lipid, status: active, level: lipid subclass, subset of lipid data: reactions, p-value: 0.05, paired data: yes.

3.9 Enzyme linked immunosorbent assay

Lipids analysed in plate-based assays were chosen based on their presence or association with lipids present in mass spectrometry based lipidomic analysis. The concentration of arachidonic acid (LSBio, Seattle, WA, USA), 5-HETE (LSBio), 12(S)-HETE (Abcam, Cambridge, UK), 15(S)-HETE (Abcam) and S1P (Echelon Bioscience, Inc., Salt Lake City, UT, USA) in the supernatant was determined using commercially available enzyme linked immunosorbent assay (ELISA) kits. Briefly, the standard (provided with ELISA kit and diluted to manufacturer's specifications to generate a standard curve) and sample (diluted to optimal concentrations as determined previously; Table 3.2) were pipetted in duplicate into a coated 96-well plate. The presence of arachidonic acid, 5-HETE, 12(S)-HETE, 15(S)-HETE or S1P were detected by competitive ELISA through the binding of the detection antibody and their presence was measured by an enzymatic reaction resulting in a colour change. The absorbance of each plate (450 nm for arachidonic acid and 5-HETE and 405 nm for 12(S)-HETE, 15(S)-HETE and S1P) was read on a plate reader (MultiSkan Spectrum; Thermo Electron Corporation, Waltham, MA, USA). The concentration of each lipid was calculated from the relevant standard curve.

Table 3.2. Dilutions used for enzyme linked immunosorbent assays

Storage	Arachidonic acid	5-HETE	12(S)-HETE	15(S)-HETE	S1P
Day 1	Neat	1/5	1/10	1/5	ND
Day 5 RT	1/5	1/10	1/10	1/5	ND
Day 5 Cold	1/5	1/10	1/10	1/5	ND
Day 14 RT	1/5	1/10	1/10	1/5	ND
Day 14 Cold	1/5	1/10	1/10	1/5	ND
PF	Neat	1/5	1/10	1/5	1/10
PT0	1/10	1/10	1/500	1/50	1/10
PT24	1/10	1/10	1/500	1/50	1/10
RS	Neat	Neat	1/10	1/5	1/10

RT: room temperature, Cold: cold-stored, PF: pre-freeze, PT0: post-thaw 0, PT24: post-thaw 24, RS: reconstitution solution, ND: not determined

3.10 Statistical analysis

Results are expressed as mean ± standard deviation (SD). Data were analysed using GraphPad Prism (GraphPad Software Inc., Version 9, La Jolla, CA, USA).

3.10.1 Cold-stored platelets

The effect of storage was assessed using a two-way repeated measures analysis of variance (ANOVA). *Post hoc* Bonferroni's multiple comparisons test was performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). A p-value of less than 0.05 was considered statistically significant.

3.10.2 Cryopreserved platelets

The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between pre-freeze, post-thaw 0 and post-thaw 24. A p-value of less than 0.05 was considered statistically significant.

Chapter 4 Results of the lipidomic characterisation of cold-stored platelet components

While historic studies have been conducted assessing the lipidome of cold-stored platelets, there have been advances in transfusion practices and improved lipidomic technologies, which justify a reassessment of the lipid profile of cold-stored platelets. In this chapter, the lipidome of the platelets and supernatant of cold-stored platelet components are described. Cold-stored platelet components were compared to room temperature stored platelet components for 14 days. The lipid profile of platelets was relatively unchanged at day 5 of storage at both temperatures, however, changes were evident at day 14, and these were exacerbated by cold storage. Further, the lipid profile of the supernatant was changed at day 5 of storage at both temperatures, and the changes stabilised at day 14 of storage.

Cold storage offers a solution to the short shelf life of conventionally stored platelets, as it can extend the storage time to somewhere in the range of 14-21 days [147, 148, 163, 197]. *In vitro* examination has shown cold-stored platelets have an increased haemostatic capability, which may be beneficial in treating active bleeding [122, 145, 147, 148, 154, 157]. While extensive efforts have been made to characterise cold-stored platelets [122, 145, 148, 154, 190, 198], only a few historic studies have examined the lipidome [51, 52]. As certain lipids have been associated with an increased haemostatic potential and adverse transfusion outcomes, a study of lipid changes over cold storage, inclusive of platelets and the supernatant, would provide a greater understanding of platelet component function and safety.

A paired study design was used, whereby room temperature and cold-stored platelet components from 8 donors were compared over a 14 day period. Australia recently transitioned from a 5 day to a 7 day platelet component shelf life. However at the time of conducting this study the shelf life was 5 days. As such, 5 days was chosen as the time point to represent the shelf life of room temperature stored platelet components. This study primarily used mass spectrometry based methods to evaluate the lipidome, although other *in vitro* testing was also carried out to complement or confirm the data provided by mass spectrometry.

4.1 Assessment of platelet number and morphology over room temperature and cold storage

The platelet components were sampled on day 1, 5 and 14 of storage, and the platelet count and MPV were obtained using an automated haematology analyser. The platelet count was significantly increased at day 5 of cold storage compared to day 1 (Figure 4.1A). While this increase was significant, the change was small, and therefore was unlikely to be clinically important. The MPV was significantly increased by day 5 of cold storage (Figure 4.1B). While no longer significant, the MPV remained higher in the cold-stored group compared to room temperature platelets at day 14. Platelet swirl was apparent in all room temperature stored components but was absent in cold-stored components. Macroscopic aggregates were not observed in any component. Additionally, the higher cell count, MPV and lack of swirl likely reflect the known cold induced morphological changes in cold-stored platelets [145].

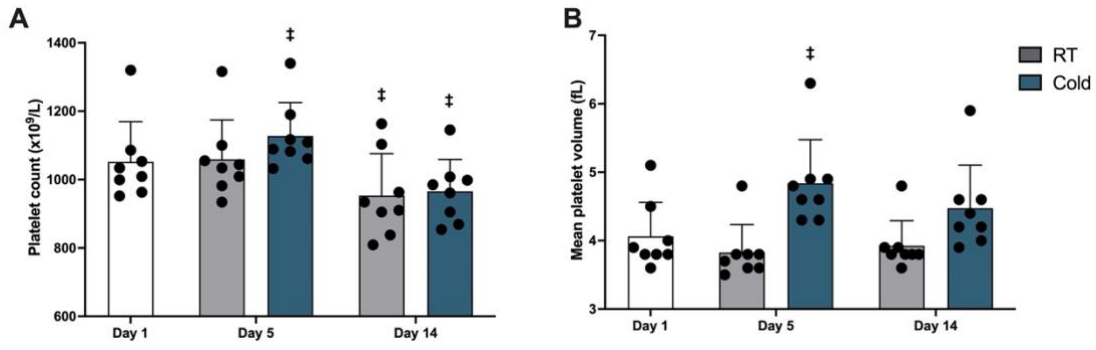

Figure 4.1. The effect of cold storage on platelet count and mean platelet volume

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The (A) platelet count and (B) mean platelet volume (MPV) were assessed using an automated haematology analyser. The data represents individual data points and mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. No statistical differences were observed between cold and room temperature at the same time point ($p>0.05$).

The externalisation of PS was assessed by flow cytometry using annexin-V. On day 1, a low proportion of platelets bound annexin-V (Figure 4.2A). By day 5, a significant increase (~4-fold) in the percentage of platelets positive for annexin-V was observed in the cold-stored platelets. However, by day 14 approximately 20 % of platelets were annexin-V positive in both the cold and room temperature groups. A similar pattern was observed for the MFI of annexin-V (Figure 4.2B). The trend towards increased externalisation of PS on cold-stored platelets at day 5 may be suggestive of increased lipid membrane changes.

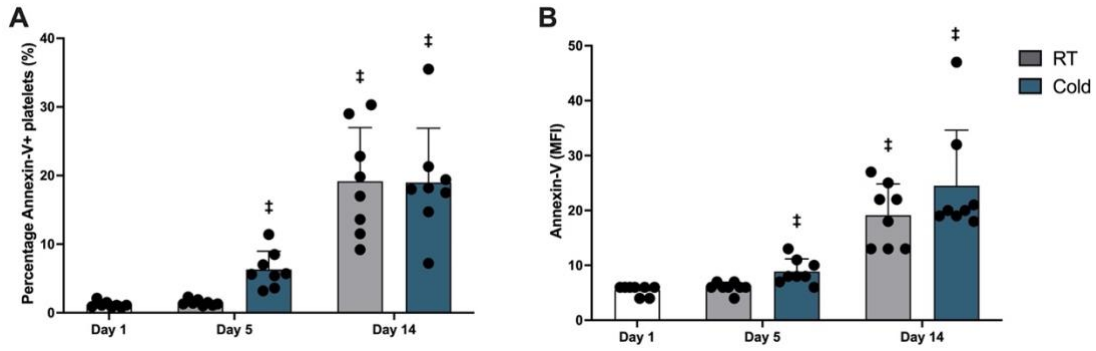

Figure 4.2. The effect of cold storage on phosphatidylserine externalisation

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. Platelets were stained with annexin-V (FITC) and (A) the percentage of annexin-V positive platelets and (B) the median fluorescence intensity (MFI) of annexin-V binding was measured by flow cytometry. The data represents individual data points and mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. No statistical differences were observed between cold and room temperature at the same time point ($p>0.05$).

4.2 Assessment of global lipidomic changes over room temperature and cold storage

The effect of storage time and temperature on the lipid profile of platelets and supernatant was assessed by LC-MS/MS. The relative abundance of lipids was determined using an internal standard. The phospholipid and sphingolipid content of the platelet and supernatant fractions was similar over 14 days, regardless of whether the platelet components were stored at room temperature or in the cold (Figure 4.3A and B).

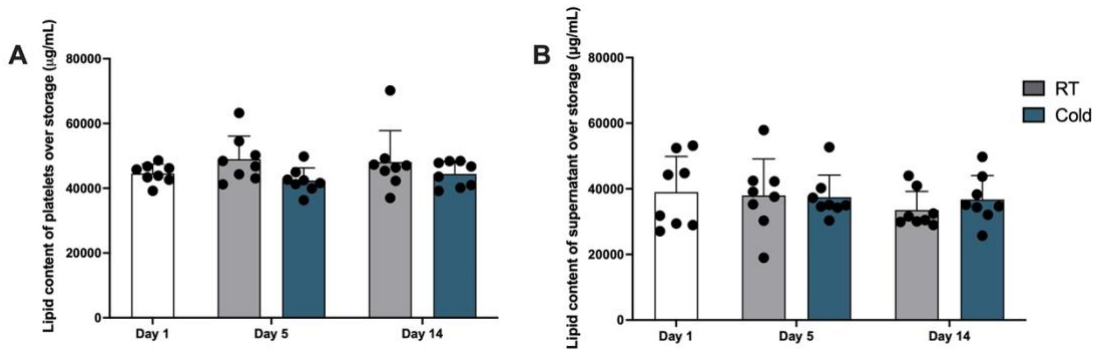

Figure 4.3. The effect of cold storage on the lipid content of platelets and supernatant

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelet and supernatant fractions were separated by differential centrifugation. The relative abundance of phospholipids and sphingolipids in the (A) platelets and (B) supernatant were determined by LC-MS/MS and compared to a known standard. The data represents individual data points and mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). No statistical differences were observed between storage time or temperature ($p>0.05$).

The composition of the phospholipid and sphingolipid classes was interrogated in the platelet and supernatant fractions. The most predominant lipid in platelets was PC (PCO was included in the PC class as it represented less than 5 % of the lipid profile), representing approximately 55% of the lipid profile (Figure 4.4A), followed by PE and PEP. Several changes were apparent in the platelet fraction, but these changes were mainly observed during extended storage (day 14). Specifically, PA, PE, PI, PS and SM were significantly increased by day 14 of cold storage, compared to day 1. Further, the increase at day 14 of cold storage for PA, PI and SM were significantly higher than room temperature stored platelets at the same time point. On the other hand, PC, LPE, PEP and ceramide were significantly decreased in platelets by day 14 of cold storage. Further, ceramide was the only lipid that was significantly increased in platelets when stored at room temperature for 14 days. Additionally, as a result of these changes ceramide and PEP were significantly different between room temperature and cold-stored platelets at day 14.

Similar to the platelet fraction, in the supernatant fraction PC was the dominant lipid (~60%, Figure 4.4B). PE and PEP represented approximately 20% and 10%, respectively. Numerous changes were apparent in the supernatant, and they were observed during the normal room temperature shelf life (5 days). There was a decrease in ceramide, PC and PEP in both room temperature and cold during storage, compared to day 1, with a trend for a larger decrease in the room temperature group at day 5. Conversely, LPA, LPE, PE, PI and PS were significantly increased in the supernatant during room temperature storage, compared to day 1. For LPE, PE and PI this increase resulted in a significant difference between the room temperature and cold stored components at day 5, that was still apparent at day 14 for LPE and PI.

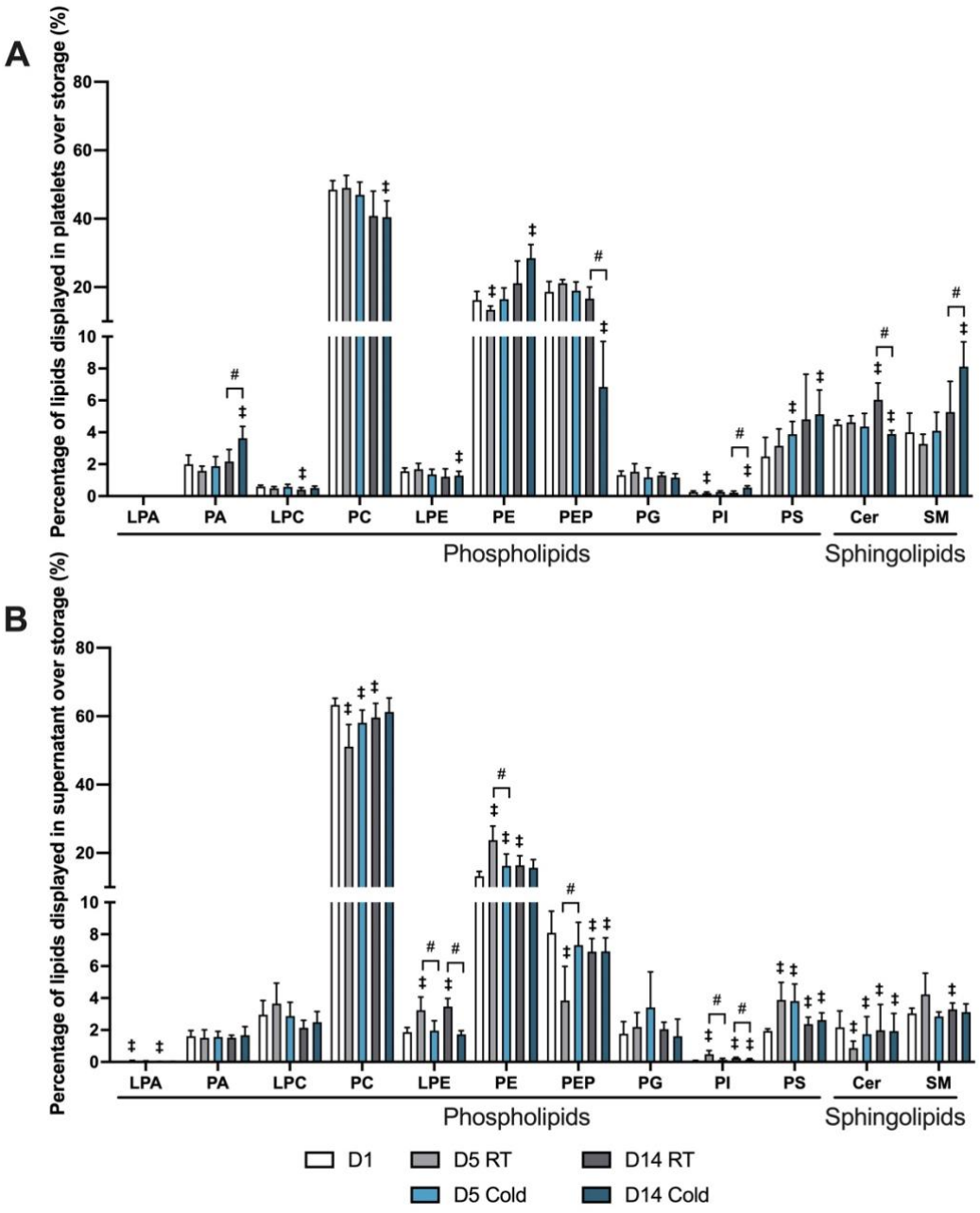
A
Percentage of lipids displayed in platelets over storage (%)
80
60
40
20
10
8
6
4
2
0
LPA
PA
LPC
PC
LPE
PE
PEP
PG
PI
PS
Cer
SM
Phospholipids
Sphingolipids
B
Percentage of lipids displayed in supernatant over storage (%)
80
60
40
20
10
8
6
4
2
0
LPA
PA
LPC
PC
LPE
PE
PEP
PG
PI
PS
Cer
SM
Phospholipids
Sphingolipids
D1
D5 RT
D14 RT
D5 Cold
D14 Cold

Figure 4.4. The effect of cold storage on phospholipids and sphingolipids

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of lipids displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time on each lipid class was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to identify specific differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ p[$p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Phosphatidylcholine was the predominant lipid class in the platelet and supernatant fractions, and it was found to be altered by storage time (Figure 4.4). As such, the shifts in the PC species resulting from room temperature and cold storage were assessed. In the platelet fraction the predominant PC species was PC(36:4) (Figure 4.5A). In general, the PC species profile was relatively stable. However, several significant differences were observed, mainly at day 14 of cold storage. There was a trend for increasing PC(34:2) and PC(36:3) in cold-stored components which was significant at day 14 compared to day 1, and when compared to room temperature storage at the same time point. Similarly, there was a trend for increasing PC(38:3) in room temperature stored components which was significant at day 14 compared to day 1, and when compared to cold storage at the same time point. Conversely, after initially remaining stable, PC(36:1) and PC(38:1) were significantly decreased at day 14 of cold storage, compared to day 1. A similar trend was seen for PC(40:5), which resulted in a significant difference between storage temperatures at day 14.

Numerous statistical differences were observed in the PC species of the supernatant fraction. As such, only significant trends and differences between storage conditions will be highlighted (Figure 4.5B). In room temperature platelets stored for 5 days, PC(34:1) and PC(36:2) were significantly increased compared to day 1 and compared to cold-stored platelets at day 5. Further, the high proportion of PC(34:1) and PC(36:2) resulted in a trend for most other PC species to be significantly lower than the day 1 and the cold-stored platelets at day 5. By day 14, the room temperature stored species were similar to day 1. In contrast, the cold-stored samples at day 14 displayed significantly increased PC(34:1) and PC(34:2) compared to day 1, and for PC(34:1) this was significantly higher than the room temperature stored group at the same time point. PC(36:1), PC(38:2) and PC(38:4) were significantly decreased at day 14 of cold storage, compared to day 1. Further, at day 14 of room temperature storage PC(38:4) was also significantly decreased compared to day 1, however the decrease was greater in cold-stored platelets.

PC(40:4) and PC(40:5) were the only species to be significantly increased at day 14 of room temperature storage, compared to day 1.

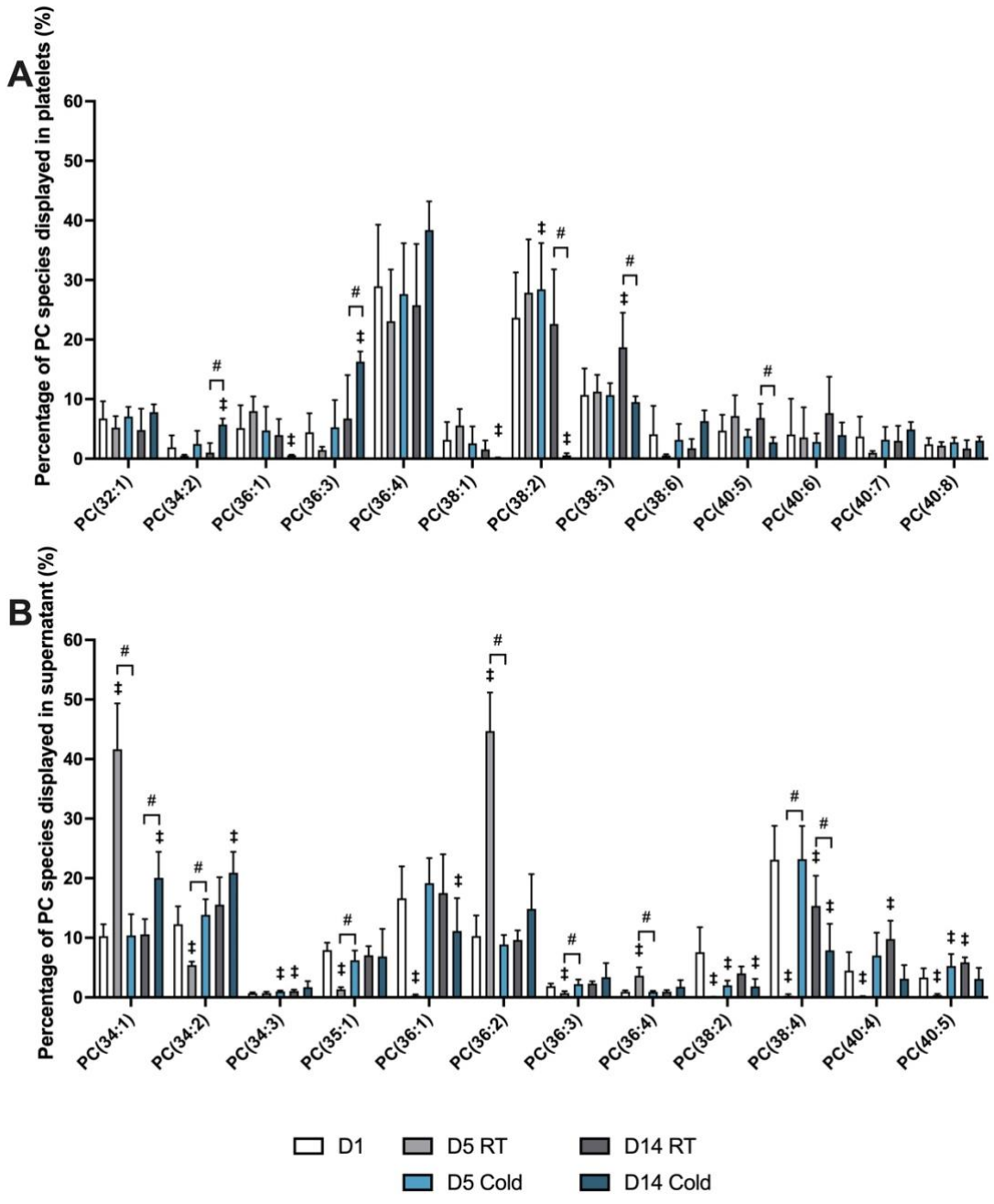
A
Percentage of PC species displayed in platelets (%)
60
50
40
30
20
10
0
PC(32:1)
PC(34:2)
PC(36:1)
PC(36:3)
PC(36:4)
PC(38:1)
PC(38:2)
PC(38:3)
PC(38:6)
PC(40:5)
PC(40:6)
PC(40:7)
PC(40:8)
B
Percentage of PC species displayed in supernatant (%)
60
50
40
30
20
10
0
PC(34:1)
PC(34:2)
PC(34:3)
PC(35:1)
PC(36:1)
PC(36:2)
PC(36:3)
PC(36:4)
PC(38:2)
PC(38:4)
PC(40:4)
PC(40:5)
D1
D5 RT
D14 RT
D5 Cold
D14 Cold

Figure 4.5. The effect of cold storage on phosphatidylcholine species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of phosphatidylcholine (PC) displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Lysophosphatidylcholine is a bioactive lipid mediator, which has been associated with adverse transfusion reactions [200, 201]. Thus, shifts in the LPC species displayed during room temperature and cold storage were assessed. In the platelet fraction, LPC(18:1) represented approximately 50 % of the LPC species (Figure 4.6A). In general, the LPC species profile was stable over cold storage. In contrast, storage at room temperature resulted in a decrease in LPC(18:0) and LPC(18:2) at day 5 and day 14, compared to day 1. For LPC(18:2), this resulted in a significant difference between the room temperature and cold-stored platelets at both day 5 and day 14. Similarly, room temperature storage resulted in a significant decrease in LPC(20:4) at day 14, compared to day 1 and cold storage at the same time point. Conversely, LPC(20:0) and LPC(O-20:0) increased across room temperature storage, compared to day 1. Further, the increase in room temperature storage at day 14 resulted in a significant difference compared to cold storage. In summary, the LPC species profile was relatively unchanged by cold storage, however, the LPC species profile was altered by room temperature storage.

In the supernatant fraction LPC(18:1) and LPC(18:2) were the most prominent LPC species, jointly accounting for approximately 80% (Figure 4.6B). Similar to the platelet fraction, the proportion of species remained relatively stable during cold storage. Further, LPC species that contributed a low proportion were unchanged until extended storage (day 14). LPC(18:1) was significantly increased at day 5 and day 14 regardless of temperature, compared to day 1, and the increase was greater in room temperature storage. On the other hand, LPC(18:2) decreased over storage at room temperature. Further, the proportion of LPC(18:2) at room temperature was significantly lower at day 14 than the cold-stored group. LPC(20:4) was significantly decreased by extended storage (day 14) regardless of temperature, compared to day 1. In summary, with the exception of LPC(18:1), the LPC species profile was relatively unchanged by cold storage until day 14, but was changed by room temperature storage by day 5.

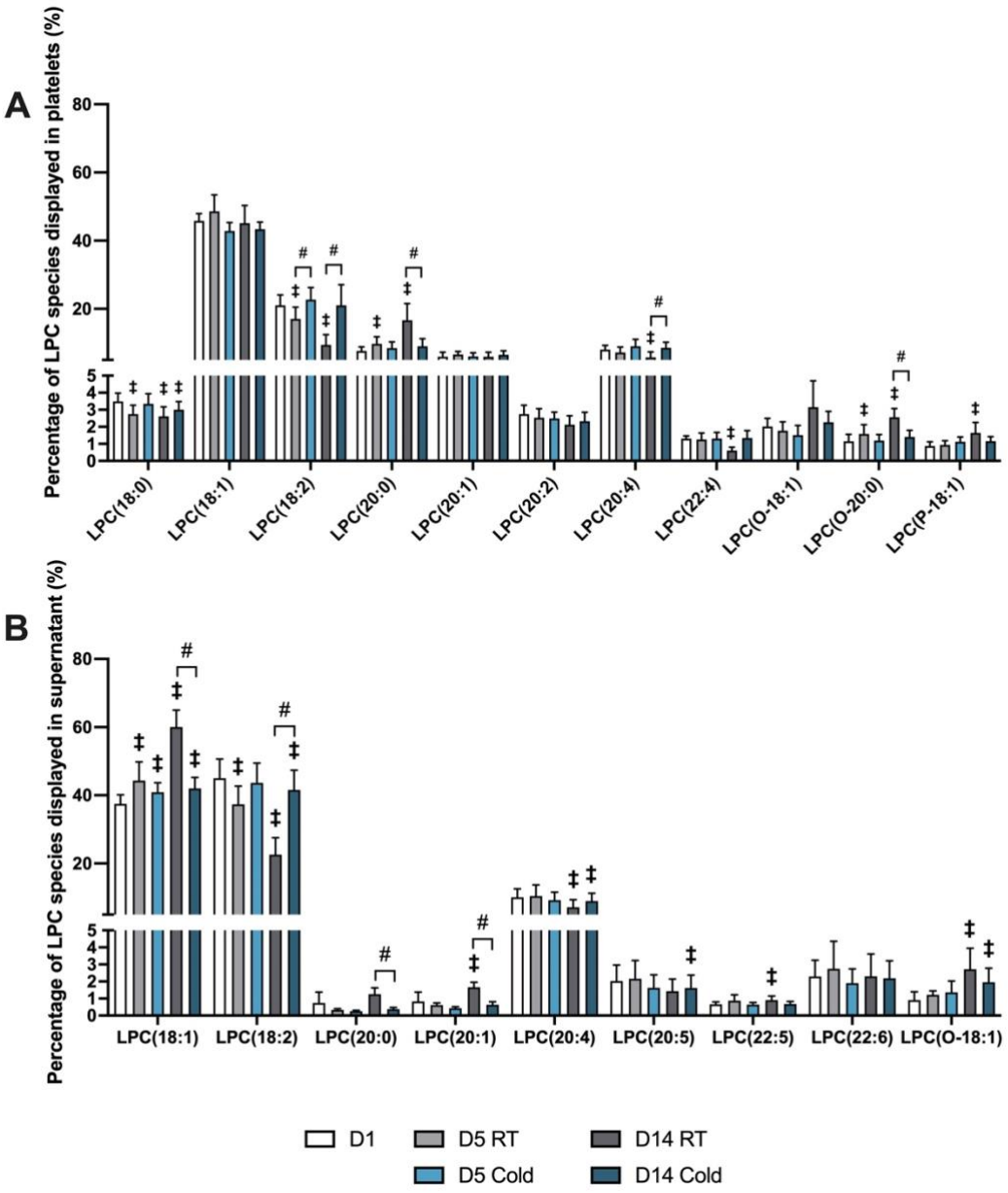
A
Percentage of LPC species displayed in platelets (%)
LPC(18:0)
LPC(18:1)
LPC(18:2)
LPC(20:0)
LPC(20:1)
LPC(20:2)
LPC(20:4)
LPC(22:4)
LPC(O-18:1)
LPC(O-20:0)
LPC(P-18:1)
B
Percentage of LPC species displayed in supernatant (%)
LPC(18:1)
LPC(18:2)
LPC(20:0)
LPC(20:1)
LPC(20:4)
LPC(20:5)
LPC(22:5)
LPC(22:6)
LPC(O-18:1)
D1
D5 RT
D5 Cold
D14 RT
D14 Cold

Figure 4.6. The effect of cold storage on lysophosphatidylcholine species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of Lysophosphatidylcholine (LPC) displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Phosphatidylethanolamine is known to support coagulation [90], and the proportion was shown to be altered by storage time and temperature (Figure 4.4). Therefore, the species profile of PE was examined. In the platelet fraction, multiple differences were found as a result of storage time and temperature, particularly at day 14 of storage. However, only key trends and differences will be highlighted (Figure 4.7A). PE(34:2), PE(36:1), PE(36:2) and PE(40:4) were present at a higher proportion at day 14 in the cold-stored components. Similarly, the proportion of PE(38:4) was significantly increased at day 14 compared to day 1 in both temperature groups. PE(38:1) and PE(40:2) showed the opposite trend, with a significant decrease observed at day 14, compared to day 1.

In the supernatant fraction, although several differences were observed, most appeared in minor species and thus only key trends and differences will be highlighted (Figure 4.7B). PE(34:1) and PE(40:1) represented the predominant species in the supernatant fraction, collectively comprising 80% of all PE species. At day 5 of storage, the proportion of PE(34:1) was significantly increased when stored in the cold, compared to day 1. By day 14 both room temperature and cold storage were higher than day 1, but no difference between the groups were observed. The proportion of PE(40:1) fluctuated, variably, over storage but remained lower than day 1. The proportion of PE(26:1) and PE(38:1) was significantly increased at day 5 when cold-stored, when compared to day 1 and day 5 samples stored at room temperature.

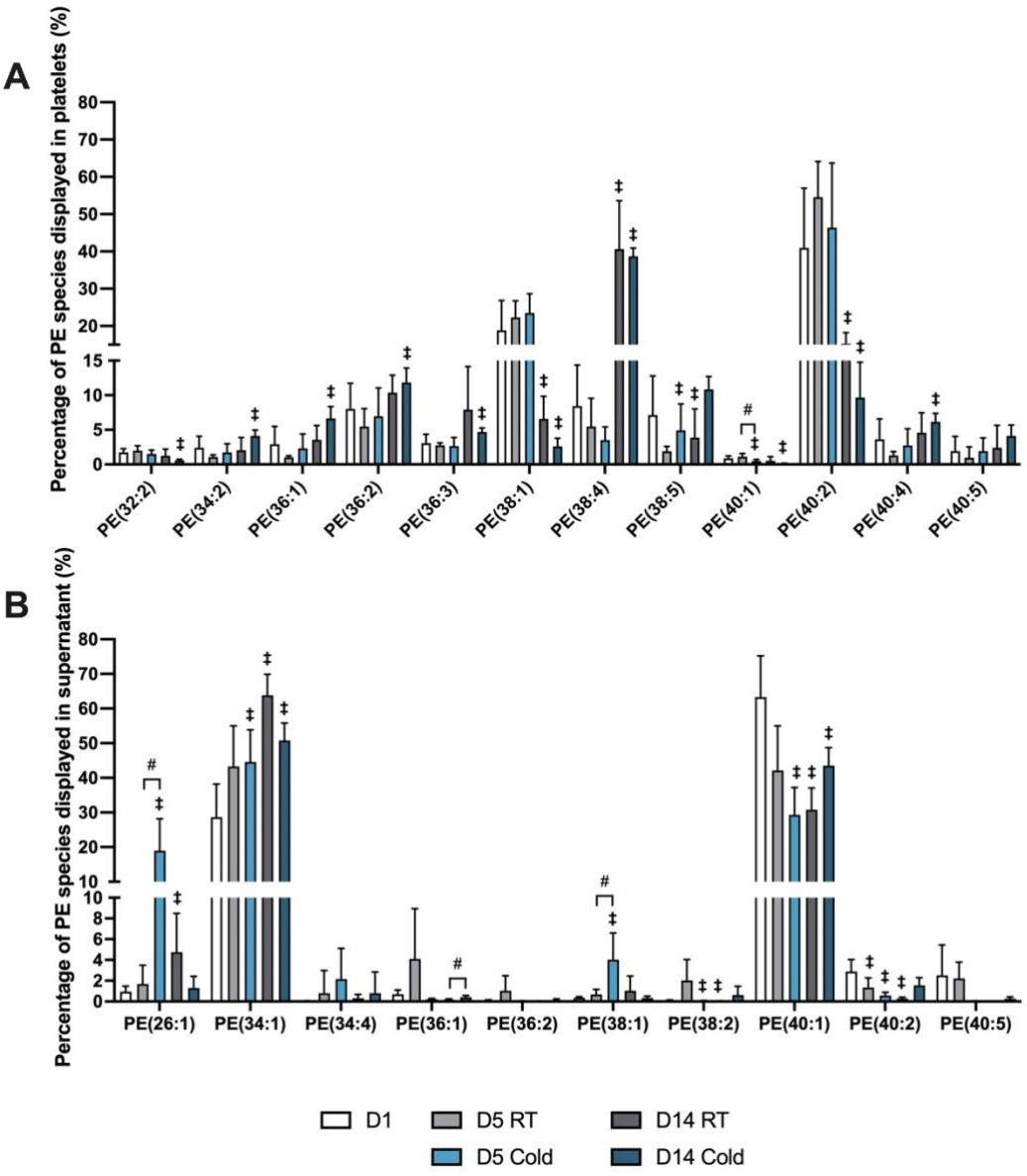
A
Percentage of PE species displayed in platelets (%)
PE(32:2)
PE(34:2)
PE(36:1)
PE(36:2)
PE(36:3)
PE(38:1)
PE(38:4)
PE(38:5)
PE(40:1)
PE(40:2)
PE(40:4)
PE(40:5)
B
Percentage of PE species displayed in supernatant (%)
PE(26:1)
PE(34:1)
PE(34:4)
PE(36:1)
PE(36:2)
PE(38:1)
PE(38:2)
PE(40:1)
PE(40:2)
PE(40:5)
D1
D5 RT
D5 Cold
D14 RT
D14 Cold

Figure 4.7. The effect of cold storage on phosphatidylethanolamine species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of phosphatidylethanolamine (PE) displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Ether linked phospholipids occur mostly in the PC and PE lipid classes [63]. Ether linked phospholipids are formed through the attachment of the alkyl chain by an ether bond to the sn-1 acyl chain (denoted by the abbreviation O) [63]. More commonly the alkyl chain is attached adjacent to a double bond, termed plasmalogen (denoted by the abbreviation P) [63]. Due to the altered structure, and thus altered function, interest in ether linked lipids has grown. Further, the PEP class was changed in the platelet and supernatant fraction (Figure 4.4). Thus, shifts in the PCO and PEP species were examined.

In the platelet fraction, the PCO species profile was relatively stable across room temperature and cold storage. PC(O-38:6) was the most prevalent PCO species, representing 30% (Figure 4.8A). There was a trend for decreasing PC(O-38:6) during cold storage, which was significant at day 14. Initially (day 5) PC(O-38:6) was significantly increased at room temperature, but at day 14 had returned to baseline. As such, at both time points PC(O-38:6) was significantly lower in the cold-stored group compared to room temperature stored platelets.

In the supernatant fraction, the most prominent PCO lipid species was PC(O-36:5) (~40%; Figure 4.8B). PC(O-36:5) was significantly higher in the cold-stored platelets at both time points, compared to day 1. There was a trend for PC(O-34:4) to increase over storage at both temperatures, and was significantly higher at day 14, compared to day 1. On the other hand, PC(O-36:1) was significantly decreased at day 5 of room temperature and cold storage compared to day 1, but this did not continue to day 14. A higher proportion of several long chain PCO species (PC(O-40:1), PC(O-40:4) and PC(O-40:5)) were detectable at day 5 during room temperature storage, but present in very low proportions at other time points.

The most prevalent PEP species in the platelet fraction was PE(P-40:1), representing approximately 80% (Figure 4.9A). The PEP profile was relatively unchanged by storage temperature, and only a few differences occurred as a result of storage time. At day 5, PE(P-40:1) was significantly increased

compared to day 1, however returned to baseline by day 14. There was a trend for PE(P-38:1) to decrease over time regardless of storage temperature, which was significant at day 5 of room temperature storage and day 14 of cold storage, compared to day 1. Similarly, PE(P-40:4) trended downward across all samples and was significantly lower at day 5 of room temperature and cold storage, compared to day 1.

The most prevalent PEP species in the supernatant fraction was PE(P-40:4), which represented approximately 60% (Figure 4.9B). Similar to the platelet fraction, PEP was relatively unchanged and differences occurred in species that represented a low proportion of the PEP profile (less than 5 %). For example, PE(P-40:6) and PE(P-40:7) were significantly increased at day 5 of room temperature storage, compared to both day 1 and cold storage at the same time point.

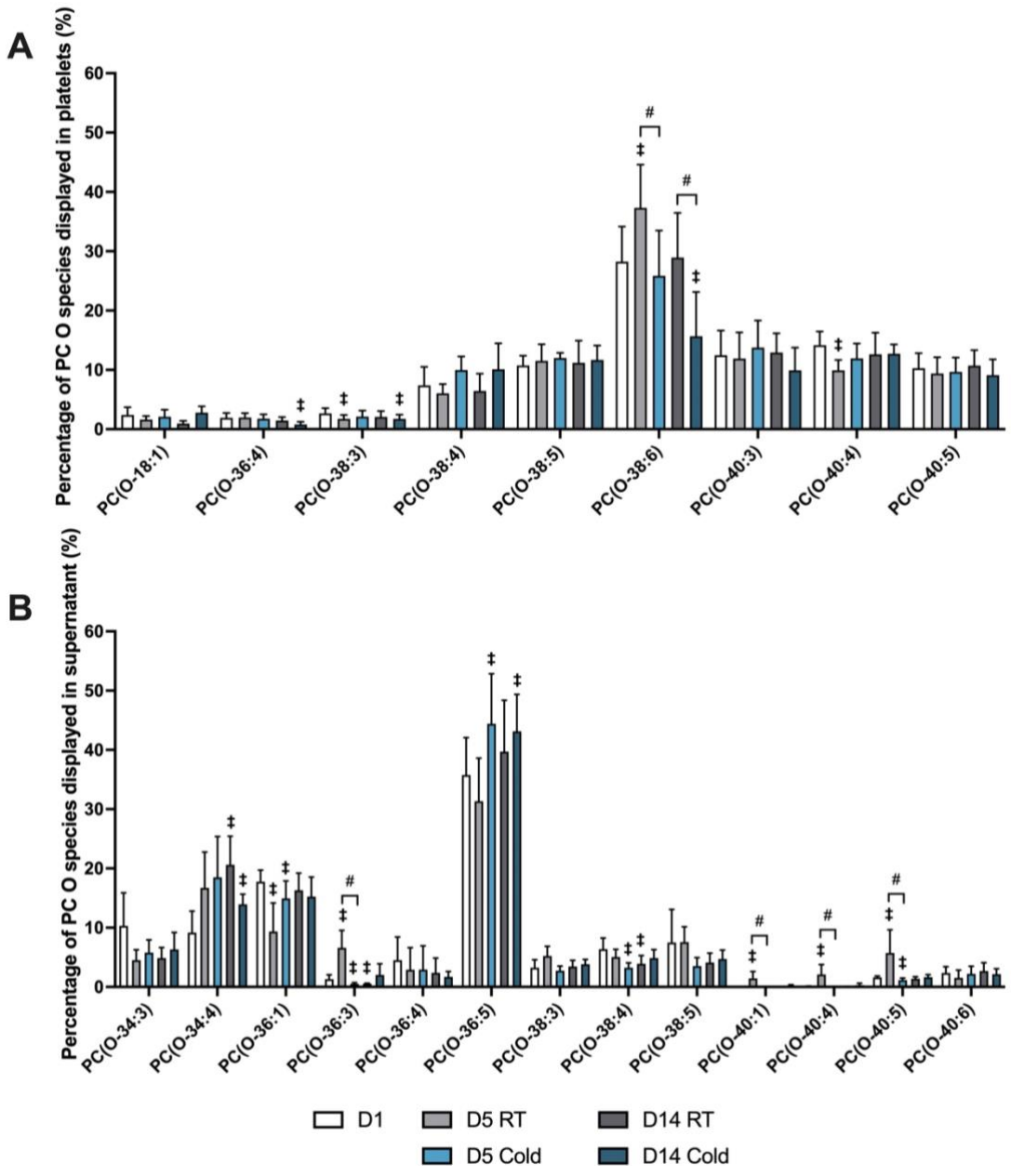
A
Percentage of PC O species displayed in platelets (%)
60
50
40
30
20
10
0
PC(O-18:1)
PC(O-36:4)
PC(O-38:3)
PC(O-38:4)
PC(O-38:5)
PC(O-38:6)
PC(O-40:3)
PC(O-40:4)
PC(O-40:5)
B
Percentage of PC O species displayed in supernatant (%)
60
50
40
30
20
10
0
PC(O-34:3)
PC(O-34:4)
PC(O-36:1)
PC(O-36:3)
PC(O-36:4)
PC(O-36:5)
PC(O-38:3)
PC(O-38:4)
PC(O-38:5)
PC(O-40:1)
PC(O-40:4)
PC(O-40:5)
PC(O-40:6)
D1
D5 RT
D14 RT
D5 Cold
D14 Cold

Figure 4.8. The effect of cold storage on ether linked phosphatidylcholine species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of ether linked phosphatidylcholine (PCO) displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

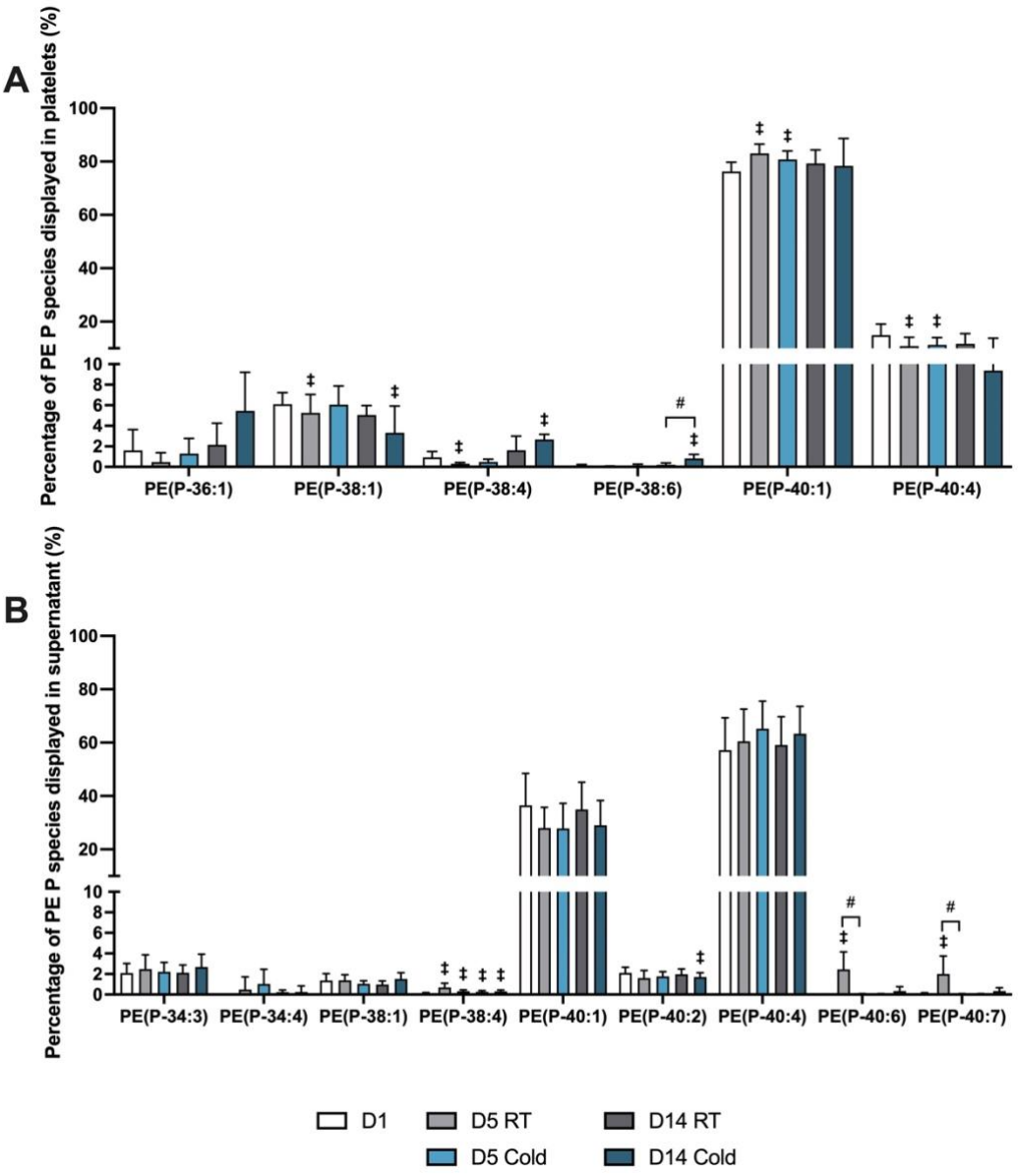
A
Percentage of PE P species displayed in platelets (%)
100
80
60
40
20
10
8
6
4
2
0
PE(P-36:1)
PE(P-38:1)
PE(P-38:4)
PE(P-38:6)
PE(P-40:1)
PE(P-40:4)
B
Percentage of PE P species displayed in supernatant (%)
PE(P-34:3)
PE(P-34:4)
PE(P-38:1)
PE(P-38:4)
PE(P-40:1)
PE(P-40:2)
PE(P-40:4)
PE(P-40:6)
PE(P-40:7)
D1
D5 RT
D5 Cold
D14 RT
D14 Cold

Figure 4.9. The effect of cold storage on ether linked phosphatidylethanolamine species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of ether linked phosphatidylethanolamine (PEP) displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Phosphatidylserine is known to facilitate coagulation [88, 90]. Further, there was an increase in the proportion of PS in platelets during cold-storage (Figure 4.4). As such, the species of PS in the platelets and supernatant were examined. In the platelet fraction, PS(36:1), PS(38:1), PS(40:1) and PS(40:3) were the most prominent species, collectively accounting for approximately 80% of the total PS (Figure 4.10A). There was a trend for increasing PS(38:1) over storage. At day 14, the proportion of PS(38:1) was significantly higher than at day 1, and the increase was greater during room temperature storage. PS(38:3) increased over cold storage and the increase was significant at day 14, compared to day 1, and was significantly higher than the room temperature group at both time points. Conversely, the proportion of PS(40:1) and PS(40:3) were significantly lower at day 14, compared to day 1. The lipids that represented < 10% of the total PS species profile were relatively constant, with only a few shifts occurring.

In the supernatant fraction (Figure 4.10B), PS(38:1) was the prominent lipid (~ 40%). When comparing changes to day 1, the majority were transient and small in magnitude. Of note, at day 5, the proportion of PS(36:1) was significantly increased and PS(40:1) was significantly decreased over room temperature storage compared cold storage.

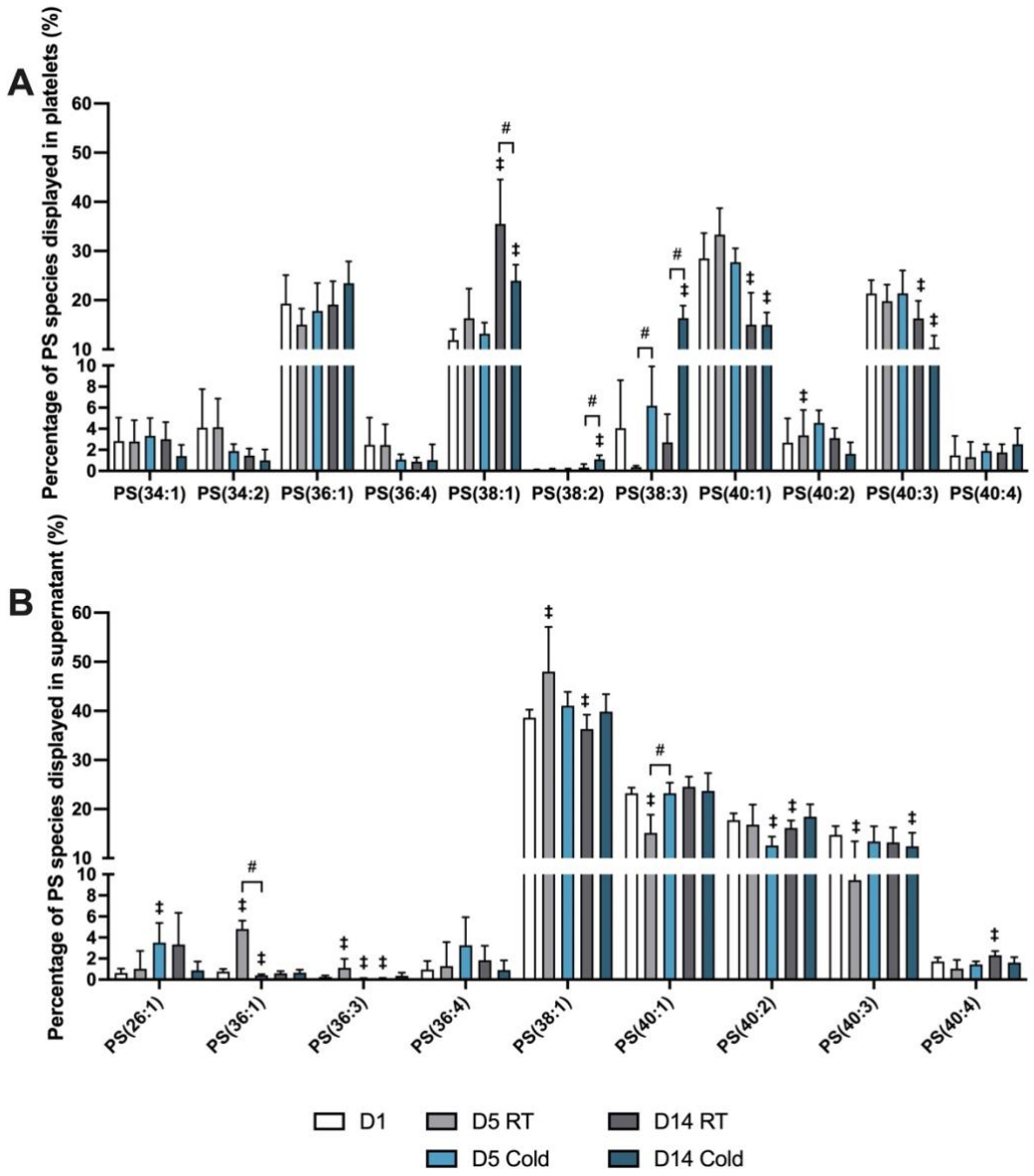
A
Percentage of PS species displayed in platelets (%)
PS(34:1)
PS(34:2)
PS(36:1)
PS(36:4)
PS(38:1)
PS(38:2)
PS(38:3)
PS(40:1)
PS(40:2)
PS(40:3)
PS(40:4)
B
Percentage of PS species displayed in supernatant (%)
PS(26:1)
PS(36:1)
PS(36:3)
PS(36:4)
PS(38:1)
PS(40:1)
PS(40:2)
PS(40:3)
PS(40:4)
D1
D5 RT
D14 RT
D5 Cold
D14 Cold

Figure 4.10. The effect of cold storage on phosphatidylserine species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The platelets and supernatant were separated by differential centrifugation. The percentage composition of phosphatidylserine (PS) displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Sphingomyelin is a precursor to the bioactive lipids, ceramide, sphingosine and sphingosine 1-phosphate [202]. Further, the percentage of SM displayed in platelets and supernatant was altered (Figure 4.4). In the platelet fraction, SM(23:0), SM(25:0) and SM(26:0) were the most prominent SM species at day 1, accounting for 80% of the SM species profile (Figure 4.11A). SM(16:0), SM(18:1) and SM(24:0) increased after cold storage and extended storage (day 14), being significantly higher at day 5 of cold storage and day 14 in both room temperature and cold-stored groups, compared to day 1. Further, cold storage induced a greater change than room temperature storage. Conversely, SM(23:0), SM(25:0) and SM(26:0) decreased over storage at both temperatures, and cold storage resulted in a greater loss of these species.

In the supernatant, SM(25:0) was the most prominent SM species (~ 50%; Figure 4.11B). The SM species profile was relatively stable when comparing storage temperatures, where only SM(20:0) and SM(25:0) were different. SM(20:0) was significantly increased at day 5 of room temperature storage, but remained similar to day 1 at other time points. SM(25:0) was significantly increased at day 5 of cold storage, compared to day 1, but was not significantly increased in room temperature samples until day 14. On the other hand, SM(18:0) was significantly decreased at day 5 and day 14 of room temperature and cold storage, compared to day 1. SM(26:0) was significantly decreased at day 14 in both room temperature and cold-stored groups, compared to day 1.

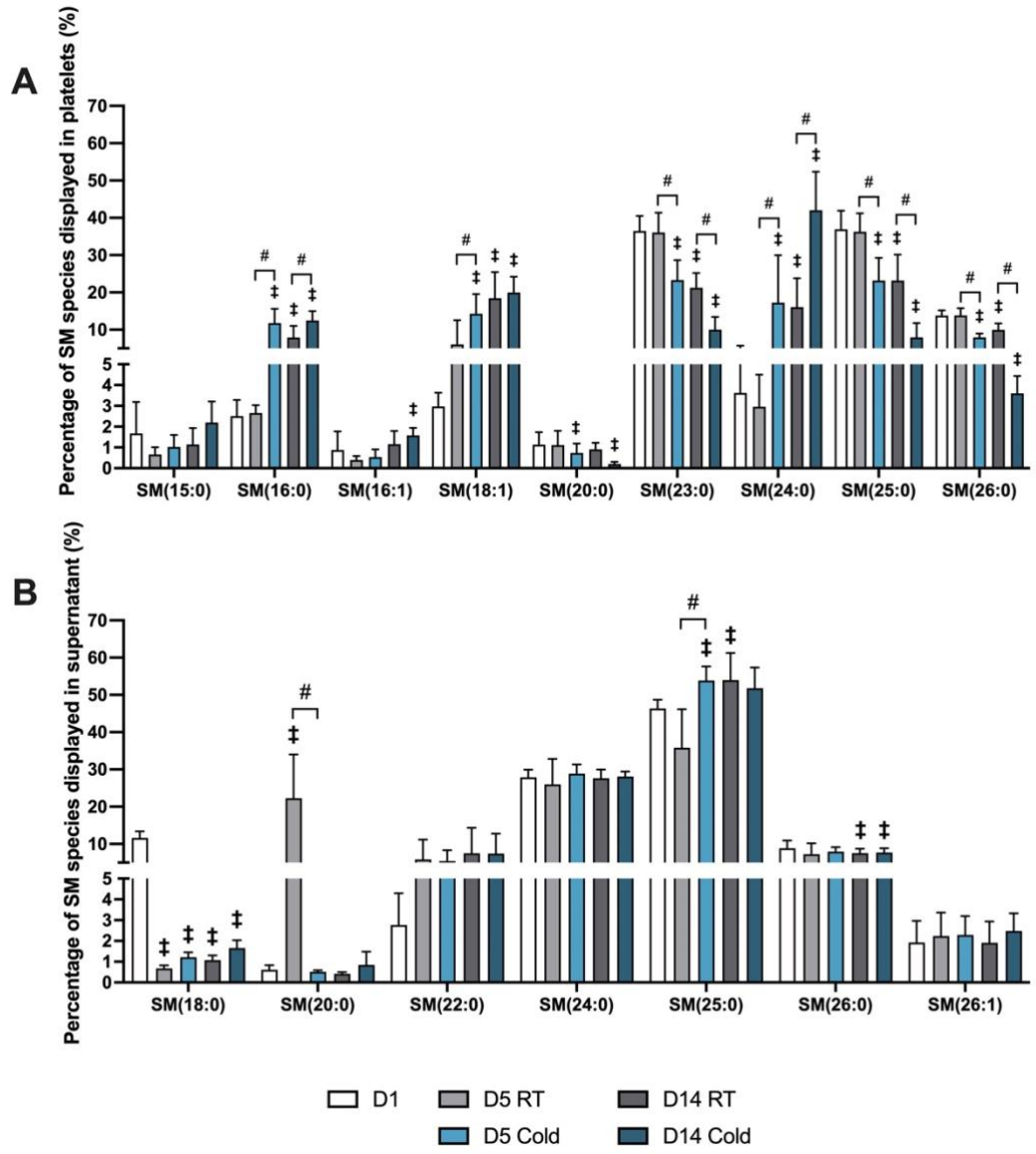

Figure 4.11. The effect of cold storage on sphingomyelin species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The percentage composition of sphingomyelin (SM) species displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

Ceramide is a known second messenger and bioactive lipid mediator, and is closely associated with oxidative stress and apoptosis [202, 203]. The percentage of total ceramide displayed was altered in the platelets and supernatant (Figure 4.4). Cer(22:0), Cer(24:0) and Cer(26:0) were the most prominent ceramide species, accounting for 70% of the ceramide profile in platelets (Figure 4.12A). In general, there was a trend for short chain ceramide species to increase over storage regardless of temperature, while long chain ceramide species tended to decrease over storage time regardless of temperature. More specifically, Cer(18:0) increased in cold-stored samples, while decreasing in room temperature stored platelets. As a result, there was a significant difference between room temperature and cold storage at day 5 and 14. Cer(22:0) was significantly increased at day 5 and day 14 of both room temperature and cold storage compared to day 1, which was more pronounced in room temperature stored samples. Cer(24:0) remained stable over cold storage, but decreased significantly during room temperature storage at day 5 and day 14, compared to day 1. Cer(26:0), Cer(32:0) and Cer(33:0) displayed a similar trend, whereby the species remained stable over the conventional storage period (day 5), but was significantly decreased after extended storage (day 14) for both room temperature and cold-stored samples, compared to day 1. Cer(30:0) and Cer(31:0) also displayed a similar trend where there was a decrease across all storage times and temperatures.

In the supernatant, Cer(30:0), Cer(31:0), Cer(32:0), Cer(33:0) and Cer(34:0) were the most prominent Cer species, totalling 85% of ceramide species (Figure 4.12B). The ceramide species that represent the highest proportion were relatively unchanged. Cer(16:0) was significantly increased at day 5 and 14 of room temperature storage compared to day 1, but remained constant over cold storage. Cer(24:0) remained stable over early time points (day 5) but was increased at day 14 of room temperature and cold storage, compared to day 1.

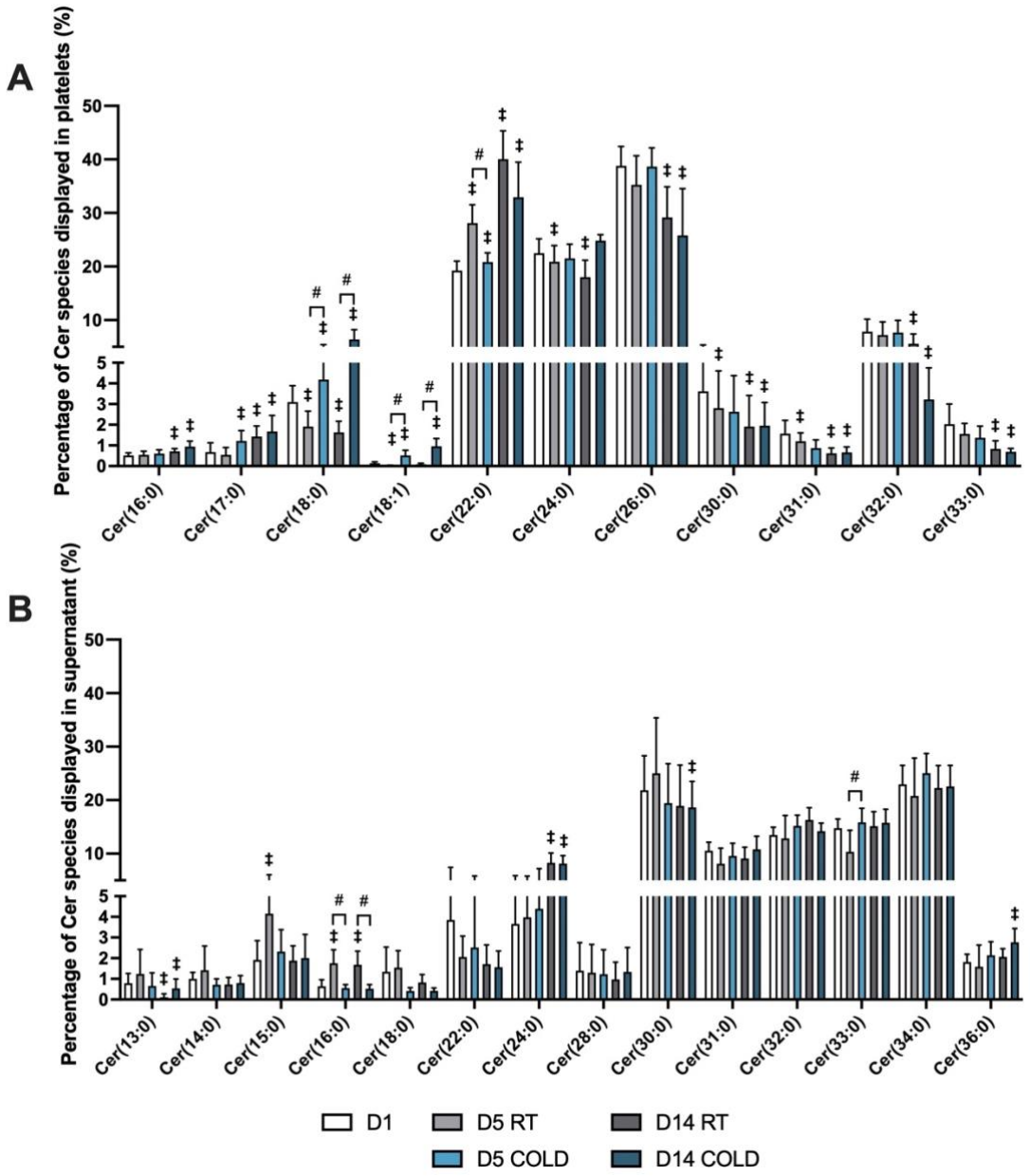
A
Percentage of Cer species displayed in platelets (%)
Cer(16:0)
Cer(17:0)
Cer(18:0)
Cer(18:1)
Cer(22:0)
Cer(24:0)
Cer(26:0)
Cer(30:0)
Cer(31:0)
Cer(32:0)
Cer(33:0)
B
Percentage of Cer species displayed in supernatant (%)
Cer(13:0)
Cer(14:0)
Cer(15:0)
Cer(16:0)
Cer(18:0)
Cer(22:0)
Cer(24:0)
Cer(28:0)
Cer(30:0)
Cer(31:0)
Cer(32:0)
Cer(33:0)
Cer(34:0)
Cer(36:0)
D1
D5 RT
D14 RT
D5 COLD
D14 COLD

Figure 4.12. The effect of cold storage on ceramide species

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The percentage composition of ceramide (Cer) species displayed in (A) platelets and the (B) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 %. The data represents mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

4.3 Assessment of bioactive lipid mediators over room temperature and cold storage

Generally, bioactive lipid mediators are defined by their ability to elicit a functional consequence [202]. In particular, several have been found to be intimately associated with platelet function [82, 202, 204]. Further, changes seen in the lipid class profile (Figure 5.4) may be the result of lipid metabolism. Lipid metabolism is known to occur in room temperature stored platelets and is known to result in the formation of bioactive lipid mediators [56, 195]. Several of these lipids were found in the supernatant by LC-MS/MS.

Although the proportion of LPA was shown to be very low and only affected by room temperature storage in the supernatant (Figure 4.4), the relative abundance was determined, as LPA is a known bioactive lipid mediator capable of producing functional changes in platelets at low concentrations [68]. The abundance of LPA was low on day 1, and remained at a similar concentration during cold storage (Figure 4.13A). In contrast, LPA was significantly higher in the room temperature samples, compared to day 1. PAF was relatively constant over storage, regardless of temperature (Figure 4.13B). 12-HETE and 15-HETE were significantly increased by room temperature storage when compared to day 1 (Figure 4.13C and D). Further, for 12-HETE there was a significant difference between room temperature stored and cold-stored samples at day 14.

The concentration of several of the bioactive lipid mediators found to be altered by LC-MS/MS and lipids within a common pathway were validated by ELISA. While LC-MS/MS was able to determine the relative abundance of bioactive lipid mediators, ELISA was used to determine the specific concentration. The concentration of arachidonic acid and 5-HETE remained relatively constant over storage, regardless of storage temperature (Figure 4.14A and B). The abundance of 12(S)-HETE and 15(S)-HETE followed the same trend, whereby the concentration was increased over storage time regardless of storage temperature, but to a greater degree during room temperature storage (Figure

4.14C and D). These results suggest cold storage suppresses either the formation or release of specific bioactive mediators.

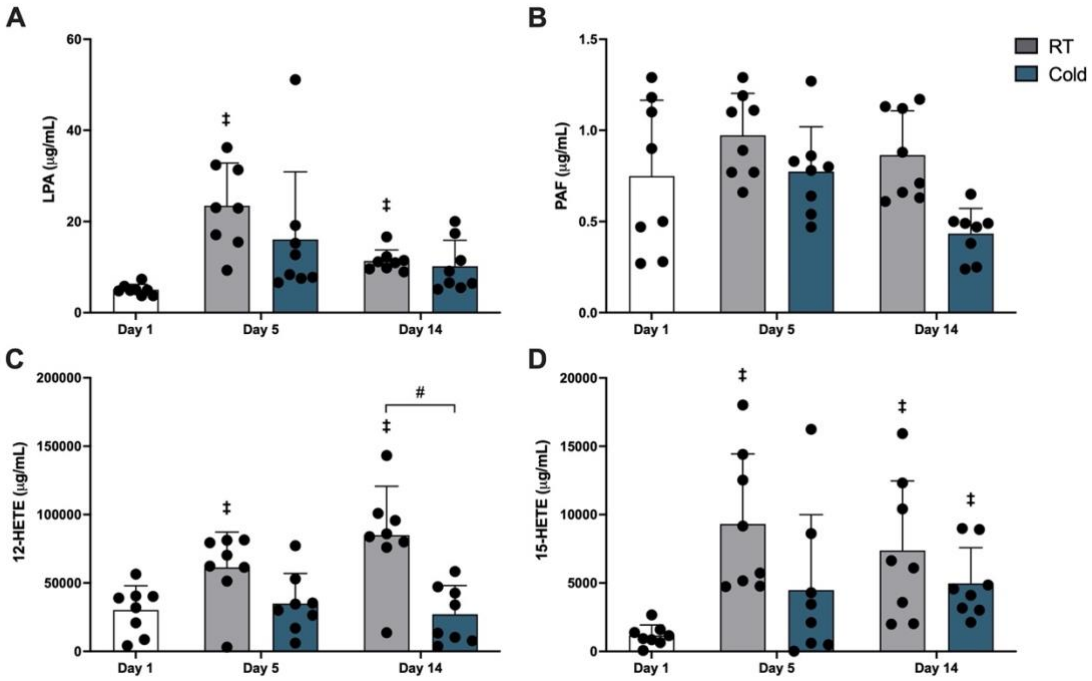

Figure 4.13. The effect of cold storage on concentration of bioactive lipid mediators in the storage solution as determined by LC-MS/MS

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The supernatant was isolated using multiple rounds of centrifugation and the relative abundance of (A) LPA, (B) platelet activating factor (PAF), (C) 12-HETE and (D) 15-HETE were determined by LC-MS/MS. The data represents individual data points, and mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

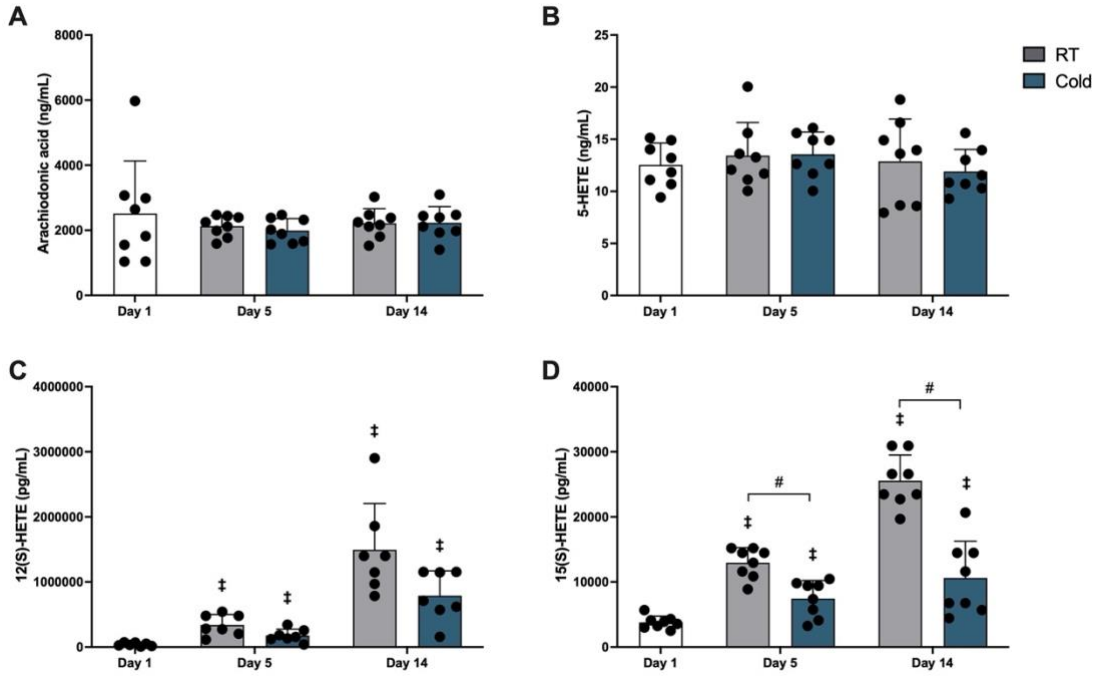

Figure 4.14. The effect of cold storage on the formation of certain bioactive lipid mediators in the storage solution as determined by ELISA

Platelet components were stored at room temperature (RT; 20-24 °C) or cold-stored (cold; 2-6 °C) and sampled on days 1, 5 and 14 post-collection. The supernatant was isolated using multiple rounds of centrifugation and the concentration of (A) arachidonic acid, (B) 5-HETE, (C) 12(S)-HETE and (D) 15(S)-HETE were determined by ELISA. The data represents individual data points, and mean (bars) + SD (error bars); n=8. The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point

4.4 Assessment of lipid dynamics and lipid pathways over room temperature and cold storage

The lipidome of platelets and the platelet component is dynamic and constantly changing, as lipids are necessary for cellular function and structure [205]. Further, lipids are able to be released or transformed by enzymatic reactions [205]. The changes observed between the platelet and supernatant fraction (Figure 4.4) may be indicative of the dynamics between elements of the platelet component. As such, an examination of the lipid ratios and lipid networks may provide a more detailed understanding of the interactions taking place within the fractions. Lipid classes were selected for comparison as ratios based on reactions and pathways that are known to occur from the literature. Further, the ratios of certain lipids were assessed where they have been suggested to impact cellular function and disease process if altered [206, 207].

In the platelet faction, the ratio of LPE/PE, PEP/PE, Cer/SM and PC/PE ratios were increased at day 5 of room temperature storage (Table 4.1), compared to day 1, which resulted in a difference between storage temperatures for LPE/PE. In contrast, the LPE/PE ratio was decreased at day 14 at both temperatures, compared to day 1. The PS/PC ratio was increased at day 5 of cold storage, compared to day 1, and continued to increase through 14 days of storage. The PS/PE and PS/PA ratios were increased at day 5, compared to day 1 regardless of storage temperature. The PA/PC ratios was increased at day 14 of cold storage, compared to day 1, which resulted in a difference between storage temperatures for PA/PC at this time point. The PEP/PE, Cer/SM and PC/PE ratios were decreased at day 14 of cold storage, compared to day 1, which resulted in a difference between storage temperatures for PEP/PE and Cer/SM at this time point.

In the supernatant fraction, the LPE/PE ratio was increased by room temperature storage, and was decreased by cold storage at day 14, compared to day 1. As such, there was a difference between storage temperatures at both day 5 and day 14. Compared to day 1, the PS/PC and LPA/PA ratios

were increased at all time points, regardless of temperature. Similarly, the ratio of PS/PE and PS/PA were increased at day 5 regardless of temperature. The Cer/SM ratio was decreased at day 5 of room temperature storage and day 14 of cold storage.

Table 4.1. Lipid class ratios of platelets and supernatant in room temperature and cold-stored platelet components

	Platelet					Supernatant				
	D1	D5 RT	D5 Cold	D14 RT	D14 Cold	D1	D5 RT	D5 Cold	D14 RT	D14 Cold
LPC/PC	0.012	0.010	0.013	0.009	0.013	0.044	0.074	0.050	0.036	0.040
LPE/PE	0.097	0.125‡	0.086#	0.072‡	0.048‡	0.055	0.116‡	0.068#	0.099‡	0.034‡#
PEP/PE	1.215	1.584‡	1.268	1.001	0.301‡#	0.247	0.161	0.243	0.196	0.137‡
PS/PC	0.051	0.064	0.081‡	0.106	0.124‡	0.030	0.075‡	0.065‡	0.040‡	0.042‡
PA/PC	0.040	0.032	0.039	0.047	0.087‡#	0.026	0.063	0.028	0.025	0.038
LPA/PA	0.003	0.004	0.003	0.003	0.002	0.009	0.036‡	0.023‡	0.023‡	0.015
Cer/SM	1.225	1.469‡	1.164	1.399	0.527‡#	0.706	0.225‡	0.594	0.587	0.623‡
PC/PE	3.099	3.731‡	3.050	2.414	1.554‡	1.982	1.945	2.003	1.712	1.210‡
PS/PE	0.148	0.233‡	0.238‡	0.219	0.175	0.058	0.130‡	0.130‡	0.066	0.049
PS/PA	1.217	1.992‡	2.175‡	2.265	1.411	1.209	2.240‡	2.315‡	1.587‡	1.514

RT = room temperature (20-24 °C); Cold = cold-stored (2-6 °C)

Values shown as mean of the ratio of the given lipid classes, n=8 in each group.

The effect of storage temperature and time was assessed using a two-way ANOVA. *Post hoc* Bonferroni's multiple comparisons test were performed to determine the differences between storage temperature (room temperature vs cold-stored at day 5 and day 14) and time (day 1 vs day 5 and day 1 vs day 14). ‡ indicates $p<0.05$ when compared to day 1. # indicates $p<0.05$ when compared to RT at the same time point.

BioPAN is an open access web-based tool that provides a statistical score for possible lipid metabolism pathways [196]. This is done by using inputted mass spectrometry data to determine relationships between lipids, that represent possible lipid substrates and lipid products capable of being catalysed by enzymes, as determined from current literature [196]. The possible reactions are scored and found to be active or suppressed [196]. A BioPAN lipid network was computed for the platelet and supernatant fractions comparing each time point to day 1 and comparing the storage temperatures at day 5 and 14. Representative examples of selected time points and storage treatments are presented in Figure 4.15A-D, and the remaining data is presented in supplementary data S.7 – S.8. The data for all days and treatments are summarised in Figure 4.16.

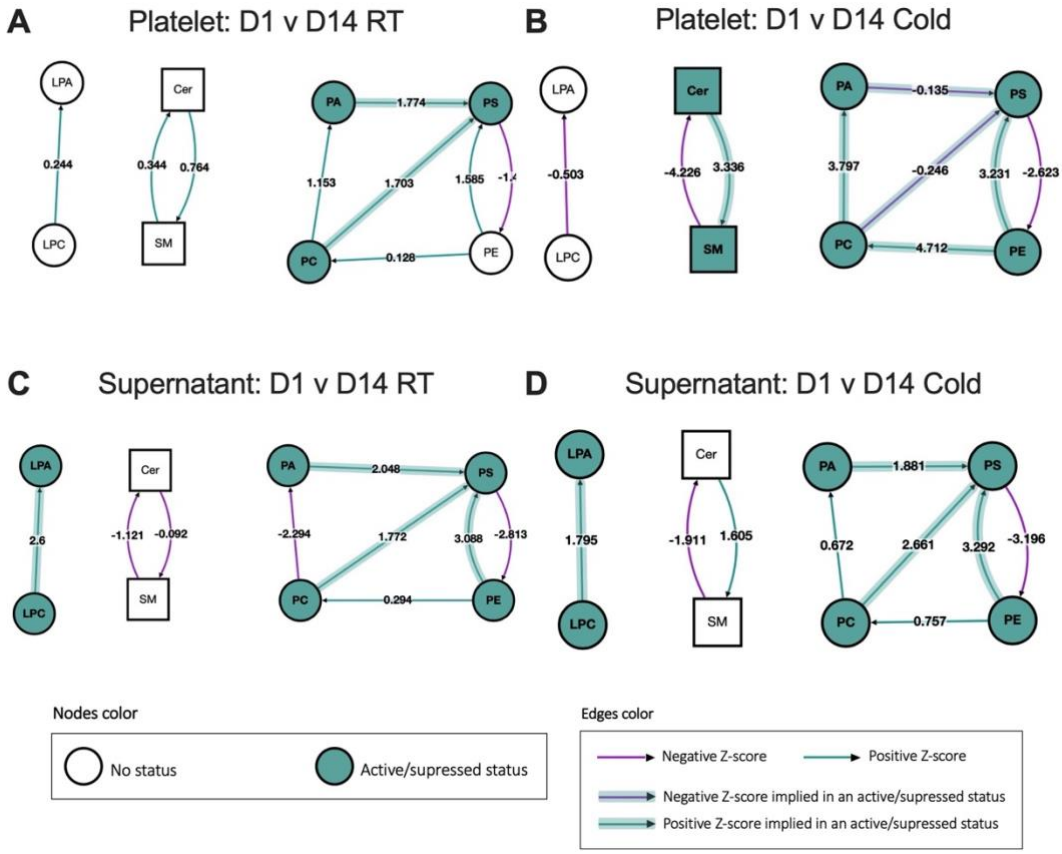

Figure 4.15. BioPAN lipid networks depicting active reactions over cold storage

The lipid networks of (A,B) platelets and the (B,C) supernatant comparing day 1 (D1) and day 14 (D14) timepoints. The networks were produced from the open access web-based tool, BioPAN [196], by inputting data obtained from mass spectrometry analysis. Networks were obtained comparing each time point, with a representative network being shown (see supplemental data, S.7-S.8).

In the platelet fraction, the conversion of PC to PA was inferred to be significantly suppressed when comparing day 1 to day 5 room temperature (Figure 4.16A). The conversion of PS to PE was inferred to be significantly suppressed when comparing day 1 to early timepoints (day 5) and to extended cold storage. The conversion of SM to Cer was inferred to be significantly suppressed when comparing day 1 to day 14 of cold storage. On the other hand, the conversion of PC to PA, PE to PC and Cer to SM were inferred to be significantly active when comparing day 1 to day 14 of cold storage. The conversion of PC to PS and PA to PS were inferred to be significantly active when comparing day 1 to early time points (day 5) and to extended room temperature storage. Between the storage conditions several reactions were significant, the conversion of PC to PA, PE to PC and Cer to SM were inferred to be significantly active between storage conditions at both time points. In contrast, the conversion of SM to Cer was inferred to be significantly suppressed between storage conditions at both time points. The PS to PE reaction was significantly suppressed between storage conditions at day 5, and the PC to PS and PA to PS reactions were inferred to be significantly suppressed between storage conditions at day 14. Reactions in the platelet fraction were differentially affected by storage time and temperature.

In the supernatant fraction, reactions differed to those observed in the platelet fraction. The conversion of LPC to LPA, PC to PS, PE to PS and PA to PS were inferred to be significantly active when comparing day 1 to all time points and temperatures (Figure 4.16B). The conversion of PE to PC was inferred to be significantly active when comparing day 1 to room temperature storage at day 5. Conversely, the conversion of PS to PE was inferred to be significantly suppressed when comparing day 1 to all time points and temperatures. The conversion of PC to PA was inferred to be significantly suppressed at day 14 of room temperature storage compared to day 1, while the conversion of SM to Cer was inferred to be significantly supressed at day 14 of cold storage compared to day 1. Between the storage conditions conversions were relatively similar, with the exception of PE to PC, which was inferred to be

significantly suppressed between room temperature and cold storage at day 5 taken together, these results suggest that reactions in the supernatant were more greatly affected by storage time, rather than temperature.

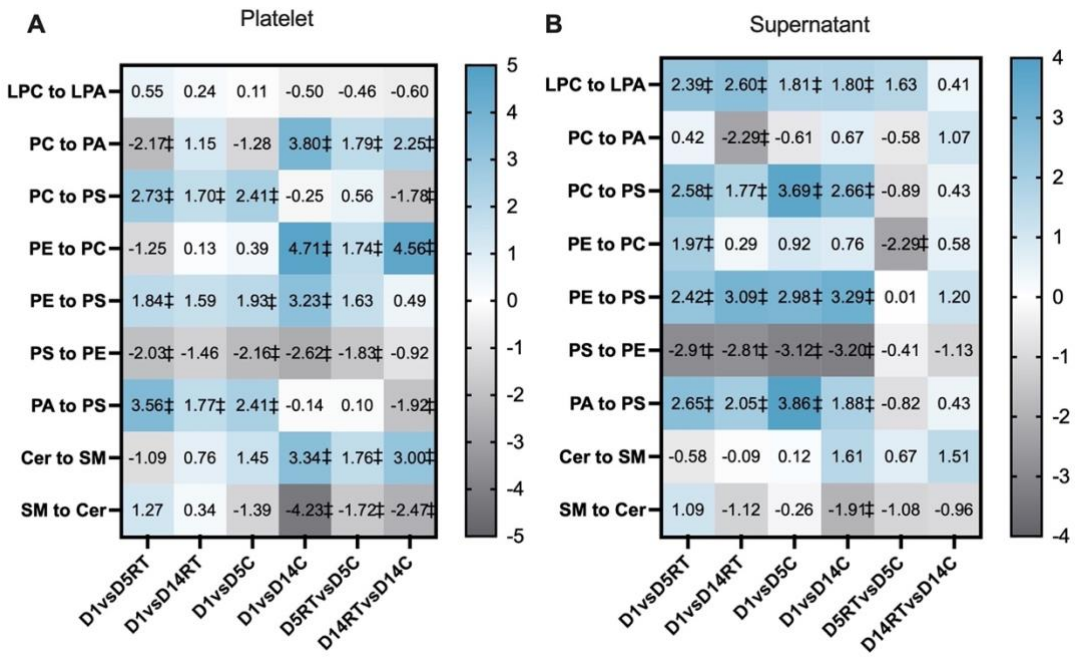

Figure 4.16. Summary of Z-scores for given reactions over room temperature and cold storage from BioPAN

Lipid networks were generated from BioPAN using data acquired by LC-MS/MS. Z-scores from the (A) platelet and (B) supernatant lipid network were summarised and are depicted as heat maps. Values shown as Z-score of the given reaction, whereby a Z-score>0 represents an active reaction and a Z-score<0 represents a suppressed reaction. A paired t-test was performed by BioPAN to determine differences between indicated groups. ‡ indicates $p<0.05$ a significant reaction (corresponding to a Z-score > 1.645 or < -1.645).

BioPAN suggests genes known to be involved in the reactions depicted in the lipid networks. After generating networks from each fraction, a list of genes was consolidated as being potentially involved in the indicated reaction (Table 4.2). The list of genes generated by the web-based tool encode proteins that facilitate the depicted reactions. Thus, this information may assist in understanding the complex interactions that are occurring, and additionally provide avenues for future research.

Table 4.2. BioPAN predicted genes to be activated or supressed in the given reaction in room temperature and cold-stored components

	Gene symbol	Gene name
LPC to LPA	-	-
PC to PA	PLD1	phospholipase D1
	PLD2	phospholipase D2
PC to PS	PTDSS1	phosphatidylserine synthase 1
PE to PC	PEMT	phosphatidylethanolamine N-methyltransferase
PE to PS	PTDSS2	phosphatidylserine synthase 2
PS to PE	PISD	phosphatidylserine decarboxylase
PA to PS	PTDSS1	phosphatidylserine synthase 1
	CDS1	CDP-diglyceride synthase 1
Cer to SM	SGMS1	sphingomyelin synthase 1
	SGMS2	sphingomyelin synthase 2
	CERT1	ceramide transfer protein 1
SM to Cer	SMPD1	sphingomyelin phosphodiesterase 1
	SMPD2	sphingomyelin phosphodiesterase 2
	SMPD3	sphingomyelin phosphodiesterase 3

Chapter 5 Results of the lipidomic characterisation of cryopreserved platelet components

To date, no studies have been conducted which assess the lipidome of cryopreserved platelets. In this chapter, the lipidome of the platelets, microparticle and supernatant fractions of cryopreserved platelets are described. Thawed platelet components were compared to paired platelet components prior to freezing. While the lipidome of the platelet fraction was not extensively altered by the cryopreservation process, post-thaw storage for 24 hours at room temperature resulted in significant changes. Further, the microparticles formed after thawing had a distinct lipid profile compared to those present prior to freezing. The lipid profile of the supernatant was primarily defined by the resuspension solution.

Cryopreservation offers an attractive alternative to conventional platelet storage, as components may be stored for at least two years [141, 142, 166]. Numerous studies have been conducted to assess both the *in vitro* and *in vivo* impact of cryopreserving platelet components [122, 145, 169, 172-174, 208]. However, the lipidome of cryopreserved platelet components has not yet been assessed. Lipids and bioactive lipid molecules have an important role in mediating haemostatic function and are associated with adverse transfusion outcomes [69, 90, 183, 186, 209-211]. Further, given that 70 % of cryopreserved platelets have externalised PS [167], it would be surprising if other lipids were not changed. As such, this study assessed the lipid profile of the platelets, microparticles and the supernatant occurring as a result of the cryopreservation process.

Data acquired in this study was assessed in a pair-wise manner, whereby the same component was tested before freezing, after thawing and after 24 hours of post-thaw storage at room temperature. This study evaluated lipidomic changes predominantly using mass spectrometry-based methodologies, however other *in vitro* testing was also performed to supplement the data provided by mass spectrometry.

5.1 Assessment of platelet number and morphology following cryopreservation

Platelets were cryopreserved, thawed, and reconstituted in a solution of 70 % SSP+/30 % plasma to match the pre-freeze composition. The platelet components were sampled prior to freezing, after thawing and after 24 hours of post-thaw storage at room temperature. The platelet count and MPV were obtained using an automated haematology analyser, and the recovery was calculated from the pre-freeze and post-thaw counts. The platelet count was significantly decreased after thawing, and continued to decrease during post-thaw storage (Figure 5.1A). As such, the average recovery was 60.4 %, which was above the minimum acceptable standard of 40 % according to the Council of Europe guidelines (Figure 5.1B) [212]. The MPV was significantly increased after thawing and remained unchanged over the post-thaw storage period (Figure 5.1C). Immediately following thawing, swirl was not observed in platelet components. However, within 1 hour of post-thaw storage at room temperature, the platelets regained the capacity to swirl. Macroaggregates were not visible in any components.

The changes to the MPV and loss of swirl seen immediately following thawing and reconstitution are indicative of morphological changes.

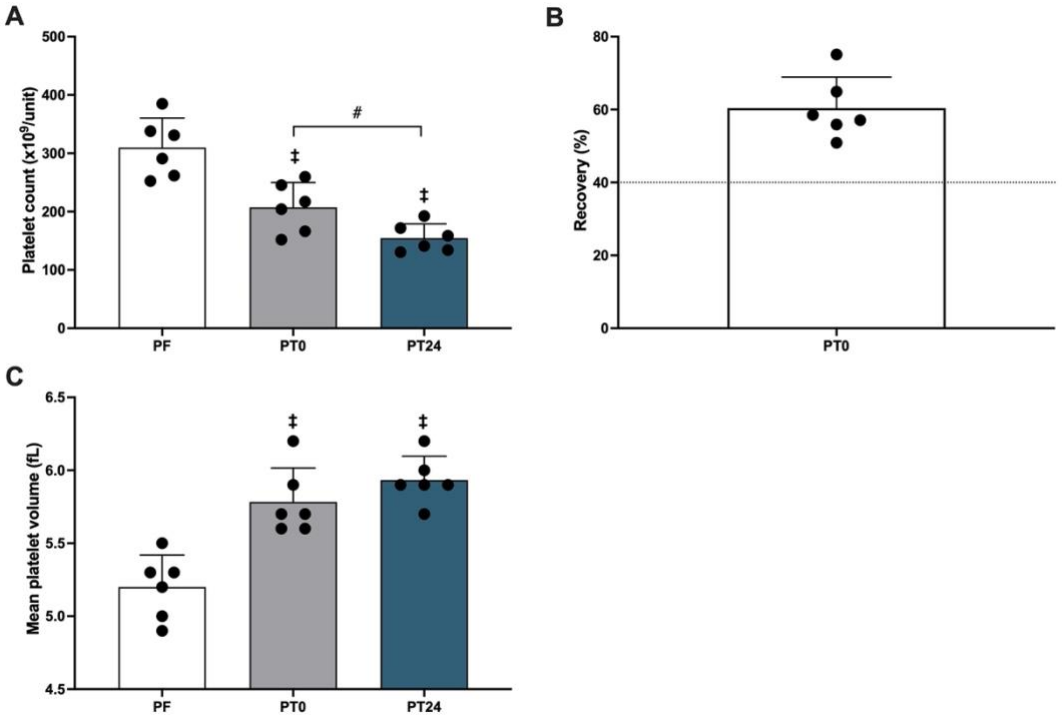

Figure 5.1. The effect of cryopreservation on the platelet count and mean platelet volume

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). The (A) platelet count was assessed using an automated haematology analyser. The (B) recovery (%) was calculated from the platelet count before and after cryopreservation. The dotted line represents the minimum acceptable standard (40 %) according to the Council of Europe guidelines [212]. The (C) mean platelet volume was assessed using an automated haematology analyser. The data represents individual data points and mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24.

The externalisation of aminophospholipids (PS and PE) and the release of microparticles with PS externalised was assessed by flow cytometry. These processes are important in mediating the coagulation process [93, 213], and are known to increase as a result of cryopreservation [178].

Annexin-V is a protein known to bind PS [214]. The percentage of platelets with PS externalised was significantly increased immediately after thawing (~63-fold, $p<0.0001$) and remained increased after the post-thaw storage period of 24 hours (~46-fold, $p=0.0005$) (Figure 5.2A).

Duramycin is a peptide known to bind PE [215, 216] and its externalisation has not previously been assessed in cryopreserved platelets. After thawing, the MFI of duramycin was significantly increased (~4.8-fold, $p=0.0005$) and was similarly maintained after 24 hours of storage (~3.9-fold, $p=0.0008$) (Figure 5.2B).

The number of annexin-V positive platelet microparticles was found to be significantly increased following cryopreservation (Figure 5.2C). Although still high, the number of microparticles decreased during the 24 hours of post-thaw storage when compared to post-thaw 0.

The increase in the percentage of platelets with PS externalised and the increase in PE externalisation indicates a change to the platelet membrane. Further, the increase in microparticles with externalised PS demonstrated the composition of the platelet component is altered by cryopreservation.

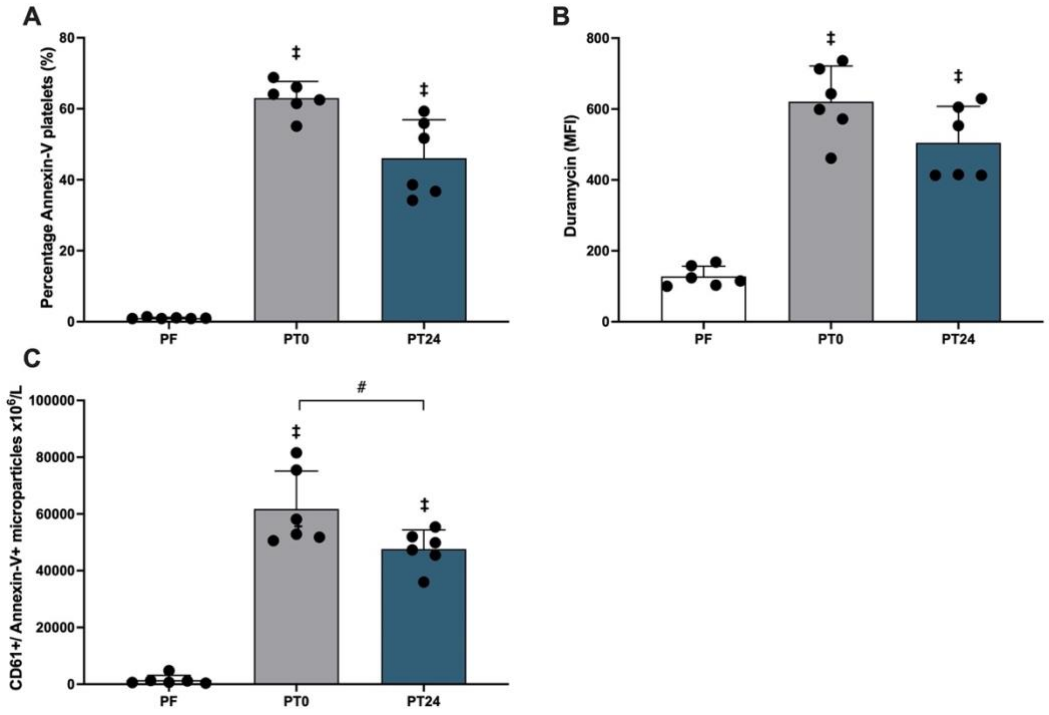

Figure 5.2. The effect of cryopreservation on the externalisation of phosphatidylserine, phosphatidylethanolamine and release of microparticles

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). (A) Samples were stained with annexin-V (FITC) and the percentage of annexin-V positive platelets was determined by flow cytometry after collecting 10 000 events. (B) Samples were stained with duramycin (Cy5) and the mean fluorescence intensity (MFI) of the platelet population was measured by flow cytometry after collecting 10 000 events. (C) Samples were stained with CD61 (APC) and annexin-V (FITC). The absolute number of microparticles was enumerated by flow cytometry and collecting 10 000 bead events. The data represents individual data points and mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24.

5.2 Assessment of the global lipidomic changes following cryopreservation

The lipid abundance has not previously been assessed in cryopreserved platelet components; however, it is known to be altered by conventional platelet storage at room temperature [56, 57]. The effect of the cryopreservation process on the lipid profile of platelets, microparticles and supernatant was assessed by LC-MS/MS. The relative abundance of phospholipids and sphingolipids was determined using an internal standard. Immediately after thawing, the abundance of lipids in platelets and microparticles did not significantly change (Figure 5.3A and B). However, during post-thaw storage at room temperature the abundance of lipids in platelets significantly increased (~1.8-fold), whereas the lipid abundance of microparticles significantly decreased (~1.4-fold) when compared to pre-freeze and post-thaw 0. The lipid content of the resuspension solution was significantly lower than the pre-freeze supernatant (Figure 5.3C). Immediately after thawing, the lipid content of supernatant was aligned with the resuspension solution, rather than the pre-freeze composition. However, while still decreased after the post-thaw storage period, the lipid content of the supernatant was no longer significant. As no difference was observed between the reconstitution solution and the post-thaw time points, it could be inferred that the differences between the pre-freeze and post-thaw samples were due to the composition of the reconstitution solution.

These results suggest that the lipid content of the platelets and microparticles was not affected by the cryopreservation process, however, were affected by the post-thaw storage period. Further, the lipid content of the supernatant was not impacted by the cryopreservation process itself, but by the composition of the reconstitution solution.

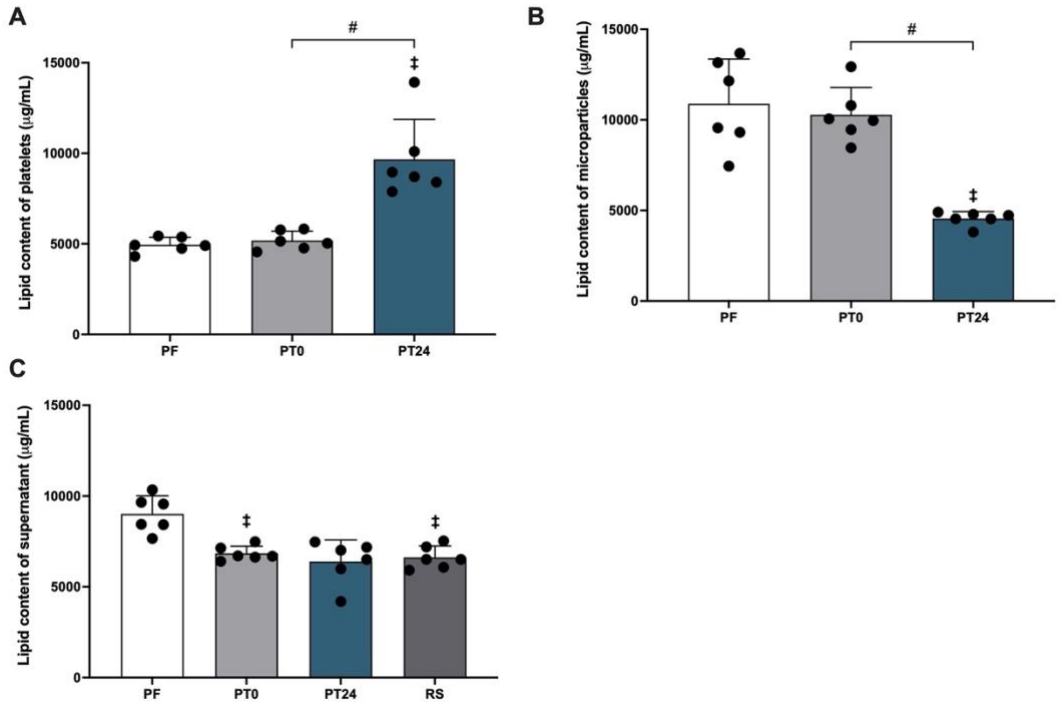

Figure 5.3. The effect of cryopreservation on the relative abundance of phospholipids and sphingolipids

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The relative abundance of phospholipids and sphingolipids in the (A) platelets, (B) microparticles and (C) supernatant were determined by LC-MS/MS. The data represents individual data points and mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24.

The lipid class composition of cryopreserved platelets was assessed by LC-MS/MS. The predominant lipid classes in the platelet fraction were PC (ether linked PC, PCO, was included with PC as it represented < 5 % of the total lipid content) and ceramide representing approximately 50% of all displayed lipids (Figure 5.4A). In contrast, the predominant lipid classes in the microparticle and supernatant fractions were PC and PE representing approximately 65% and 50% of displayed lipids, respectively (Figure 5.4B and C).

Immediately following thawing, the lipid profile of platelets was not significantly different to that prior to freezing, with the exception of PE. PE was significantly decreased immediately after thawing, compared to pre-freeze. However, after the 24 hour post-thaw storage period, PA and LPC were significantly lower than the pre-freeze and post-thaw 0 groups. Conversely, the post-thaw storage period resulted in a significant increase in PC, when compared to post-thaw 0.

The lipid profile of the microparticle fraction was affected by the cryopreservation process. After thawing, the microparticles had an altered proportion of LPA, PA LPC, LPE and PG compared to pre-freeze, and the difference was maintained through the post-thaw storage period. LPA, LPC and LPE were significantly decreased, while PA and PG were significantly increased in microparticles formed after thawing. Further, a significant increase in ceramide was observed during the 24 hour post-thaw storage period. These results suggest that microparticles formed as a result of the cryopreservation process have a different lipid profile to those present prior to freezing.

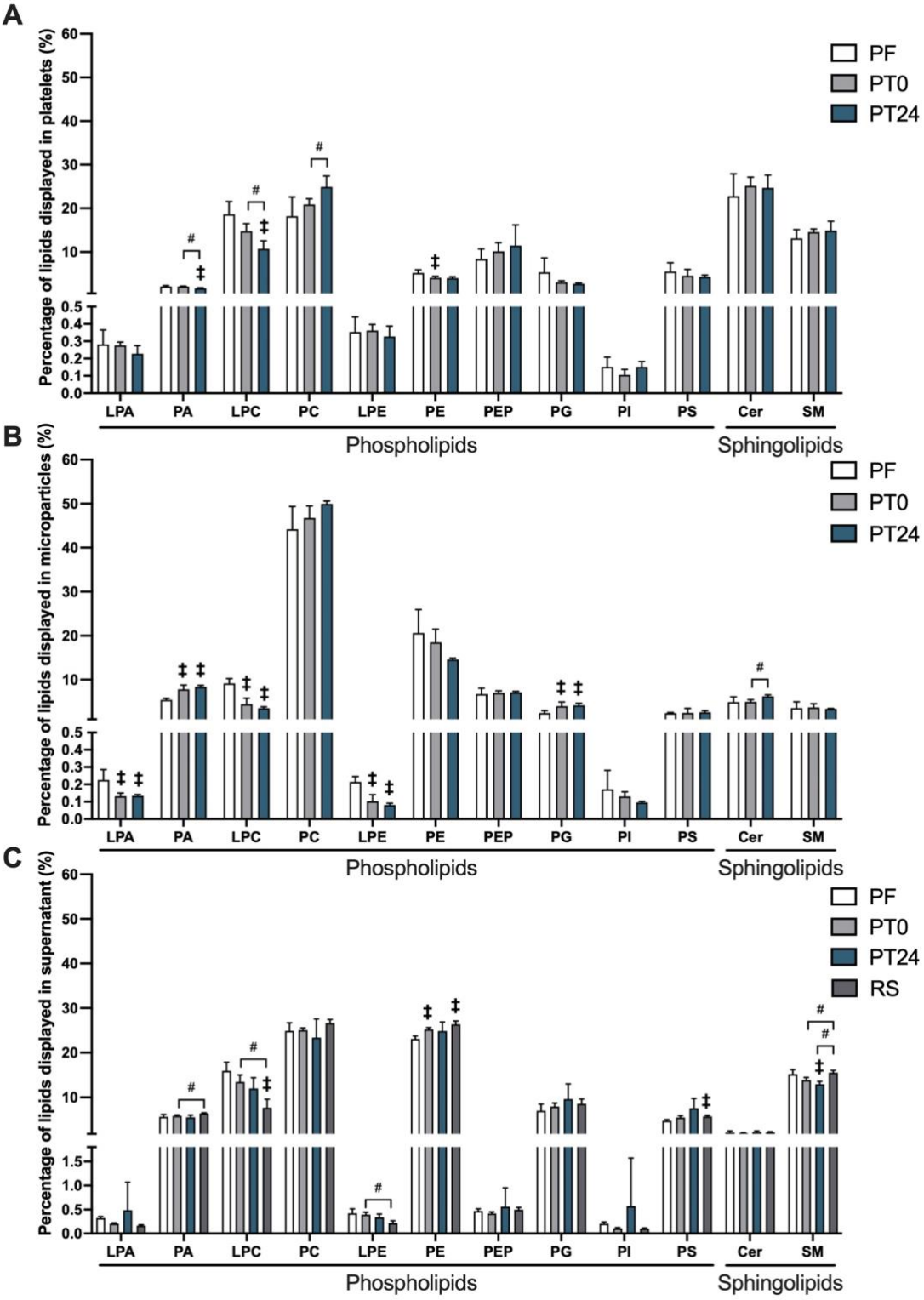
A
Percentage of lipids displayed in platelets (%)
PF
PT0
PT24
LPA
PA
LPC
PC
LPE
PE
PEP
PG
PI
PS
Cer
SM
Phospholipids
Sphingolipids
B
Percentage of lipids displayed in microparticles (%)
PF
PT0
PT24
LPA
PA
LPC
PC
LPE
PE
PEP
PG
PI
PS
Cer
SM
Phospholipids
Sphingolipids
C
Percentage of lipids displayed in supernatant (%)
PF
PT0
PT24
RS
LPA
PA
LPC
PC
LPE
PE
PEP
PG
PI
PS
Cer
SM
Phospholipids
Sphingolipids

Figure 5.4. The effect of cryopreservation on the phospholipids and sphingolipids

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of phospholipids and sphingolipids displayed in the (A) platelets, (B) microparticles and (C) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

The cryopreservation process and post-thaw storage altered the lipid profile of the supernatant. The resuspension solution was tested prior to the addition of the thawed platelets to determine a baseline for samples taken after thawing. It was found that the resuspension solution contained less LPC than the pre-freeze supernatant. Once thawed, the proportion of LPC and LPE increased compared to the resuspension solution, indicating release from the platelets or microparticles, or liberation of lysophospholipid species may have occurred during thawing. Conversely, the resuspension solution contained significantly more PE and PS than the pre-freeze supernatant, such that the PE after thawing was higher than pre-freeze. While not significant compared to pre-freeze, there was a decreased proportion of PA and SM immediately after thawing compared to the resuspension solution, indicating an uptake by the platelet or microparticle, or catabolism of these lipid classes. The proportion of SM continued to decrease over the post-thaw storage period, compared to immediately after thawing. These changes suggest that the composition of the resuspension solution is affecting the lipid composition of the supernatant after thawing.

The trends seen in each fraction may be suggestive of the mutability of the lipid profile of platelet components. For example, in the platelet fraction PA was decreased in thawed samples, but was increased in the microparticle fraction after thawing. Similarly, in the supernatant fraction LPC was increased after thawing, compared to the resuspension solution, but was decreased in the platelet and microparticle fractions after thawing.

PC is the major constituent of platelet lipid membranes [55, 57]. Given that a change in total PC was seen after post-thaw storage, shifts in the PC species profile that may be occurring as a result of cryopreservation were further examined. In platelets, PC(36:1) was the dominant species (Figure 5.5A), representing 80% of the PC species. Immediately after thawing, the profile of PC species were relatively stable. After the post-thaw storage period, PC(32:4) and PC(40:6) were significantly decreased compared to pre-freeze and post-thaw 0.

In the microparticles, a greater amount of diversity in the number of PC species displayed was observed compared to the platelet fraction (Figure 5.5B). PC(36:1), PC(38:2), PC(38:4) and PC(40:4) were significantly decreased immediately after thawing, however, the decrease was transient and returned to a similar level similar to pre-freeze over the post-thaw storage period. In contrast, initially after thawing, PC(36:5) and PC(38:3) were unchanged, but post-thaw storage resulted in a significant increase compared to pre-freeze. PC(40:5) was the only PC species in the microparticle fraction to be significantly increased in post-thaw samples compared to pre-freeze, with a dramatic increase observed immediately after thawing.

In the supernatant fraction, the PC species profile was relatively unchanged by cryopreservation or storage. PC(36:3), PC(38:3) and PC(38:4) were the most prominent, cumulatively accounting for approximately 85% of PC species (Figure 5.5C). PC(36:1) was the only species to be significantly changed, although the magnitude was small. The proportion of PC(36:1) in the resuspension solution was significantly less than the pre-freeze supernatant, resulting in a lower proportion of PC(36:1) immediately at post-thaw.

Subtle shifts were seen in the PC species profile of platelets and the supernatant. It was apparent that the profile of PC species in microparticles was more diverse than their parent platelets, and that the microparticles present after thawing had a different PC profile to the microparticles present prior the freezing.

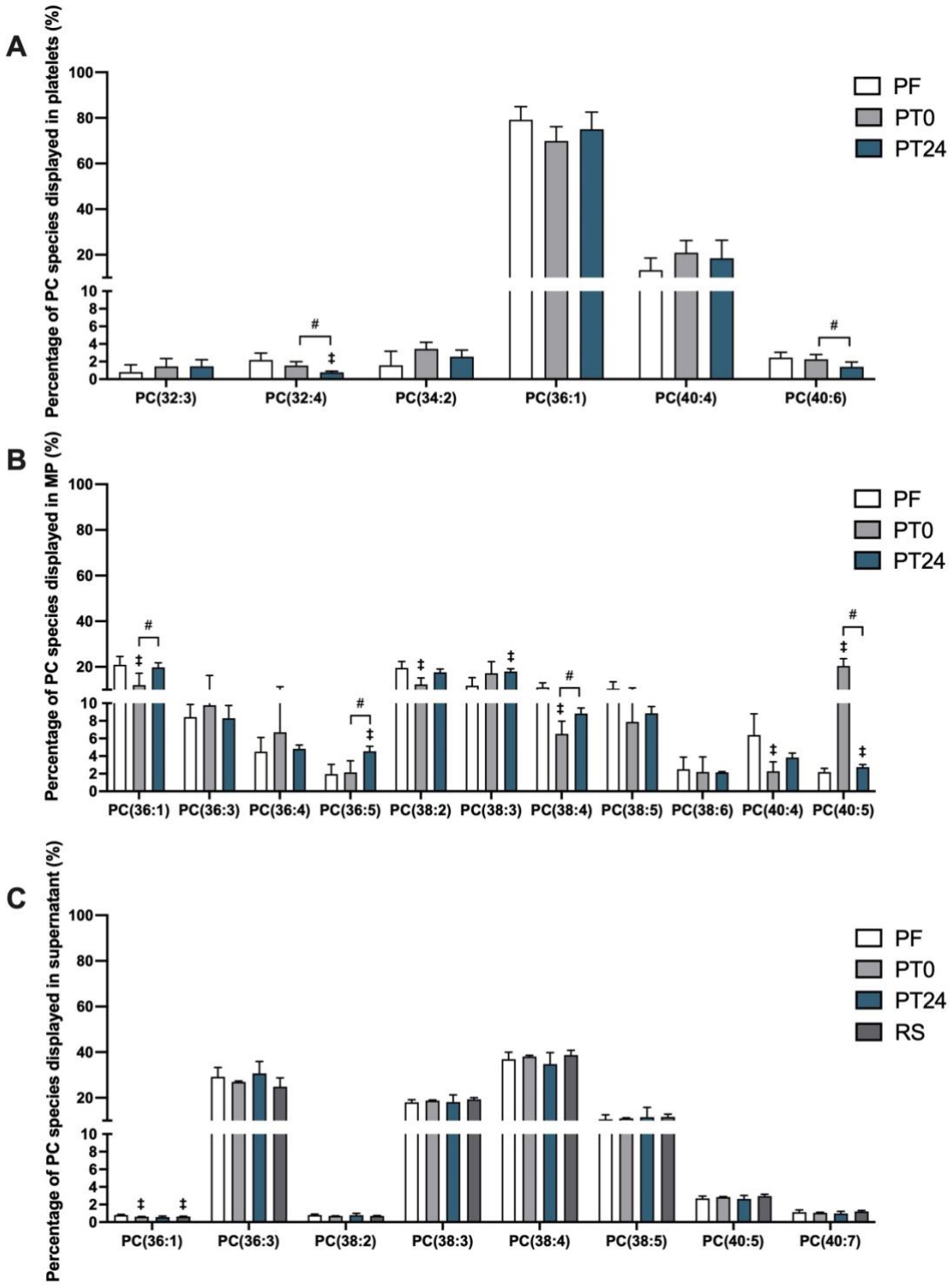
A
Percentage of PC species displayed in platelets (%)
PF
PT0
PT24
PC(32:3)
PC(32:4)
PC(34:2)
PC(36:1)
PC(40:4)
PC(40:6)
B
Percentage of PC species displayed in MP (%)
PF
PT0
PT24
PC(36:1)
PC(36:3)
PC(36:4)
PC(36:5)
PC(38:2)
PC(38:3)
PC(38:4)
PC(38:5)
PC(38:6)
PC(40:4)
PC(40:5)
C
Percentage of PC species displayed in supernatant (%)
PF
PT0
PT24
RS
PC(36:1)
PC(36:3)
PC(38:2)
PC(38:3)
PC(38:4)
PC(38:5)
PC(40:5)
PC(40:7)

Figure 5.5. The effect of cryopreservation on the phosphatidylcholine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of phosphatidylcholine (PC) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 % for any time point. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

Lysophosphatidylcholine is a potent mediator of platelet function, known to activate platelets and mediate pro-inflammatory pathways [217-219]. Further, LPC has been associated with adverse transfusion reactions [220, 221]. The LPC class was altered in each fraction (Figure 5.4) and as such, the LPC species were interrogated further.

LPC(18:0) represented 50 % of LPC species in the platelet fraction, but was not significantly altered by the cryopreservation process (Figure 5.6A). Immediately after thawing, LPC(18:2) was significantly increased, however it decreased during post-thaw storage period and was significantly different compared to post-thaw 0. LPC(20:0) and LPC(O-18:0) did not change immediately after thawing, but were significantly increased at post-thaw 24 compared to pre-freeze and post-thaw 0.

The microparticles present following the cryopreservation process displayed several shifts in the LPC species (Figure 5.6B). There was a trend for a gradual increase in the saturated species, LPC(18:0) and LPC(20:0), after thawing and after post-thaw storage. Conversely, there was a trend for unsaturated species, LPC(18:1), LPC(18:2), LPC(20:4) and LPC(22:6), to decrease after thawing, with a greater decrease observed in the microparticles at post-thaw 24.

LPC(16:0) was the most prominent LPC species in the supernatant, representing approximately 50 % of the LPC species (Figure 5.6C). In most cases, the resuspension solution was significantly different to the supernatant prior to freezing, with LPC(18:0) being higher and LPC(18:1), LPC(18:2), LPC(20:4) and LPC(22:6) being lower in the resuspension solution compared to pre-freeze. Despite LPC(18:0) being higher in the resuspension solution, LPC(18:0) was significantly decreased after thawing compared to pre-freeze and the resuspension solution. Conversely, there was a trend for LPC(18:1), LPC(18:2) and LPC(20:4) to increase in post-thaw samples compared to the resuspension solution. These results suggest the LPC species profile changes after thawing as it no longer similar to the resuspension solution. After thawing,

processes are occurring that make the LPC profile of the resuspension solution more similar to the supernatant pre-freeze.

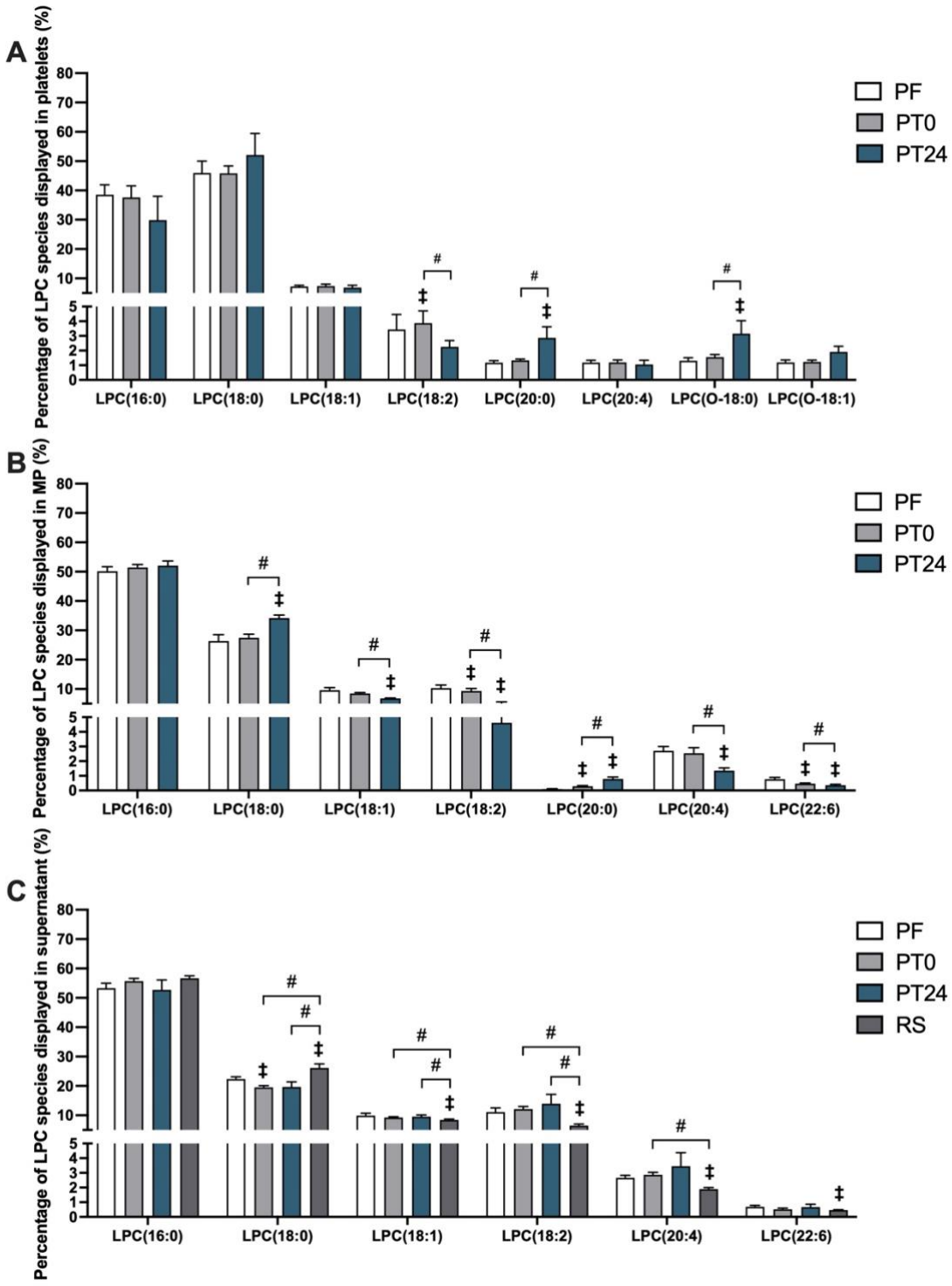
A
Percentage of LPC species displayed in platelets (%)
PF
PT0
PT24
LPC(16:0)
LPC(18:0)
LPC(18:1)
LPC(18:2)
LPC(20:0)
LPC(20:4)
LPC(O-18:0)
LPC(O-18:1)
B
Percentage of LPC species displayed in MP (%)
PF
PT0
PT24
LPC(16:0)
LPC(18:0)
LPC(18:1)
LPC(18:2)
LPC(20:0)
LPC(20:4)
LPC(22:6)
C
Percentage of LPC species displayed in supernatant (%)
PF
PT0
PT24
RS
LPC(16:0)
LPC(18:0)
LPC(18:1)
LPC(18:2)
LPC(20:4)
LPC(22:6)

Figure 5.6. The effect of cryopreservation on the lysophosphatidylcholine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of lysophosphatidylcholine (LPC) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 % for any time point. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

Phosphatidylethanolamine is known to support coagulation [90]. Further, PE was shown to be externalised (Figure 5.2) and there were alterations to the PE class (Figure 5.4). As such, the profile of PE species was further interrogated.

For the PE species profile of platelets, PE(36:1) represented the most abundant lipid (Figure 5.7A). PE(38:5) was unchanged immediately after thawing but was decreased significantly after the 24 hour storage period. Conversely, PE(40:2) was significantly increased in post-thaw samples, with a greater increase observed at post-thaw 24.

PE(40:1) was the most abundant lipid of the PE species in the microparticle fraction, representing approximately 80% of PE species (Figure 5.7B). The displayed PE species in the microparticle fraction were relatively unchanged by cryopreservation.

The PE species profile of the supernatant was more diverse in the number of species displayed than the platelet and microparticle fractions (Figure 5.7C). However, no significant changes were observed after thawing or after the post-thaw storage period and the resuspension solution was similar to the pre-freeze supernatant.

The PE profile of the supernatant was more diverse than the platelet and microparticle fractions. Further, despite the externalisation of PE and the changes in the PE class, the species profiles were relatively stable, particularly in the microparticle and supernatant fractions.

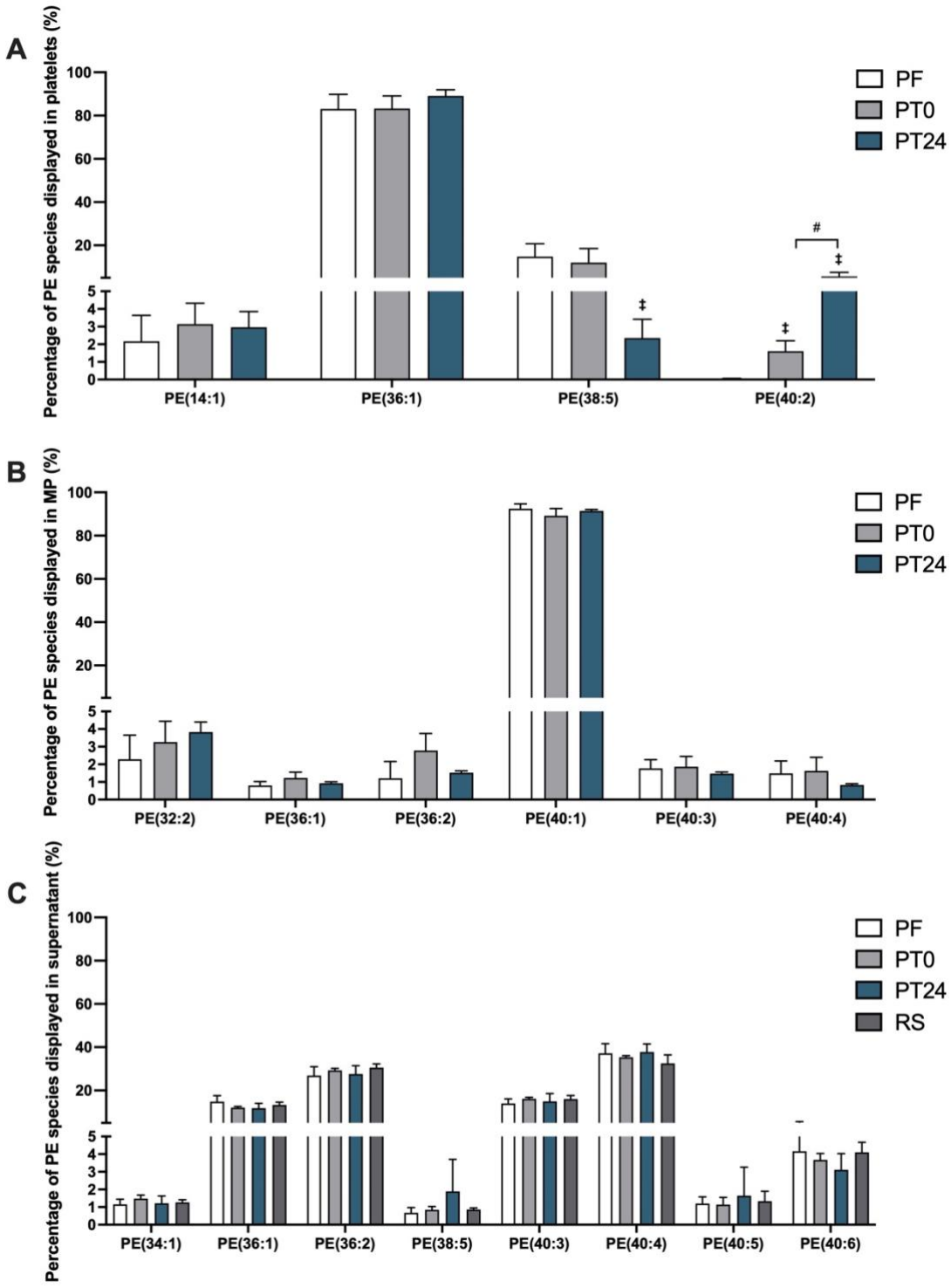
A
Percentage of PE species displayed in platelets (%)
PF
PT0
PT24
PE(14:1)
PE(36:1)
PE(38:5)
PE(40:2)
B
Percentage of PE species displayed in MP (%)
PF
PT0
PT24
PE(32:2)
PE(36:1)
PE(36:2)
PE(40:1)
PE(40:3)
PE(40:4)
C
Percentage of PE species displayed in supernatant (%)
PF
PT0
PT24
RS
PE(34:1)
PE(36:1)
PE(36:2)
PE(38:5)
PE(40:3)
PE(40:4)
PE(40:5)
PE(40:6)

Figure 5.7. The effect of cryopreservation on the phosphatidylethanolamine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of phosphatidylethanolamine (PE) species in the (B) platelets, (C) microparticles and (D) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 % for any time point. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

The role of LPE remains relatively unknown in platelets, however, in other cell types it has been shown that LPE may elicit a chemotactic response and promote calcium signalling [222, 223]. Further, LPE was altered by the cryopreservation process in the microparticle and supernatant fraction (Figure 5.4), and therefore the LPE species displayed were examined further. In the platelet fraction, the LPE species profile was not significantly altered by the cryopreservation process or by the post-thaw storage period (Figure 5.8A).

The number of LPE species present in the microparticles and the supernatant was far more diverse than the platelet fraction (Figure 5.8B and C). LPE(18:0) and LPE(P-18:0) were the most prominent LPE species in the microparticle fraction, cumulatively accounting for approximately 80 % of the LPE species. In general, LPE species, including LPE(18:0), LPE(20:3), LPE(20:4) and LPE(22:1) were significantly decreased immediately after thawing and continued to decrease over the post-thaw storage period. In contrast, the ether linked LPE species, LPE(P-16:0) and LPE(P-18:0), were significantly increased in post-thaw samples, and the increase over the 24 hour storage period was greater for LPE(P-18:0).

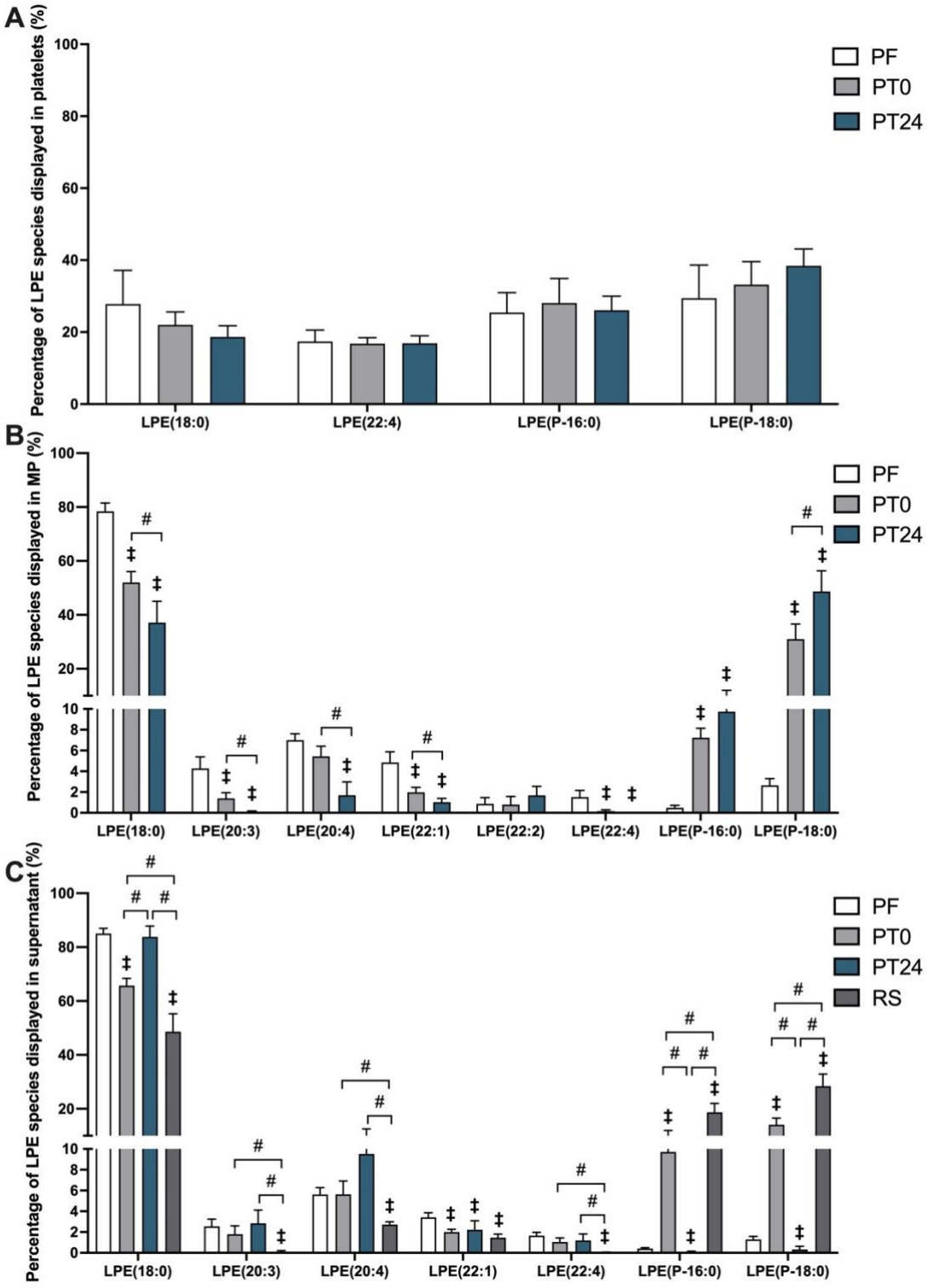
A
Percentage of LPE species displayed in platelets (%)
PF
PT0
PT24
LPE(18:0)
LPE(22:4)
LPE(P-16:0)
LPE(P-18:0)
B
Percentage of LPE species displayed in MP (%)
PF
PT0
PT24
LPE(18:0)
LPE(20:3)
LPE(20:4)
LPE(22:1)
LPE(22:2)
LPE(22:4)
LPE(P-16:0)
LPE(P-18:0)
C
Percentage of LPE species displayed in supernatant (%)
PF
PT0
PT24
RS
LPE(18:0)
LPE(20:3)
LPE(20:4)
LPE(22:1)
LPE(22:4)
LPE(P-16:0)
LPE(P-18:0)

Figure 5.8. The effect of cryopreservation on the lysophosphatidylethanolamine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of lysophosphatidylethanolamine (LPE) species in the (B) platelets, (C) microparticles and (D) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 % for any time point. The data represents individual data points, and mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

In the supernatant fraction, LPE(18:0) was the most prominent LPE species, accounting for approximately 70 % of PE species. Several significant changes were observed in the LPE profile of the supernatant, however, only the key trends will be discussed. Overall, the resuspension solution contained significantly less LPE species (LPE(18:0), LPE(20:3), LPE(20:4), LPE(22:1) and LPE(22:4)) than the pre-freeze supernatant. Immediately following thawing, several of the LPE species had increased (LPE(18:0), LPE(20:3), LPE(20:4) and LPE(22:4)) in the supernatant and continued to increase over the post-thaw storage period, compared to the resuspension solution. On the other hand, a greater proportion of ether linked LPE species (LPE(P-16:0) and LPE(P-18:0)) was present in the resuspension solution compared to pre-freeze. Immediately after thawing, the proportion of ether linked LPE species declined compared to the resuspension solution, and continued to decline after the storage period.

Interestingly, significant changes in the microparticle fraction appear to be related to the significant changes occurring in the supernatant fraction, particularly at the post-thaw 24 hour time point. For instance, microparticles present after the 24 hour storage period had significantly reduced LPE(20:4), while in the supernatant fraction LPE(20:4) was significantly increased at this time point.

These results suggest the profile of LPE species in the microparticles present after thawing was different to the profile of the LPE species in the microparticles present prior to freezing, and there may be a relationship between the resuspension solution and supernatant, and the microparticles.

Ether linked phospholipids occur mostly in the PC and PE class [63]. The moiety is typified by the attachment of the alkyl chain by an ether bond to the sn-1 acyl chain (denoted by the abbreviation O) [63]. More commonly the alkyl chain is attached adjacent to a double bond, termed plasmalogen (denoted by the abbreviation P) [63]. Ether linked phospholipids were not significantly altered (Figure 5.4), however, there is growing interest in these lipid classes.

The most prominent PCO species in the platelet fraction were PC(O-38:4) and PC(O-40:3), cumulatively accounting for approximately 80 % of PCO species (Figure 5.9A). PC(O-40:3) was the only species to be altered by cryopreservation in the platelet fraction, which increased significantly in post-thaw samples.

The PCO species profile of microparticles was more diverse in the number of PCO species displayed than the platelet fraction (Figure 5.9B). PC(O-18:1) and PC(O-38:5) were significantly decreased in post-thaw samples. Immediately after thawing, PC(O-20:1) was significantly decreased, however, it increased after the 24 hour storage period. Conversely, PC(O-40:3) was significantly increased in post-thaw samples.

PC(O-36:2) was the most abundant PCO species in the supernatant fraction, representing 55 % of the PCO species (Figure 5.9C). The species profile was relatively stable, with no changes observed between pre-freeze and post-thaw conditions. However, PC(O-20:1) was higher in the resuspension solution than in the pre-freeze supernatant and a loss of this species was observed after thawing.

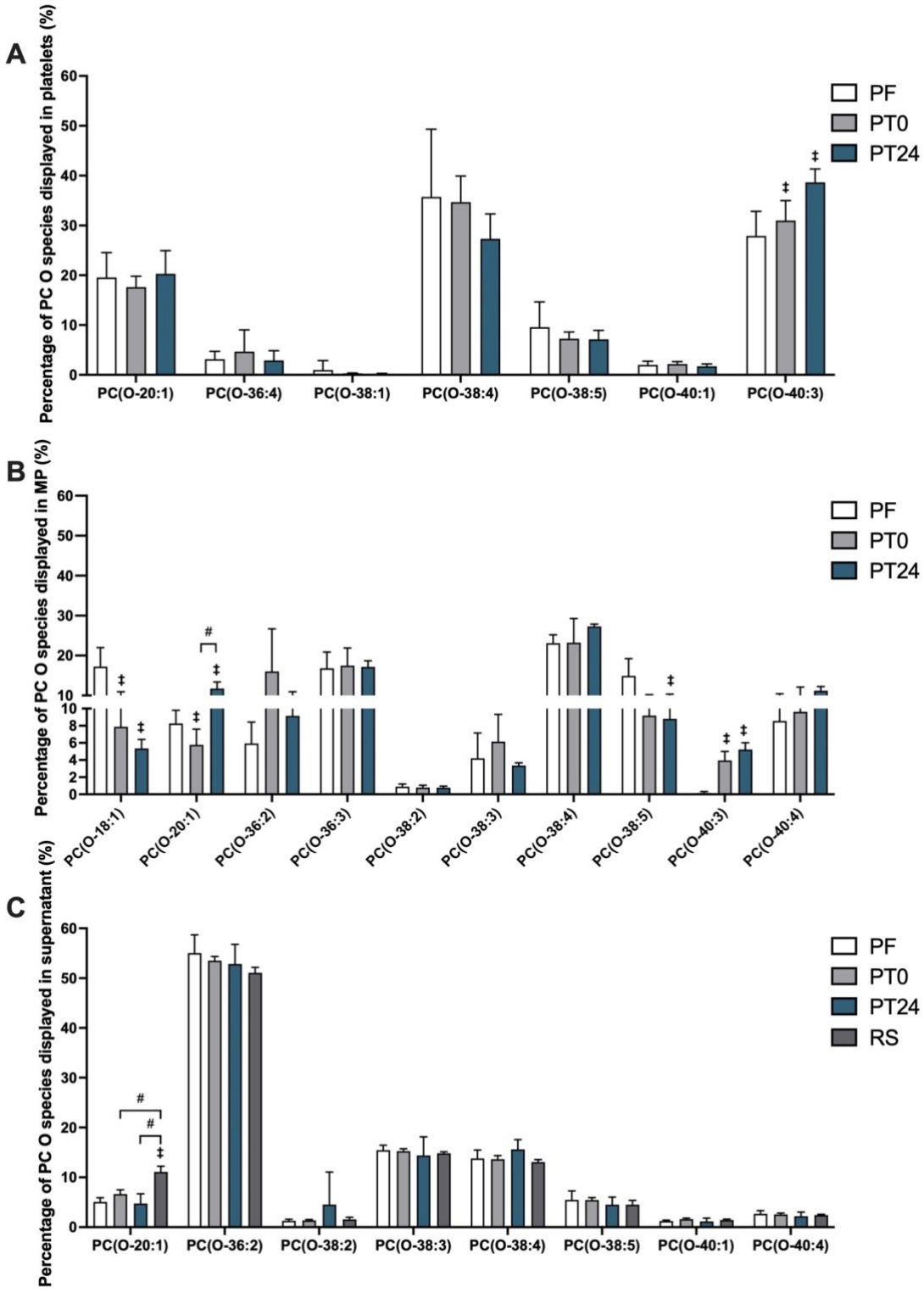
A
Percentage of PC O species displayed in platelets (%)
PF
PT0
PT24
PC(O-20:1)
PC(O-36:4)
PC(O-38:1)
PC(O-38:4)
PC(O-38:5)
PC(O-40:1)
PC(O-40:3)
B
Percentage of PC O species displayed in MP (%)
PF
PT0
PT24
PC(O-18:1)
PC(O-20:1)
PC(O-36:2)
PC(O-36:3)
PC(O-38:2)
PC(O-38:3)
PC(O-38:4)
PC(O-38:5)
PC(O-40:3)
PC(O-40:4)
C
Percentage of PC O species displayed in supernatant (%)
PF
PT0
PT24
RS
PC(O-20:1)
PC(O-36:2)
PC(O-38:2)
PC(O-38:3)
PC(O-38:4)
PC(O-38:5)
PC(O-40:1)
PC(O-40:4)

Figure 5.9. The effect of cryopreservation on the ether linked phosphatidylcholine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of ether linked phosphatidylcholine (PCO) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

The PEP species profile was stable in the platelets following cryopreservation and over the storage period (Figure 5.10A). In the microparticle fraction, PE(P-40:2) was the most prominent PEP species, accounting for approximately 75 % of the PEP species (Figure 5.10B). The PEP species that accounted for majority of the microparticle profile (~95 %; PE(P-40:2) and PE(P-40:4)) were unchanged by the cryopreservation process. However, a significant increase in PE(P-36:4) was observed after thawing.

In the supernatant fraction, PE(P-40:6) was the most abundant, accounting for approximately 60 % of the PEP species (Figure 5.10C). PE(P-40:6) was significantly lower in the resuspension solution compared to the supernatant prior to freezing, and immediately after thawing remained significantly decreased. Conversely, there was a greater amount of PE(P-38:4) in the resuspension solution compared to the pre-freeze supernatant. After post-thaw storage, PE(P-38:4) decreased significantly compared to the resuspension solution.

The profile of ether linked PC and PE of platelets, microparticles and the supernatant fraction were relatively stable. These results suggest the ether linked phospholipid profile was not significantly altered by cryopreservation or post-thaw storage

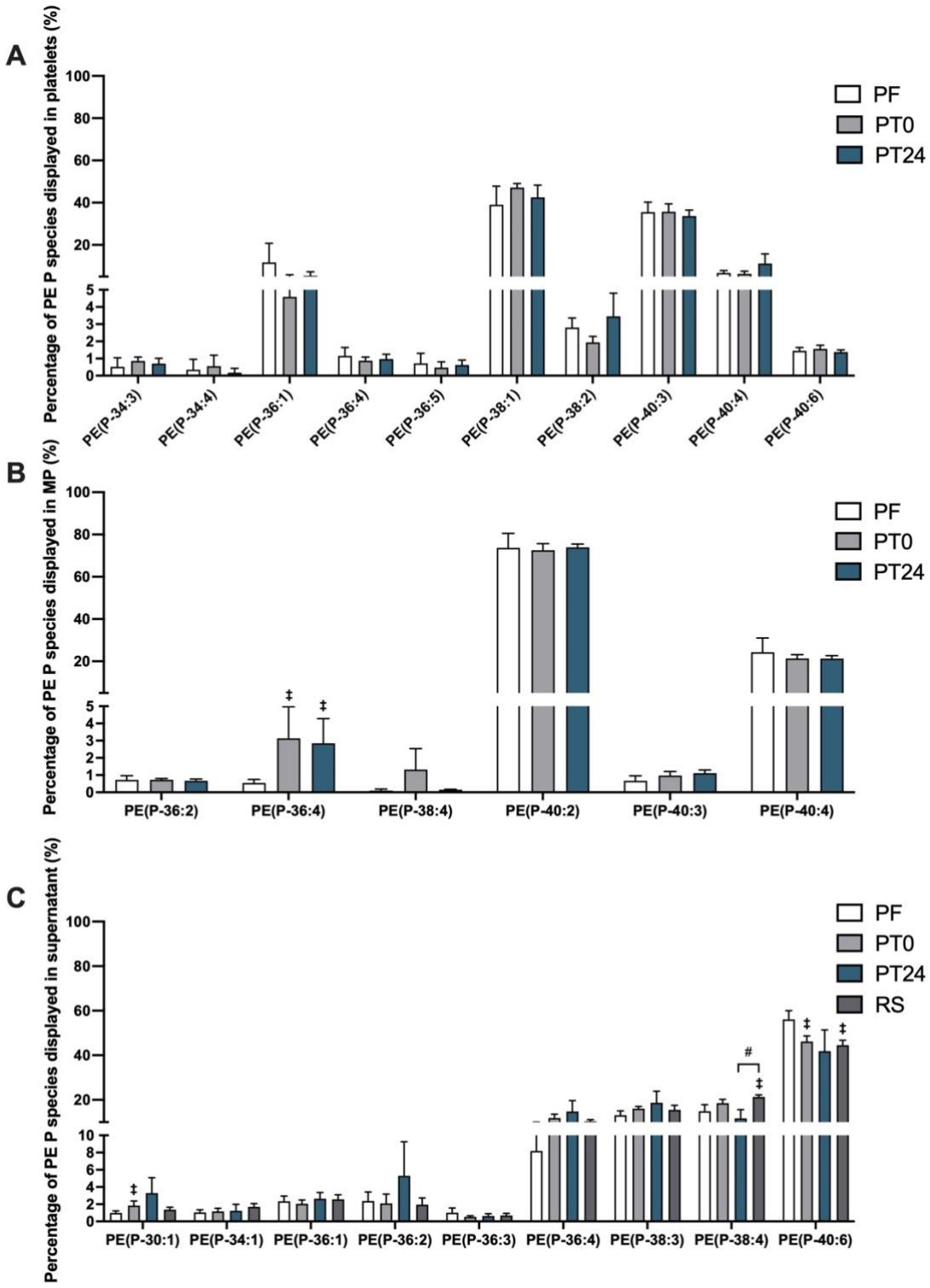
A
Percentage of PE P species displayed in platelets (%)
PF
PT0
PT24
PE(P-34:3)
PE(P-34:4)
PE(P-36:1)
PE(P-36:4)
PE(P-36:5)
PE(P-38:1)
PE(P-38:2)
PE(P-40:3)
PE(P-40:4)
PE(P-40:6)
B
Percentage of PE P species displayed in MP (%)
PF
PT0
PT24
PE(P-36:2)
PE(P-36:4)
PE(P-38:4)
PE(P-40:2)
PE(P-40:3)
PE(P-40:4)
C
Percentage of PE P species displayed in supernatant (%)
PF
PT0
PT24
RS
PE(P-30:1)
PE(P-34:1)
PE(P-36:1)
PE(P-36:2)
PE(P-36:3)
PE(P-36:4)
PE(P-38:3)
PE(P-38:4)
PE(P-40:6)

Figure 5.10. The effect of cryopreservation on the ether linked phosphatidylethanolamine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of ether linked phosphatidylethanolamine (PEP) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents individual data points, and mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

The cryopreservation process is known to increase the number of platelets and microparticles with externalised PS (Figure 5.2) [178]. Despite the PS class being relatively stable (Figure 5.4), the PS species profile was examined due to the importance of PS in the coagulation process [88, 90].

PS(34:1) and PS(36:1) represented the most prominent PS species in the platelet fraction, together accounting for approximately 80 % (Figure 5.11A). The PS species did not change immediately after thawing, however, significant alterations were observed at post-thaw 24. PS(34:1) was significantly increased, and PS(36:1) and PS(38:4) were significantly decreased, compared to pre-freeze.

There was a greater number of PS species displayed in the microparticles, than the platelet fraction, and changes in the proportion of species were seen immediately after thawing (Figure 5.11B). More specifically, short chain PS species, PS(34:1) and PS(36:1), were significantly increased in thawed samples compared to pre-freeze. On the other hand, the long chain PS species, PS(40:3) and PS(40:4), were significantly decreased after thawing compared to pre-freeze.

In the supernatant fraction, PS(38:1) represented the most abundant PS species (Figure 5.11C). The PS species remained relatively stable in the supernatant in all conditions tested.

The PS profile of platelets and the supernatant were relatively stable. Further, a greater number of PS species were observed in the microparticle and supernatant fractions compared to the platelet fraction.

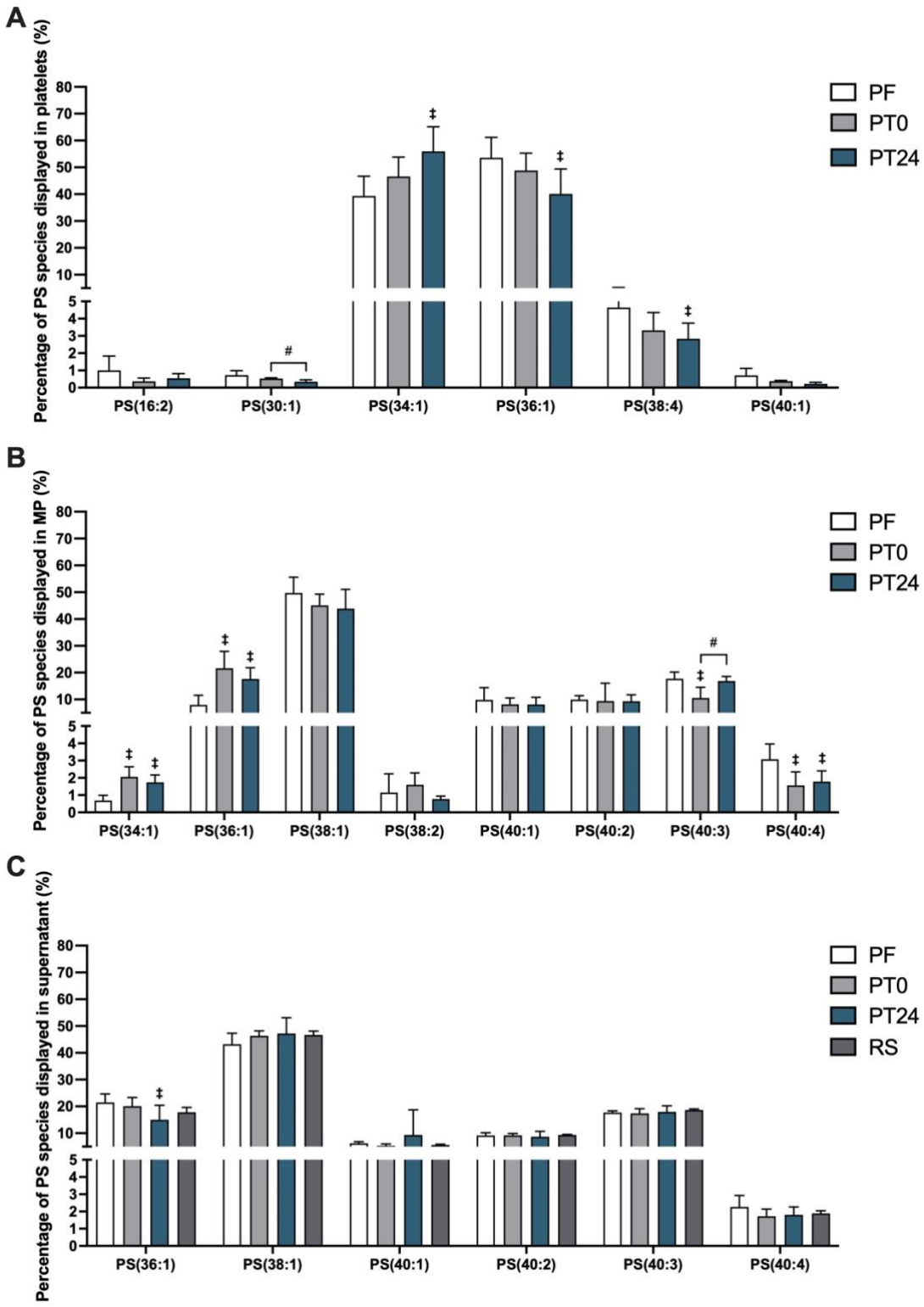
A
Percentage of PS species displayed in platelets (%)
PF
PT0
PT24
PS(16:2)
PS(30:1)
PS(34:1)
PS(36:1)
PS(38:4)
PS(40:1)
B
Percentage of PS species displayed in MP (%)
PF
PT0
PT24
PS(34:1)
PS(36:1)
PS(38:1)
PS(38:2)
PS(40:1)
PS(40:2)
PS(40:3)
PS(40:4)
C
Percentage of PS species displayed in supernatant (%)
PF
PT0
PT24
RS
PS(36:1)
PS(38:1)
PS(40:1)
PS(40:2)
PS(40:3)
PS(40:4)

Figure 5.11. The effect of cryopreservation on the phosphatidylserine species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of phosphatidylserine (PS) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents lipid species that exceed 1 % for any time point. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

Phosphatidic acid is metabolised during platelet activation, resulting in the formation of LPA [97, 224]. LPA is a potent bioactive lipid mediator, known to activate platelets and induce shape change even at low concentrations [68]. Significant changes were seen in PA and LPA of the platelet, microparticle and supernatant fractions (Figure 5.4), leading to the investigation of PA species and LPA species.

PA(32:1) was the most prominent PA species in the platelet fraction, accounting for approximately 35% (Figure 5.12A). PA(32:1) was significantly decreased after the post-thaw storage period compared to pre-freeze. Similarly, PA(38:4) was significantly decreased in post-thaw samples compared to pre-freeze. Conversely, PA(34:4) and PA(40:1) were significantly increased in post-thaw samples.

In the microparticle fraction, PA(40:2) was the most abundant PA species, representing approximately 65% of PA species (Figure 5.12B). The PA species in the microparticles remained relatively similar prior to freezing, after thawing and after post-thaw storage.

PA(38:1) and PA(38:4) comprised the majority of the PA species in the supernatant fraction, totalling 90% (Figure 5.12C). The PA species profile of the supernatant and resuspension solution was relatively stable, with only a few changes being observed after thawing. PA(38:7) was significantly increased immediately after thawing, when compared to the resuspension solution. Conversely, PA(40:1) was significantly decreased after the 24 hour storage period, compared to pre-freeze.

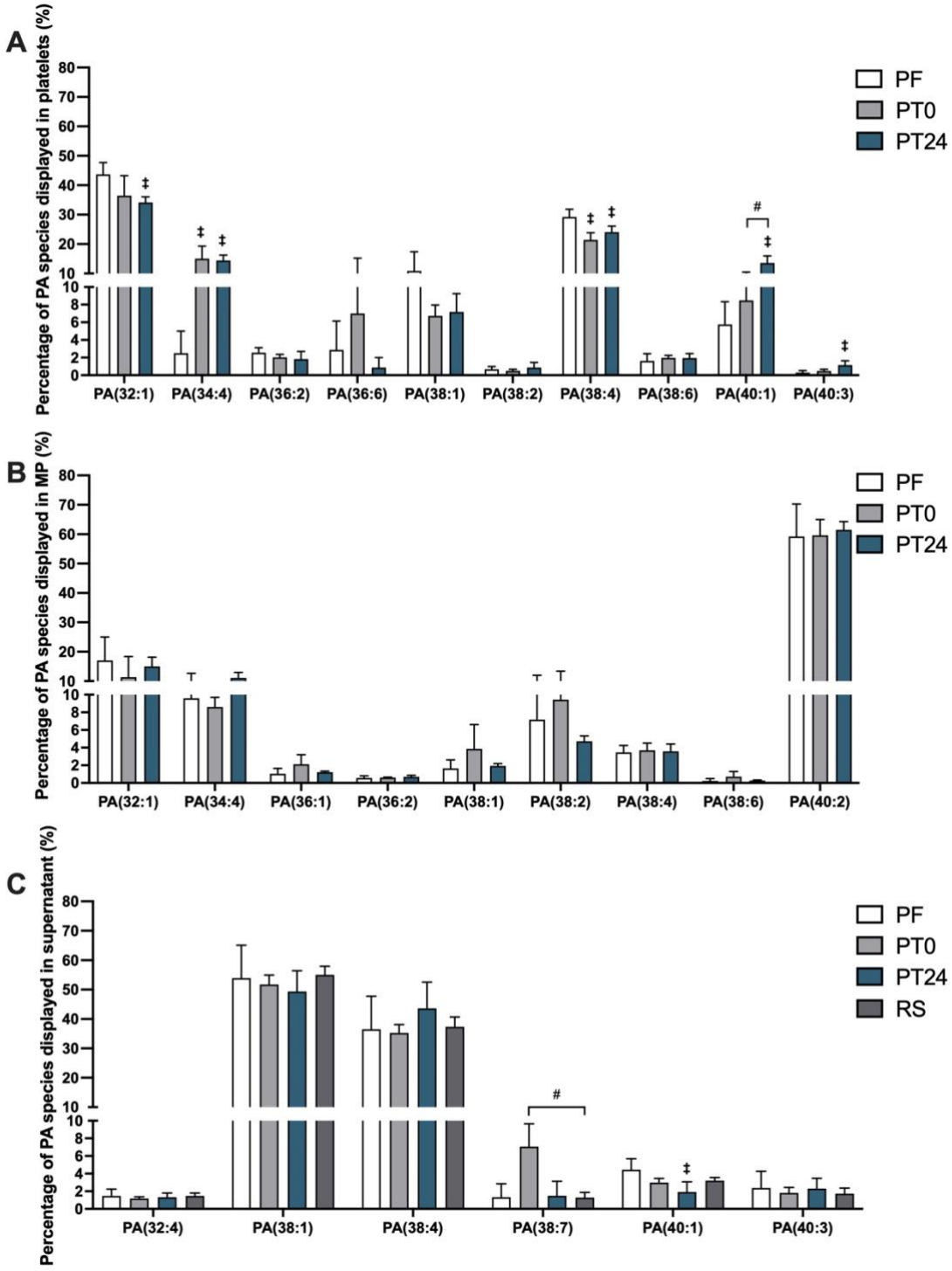
A
Percentage of PA species displayed in platelets (%)
PF
PT0
PT24
PA(32:1)
PA(34:4)
PA(36:2)
PA(36:6)
PA(38:1)
PA(38:2)
PA(38:4)
PA(38:6)
PA(40:1)
PA(40:3)
B
Percentage of PA species displayed in MP (%)
PF
PT0
PT24
PA(32:1)
PA(34:4)
PA(36:1)
PA(36:2)
PA(38:1)
PA(38:2)
PA(38:4)
PA(38:6)
PA(40:2)
C
Percentage of PA species displayed in supernatant (%)
PF
PT0
PT24
RS
PA(32:4)
PA(38:1)
PA(38:4)
PA(38:7)
PA(40:1)
PA(40:3)

Figure 5.12. The effect of cryopreservation on the phosphatidic acid species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of phosphatidic acid (PA) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

In the platelet fraction, LPA(22:1) was the most prominent LPA species representing approximately 50% of LPA species (Figure 5.13A). LPA(22:1) was significantly decreased immediately after thawing and continued to decrease over the post-thaw storage period. Conversely, LPA 20:0 was significantly increased after the post-thaw storage period when compared to pre-freeze.

LPA(16:1) and LPA(22:1) were the most abundant LPA species in the microparticle fraction, together accounting for 90 % of LPA species (Figure 5.13B). LPA(16:1) and LPA(18:3) were significantly increased in post-thaw samples. In contrast, LPA(22:1) significantly decreased in post-thaw samples.

LPA(22:1) was the most abundant LPA species in the supernatant fraction (Figure 5.13C). There was significantly less LPA(22:1) in the resuspension solution than in the pre-freeze supernatant and, immediately after thawing LPA(22:1) remained significantly lower compared to pre-freeze. On the other hand, there was significantly more LPA(20:0) in the resuspension solution compared to the pre-freeze supernatant. Further, immediately after thawing LPA(20:0) remained higher compared to pre-freeze.

To summarise the PA and LPA species profiles, only a limited number of LPA species were identified in each fraction. Further, despite the changes in the PA and LPA class, the species profiles were relatively stable, particularly in the microparticle and supernatant fractions.

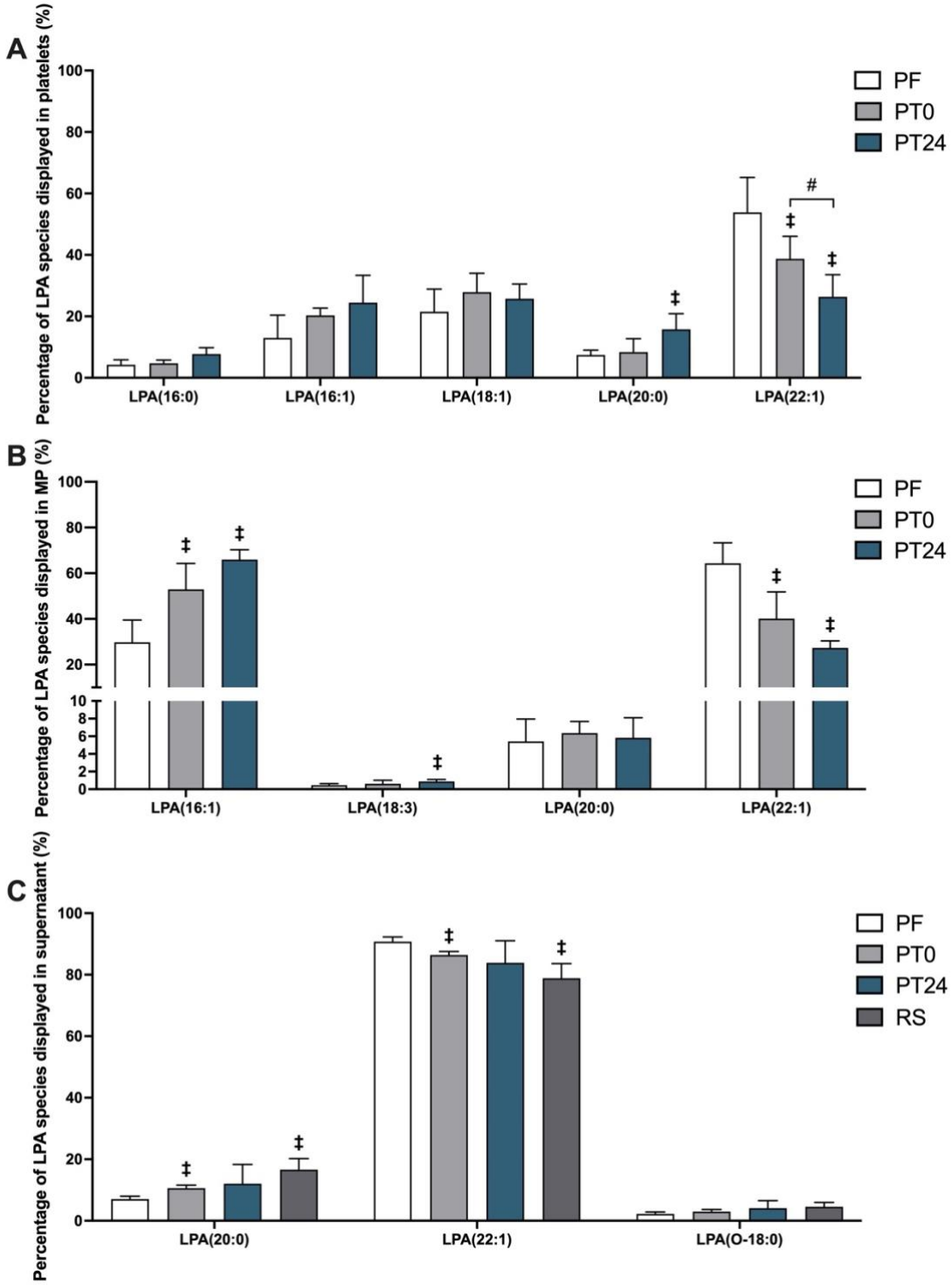
A
Percentage of LPA species displayed in platelets (%)
100
80
60
40
20
0
PF
PT0
PT24
#
‡
LPA(16:0)
LPA(16:1)
LPA(18:1)
LPA(20:0)
LPA(22:1)
B
Percentage of LPA species displayed in MP (%)
100
80
60
40
20
10
8
6
4
2
0
PF
PT0
PT24
‡
LPA(16:1)
LPA(18:3)
LPA(20:0)
LPA(22:1)
C
Percentage of LPA species displayed in supernatant (%)
100
80
60
40
20
0
PF
PT0
PT24
RS
‡
LPA(20:0)
LPA(22:1)
LPA(O-18:0)

Figure 5.13. The effect of cryopreservation on the lysophosphatidic acid species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of lysophosphatidic acid (LPA) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

Sphingomyelin is a precursor to the bioactive lipids, ceramide, sphingosine and sphingosine 1-phosphate [202]. Ceramide is a bioactive lipid mediator and second messenger, and is closely associated with oxidative stress and apoptosis [202, 203]. As such, although sphingomyelin and ceramide were not significantly altered following cryopreservation, an investigation into the species displayed was justified.

In the platelet fraction, SM(24:1) was the most prominent sphingomyelin species, representing 50 % of the sphingomyelin species (Figure 5.14A). SM(24:1) was unchanged immediately after cryopreservation but was significantly decreased after post-thaw storage, when compared to pre-freeze and post-thaw 0. In contrast, SM(14:0) and SM(15:0) were significantly increased immediately after thawing.

SM(22:0) and SM(24:0) were the most abundant sphingomyelin species of the microparticle fraction, together accounting for 40 % of sphingomyelin species (Figure 5.14B). The sphingomyelin species profile of microparticles was relatively stable, with the exception of SM(16:1). SM(16:1) was significantly decreased after post-thaw storage for 24 hours compared to pre-freeze.

SM(22:0) and SM(24:1) were the most prominent sphingomyelin species in the supernatant fraction, cumulatively accounting for 60% of sphingomyelin species (Figure 5.14C). The profile of sphingomyelin species present in the pre-freeze supernatant was similar to the resuspension solution and was unchanged after thawing or post-thaw storage for 24 hours.

The sphingomyelin profile of platelets, microparticles and the supernatant were stable after thawing. These results suggest the sphingomyelin profile was not significantly altered by cryopreservation or post-thaw storage.

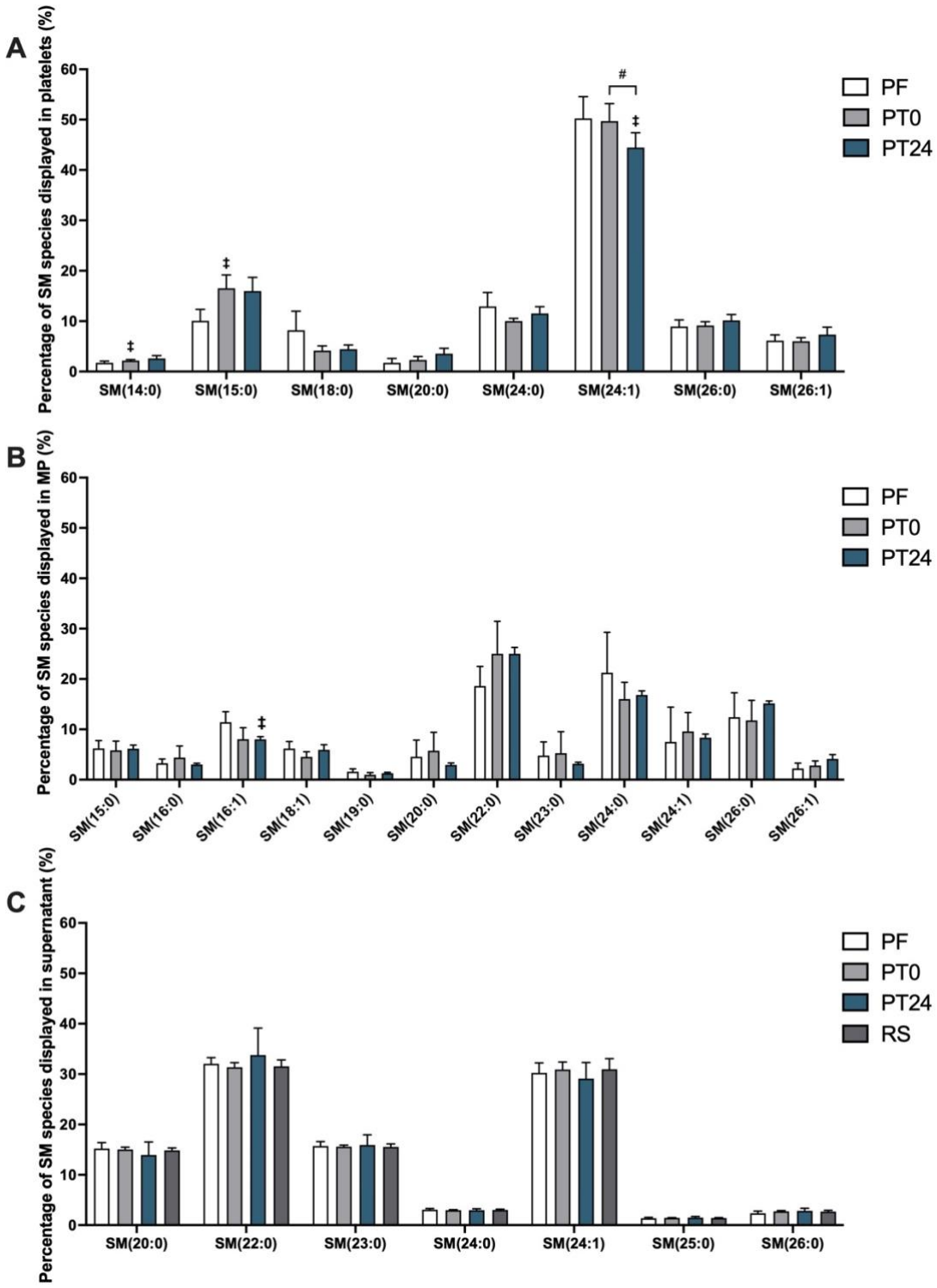
A
Percentage of SM species displayed in platelets (%)
PF
PT0
PT24
SM(14:0)
SM(15:0)
SM(18:0)
SM(20:0)
SM(24:0)
SM(24:1)
SM(26:0)
SM(26:1)
B
Percentage of SM species displayed in MP (%)
SM(15:0)
SM(16:0)
SM(16:1)
SM(18:1)
SM(19:0)
SM(20:0)
SM(22:0)
SM(23:0)
SM(24:0)
SM(24:1)
SM(26:0)
SM(26:1)
C
Percentage of SM species displayed in supernatant (%)
PF
PT0
PT24
RS
SM(20:0)
SM(22:0)
SM(23:0)
SM(24:0)
SM(24:1)
SM(25:0)
SM(26:0)

Figure 5.14. The effect of cryopreservation on the sphingomyelin species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of sphingomyelin (SM) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

Cer(22:0) was the most abundant ceramide species in the platelet fraction (Figure 5.15A). The ceramide species profile remained relatively stable following cryopreservation. Cer(18:0) was the only species to be altered, which was significantly increased after post-thaw storage compared to post-thaw 0.

The ceramide species profile of microparticles was extensively altered by the cryopreservation process (Figure 5.15B). Microparticles that formed after thawing contained a significantly increased proportion of shorter chain ceramide species (Cer(20:0), Cer(22:0), Cer(24:0), Cer(26:0) and Cer(26:1)). In contrast, the longer chain ceramide species (Cer(28:0), Cer(30:0), Cer(31:0) and Cer(33:0)) were decreased in the post-thaw microparticles, compared to pre-freeze. With the exception of Cer(24:0), the ceramide profile of microparticles immediately following thawing was maintained through the storage period.

Cer(24:1) was the most prominent ceramide in the supernatant fraction, representing 30% of the ceramide species (Figure 5.15C). The ceramide species profile of the resuspension solution was similar to the supernatant prior to freezing, and did not change after thawing or after the post-thaw storage period of 24 hours.

The ceramide profile of platelets and the supernatant was relatively stable. However, these results suggest the microparticles formed after thawing have a different ceramide profile than microparticles formed prior to freezing.

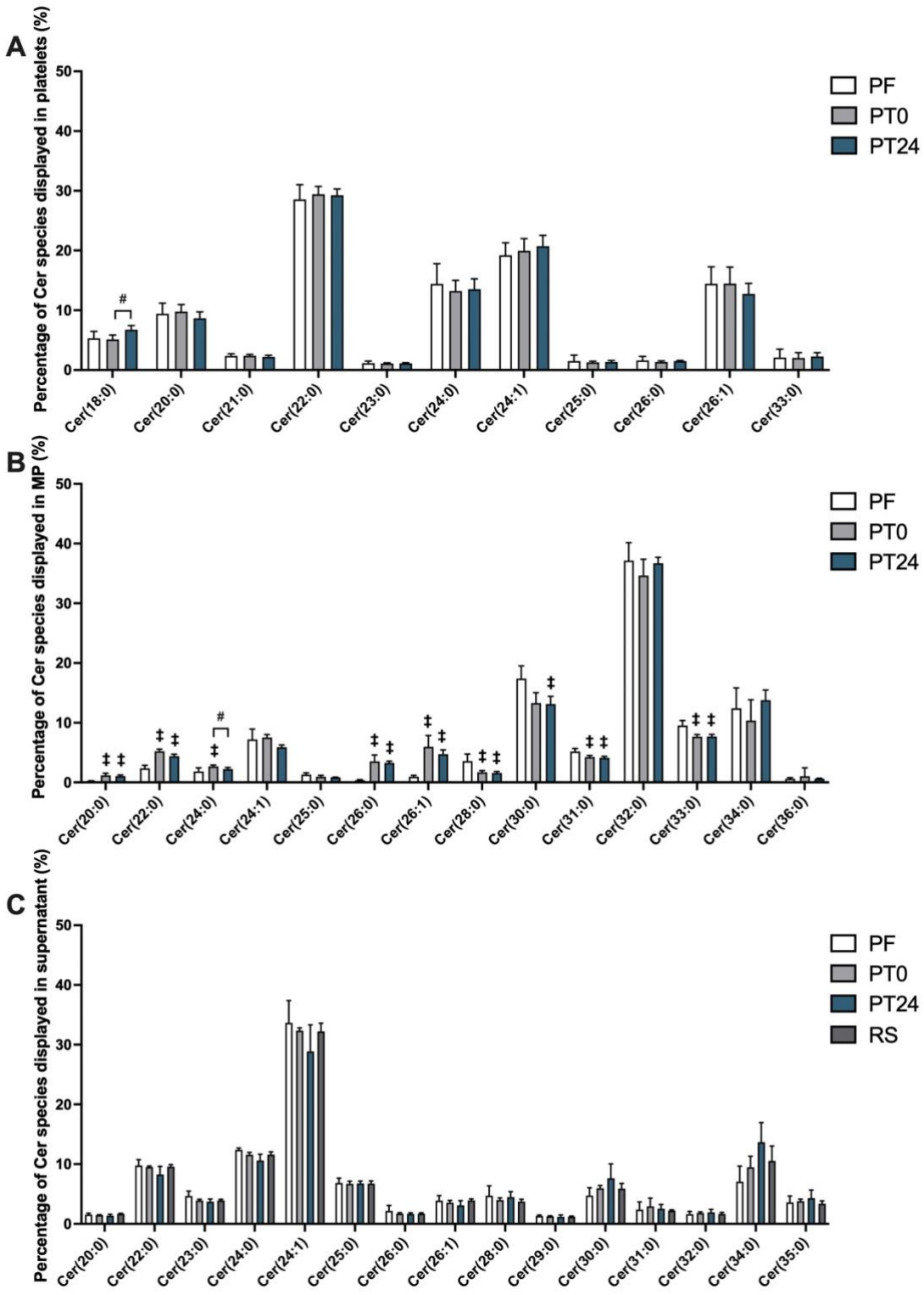
A
Percentage of Cer species displayed in platelets (%)
PF
PT0
PT24
Cer(18:0)
Cer(20:0)
Cer(21:0)
Cer(22:0)
Cer(23:0)
Cer(24:0)
Cer(24:1)
Cer(25:0)
Cer(26:0)
Cer(26:1)
Cer(33:0)
B
Percentage of Cer species displayed in MP (%)
PF
PT0
PT24
Cer(20:0)
Cer(22:0)
Cer(24:0)
Cer(24:1)
Cer(25:0)
Cer(26:0)
Cer(26:1)
Cer(28:0)
Cer(30:0)
Cer(31:0)
Cer(32:0)
Cer(33:0)
Cer(34:0)
Cer(36:0)
C
Percentage of Cer species displayed in supernatant (%)
PF
PT0
PT24
RS
Cer(20:0)
Cer(22:0)
Cer(23:0)
Cer(24:0)
Cer(24:1)
Cer(25:0)
Cer(26:0)
Cer(26:1)
Cer(28:0)
Cer(29:0)
Cer(30:0)
Cer(31:0)
Cer(32:0)
Cer(34:0)
Cer(35:0)

Figure 5.15. The effect of cryopreservation on the ceramide species

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The platelets, microparticles and supernatant were separated by differential centrifugation. The percentage composition of ceramide (Cer) species in the (A) platelets, (B) microparticles (MP) and (C) supernatant were determined by LC-MS/MS. The data represents mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 or RS and PT24 to RS.

5.3 Assessment of bioactive lipid mediators following cryopreservation

Bioactive lipid mediators have been shown to effect transfusion outcomes [186, 209, 225]. Further, several of the mechanisms that could provide an explanation for the changes seen in the lipid classes (Figure 5.4), are known to result in the formation of bioactive lipid mediators [61, 83]. As such, the abundance of several of these bioactive lipid mediators were assessed by ELISA in the supernatant before freezing, after thawing, after 24 hours of storage, as well as in the solution used to reconstitute thawed platelets. The concentration of arachidonic acid and 5-HETE were low in the reconstitution solution (Figure 5.16A and B). After thawing and after the post-thaw storage period, arachidonic acid and 5-HETE were significantly increased compared to the resuspension solution. The concentration of 12(S)-HETE was low in both the reconstitution solution and pre-freeze, but was significantly increased after thawing and after the post-thaw storage period (Figure 5.16C). The concentration of 15(S)-HETE and S1P was low in the reconstitution solution compared to the supernatant prior to freezing (Figure 5.16 D and E). After thawing and after the post-thaw storage period, 15(S)-HETE and S1P were significantly increased compared to the resuspension solution. However, only the concentration of 15(S)-HETE was significantly different to the pre-freeze concentration.

In all cases, the reconstitution solution contained very low concentrations of these bioactive lipids, indicating the reconstitution solution did not substantially contribute to the observed post-thaw changes. However, after thawing the concentration of these bioactive lipid mediators increased, suggesting the cryopreservation process may increase the formation or release of certain bioactive lipid mediators.

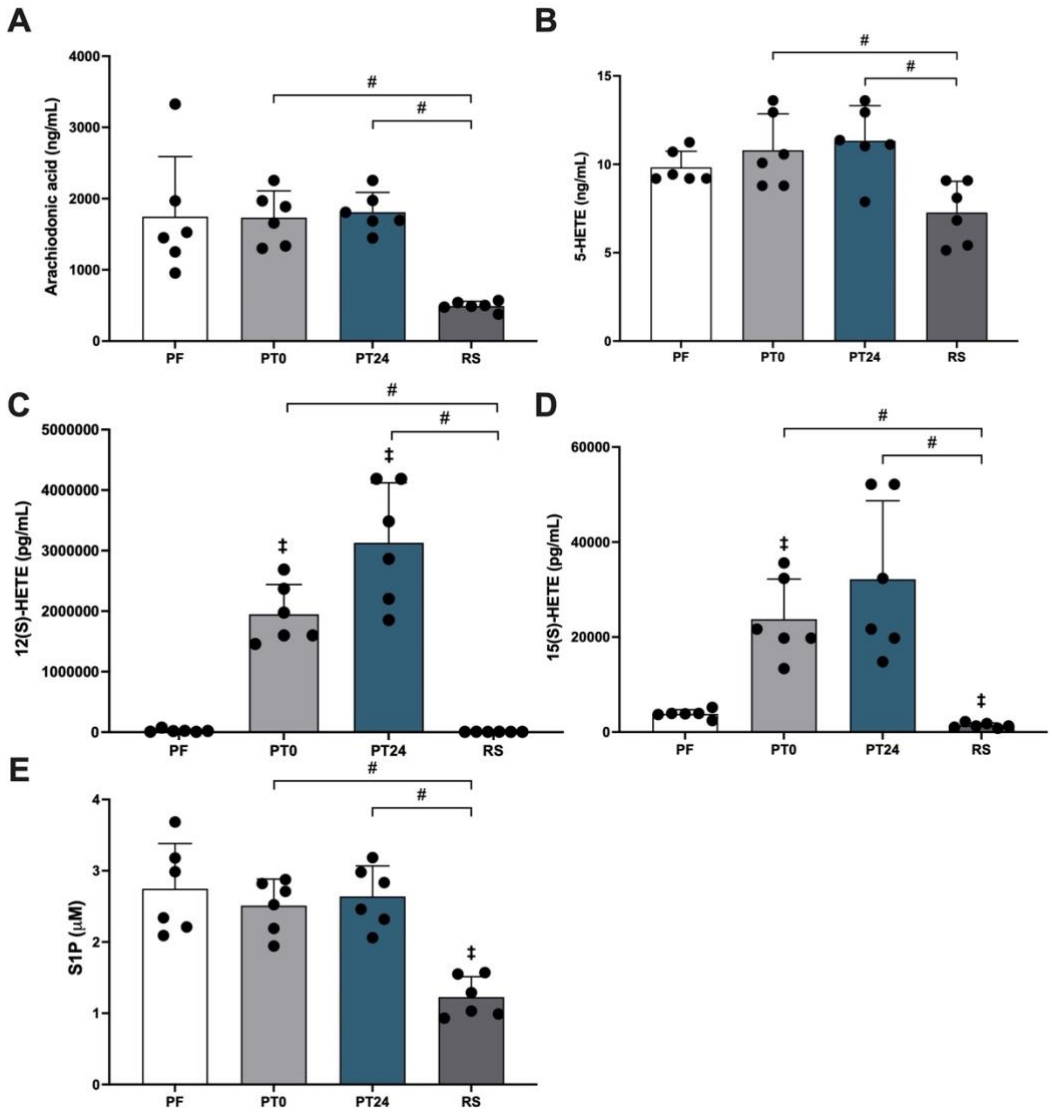

Figure 5.16. Th effect of cryopreservation on the presence of certain bioactive lipid mediators in the supernatant of platelet components

Platelet components were sampled before freezing (PF), immediately after thawing (PT0) and 24 hours after thawing (PT24). In addition, the reconstitution solution (RS) was sampled as a control. The supernatant was isolated using multiple rounds of centrifugation and the concentration of (A) arachidonic acid, (B) 5-HETE, (C) 12(S)-HETE, (D) 15(S)-HETE and (E) S1P were determined by ELISA. The data represents individual data points, and mean (bars) + SD (error bars); n=6. The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when comparing PT0 to PT24 and PT0 or PT24 to RS.

5.4 Assessment of lipid dynamics and lipid pathways following cryopreservation

The lipid profile of any cell is inherently dynamic. As lipids act as both structural and signalling molecules, and can be transformed by enzymatic reactions [205], assessing the lipid class ratios and performing pathway analysis may give a more nuanced insight into the lipid dynamics taking place. Lipid classes were selected for comparison as ratios based on known metabolic pathways and based on literature, where it has been suggested an altered lipid ratio may impact cellular function and disease process [206, 207].

In the platelet fraction, the LPC/PC and PA/PC ratios were decreased after post-thaw storage compared to pre-freeze and post-thaw 0 (Table 5.1). On the other hand, the PC/PE ratio was increased after post-thaw storage compared to pre-freeze. In the microparticle fraction, the LPC/PC, LPE/PE and LPA/PA ratios were decreased after thawing and after the post-thaw storage period. Conversely, the PA/PC and PC/PE ratios increased after thawing and post-thaw storage. In the supernatant fraction, LPC/PC and LPE/PE ratios were lower in the resuspension solution. Similarly, the Cer/SM ratio was decreased after post-thaw storage compared to pre-freeze.

Table 5.1. Lipid class ratios of platelets, microparticles and supernatant from cryopreserved platelet components

	Platelet			Microparticle			Supernatant			
	PF	PT0	PT24	PF	PT0	PT24	PF	PT0	PT24	RS
LPC/PC	1.073	0.677	0.428‡#	0.203	0.093‡	0.070‡	0.653	0.538	0.523	0.292‡#
LPE/PE	0.070	0.088	0.083	0.012	0.006	0.005‡	0.018	0.017	0.012	0.010‡#
PEP/PE	1.737	2.683	2.902	0.362	0.383	0.488	0.020	0.020	0.025	0.020
PS/PC	0.325	0.220	0.175	0.053	0.053	0.052	0.192	0.227	0.352	0.222
PA/PC	0.118	0.097	0.067#	0.118	0.170‡	0.167‡	0.228	0.235	0.242	0.242
LPA/PA	0.135	0.132	0.135	0.040	0.017‡	0.018‡	0.057	0.035	0.093	0.028
Cer/SM	1.715	1.725	1.675	1.658	1.348	1.842	0.135	0.142	0.170‡	0.140
PC/PE	3.743	5.342	6.380‡	2.367	2.534	3.431‡	1.071	0.989	0.957	1.011
PS/PE	1.086	1.184	1.098	0.123	0.127	0.173	0.205	0.222	0.306	0.222
PS/PA	2.703	2.320	2.603	0.443	0.313	0.304	0.852	0.954	1.415	0.912

PF = pre-freeze; PT0 = post-thaw 0; PT24 = post-thaw 24; RS = resuspension solution
Values shown as mean of the ratio of the given lipid classes, n=6 in each group.
The effect of cryopreservation was assessed using a one-way repeated measures ANOVA. *Post hoc* Bonferroni's multiple comparisons test was performed to determine differences between all groups. ‡ indicates $p<0.05$ when compared to PF. # indicates $p<0.05$ when compared to PT0. * indicates $p<0.05$ when compared PT24.

BioPAN, as mentioned in the previous chapter, is an open access web-based tool that provides a statistical score for possible lipid metabolism pathways [196, 226]. This is achieved by establishing relationships between lipid substrates and lipid products, present in a user's data, and the enzymes involved the reaction, and determining if the reaction is active or suppressed [196]. The information used to assess these relationships is sourced from current literature [196]. BioPAN lipid networks were generated to assess the changes in each fraction following cryopreservation and storage. Representative lipid networks depicting active reactions between pre-freeze to post-thaw samples are shown in Figure 5.17A, B, C and D, and the remaining data is presented in supplementary data S.9 – S.10. The data for all sample points and fractions has been summarised in Figure 5.18.

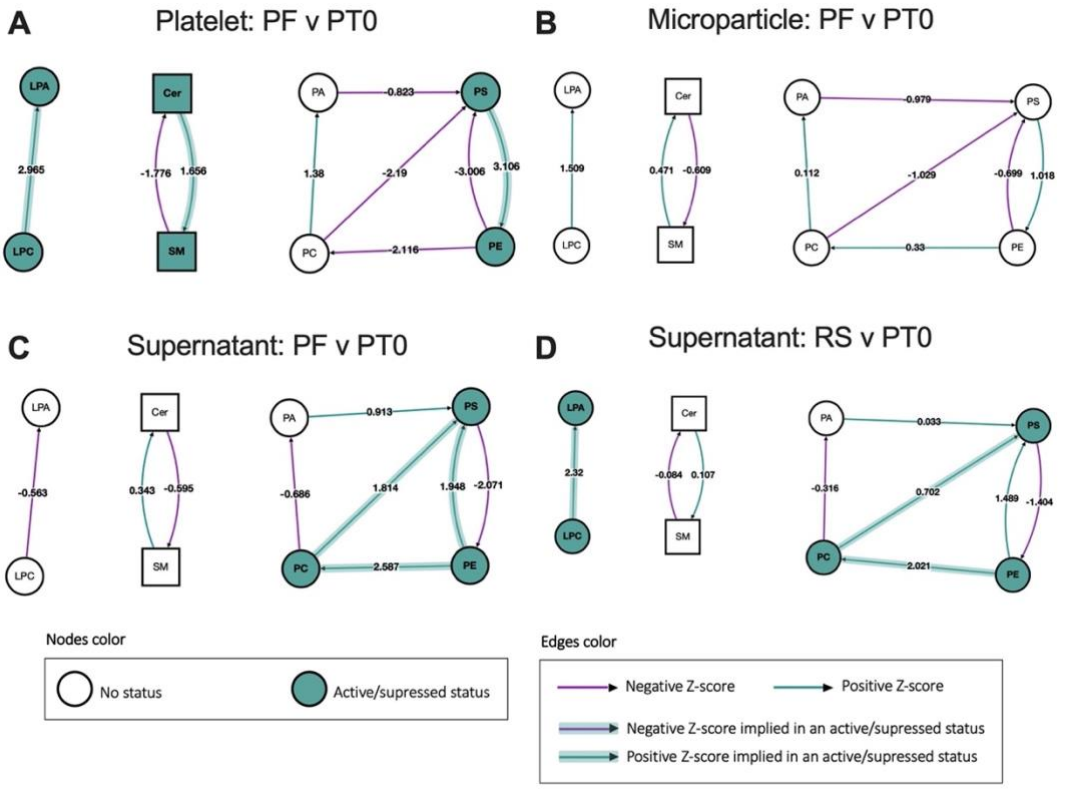

Figure 5.17. BioPAN lipid networks depicting active reactions following cryopreservation

The lipid networks of (A) platelets, (B) microparticles, (C) supernatant and the (D) resuspension solution comparing pre-freeze (PF) and post-thaw 0 (PT0) time points. The networks were produced using BioPAN, an open access web-based tool (https://www.lipidmaps.org/biopan/) [196], by inputting data obtained from mass spectrometry analysis. Representative lipid are shown, networks were obtained comparing each time point (see supplemental data, S.9-S.10).

In the platelet fraction, the conversion of LPC to LPA, PS to PE and Cer to SM were inferred to be significantly active when comparing pre-freeze to either time point post-thaw (Figure 5.18A). On the other hand, the conversion of PC to PS, PE to PC, PE to PS and SM to Cer were inferred to be significantly suppressed when comparing pre-freeze to post-thaw time points.

In the microparticle fraction, the majority of the significant changes were observed when comparing to post-thaw 24 (Figure 5.18B). Specifically, the conversion of LPC to LPA was inferred to be significantly active when comparing pre-freeze to the post-thaw 24. The conversion of PC to PA and PC to PS were inferred to be significantly active when comparing pre-freeze and post-thaw 0 to post-thaw 24. Conversely, the conversion of PA to PS and PE to PC were inferred to be significantly suppressed when making post-thaw 24 comparisons.

In the supernatant fraction, the conversion of LPC to LPA was inferred to be significantly active in the pre-freeze sample compared to the resuspension solution (Figure 5.18C). Conversely, the conversion of PC to PS and PE to PC were inferred to be significantly suppressed pre-freeze compared to the resuspension solution. After thawing, the conversion of PC to PS, PE to PC, PE to PS and SM to Cer were inferred to be significantly active when comparing pre-freeze to post-thaw time points, and PS to PE and Cer to SM were inferred to be significantly suppressed when comparing pre-freeze to post-thaw time points. The conversion of Cer to SM was inferred to be significantly suppressed and the conversion of SM to Cer was inferred to be significantly active after post-thaw storage compared to post-thaw 0. Further, the conversion of LPC to LPA, PE to PC, PA to PS and SM to Cer were inferred to be significantly active in post-thaw samples compared to the resuspension solution. Conversely, Cer to SM was inferred to be significantly suppressed post-thaw compared to the resuspension solution.

Several reactions were found to be significantly activated or supressed when comparing each time point of the cryopreservation process. Further, the use

of lipid network pathways allows for visualisation of relationships between different lipids classes.

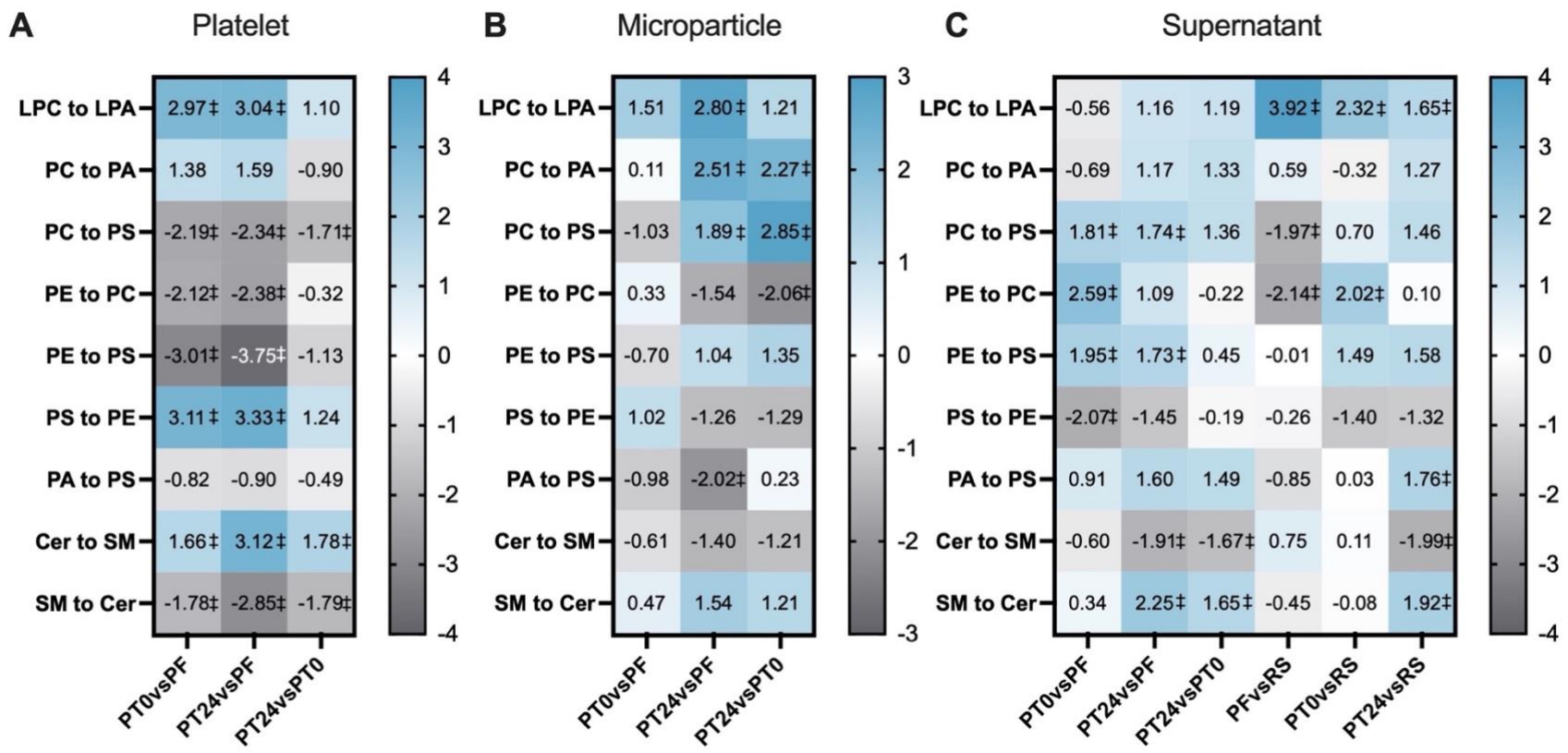

Figure 5.18. Summary of Z-scores for given reactions from cryopreserved platelet components obtained from BioPAN

Lipid networks were generated from BioPAN using data acquired by LC-MS/MS. Z-scores from the (A) platelet, (B) microparticle and (C) supernatant lipid networks were summarised and are depicted as heat maps. Values shown as Z-score of the given reaction, whereby a Z-score>0 represents an active reaction and a Z-score<0 represents a suppressed reaction. A paired t-test was performed by BioPAN to determine differences between indicated groups. ‡ indicates $p<0.05$ a significant reaction (corresponding to a Z-score > 1.645 or < -1.645)

Genes known to be involved in the reactions depicted in the lipid networks are suggested by BioPAN. After generating networks from each fraction comparing cryopreservation and storage, a list of genes was consolidated as being potentially involved in the indicated reaction (Table 5.2). The list of genes generated by the web-based tool encode proteins that facilitate the depicted reactions. Thus, this information may assist in understanding complex interactions occurring between fractions, and additionally provide avenues for future research.

Table 5.2. BioPAN predicted genes to be activated or supressed in the given reaction in cryopreserved platelet components

	Gene symbol	Gene name
LPC to LPA	-	-
PC to PA	-	-
PC to PS	PTDSS1	phosphatidylserine synthase 1
PE to PC	PEMT	phosphatidylethanolamine N-methyltransferase
PE to PS	PTDSS2	phosphatidylserine synthase 2
PS to PE	PISD	phosphatidylserine decarboxylase
PA to PS	-	-
Cer to SM	SGMS1	sphingomyelin synthase 1
	SGMS2	sphingomyelin synthase 2
	CERT1	ceramide transfer protein 1
SM to Cer	SMPD1	sphingomyelin phosphodiesterase 1
	SMPD2	sphingomyelin phosphodiesterase 2
	SMPD3	sphingomyelin phosphodiesterase 3

Chapter 6 Discussion of the lipidome of alternatively stored platelets

The results from this study outlined changes to the phospholipid and sphingolipid profile during cold storage and following cryopreservation, which are further discussed in this chapter. The changes observed in the cold-stored platelet components lipid profile may have arisen due to exchanges of lipids between fractions and altered lipid metabolism; whereas the changes occurring in the lipid profile following cryopreservation are likely the result of lipid uptake from lipoproteins, microparticle formation and platelet or microparticle degradation. Functionally, the observed changes are associated with coagulation, apoptosis, inflammation and signalling. The changes occurring in both cold-stored and cryopreserved platelet components may affect platelet function once these components are transfused. As such, further avenues of study which are required to more comprehensively understand the functional implications of these changes have been outlined.

6.1 Addressing challenges of platelet storage

The current requirement for platelet storage in Australia is at room temperature in gas permeable bags, with constant agitation for a maximum of seven days [108, 130, 131]. These conditions limit the shelf life to 7 days, in order to minimise the risk of bacterial proliferation and to lessen the effects of the PSL [132].

The storage requirements of platelets limit their availability and can result in supply difficulties. When supplying rural, remote and military locations the lengthy transport times impact product quality and unpredictable demand can result in product wastage [131, 141-144]. The inability to readily supply platelets in these locations can result in a failure to meet current standards of resuscitative care [141, 144]. Alternative storage techniques, such as cold storage and cryopreservation, could resolve some issues surrounding conventionally stored platelets.

Cold storage of platelets involves storing platelets in a refrigerator (2-6 °C), without agitation, and is purported to extend the shelf life up to 2-3 weeks [145-148]. Cold storage is an attractive solution due to the extended shelf life, less burdensome storage and transport logistics, lower risk of bacterial proliferation and enhanced haemostatic capabilities [163-165]. Internationally, cold-stored platelets are currently being used in civilian hospitals on a limited basis and have been used within military settings [163-165]. However, cold-stored platelets are not yet approved for use in Australia.

The effect of cold storage has previously been assessed in the context of changes to platelet metabolism, surface receptor expression and the proteome [122, 148, 153-156]. While the lipidome of room temperature stored platelets has previously been reported [51, 52], only historic studies of the lipidome of cold-stored platelets have been conducted [56, 57]. A reassessment of the lipidome of cold-stored and room temperature stored platelets was justified as advances in transfusion medicine and in mass

spectrometry technology have occurred since the com3pletion of these studies.

Platelet cryopreservation is an appealing alternative to room temperature storage, especially for supplying austere locations as it can extend the shelf life to at least two years [141, 142, 166]. Platelet cryopreservation involves the addition of DMSO and freezing at -80 °C [142]. Cryopreserved platelets have been extensively used in military settings [166, 171]. Further, several clinical trials are underway to extend their use to support surgical bleeding in a civilian context [142, 170-174].

Extensive *in vitro* assessments of cryopreserved platelets have been conducted to assess changes to metabolism, surface receptor expression and the proteome. Central to this project, cryopreserved platelets externalise PS and result in the formation of a high number of PS-expressing microparticles [167, 168, 175-179]. Despite this knowledge, no lipidomic investigations have been performed.

Understanding changes to the lipid profile of alternatively stored platelet components is important as even subtle lipid changes are known to be associated with altered platelet function, platelet clearance and component safety and efficacy [56, 57, 137, 182].

6.2 Assessment of the lipid profile of cold-stored platelets

This study assessed the lipidomic changes occurring in platelets that were stored at either room temperature or cold-stored. Storage at room temperature limits the shelf life to between 5 and 7 days, depending on the institutional regulations, with Australia recently transitioning from a 5 day to a 7 day shelf life. However, at the time of conducting this study the shelf life was 5 days, which is why this time point was assessed. Similarly, day 14 represents the feasible shelf life for cold-stored platelet components, and thus was chosen as the later time point.

It has previously been noted that differences in the method of production, storage containers, storage solutions and temperature of platelet components differentially affects their *in vitro* quality [118, 145, 159, 227-229]. The lipidome of room temperature stored platelet components collected by apheresis in 100 % plasma has previously been assessed [56, 57]. Similarly, studies have been performed examining the lipidome of cold-stored platelet components produced from whole blood in 100 % plasma [51, 52]. In contrast, this study examined the effect of cold storage on platelet components collected by apheresis and stored in 40 % plasma/60 % SSP+, as is standard practice in our institute. As the contribution of plasma lipids and plasma lipoproteins is known to impact the platelet lipidome [57, 98, 230], it was anticipated that the lower proportion of plasma may result in lipid changes over storage. However, this study demonstrated that the total lipid content of the platelet and supernatant fractions remained unchanged over storage (Figure 4.3), regardless of storage temperature. From the literature, there is conflicting evidence regarding the effect of storage on the lipid content, which is likely due to the differences in study design mentioned above [118, 145, 159, 227-229]. Specifically, some studies have demonstrated a decrease in the lipid content of platelets [51, 52, 57], while others demonstrate no change [56].

While the total lipid content was not altered, changes were observed in the composition of the phospholipid and sphingolipid profile. The lipid profile of the platelet fraction was stable during early storage (5 days), but by day 14, alterations to the lipid profile were observed, and these changes were exacerbated by cold storage. In contrast, the proportion of many lipid classes were altered in the supernatant at day 5 at both storage temperatures. However, by day 14, the proportions were more similar to those seen at day 1, particularly in the cold-stored samples.

6.2.1 Changes to the lipid profile of platelets following cold storage

The phospholipid and sphingolipid profile of platelets stored in additive solution was similar to previously reported data of platelets in plasma [55-57], and this was relatively unaffected by cold storage during early time points (5 days). However, alterations in the proportion of specific lipid classes were evident during extended cold storage, specifically in the PA, PC, PE, PEP, PI, PS, ceramide and SM lipid classes (Figure 4.4). The increases in PE and SM may be the result of incorporation from plasma lipoproteins into the platelets [98, 230], as plasma lipoproteins are rich in PC, PE and SM [231]. This is purported to take place via selective endocytosis or facilitated by protein interaction [232]. Further, the regulation and mechanism facilitating lipid uptake is variable and dependant on lipoprotein type [232]. Specifically, GPIV (CD36) supports the transfer of PC and SM [232-234], and cytosolic phospholipase A_2 supports the transfer of PE [235]. Cold-stored platelets have been shown to have lower expression of GPIV, and over storage (at both room temperature and cold) the abundance of GPIV (CD36) is reduced [153]. Further, the transfer of lipids from lipoproteins is temperature dependent [98, 99, 236]. Thus, the cold and storage related reduction of GPIV may alter lipoprotein incorporation, resulting in the reduced proportion of PC in cold-stored platelets at later time points.

In addition to the decreased transfer of PC from lipoproteins [232-235], the results from this study (Table 4.1 and Figure 4.16) suggest PC lipid metabolism was altered at day 14 of cold storage. Specifically, the conversion of PC to PA was significantly activated over extended cold storage, and the ratio of PA/PC was significantly increased. Phospholipase D_1 and phospholipase D_2 are the enzymes responsible for this conversion [237]. In platelets, phospholipase D activity is typically increased by agonists responsible for activating platelets, such as thrombin, ADP and collagen, which act through signal transduction pathways, such as G coupled protein receptors [237]. During cold storage platelets are known to have increased markers of platelet activation, including activated integrin αIIbβ3, P-selectin, externalised PS [153, 157] and increased intracellular calcium levels [238]. Therefore, these data suggest cold induced platelet activation may increase

the activity of phospholipase D, however, additional studies are required to confirm this.

The proportion of PA was increased during extended cold storage. This may be due to the altered PA and PC metabolism (Table 4.1 and Figure 4.16), as mentioned above. While PA has limited cellular functions, PA can be catabolised to the bioactive lipid mediator LPA [68, 239]. Activated platelets are known to release LPA, and LPA has been shown to induce platelet activation, aggregation and induce immune responses [68, 239-241]. Interestingly, the concentration of LPA in the supernatant was significantly increased by room temperature storage but not cold storage (Figure 4.4 and 4.13). Platelets do not store LPA within their granules, as they do with other bioactive mediators; rather it is produced *de novo* from PA and lysophospholipids, and released to the surrounding plasma during activation [241, 242]. Therefore, the increased PA, seen in the cold-stored platelets, may represent a reservoir to be catabolised to LPA and released once platelets are activated during haemostasis.

The externalisation of aminophospholipids, PS and PE, is essential to support normal haemostatic function [33]. The externalisation of PS mediates the interactions with the tenase and prothrombinase complexes of the coagulation system [33], while the externalisation of PE improves the catalytic ability of PS by increasing the affinity of the membrane for hydrophobic coagulation factors [33]. As demonstrated in this study, PS externalisation was increased in cold-stored platelets in a time dependent manner (Figure 4.2), which is in line with results from other studies [153, 154]. Further, subtle changes in arachidonic acid (20:4) containing PE, PEP and PS species may contribute to improved haemostatic function [96]. The proportion of PS and PE was significantly increased by extended (14 days) cold storage (Figure 4.4). Further,an increase in PE and PEP species in which one of their fatty acyl tails could be arachiodonic acid (20:4), such as PE(38:4), PE(40:4) and PE(P-38:4) was seen (Figure 4.7 and 4.9). However, somewhat surprisingly, there was no

change to the arachidonic acid containing PS species (Figure 4.10). These subtle shifts, particularly in the PE species, may contribute to the increased haemostatic profile typified by cold-stored platelets [122, 154].

In addition to the increased proportion of PS in cold-stored platelets, lipid metabolism of PS was also altered. An explanation for the increase in PS may be related to a dysfunction in the base exchange process. PS synthesis occurs in the endoplasmic reticulum, in a specialised area known as the mitochondria-associated membranes (MAM) [243, 244]. PS is synthesised from PC and PE by the action of PS synthase 1 and PS synthase 2, respectively [245]. From here PS is transported to the mitochondria for PE synthesis by PS decarboxylation; this process is ATP dependent and limited by its availability [246]. From the lipid metabolism analysis (Table 4.1 and Figure 4.16), it can be seen that the reactions converting PC to PS and PE to PS were significantly active in cold-stored samples, suggesting PS synthase 1 and PS synthase 2 activity were increased. In contrast, the PS to PE reaction was significantly suppressed, suggesting PS decarboxylase activity was decreased. It would then be anticipated that this would result in a decrease in the proportion of PE in platelets, however, this was not observed. It is likely that the transfer of PE from lipoproteins is sufficient to negate this.

The function of a LPC species is dependent on the fatty acyls tail [247-253]. Although the proportion of LPC in platelets was only decreased by extended room temperature storage (Figure 4.4), the LPC species profile within platelets was mainly altered by room temperature storage (Figure 4.6). LPC(18:0), a pro-inflammatory LPC species [247-251], was decreased by room temperature storage and extended cold storage. Further, LPC(20:4), an anti-inflammatory LPC species [252, 253], was decreased by extended room temperature storage. Although other changes were observed, the role of those LPC species in modulating platelet function has not been specifically evaluated. Regardless, this data suggests that extended room temperature

storage alters the LPC species profile of platelets, and the immunoregulatory role of platelets requires further investigation.

Sphingolipids, such as ceramide and S1P, are potent bioactive lipid mediators [65, 66, 69, 71]. The catabolism of SM is considered to be an essential route by which these bioactive lipid mediators are formed [66]. Sphingolipid metabolism is unique in platelets as they lack the enzymes necessary for *de novo* synthesis and S1P catabolism (Figure 6.1) [66, 254]. Pienimaeki-Roemer *et al.* hypothesised that during conventional room temperature storage ceramide is generated by the transmembrane cycling and salvage pathway [56, 255]. In this pathway, S1P is catabolised to sphingosine by S1P phosphatase and sphingosine is catabolised to ceramide by ceramide synthase [56, 255]. Similarly, data presented in this study showed ceramide was significantly increased in platelets stored for 14 days at room temperature, and SM remained constant (Figure 4.4). Further, work previously done in our laboratory has shown S1P to be significantly decreased in the supernatant of platelet components stored at room temperature [256]. Taken together it could be hypothesised that S1P was consumed during extended storage to form ceramide. In contrast, extended cold storage of platelets resulted in a significant decrease in ceramide, while SM was significantly increased. These results suggest sphingolipid metabolism differs in room temperature and cold-stored platelets.

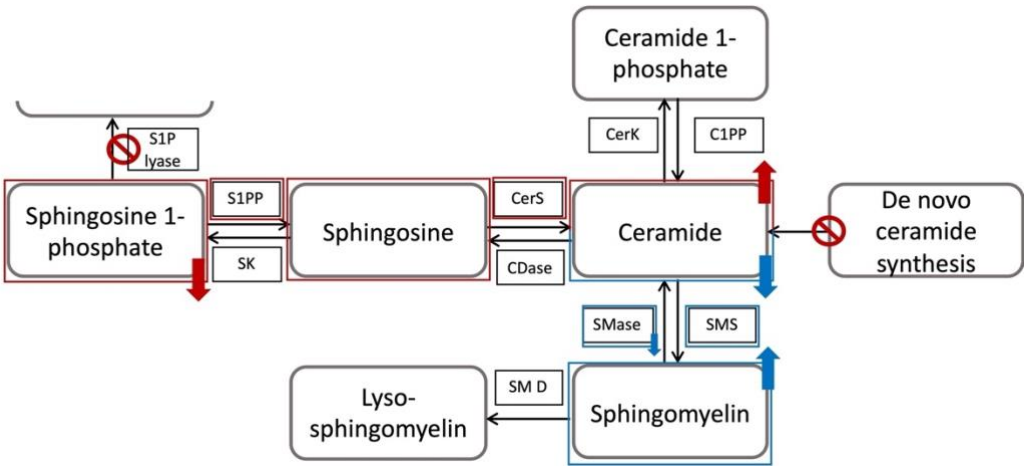

Figure 6.1. Simplified pictogram of the hypothesised sphingolipid metabolism in room temperature and cold-stored platelets

During room temperature storage (highlighted in red) it is hypothesised sphingosine 1-phosphate (S1P) is catabolised to sphingosine by S1P phosphatase (S1PP), and sphingosine is then catabolised to ceramide by ceramide synthase (CerS). On the other hand, during cold storage (highlighted in blue) it is hypothesised ceramide is catabolised by sphingomyelin synthase (SMS) to form sphingomyelin. Notably platelets lack the ability to synthesise ceramide *de novo* and lack the enzyme S1P lyase. SK = sphingosine kinase, CDase = ceramidase, SMase = sphingomyelinase SM D = SM deaclyase, CerK = ceramide kinase, C1PP = ceramide 1-phosphate phosphatase (Image by author).

Ceramide is proposed to mediate apoptosis and has been associated with the Bcl-2 pathway [207]. Conversely, S1P promotes cell survival [207]. This balance between ceramide and S1P, termed the “sphingolipid rheostat”, is thought to be a determining factor of cell fate [207]. Given the difference in the proportion of ceramide at day 14 in room temperature and cold-stored platelets (Figure 4.4), the sphingolipid rheostat and cell fate may be altered in platelets under different storage temperatures. During extended room temperature storage, disruption in the balance of the sphingolipid rheostat may result in the increase in apoptotic signalling seen in room temperature platelets [257]. Conversely, the reduction in ceramide in cold-stored platelets may have a role in delaying the progression of apoptosis seen in cold-stored platelets [257]. These results present interesting avenues for future investigation, particularly given the stark differences observed between storage temperatures.

While the ceramide-S1P ratio is proposed to determine cell fate [207], certain ceramide species have identified roles in apoptosis [258-261]. Specifically, Cer(16:0) and Cer(18:0) increase during the early stages of apoptosis, while Cer(22:0) and Cer(24:0) are generated during later stages of apoptosis [260]. These lipid species are involved in the formation of channels in the mitochondrial membrane and release of apoptotic proteins [258-261]. Further, Cer(16:0) and Cer(18:0) have been shown to have an affinity for the anti-apoptotic protein, Bcl-xL [260]. By interacting with Bcl-xL it is thought that mitochondrial permeabilisation by Cer(16:0) and Cer(18:0) is prevented, and additional apoptotic signalling is arrested [260]. Interestingly, Cer(16:0) and Cer(18:0) were increased by extended and cold storage (Figure 4.12), while Cer(22:0) was increased to a greater extent by room temperature storage. It has previously been shown that room temperature storage results in decreased Bcl-xL, increased Bax and the accumulation of apoptotic proteins, however, these processes are limited in cold-stored components [257, 262]. Therefore, changes in the ceramides species profile supports the concept that cold storage of platelets may delay apoptosis.

6.2.2 Changes to the lipid profile of the supernatant following cold storage

The phospholipid and sphingolipid profile of the supernatant of platelet components stored in additive solution was similar to previously reported data from platelets in plasma [55-57]. Changes to the profile of these supernatant lipids occurred during early time points (5 days) regardless of storage temperature, and changes stabilised at day 14 of storage (Figure 4.4). More specifically changes occurred in the LPA, PC, LPE, PE, PEP, PI, PS, ceramide and SM lipid classes. Similar to the platelet fraction, changes to the lipid classes of the supernatant are likely the result of a multitude of factors, including uptake of lipoproteins, release of microparticles and enzymatic conversion of lipid classes. Additionally, the changes observed to occur during 5 days of storage in this study are in agreement with changes observed in other studies occurring over 5 days of room temperature storage [56, 57].

The plasma is known to contain PC and LPC, and the proportions of these lipids may be changed by conventional platelet storage [57]. In this study, the proportion of PC in the supernatant was decreased by room temperature storage and by cold storage for 5 days (Figure 4.4). However, there was no change in proportion of LPC (Figure 4.4). Further, in terms of lipid metabolism analysis (Table 4.1 and Figure 4.16), there was no change in the ratio of LPC/PC, and the computed BioPAN lipid network could not determine the conversion of PC to LPC, as free fatty acids were not assessed as a part of the lipid profile. However, the enzyme responsible for converting PC to LPC, lectin-cholesterol acyltransferase (LCAT), is present in plasma [263]. Functionally, an accumulation of LPC in the plasma of platelet components has been associated with TRALI [184, 187, 225, 264]. This study examined platelet components stored in additive solution, which is standard practice in Australia. The use of additive solution may reduce the presence of enzymes such as LCAT which is responsible for the formation LPC. Assessing the presence of enzymes responsible for the formation of bioactive mediators, particularly those associated with adverse transfusion reactions, may be of

interest in understanding the risk of transfusion reactions in room-temperature and cold-stored platelet products and the impact of using additive solutions.

Although the overall proportion of LPC in the supernatant remained similar throughout storage, specific LPC species were altered (Figure 4.6). As mentioned in the context of the platelets, the function of different LPC species is associated with the fatty acyl tail length [247-253]. In the supernatant fraction, differences in pro-inflammatory LPC species, such as LPC(18:1), were increased by storage and the increase was greater in room temperature stored components [247-251]. Additionally, anti-inflammatory LPC species (LPC(20:4)) were decreased by extended storage (day 14) [252, 253]. Thus, this data would suggest that the supernatant of platelet components stored for an extended period may have an impact on the inflammatory status of the platelet component.

In addition to LPC, the lysophospholipids LPA and LPE are known constituents of human plasma [247]. The proportion of LPA and LPE were significantly increased by room temperature storage (Figure 4.4). While LPA has previously been shown to increase in the supernatant of room temperature stored platelet components [56], LPE has not been assessed in stored platelets. LPA and LPE are both capable of interacting with numerous receptors and, thus, eliciting a variety of cellular responses [222, 239, 247, 265, 266]. LPA mediates cell migration, cytoskeletal reorganisation, cytokine release and cell survival [239, 242]. Further, LPE is proposed to affect cell migration, differentiation, proliferation and mobilisation of calcium stores [222, 247, 265, 266]. The functional impact of the increases in LPA and LPE once transfused is unknown, and these results suggest that further investigations are warranted.

Transfusion of components containing a high concentration of polar (LPC and PAF) and non-polar (12-HETE and 15-HETE) bioactive lipid mediators has been associated with TRALI [184, 187, 225, 264]. The abundance of PAF remained constant in the supernatant fraction during extended storage (Figure

4.13). While supernatant PAF has been shown to be increased during storage of platelets in plasma [187, 201, 267, 268], as previously mentioned the use of additive solution may reduce the presence of enzymes, such as phospholipase A_2, which are responsible for the formation of PAF [61, 64, 263]. Non-polar bioactive lipids have been shown to accumulate in stored blood products, including red blood cells and room-temperature stored platelets [186, 209]. This study demonstrates that 12-HETE and 15-HETE accumulate in platelets stored in additive solution regardless of storage temperature (Figure 4.14). However, there was a trend towards reduced accumulation of these lipids during cold storage, which again, may reflect the requirement for enzymatic processes which are inhibited at colder temperatures [186].

In summary, the sphingolipid and phospholipid profile of platelets and the supernatant was differentially altered by storage time and temperature. It is apparent that room temperature and cold storage results in differences to lipid classes and species associated with coagulation, apoptosis and inflammation. It is likely these changes may have functional effects once the components are transfused.

6.3 Assessment of the lipid profile of cryopreserved platelet components

This study assessed the lipidomic changes occurring in platelets immediately following cryopreservation and after 24 hours of storage at room temperature. Numerous studies have determined the *in vitro* characteristics of cryopreserved platelets components [167, 175, 179]. The platelet count, mean platelet volume and post-thaw recovery of the components used in this study were similar to previously published data (Figure 5.1) [167, 175, 179].

Cryopreservation of platelets significantly increases PS externalisation and the release of PS-expressing microparticles that contribute to clot formation [178]. The processes resulting in PS externalisation also result in the externalisation of PE [33, 88, 89], although this has not previously been examined in the context of cryopreserved platelets. This study confirmed that cryopreservation induced the externalisation of PS and PE and the formation of microparticles with externalised PS (Figure 5.2). Interestingly, the proportion of PS was not different in the platelets and microparticles after thawing, despite the externalisation. In line with previous reports [122, 178], the number of microparticles with externalised PS was reduced following post-thaw storage at room temperature (Figure 5.2).

The effect of cryopreservation on the lipid profile of the platelet, microparticle and supernatant fractions of buffy coat derived platelet components was assessed. The fractions were assessed discretely as it is known that transfer of lipids occurs between platelets, microparticles, plasma lipids and lipoproteins [56, 57, 98, 269]. This study demonstrated that the lipid content of the platelets and microparticles remained unchanged immediately following thawing (Figure 5.3). However, after post-thaw storage at room temperature for 24 hours, the total lipid content of platelets increased two-fold, which coincided with a decrease in the lipid content of microparticles. The differences occurring after post-thaw storage in the platelets and microparticles, may be due an interaction between the fractions. Platelets are capable of re-

internalising microparticles [270-272], and it has been suggested that this process is partially mediated by Toll Like Receptor 4 [270]. Work previously performed in our laboratory has shown Toll Like Receptor 4 is increased after post-thaw storage at room temperature [273]. The decrease in lipid content of microparticles following post-thaw storage was consistent with the reduction in microparticles with exposed PS, as observed by flow cytometry (Figure 5.2). Given that platelet microparticles play a key role in haemostasis [178], understanding how long that microparticles persist during post-thaw storage and the processes by which microparticles are internalised may be of interest.

To prepare cryopreserved platelets for transfusion, the thawed platelet hyperconcentrate is resuspended in a volume of an appropriate solution, usually 100% plasma, saline or SSP+ [167, 172]. In this study, a volume of 30 % plasma/70 % SSP+ was used as the resuspension solution, as this is the standard storage solution for platelet components produced from whole blood in our institution. This was done to ensure the plasma content was similar between pre-freeze and post-thaw samples, and that differences in lipid profile could be attributed to thawing and/or post-thaw storage. The lipid content of the supernatant of the thawed and resuspended platelets (post-thaw 0 and post-thaw 24) was the same as the resuspension solution, indicating the lipid content was not impacted by the thawing process but by the addition of the resuspension solution (Figure 5.3). However, the lipid content of post-thaw supernatant was lower than the lipid content of the pre-freeze supernatant. An explanation for the higher lipid content of the supernatant prior to freezing may be due to the activation associated changes occurring during collection and manufacturing of the buffy coat derived platelet component [133, 134, 274].

The phospholipid and sphingolipid profile of the platelets, microparticles and supernatant (Figure 5.4) showed similarities to previously reported profiles of fresh and conventionally stored platelets [56, 57]. In general, the phospholipid and sphingolipid profile of platelets was not extensively altered immediately after thawing. However, the 24 hour post-thaw storage period resulted in

significant changes. In contrast, the phospholipid and sphingolipid profile of microparticles formed after thawing were distinct from those present prior to freezing. Further, the resuspension solution impacted the lipidome of the supernatant. The changes observed after thawing and post-thaw storage may be the result of several mechanisms occurring in concert.

6.3.1 Changes to the lipid profile of platelets following cryopreservation

The phospholipid and sphingolipid profile of platelets was unchanged immediately after thawing (Figure 5.4A). This result was somewhat surprising, as changes to the platelet shape, phenotype and metabolism are observable immediately after thawing [122, 178, 275]. However, several changes were observed after the 24 hour post-thaw storage period, including to the PA, LPC and PC classes. An explanation for the delay in observable lipid changes may be the time and temperature dependence of the reactions thought to alter lipid profiles of platelets [56, 57], including the transfer of plasma lipids and lipoproteins [98, 99, 236]. When used clinically, the post-thaw shelf life of cryopreserved platelets is between 4 and 6 hours [173], this data suggests that an extension of the post-thaw shelf life may be undesirable as changes to the platelet lipid profile were only observed after 24 hours of storage.

The proportion of PC was significantly increased in the platelet fraction after post-thaw storage (Figure 5.4A). Additionally, although not significant, there was a trend for decreased PC in the supernatant fractions after the post-thaw storage period. Similarly, the proportion of PE was decreased in the platelet fraction immediately after thawing, but was increased in the supernatant at the same time point. PC, PE and SM can be rapidly transferred from plasma lipoproteins to platelets [98, 236]. This is purported to take place via selective endocytosis or facilitated by protein interaction [232]. Further, the regulation and process by which lipid uptake occurs is variable and dependant on lipoprotein type [232], with the mechanisms facilitating endocytosis of PC differing to those supporting PE. More specifically, GPIV (CD36) supports the

transfer of PC from plasma lipoproteins to platelets [232-234], while cytosolic phospholipase A_2 facilitates the transfer of PE from plasma lipoproteins to platelets [235]. Given this, the selective uptake of PC could be reasonable. Further, the transfer of lipids is temperature dependent, and known to increase when platelets are activated [98, 99, 236]. Following thawing, platelets were stored at room temperature for 24 hours during which markers for platelet activation have been shown to increase [167]. Thus, it may be likely that the transfer of lipids from lipoproteins is increased during the 24 hour storage period, resulting in an increase in the proportion of certain lipid classes.

The proportion of LPC was decreased following post-thaw storage and the LPC species profile of platelets was altered following thawing and post-thaw storage (Figure 5.6A). LPC has different functions, and LPC species may be pro-inflammatory or anti-inflammatory depending on the fatty acyl tail [247-253]. LPC(18:2), LPC(20:0) and LPC(O-18:0) were altered during post-thaw storage at room-temperature, however, the role of these particular species in immune modulation have not been assessed.

The proportion of PE was decreased and there was no change in the proportion of PS in platelets following thawing, despite the increase in PE and PS externalisation (Figure 5.4A). Externalisation of PE and PS is essential to support normal haemostatic function [33, 89]. PS localises the tenase and prothrombinase complexes of the coagulation cascade for thrombin formation [33, 95], and PE increases the affinity of coagulation proteins to the membrane [33]. Further, pro-coagulant activity can also be modulated by PE, PEP and PS species, whereby long chain fatty acyls, specifically arachidonic acid (20:4), provide better support for tissue factor dependent thrombin generation compared to short chain fatty acyls [96]. The species profile of PE, PEP and PS were relatively stable following cryopreservation, compared to pre-freeze (Figure 5.7A, 5.10A and 5.11A). Given the increased externalisation of PE and PS and the increased haemostatic activity of cryopreserved platelets [122, 175], the stability of the PE, PEP and PS species was somewhat surprising.

Ceramide and ceramide species are known to regulate apoptotic processes [203, 207]. More specifically, an increase in ceramide is thought to promote apoptosis [203, 207]. Additionally, several ceramide species (Cer(16:0), Cer(18:0), Cer(22:0) and Cer(24:0)) are known to induce apoptosis by disrupting the mitochondria [258-261]. In this study, the proportion of ceramide was not altered and no change was seen in the ceramide species profile of platelets immediately after thawing (Figure 5.4A and 5.15A). This was somewhat surprising, as the mitochondrial membrane potential is depolarised following cryopreservation [168]. However, it has been suggested that changes to cryopreserved platelets such as externalisation of PS, typically viewed as apoptotic, are actually associated with pro-coagulant function rather than apoptosis [210, 276]. Similarly, this data would suggest the ceramide profile of cryopreserved platelet does not promote apoptosis. However, the only change observed after the 24 hour storage period was an increase in Cer(18:0), which is thought to induce apoptosis. This change towards apoptosis could provide further support that an extension of the post-thaw shelf life beyond 4-6 hours may not be advantageous.

6.3.2 Changes to the lipid profile of microparticles following cryopreservation

Microparticle function is understood to depend on the mechanism of generation, which determines the microparticle number, size, lipid and protein profile, and packaging of other molecules [37, 48]. The phospholipid and sphingolipid profile of microparticles present after thawing was significantly different to the microparticles present prior to freezing (Figure 5.4B). Given the role microparticles play *in vivo* in the transport of molecules, cell to cell communication, inflammation, haemostasis and in disease processes [36, 47], a significant change in the phospholipid and sphingolipid profile may indicate a change in the function of these microparticles *in vivo*. In particular, the proportion of LPA, PA, LPC, LPE and PG was altered in the microparticles generated after cryopreservation. This aligns with previous data which demonstrates that microparticles formed following cryopreservation have a

different membrane and cytoskeletal protein profile to those present prior to freezing [180]. In addition, microparticles formed following cryopreservation have a different phospholipid and sphingolipid profile to their parent platelets (Figure 5.4B). This would support the hypothesis that microparticles formed following cryopreservation are the result of selective packaging, and are not just smaller platelets [28, 180, 277]. Further, the number of microparticles is greatly increased following cryopreservation, thus the functional outcome of even minor changes in the lipid profile is likely exaggerated.

Lipids and lipid metabolism, specifically sphingolipids and PA, are associated with the formation of microparticles [81, 278-281]. It has been shown that inhibition of sphingomyelinase, the enzyme responsible for conversion of SM to ceramide, significantly reduces the formation of microparticles in various cell types, and during storage of red blood cells [81, 280, 282]. However, it has also been shown that inhibition of sphingomyelinase increases the formation of microparticles [278]. Data presented in this thesis, shows that sphingomyelinase activity was significantly suppressed in platelets (Figure 5.18), and the proportion of ceramide and SM did not change (Figure 5.4A). Regardless of the differences, these previous studies and the results of this study suggest sphingolipids and sphingomyelinase may have a role in regulating microparticle formation. Similarly, it is hypothesised that the activity of phospholipase D may regulate the formation of microparticles, and PA, the main product of phospholipase D activity, may also have a function in microparticle formation [283, 284]. PA is thought to alter the membrane curvature, making it more likely to undergo fusion or fission events [285]. Further, it is proposed that PA could co-localise the proteins thought to be associated with microparticle formation [283]. Interestingly, in this study PA was increased in microparticles formed after thawing (Figure 5.4B). These data provide interesting avenues into the biogenesis of platelet microparticles, and potentially propose different mechanisms by which formation occurs depending on storage condition.

Lysophospholipids are known to be a constituent of microparticles [56, 286-288]. *In vivo*, unbound LPC and LPE remains in circulation temporarily, as they are readily degraded by lysophospholipases. Therefore, microparticles are proposed to be essential transporters of LPC, LPE, and LPA [247, 289-292]. In this study the proportion of LPA, LPC and LPE was reduced in the microparticles after thawing (Figure 5.4B). However, this decrease may be offset by the substantial increase in microparticle number. Further, the microparticles released following thawing had a different profile of LPA, LPC and LPE species compared to those present before freezing and, as they are bioactive lipid mediators, this may alter their functional potential [247].

LPC species have been shown to have both pro-inflammatory and anti-inflammatory functions, depending on the fatty acyl chain length [247-253]. Microparticles formed during post-thaw storage contain increased LPC(18:0), which is pro-inflammatory; while a decrease in anti-inflammatory LPC species (LPC(20:4) and LPC(22:6)) was observed (Figure 5.6B) [247-253]. Similarly, the LPC species profile of the supernatant after thawing was quite different to the resuspension solution and was more similar to the pre-freeze LPC profile (Figure 5.6C). However, the only differences between pre-freeze and post-thaw supernatant was an alteration in the pro-inflammatory LPC species, LPC(18:0) [247-253], which was decreased. It may be that processes, such as enzymatic conversion or lipid exchange, were occurring between platelets, microparticles and the supernatant that resulted in shifts to the LPC species profile of microparticles and the supernatant. Further, the LPC species profile of microparticles appears to have an association with the LPC species profile of the supernatant, for example with LPC(18:0), LPC(18:1), LPC(18:2) and LPC(20:4), a decrease in one fraction was paralleled by an accompanying increase in the other fraction (Figure 5.6B and C). The enzymes responsible for the formation of LPC (phospholipase A_2) has previously been found in platelets and microparticles from room temperature stored platelet components [195]. Further, lipoproteins are known to contain LPC [231]. Taken together, these results suggest that microparticles formed after thawing

and the post-thaw supernatant may differentially promote inflammation. Further, LPC is closely associated with TRALI [184, 185, 225] and, given the high number of microparticles present in cryopreserved platelets and the ability of microparticles to transfer lipid species to other cell types [288], the immunomodulatory capacity of cryopreserved platelet components is another area of active investigation in our group [273].

Specific LPE species are proposed to differentially affect cell migration, differentiation, proliferation and mobilisation of calcium stores [222, 247, 265, 266]. In this study, the LPE species profile of microparticles and the supernatant was considerably altered after thawing and after storage (Figure 5.8B and C). Specifically, LPE species, LPE(18:0) and LPE(P-18:0), associated with calcium mobilisation [265, 266], were differentially altered in the microparticle and supernatant fractions by cryopreservation (Figure 5.8). Immediately after thawing, LPE(18:0) was decreased and LPE(P-18:0) was increased. Calcium mobilisation is necessary for the activation associated changes occurring in platelets [19, 21]. Additionally, a relationship between the LPE species profile of microparticles and LPE species profile of the supernatant is evident, where for example LPE(18:0) and LPE(20:4) increased in the supernatant fraction, while concomitantly decreasing in the microparticle fraction (Figure 5.8B and C). The physiological functions of LPE and LPE species have only recently begun to be discerned [222, 247, 265, 266] and as such the presence of LPE within platelet components is relatively novel. Given this, further investigations into the functional effects of LPE species, particularly those altered by cryopreservation, are warranted.

The ceramide species profile of microparticles has been associated with mediating TRALI [281]. Platelet components containing microparticles with increased long chain ceramides (Cer(16:0), Cer(18:0) and Cer(20:0)) induced TRALI in animal models and resulted in characteristics of TRALI in pulmonary cell models [281]. Other studies have shown that long chain ceramides are more injurious than very long chain ceramides [261, 281, 293]. In this study, the ceramide species within the microparticles before freezing and after

thawing were distinct (Figure 5.15B). Specifically, some long chain ceramides, including Cer(20:0), were increased and other ceramide species (Cer(28:0), Cer(30:0), Cer(31:0), Cer(33:0)) were decreased in post-thaw microparticles. Given the significant increase in the number of microparticles and a ceramide profile that may be considered more injurious after thawing, further work is required to determine if a relationship between the ceramide species profile of microparticles and transfusion reactions exists.

6.3.3 Changes to the lipid profile of the supernatant following cryopreservation

The lipid profile of the post-thaw supernatant was primarily altered by the addition of the resuspension solution. More specifically, changes were seen in the proportion of PA, LPC, LPE and SM post-thaw compared to the resuspension solution (Figure 5.4C). In addition, to the resuspension solution altering the proportion of several lipid classes, the lipid species of these classes were also affected. Plasma contains lipids and lipoproteins, which are known to interact with the platelet lipidome [57, 98, 230]. As previously mentioned, the observed changes are likely the result of endocytosis and the exocytotic process [98, 99, 235, 236]. Similar processes have been shown to alter the cytokine composition of the storage solution of cryopreserved components as platelets release their granule contents and as platelet degradation occurs [122, 273, 294].

Transfusion of blood components containing high concentrations of bioactive lipid mediators, including 5-HETE, 12-HETE and 15-HETE, have been associated with TRALI [82, 186, 209, 295]. While the abundance of 5-HETE did not change following thawing, 12-HETE and 15-HETE were dramatically increased (Figure 5.16). These bioactive lipid mediators have been shown to accumulate in blood products during conventional storage [183, 185, 264], however, this is the first instance in which they have been assessed following cryopreservation. Further, the increase of 12-HETE and 15-HETE occurs rapidly after thawing and resuspension and continues to accumulate during

storage at room temperature. It is important to note that 12-HETE and 15-HETE were essentially undetectable in the resuspension solution, and therefore this was not a contributing factor to the observed increase after thawing. As such, further investigations of these bioactive lipid mediators in the context of platelet cryopreservation, inclusive of their role in the transfusion reactions, should be pursued.

Reduced levels of S1P have been shown to be associated with increased sepsis induced organ failure [211]. The concentration of S1P was similar before freezing and after thawing, however, S1P was lower in the resuspension solution (Figure 5.16). This increase following thawing and resuspension was likely due to release from granules, as S1P is known to be stored within the granules of platelets and released during activation [296]. Given that transfusion of cryopreserved platelets are indicated for the treatment of trauma-associated bleeding [171, 297], it is likely beneficial that the S1P concentration is not different between fresh and cryopreserved platelets. Further, S1P is known to have a counter-regulatory affect to ceramide and has been shown to reduce TRALI associated lung injury [207, 281]. Taking into consideration the changes to the ceramide species profile of microparticles and the increased concentration of 12-HETE and 15-HETE, a better understanding of the role of S1P in cryopreserved platelet components may be of interest.

In summary, the sphingolipid and phospholipid profile of platelets, microparticles and the supernatant was differentially altered by the cryopreservation process. Surprisingly, the phospholipid and sphingolipid profile of platelets was relatively unchanged immediately after thawing, but the proportion of several lipid classes was altered by post-thaw storage at room temperature. The lipid profile of microparticles formed following thawing was different to that present prior to freezing. Further, the lipid profile of the resuspension solution is the primary factor responsible for differences in the lipid profile of the post-thaw supernatant. Changes to the lipid profile of platelets, microparticles and supernatant occurred in lipids associated with

coagulation, inflammation and signalling. These changes may have functional effects once the components are transfused, and more in-depth studies should be conducted.

6.4 Limitations

While novel findings regarding the phospholipid, sphingolipid and downstream bioactive lipid mediator profile of conventionally and alternatively stored platelet components have been presented, some limitations should be considered.

This study examined cryopreserved platelets before freezing and after thawing, with an average frozen storage time of 23 days. However, in a clinical setting cryopreserved platelets can be stored for up to 2 years [141, 142, 166], as such there is a discrepancy in the length of time cryopreserved platelets were frozen in this study compared to what might be used in a clinical setting. The effect of frozen storage time on the lipid profile of platelet components remains unknown. Understanding the effect of frozen storage time on the lipid profile of platelet components may be of interest.

In order to understand the changes occurring as a result of cryopreservation, once thawed, the thawed platelets were resuspended in a unit of 30 % plasma/70 % SSP+. However, when used clinically, as in the CLIP-II clinical trial [173], cryopreserved platelet components are resuspended in a unit of freshly thawed plasma. Therefore, the lipid profile of platelets used in the clinical situation may differ. However, it is important to note that the majority of lipid changes were only observed after post-thaw storage at room temperature (PT24) and the CLIP-II platelets are transfused within 4 hours of thawing.

This study focused on changes to the sphingolipid and phospholipid profile of platelets, as these lipids represent the majority of lipids that make up the profile of platelets, microparticles and plasma. However, this study did not examine cholesterol or cholesterol ester. Cholesterol and cholesterol ester are important for platelet structure and function [59, 72, 74, 75, 77], and the

contribution of these lipid classes to the lipid profile of platelets stored at room temperature for 5 days is known [56, 57]. Understanding the changes to cholesterol and cholesterol ester following alternative storage would be of interest.

6.5 Future directions

The results generated provide a greater understanding of the cold-stored and cryopreserved platelet product. However, more research is required to develop a comprehensive understanding of the changes occurring and the processes mediating these changes, and to understand how the alterations observed impact the function of the platelet component once transfused.

Microparticles were not investigated in the cold study, however, their contribution to the overall efficacy of room temperature and cold-stored platelet component is well known [122, 148, 198, 298]. As was seen in this study, cryopreservation yielded microparticles with a distinct lipid profile and interactions between the microparticle fraction and other fractions were observed. Therefore, studies investigating the impact of room temperature and cold storage on the lipidome of microparticles would be of interest, particularly given that cold storage has been shown to release a greater number of annexin-V positive microparticles [122].

There is evidence that the formation of lipid rafts may be temperature dependent [299, 300], but this has not yet been investigated in the context of cold-stored or cryopreserved platelet components. Further, lipid rafts appear to have functional roles [72]. As was seen in this study, several lipid classes enriched in lipid rafts were altered by alternative platelet storage, including SM. SM is highly enriched in lipid rafts, and known to coalesce when exposed to the cold [80, 300]. As such, studies investigating the impact of storage on lipid rafts may be of interest [78].

Ceramides are thought to have specific roles in mediating apoptosis [207, 258-261]. Results from this study indicate the ceramide species profile of cold-

stored platelets were altered and may be reflective of early or suspended apoptosis. Work previously performed in our laboratory, and by others, has indicated a difference in the accumulation of apoptotic proteins, which suggests that cold-storage of platelets may delay apoptosis [257, 262]. Given this, the association between ceramide and apoptosis in cold-stored platelets presents an area for future research to improve our understanding of the progression of apoptosis.

Bioactive lipid mediators are important modulators of cellular function, immune responses and are associated with TRALI [69, 82, 223]. During this study, several bioactive lipid mediators were examined (LPA, LPC, LPE, ceramide, 12-HETE, 15-HETE, S1P) and found to be altered during alternative platelet storage. Given the role of bioactive lipid mediators in the modulation of immune responses [69, 82, 223], expanding the profile of bioactive lipid mediators examined to include, for example ceramide 1-phosphate, leukotrienes, resolvins and lipoxins [82, 301] may be beneficial. In this study, an internal standard was used which contains deuterium labelled lipids from all of the major lipid classes and data was searched against lipid libraries. A more targeted approach could be employed where standards for bioactive lipids of interest are used and LC-MS/MS data can then be more accurately searched for and matched [195]. Further, many bioactive lipids, although they have not been directly examined in the context of platelet components, are capable of controlling cellular activity, including apoptosis, release of chemokines and cytokines, mobilisation of calcium and cytoskeletal reorganisation [201, 207, 247, 248, 250, 260, 261]. As such, it would be of interest to further investigate bioactive lipid mediators derived from alternatively stored platelet components.

Lipids are known to have specific functions and mediate cellular function [96, 222, 249, 260]. As was shown in this study, the lipid profile of cold-stored and cryopreserved components was different to conventionally stored platelet components. Initially, it was planned that the functional effect of the lipids that were altered would be assessed in this study. It was anticipated that platelet

component fractions and isolated lipids would be co-cultured with primary endothelial and immune cells to model an *in vitro* transfusion [302]. Changes to cytokines, chemokines and surface receptor profile of the endothelial cells and leukocytes could be assessed by plate-based assay and flow cytometry [303]. Although such a study was unable to be completed as a part of this thesis (due to the reasons outlined in the COVID impact statement), assessing the function of changed lipids would provide valuable information to better understand alternatively stored platelet components.

6.6 Concluding remarks

The research presented in this thesis aimed to characterise the lipidome of stored platelet components. Lipids and bioactive lipid mediators have an essential role in platelet function and the ability to impact the clinical role of platelet components. As was shown in this study, alternative storage resulted in changes to lipids associated with coagulation, apoptosis, signalling and inflammation. This research has furthered the characterisation of alternatively stored platelet components and has provided a greater understanding of the processes occurring during storage. In doing so, this work has recognised areas for future research, including information relevant for understanding adverse transfusion reactions. By broadening the understanding of alternatively stored platelet components this work may be beneficial in supporting their utility. The use of alternatively stored platelet components would resolve the issues surrounding conventionally stored platelets, and allow appropriate resuscitative care to be provided in rural, remote and military locations.

Supplemental data

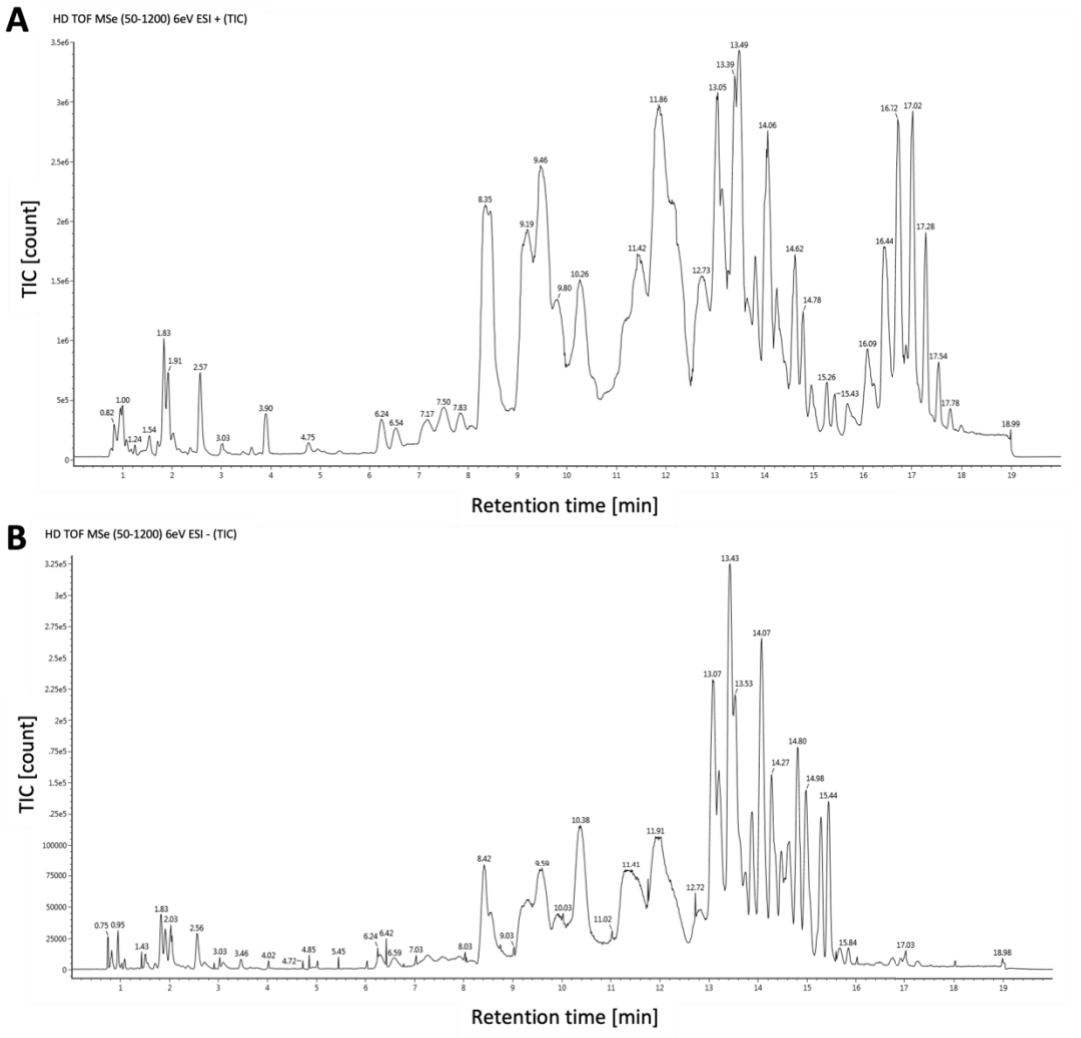

Figure S.1. Representative total ion chromatograms from platelet samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from post thaw 0 platelets are shown.

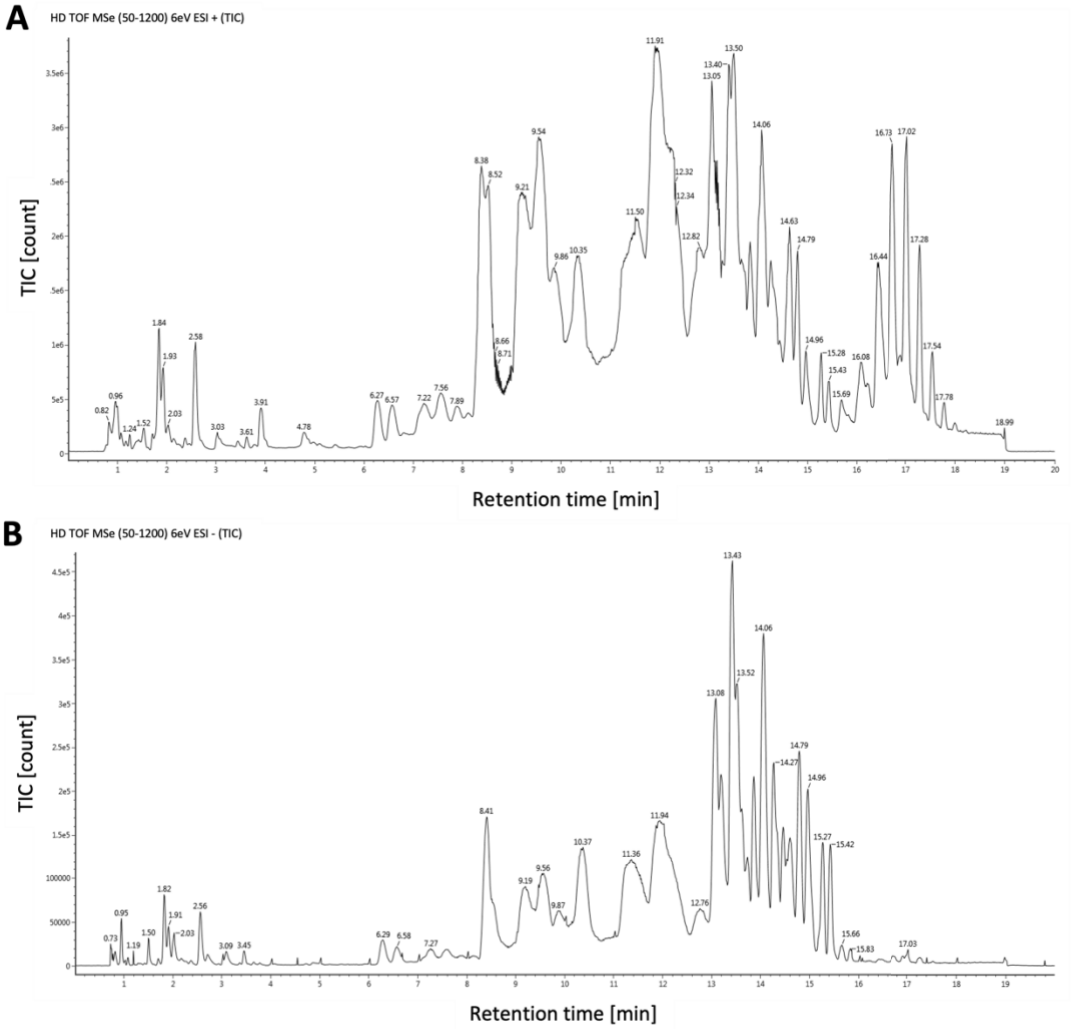

Figure S.2. Representative total ion chromatograms from platelet samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from post thaw 24 platelets are shown.

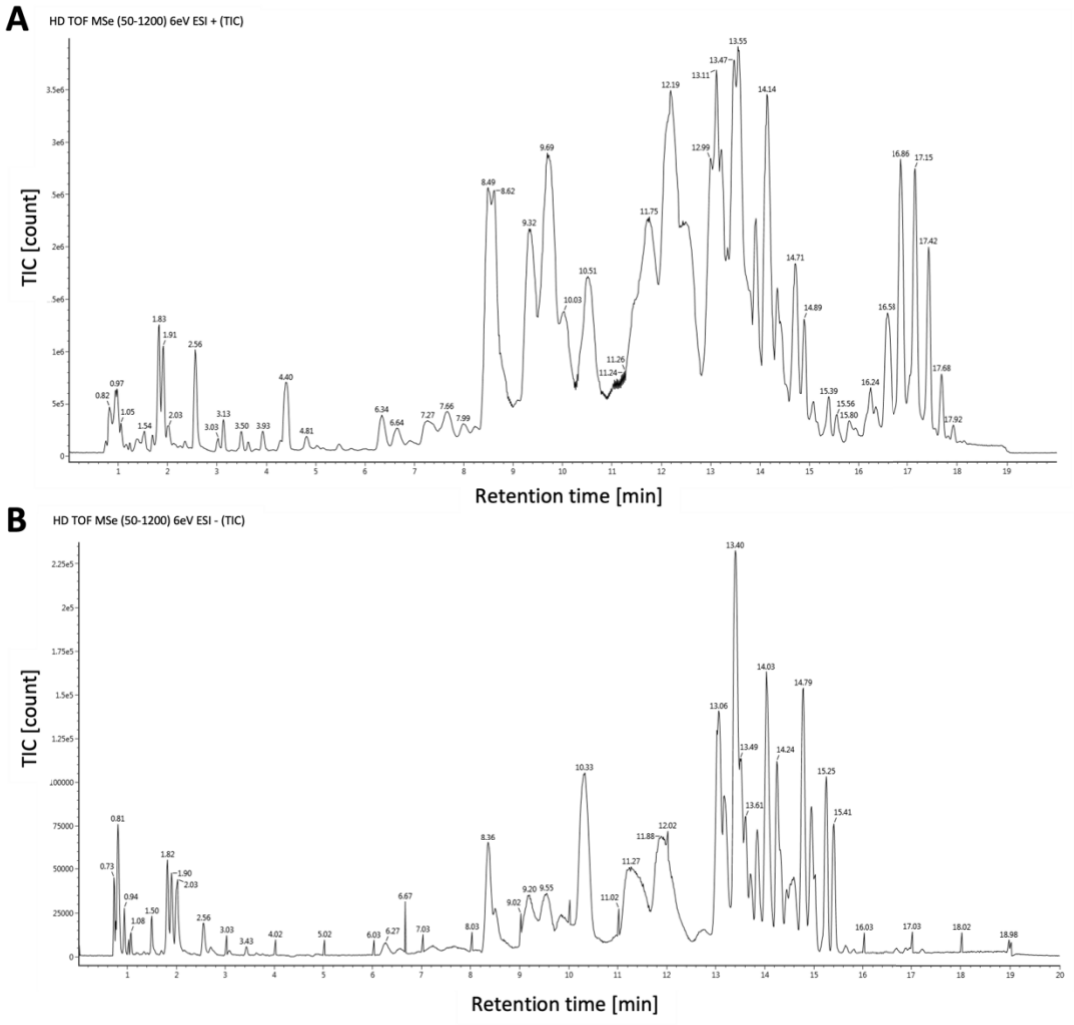

Figure S.3. Representative total ion chromatograms from platelet samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from platelets following 5 days of cold storage are shown.

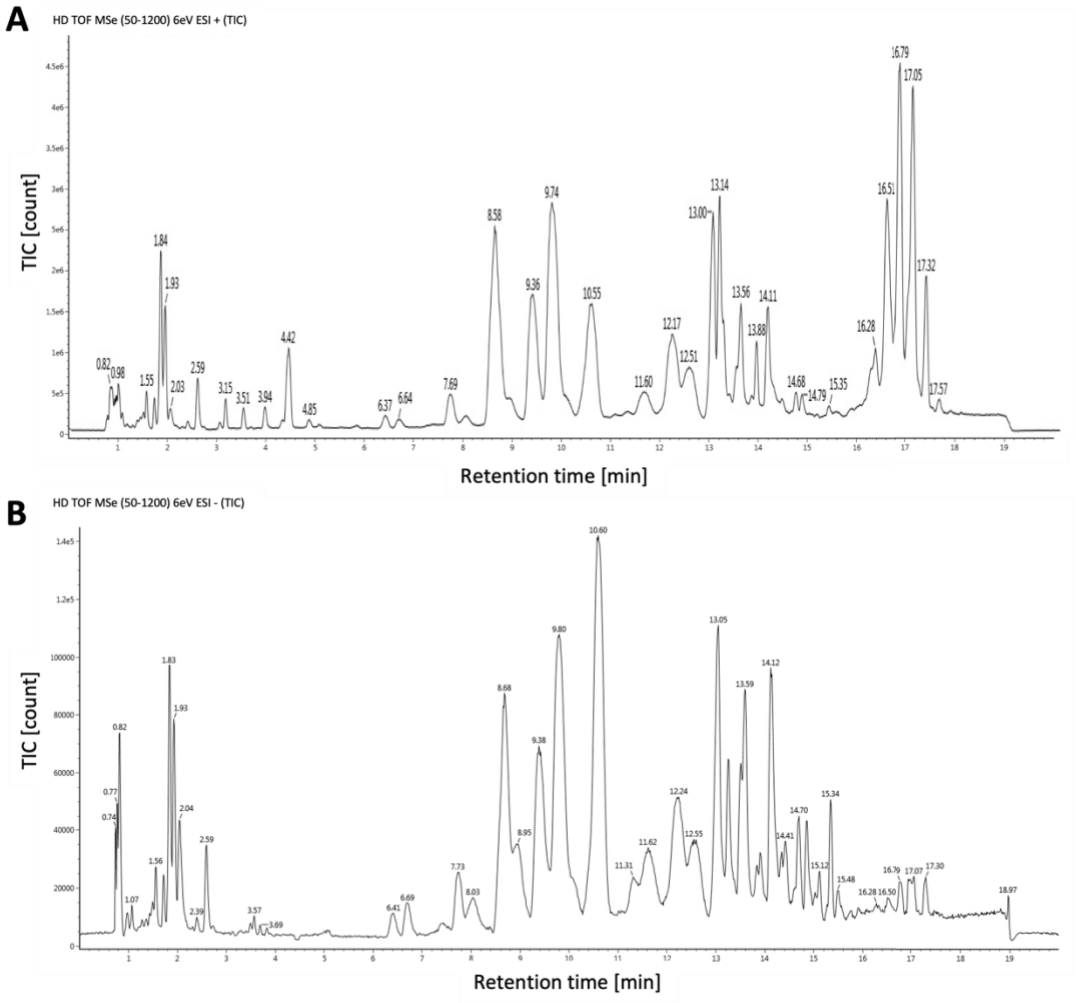

Figure S.4. Representative total ion chromatograms from supernatant samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from the day 1 supernatant are shown.

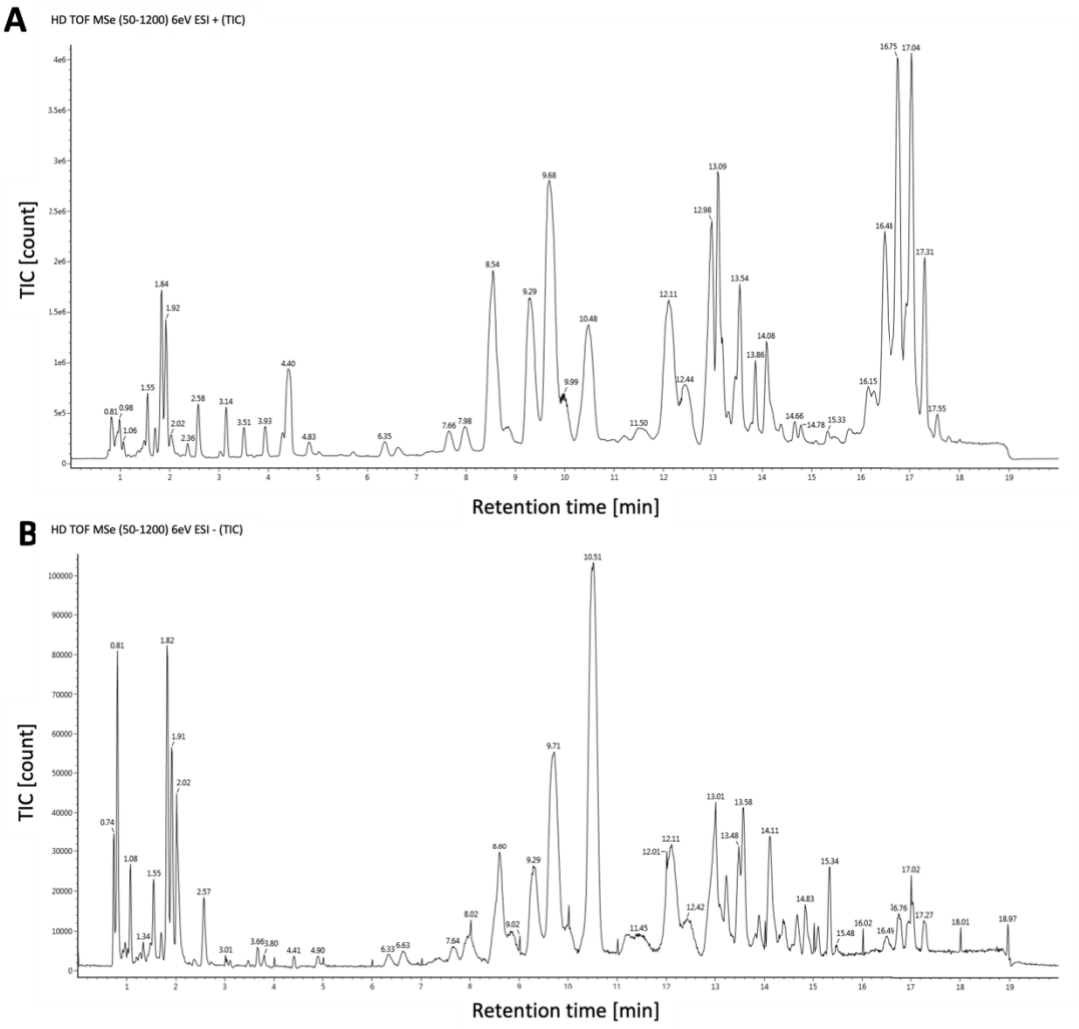

Figure S.5. Representative total ion chromatograms from supernatant samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from the supernatant following 14 days of cold storage are shown.

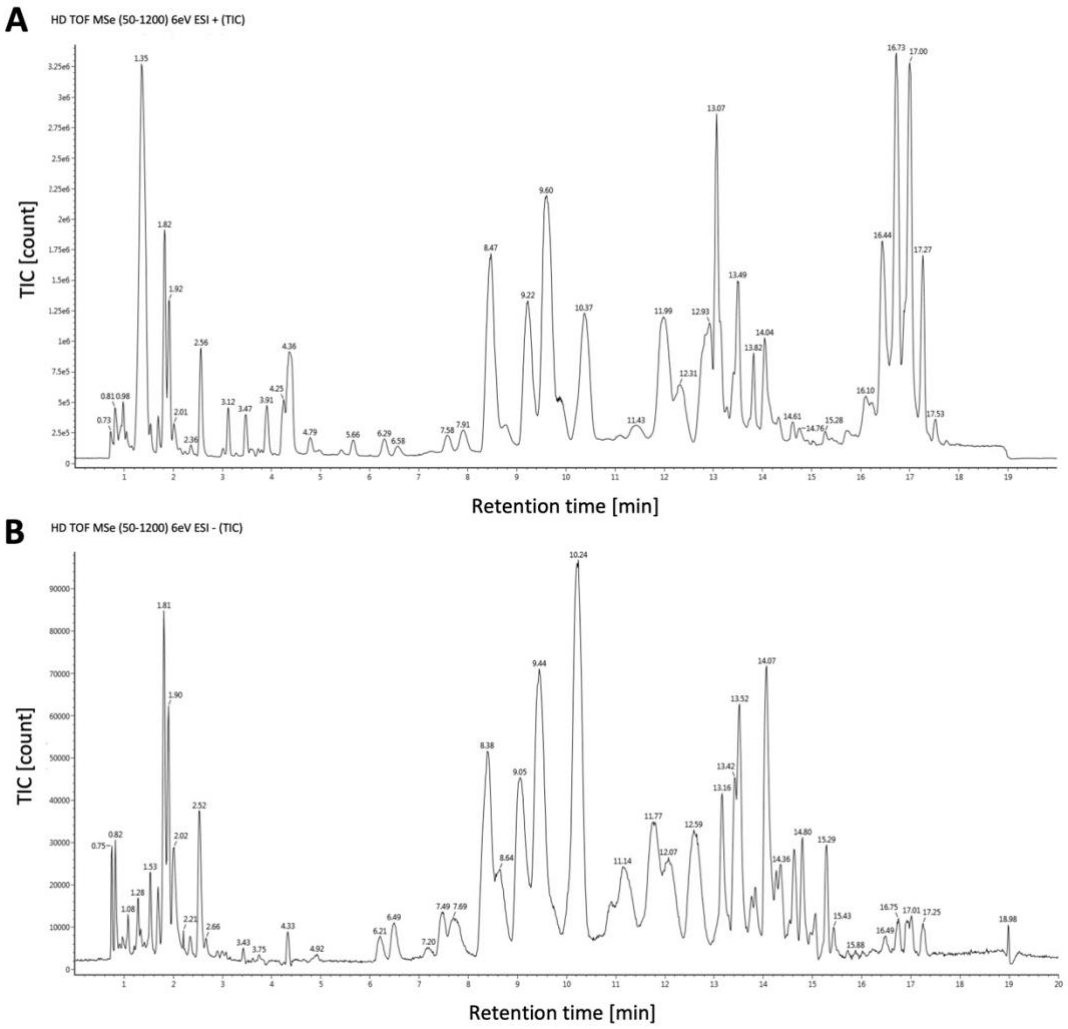

Figure S.6. Representative total ion chromatograms from supernatant samples

Total ion chromatograms (TIC) from LC-MS/MS acquisition in (A) positive and (B) negative mode. The chromatograms were obtained from the mass spectrometer. Representative chromatograms from the supernatant following 14 days of room temperature storage are shown.

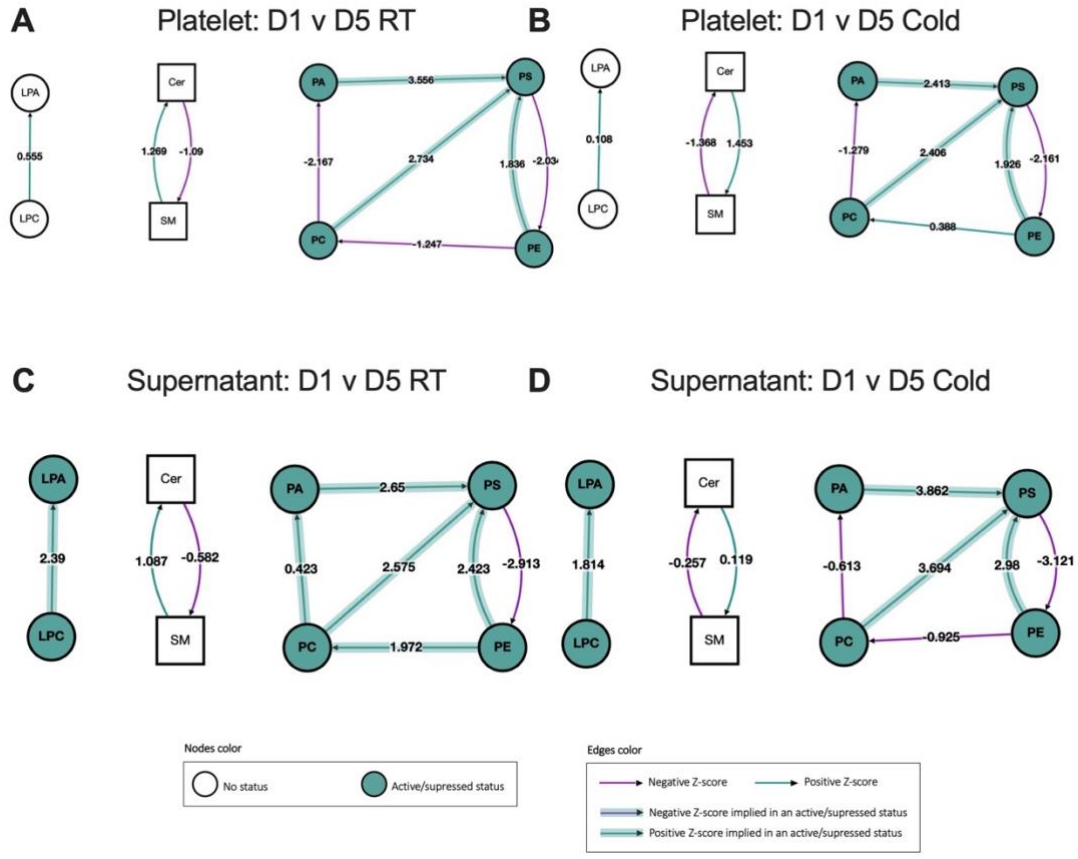

Figure S.7. BioPAN lipid networks depicting active reactions over storage

The lipid networks of (A) platelets and the (B) supernatant comparing day 1 (D1) and day 5 (D5) timepoints. The networks were produced from the open access web-based tool, BioPAN [196], by inputting data obtained from mass spectrometry analysis.

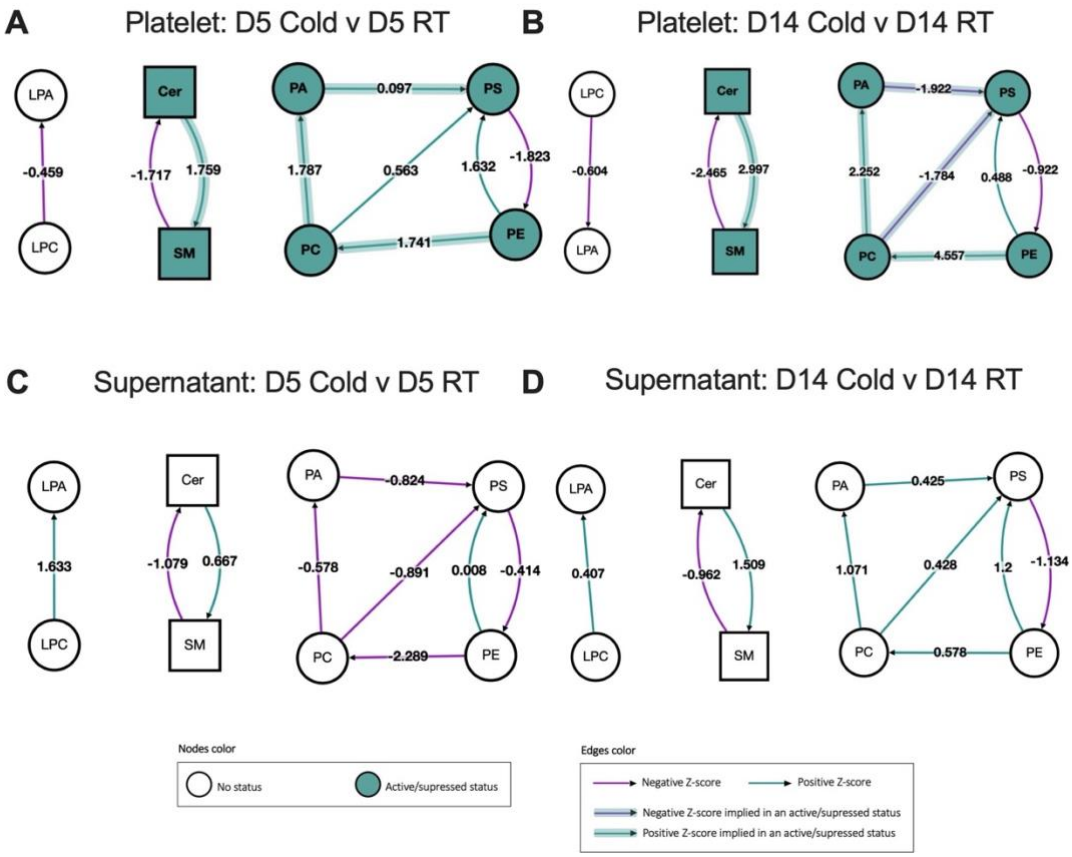

Figure S.8. BioPAN lipid networks depicting active reactions over storage

The lipid networks of (A) platelets and the (B) supernatant comparing day 1 (D1) and day 5 (D5) timepoints. The networks were produced from the open access web-based tool, BioPAN [196], by inputting data obtained from mass spectrometry analysis.

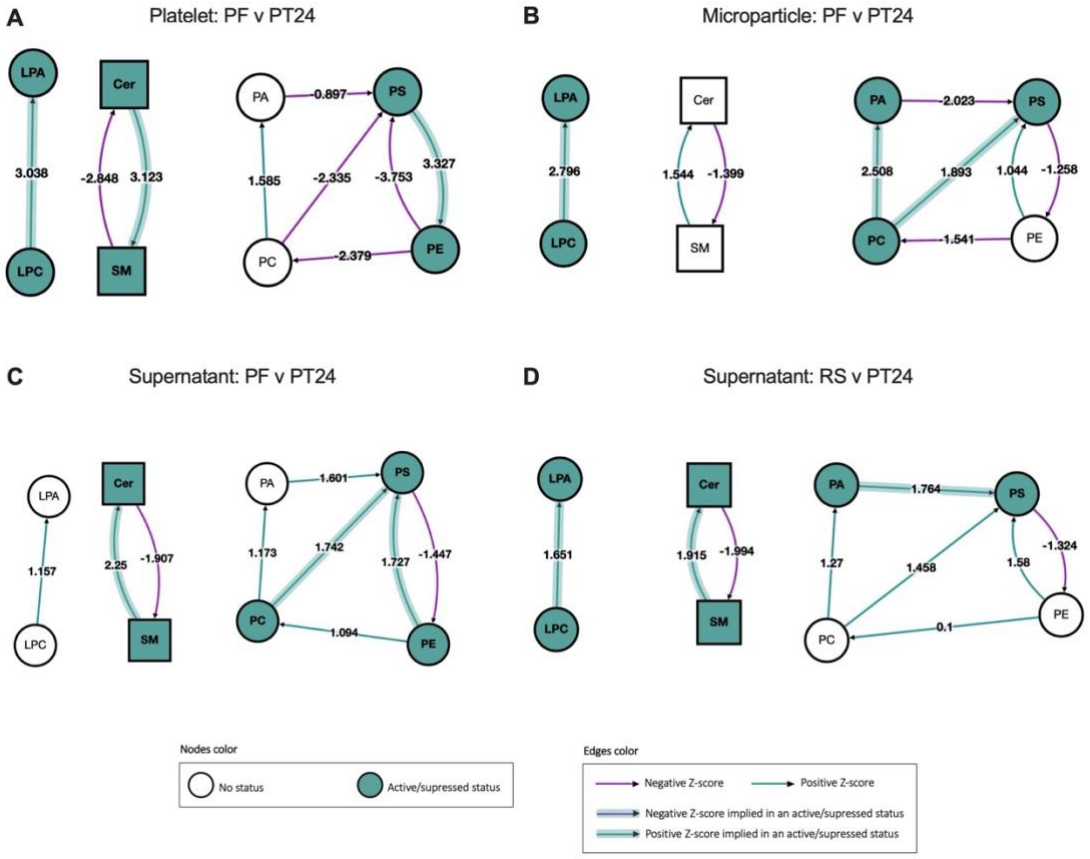

Figure S.9. BioPAN lipid networks depicting active reactions following post-thaw storage

The lipid networks of (A) platelets, (B) microparticles, (C) supernatant and the (D) resuspension solution comparing the pre-freeze (PF) and post-thaw storage (PT24) time points. The networks were produced from the open access web-based tool, BioPAN [196], by inputting data obtained from mass spectrometry analysis.

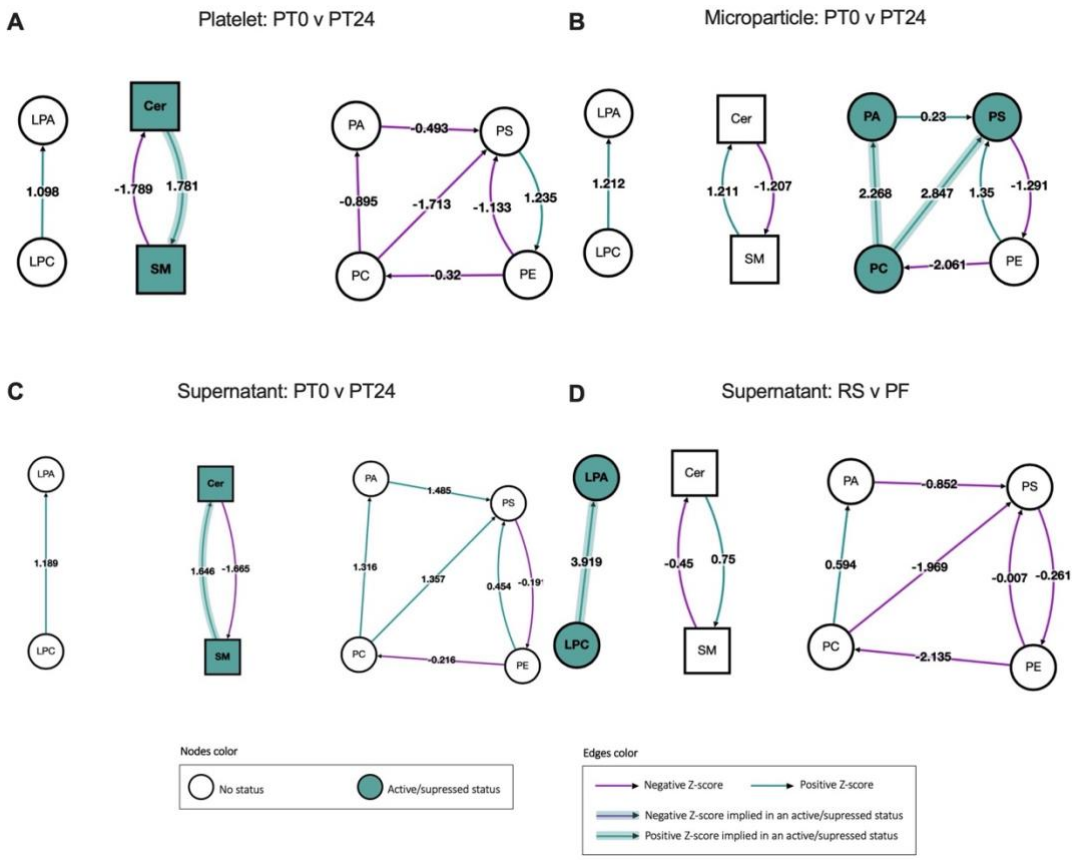

Figure S.10. BioPAN lipid networks depicting active reactions following thawing and post-thaw storage

The lipid networks of (A) platelets, (B) microparticles, (C) supernatant and the (D) resuspension solution comparing the post-thaw 0 (PT0) and post-thaw storage (PT24) time points. The networks were produced from the open access web-based tool, BioPAN [196], by inputting data obtained from mass spectrometry analysis.

References

1 Italiano JE, Jr.: Unraveling mechanisms that control platelet production. *Seminars in Thrombosis and Hemostasis* 2013; 39: 15-24.

2 Machlus KR, Thon JN, Italiano JE, Jr.: Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. *British Journal of Haematology* 2014; 165: 227-36.

3 Thon JN, Montalvo A, Patel-Hett S, Devine MT, Richardson JL, Ehrlicher A, Larson MK, Hoffmeister K, Hartwig JH, Italiano JE, Jr.: Cytoskeletal mechanics of proplatelet maturation and platelet release. *The Journal of Cell Biology* 2010; 191: 861-74.

4 Machlus KR, Italiano JE, Jr.: The incredible journey: from megakaryocyte development to platelet formation. *Journal of Cell Biology* 2013; 201: 785-96.

5 Ogawa M: Differentiation and proliferation of hematopoietic stem cells. *Blood* 1993; 81: 2844-53.

6 Hoffman R: Regulation of megakaryocytopoiesis. *Blood* 1989; 74: 1196-212.

7 Italiano JE, Jr., Lecine P, Shivdasani RA, Hartwig JH: Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *Journal of Cell Biology* 1999; 147: 1299-312.

8 Wang B, Zheng J: Platelet generation in vivo and in vitro. *Springerplus* 2016; 5: 787.

9 Junt T, Schulze H, Chen Z, Massberg S, Goerge T, Krueger A, Wagner DD, Graf T, Italiano JE, Shivdasani RA, von Andrian UH: Dynamic visualization of thrombopoiesis within bone marrow. *Science* 2007; 317: 1767.

10 Ghoshal K, Bhattacharyya M: Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. *The Scientific World Journal* 2014; 2014: 781857.

11 Davì G, Patrono C: Platelet activation and atherothrombosis. *The New England Journal of Medicine* 2007; 357: 2482-94.

12 Rendu F, Brohard-Bohn B: The platelet release reaction: granules' constituents, secretion and functions. *Platelets* 2001; 12: 261-73.

13 Nurden AT: Platelet membrane glycoproteins: a historical review. *Seminars in Thrombosis and Hemostasis* 2014; 40: 577-84.

14 Gremmel T, Frelinger AL, 3rd, Michelson AD: Platelet physiology. *Seminars in Thrombosis and Hemostasis* 2016; 42: 191-204.

15 Selvadurai MV, Hamilton JR: Structure and function of the open canalicular system - the platelet's specialized internal membrane network. *Platelets* 2018; 29: 319-25.

16 Varga-Szabo D, Pleines I, Nieswandt B: Cell adhesion mechanisms in platelets. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2008; 28: 403-12.

17 Holinstat M: Normal platelet function. *Cancer Metastasis Reviews* 2017; 36: 195-8.
18 Sang Y, Roest M, de Laat B, de Groot PG, Huskens D: Interplay between platelets and coagulation. *Blood Reviews* 2020: 100733.
19 Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H: Platelets at work in primary hemostasis. *Blood Reviews* 2011; 25: 155-67.
20 Tomaiuolo M, Brass LF, Stalker TJ: Regulation of platelet activation and coagulation and its role in vascular injury and arterial thrombosis. *Interventional Cardiology Clinics* 2017; 6: 1-12.
21 Varga-Szabo D, Braun A, Nieswandt B: Calcium signaling in platelets. *Journal of Thrombosis and Haemostasis* 2009; 7: 1057-66.
22 Dachary-Prigent J, Pasquet JM, Freyssinet JM, Nurden AT: Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca2+-ATPase inhibitors. *Biochemistry* 1995; 34: 11625-34.
23 Gitz E, Koopman CD, Giannas A, Koekman CA, van den Heuvel DJ, Deckmyn H, Akkerman JW, Gerritsen HC, Urbanus RT: Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Ibalpha. *Haematologica* 2013; 98: 1810-8.
24 Sharda A, Flaumenhaft R: The life cycle of platelet granules. *F1000Research* 2018; 7: 236.
25 Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, Housman DE, Graybiel AM: CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. *Nature Medicine* 2004; 10: 982-6.
26 Sorrentino S, Studt JD, Medalia O, Tanuj Sapra K: Roll, adhere, spread and contract: structural mechanics of platelet function. *European Journal of Cell Biology* 2015; 94: 129-38.
27 Skeaff CM, Holub BJ: Altered phospholipid composition of plasma membranes from thrombin-stimulated human platelets. *Biochimica et Biophysica Acta* 1985; 834: 164-71.
28 Freyssinet JM, Toti F: Formation of procoagulant microparticles and properties. *Thrombosis Research* 2010; 125: S46-8.
29 Harrison P, Cramer EM: Platelet alpha-granules. *Blood Reviews* 1993; 7: 52-62.
30 Blair P, Flaumenhaft R: Platelet alpha-granules: basic biology and clinical correlates. *Blood Reviews* 2009; 23: 177-89.
31 Li Z, Delaney MK, O'Brien KA, Du X: Signaling during platelet adhesion and activation. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2010; 30: 2341-9.
32 Periayah MH, Halim AS, Mat Saad AZ: Mechanism action of platelets and crucial blood coagulation pathways in hemostasis. *International Journal of Hematology-oncology and Stem Cell Research* 2017; 11: 319-27.
33 Heemskerk JW, Bevers EM, Lindhout T: Platelet activation and blood coagulation. *Thrombosis and Haemostasis* 2002; 88: 186-93.

34 Hawiger J: Formation and regulation of platelet and fibrin hemostatic plug. *Human Pathology* 1987; 18: 111-22.
35 Burnouf T, Chou ML, Goubran H, Cognasse F, Garraud O, Seghatchian J: An overview of the role of microparticles/microvesicles in blood components: are they clinically beneficial or harmful? *Transfusion and Apheresis Science* 2015; 53: 137-45.
36 Burnouf T, Goubran HA, Chou ML, Devos D, Radosevic M: Platelet microparticles: detection and assessment of their paradoxical functional roles in disease and regenerative medicine. *Blood Reviews* 2014; 28: 155-66.
37 Cognasse F, Hamzeh-Cognasse H, Laradi S, Chou M-L, Seghatchian J, Burnouf T, Boulanger C, Garraud O, Amabile N: The role of microparticles in inflammation and transfusion: a concise review. *Transfusion and Apheresis Science* 2015; 53: 159-67.
38 van Niel G, D'Angelo G, Raposo G: Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology* 2018; 19: 213-28.
39 Théry C, Ostrowski M, Segura E: Membrane vesicles as conveyors of immune responses. *Nature Reviews Immunology* 2009; 9: 581.
40 Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ: Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 1999; 94: 3791-9.
41 Siljander PRM: Platelet-derived microparticles – an updated perspective. *Thrombosis Research* 2011; 127: S30-S3.
42 Garcia BA, Smalley DM, Cho H, Shabanowitz J, Ley K, Hunt DF: The platelet microparticle proteome. *Journal of Proteome Research* 2005; 4: 1516-21.
43 Biro E, Akkerman JW, Hoek FJ, Gorter G, Pronk LM, Sturk A, Nieuwland R: The phospholipid composition and cholesterol content of platelet-derived microparticles: a comparison with platelet membrane fractions. *Journal of Thrombosis and Haemostasis* 2005; 3: 2754-63.
44 Risitano A, Beaulieu LM, Vitseva O, Freedman JE: Platelets and platelet-like particles mediate intercellular RNA transfer. *Blood* 2012; 119: 6288-95.
45 Flaumenhaft R, Dilks JR, Richardson J, Alden E, Patel-Hett SR, Battinelli E, Klement GL, Sola-Visner M, Italiano JE, Jr.: Megakaryocyte-derived microparticles: direct visualization and distinction from platelet-derived microparticles. *Blood* 2009; 113: 1112-21.
46 Morel O, Morel N, Jesel L, Freyssinet JM, Toti F: Microparticles: a critical component in the nexus between inflammation, immunity, and thrombosis. *Seminars in Immunopathology* 2011; 33: 469-86.
47 Lynch SF, Ludlam CA: Plasma microparticles and vascular disorders. *British Journal of Haematology* 2007; 137: 36-48.

48 Skotland T, Sandvig K, Llorente A: Lipids in exosomes: current knowledge and the way forward. *Progress in Lipid Research* 2017; 66: 30-41.
49 Aatonen MT, Öhman T, Nyman TA, Laitinen S, Grönholm M, Siljander PRM: Isolation and characterization of platelet-derived extracellular vesicles. *Journal of Extracellular Vesicles* 2014; 3: 24692.
50 Broekman MJ, Ward JW, Marcus AJ: Phospholipid metabolism in stimulated human platelets: changes in phosphatidylinositol, phosphatidic acid and lysophospholipds. *Journal of Clinical Investigation* 1980; 66: 275-83.
51 Hamid MA, Kunicki TJ, Aster RH: Lipid composition of freshly prepared and stored platelet concentrates. *Blood* 1980; 55: 124-30.
52 Okuma M, Steiner M, Baldini M: Lipid content and in vitro incorporation of free fatty acids into lipids of human platelets: the effect of storage at 4 degrees C. *Blood* 1971; 38: 27-38.
53 van Meer G, Voelker DR, Feigenson GW: Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology* 2008; 9: 112-24.
54 Murakami M: Lipid mediators in life science. *Experimental Animals* 2011; 60: 7-20.
55 Leidl K, Liebisch G, Richter D, Schmitz G: Mass spectrometric analysis of lipid species of human circulating blood cells. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 2008; 1781: 655-64.
56 Pienimaeki-Roemer A, Ruebsaamen K, Boettcher A, Orso E, Scherer M, Liebisch G, Kilalic D, Ahrens N, Schmitz G: Stored platelets alter glycerophospholipid and sphingolipid species, which are differentially transferred to newly released extracellular vesicles. *Transfusion* 2013; 53: 612-26.
57 Ruebsaamen K, Liebisch G, Boettcher A, Schmitz G: Lipidomic analysis of platelet senescence. *Transfusion* 2010; 50: 1665-76.
58 Cohen P, Derksen A: Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. *British Journal of Haematology* 1969; 17: 359-71.
59 Owen JS, Hutton RA, Day RC, Bruckdorfer KR, McIntyre N: Platelet lipid composition and platelet aggregation in human liver disease. *Journal of Lipid Research* 1981; 22: 423-30.
60 Min SH, Abrams CS: Regulation of platelet plug formation by phosphoinositide metabolism. *Blood* 2013; 122: 1358-65.
61 McKean ML, Smith JB, Silver MJ: Formation of lysophosphatidylcholine by human platelets in response to thrombin. Support for the phospholipase A2 pathway for the liberation of arachidonic acid. *Journal of Biological Chemistry* 1981; 256: 1522-4.
62 Slatter DA, Aldrovandi M, O'Connor A, Allen SM, Brasher CJ, Murphy RC, Mecklemann S, Ravi S, Darley-Usmar V, O'Donnell VB: Mapping the human platelet lipidome reveals cytosolic phospholipase A2 as a

regulator of mitochondrial bioenergetics during activation. *Cell Metabolism* 2016; 23: 930-44.
63 Dean JM, Lodhi IJ: Structural and functional roles of ether lipids. *Protein & Cell* 2018; 9: 196-206.
64 Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM: Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. *Critical Reviews in Clinical Laboratory Sciences* 2003; 40: 643-72.
65 Futerman AH, Hannun YA: The complex life of simple sphingolipids. *EMBO Reports* 2004; 5: 777-82.
66 Tani M, Ito M, Igarashi Y: Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cellular Signalling* 2007; 19: 229-37.
67 Hannun YA, Obeid LM: Sphingolipids and their metabolism in physiology and disease. *Nature Reviews Molecular Cell Biology* 2017; 19: 175.
68 Siess W: Athero- and thrombogenic actions of lysophosphatidic acid and sphingosine-1-phosphate. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 2002; 1582: 204-15.
69 Rauch BH: Sphingosine 1-phosphate as a link between blood coagulation and inflammation. *Cellular Physiology and Biochemistry* 2014; 34: 185-96.
70 Fyrst H, Saba JD: An update on sphingosine-1-phosphate and other sphingolipid mediators. *Nature Chemical Biology* 2010; 6: 489.
71 Pulli I, Asghar MY, Kemppainen K, Tornquist K: Sphingolipid-mediated calcium signaling and its pathological effects. *Biochimica et Biophysica Acta - Molecular Cell Research* 2018; 1865: 1668-77.
72 Pike LJ: Lipid rafts: bringing order to chaos. *Journal of Lipid Research* 2003; 44: 655-67.
73 Marcus AJ, Ullman HL, Safier LB: Lipid composition of subcellular particles of human blood platelets. *Journal of Lipid Research* 1969; 10: 108-14.
74 Krause MR, Regen SL: The structural role of cholesterol in cell membranes: from condensed bilayers to llpid rafts. *Accounts of Chemical Research* 2014; 47: 3512-21.
75 Owen JS, Bruckdorfer KR, Day RC, McIntyre N: Decreased erythrocyte membrane fluidity and altered lipid composition in human liver disease. *Journal of Lipid Research* 1982; 23: 124-32.
76 Shattil SJ, Anaya-Galindo R, Bennett J, Colman RW, Cooper RA: Platelet hypersensitivity induced by cholesterol incorporation. *Journal of Clinical Investigation* 1975; 55: 636-43.
77 Munro S: Lipid rafts: elusive or illusive? *Cell* 2003; 115: 377-88.
78 Sezgin E, Levental I, Mayor S, Eggeling C: The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nature Reviews Molecular Cell Biology* 2017; 18: 361-74.

79 Moscardo A, Valles J, Latorre A, Santos MT: The association of thromboxane A2 receptor with lipid rafts is a determinant for platelet functional responses. *FEBS letters* 2014; 588: 3154-9.
80 Bodin S, Tronchere H, Payrastre B: Lipid rafts are critical membrane domains in blood platelet activation processes. *Biochimica et Biophysica Acta* 2003; 1610: 247-57.
81 Pollet H, Conrard L, Cloos A-S, Tyteca D: Plasma membrane lipid domains as platforms for vesicle biogenesis and shedding? *Biomolecules* 2018; 8: 94.
82 Yeung J, Hawley M, Holinstat M: The expansive role of oxylipins on platelet biology. *Journal of Molecular Medicine* 2017; 95: 575-88.
83 Derksen A, Cohen P: Patterns of fatty acid release from endogenous substrates by human platelet homogenates and membranes. *Journal of Biological Chemistry* 1975; 250: 9342-7.
84 Chandrasekharan JA, Marginean A, Sharma-Walia N: An insight into the role of arachidonic acid derived lipid mediators in virus associated pathogenesis and malignancies. *Prostaglandins & Other Lipid Mediators* 2016; 126: 46-54.
85 Serhan CN, Petasis NA: Resolvins and protectins in inflammation resolution. *Chemical Reviews* 2011; 111: 5922-43.
86 Eichmann TO, Lass A: DAG tales: the multiple faces of diacylglycerol--stereochemistry, metabolism, and signaling. *Cellular and Molecular Life Sciences* 2015; 72: 3931-52.
87 Fukami K, Inanobe S, Kanemaru K, Nakamura Y: Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *Progress in Lipid Research* 2010; 49: 429-37.
88 Zwaal RF, Schroit AJ: Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997; 89: 1121-32.
89 Zwaal RFA, Comfurius P, Van Deenen LLM: Membrane asymmetry and blood coagulation. *Nature* 1977; 268: 358.
90 Tavoosi N, Davis-Harrison RL, Pogorelov TV, Ohkubo YZ, Arcario MJ, Clay MC, Rienstra CM, Tajkhorshid E, Morrissey JH: Molecular determinants of phospholipid synergy in blood clotting. *Journal of Biological Chemistry* 2011; 286: 23247-53.
91 Lhermusier T, Chap H, Payrastre B: Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome. *Journal of Thrombosis and Haemostasis* 2011; 9: 1883-91.
92 Op den Kamp JA: Lipid asymmetry in membranes. *Annual Review of Biochemistry* 1979; 48: 47-71.
93 Zwaal RF, Comfurius P, Bevers EM: Surface exposure of phosphatidylserine in pathological cells. *Cellular and Molecular Life Sciences* 2005; 62: 971-88.
94 Daleke DL: Regulation of transbilayer plasma membrane phospholipid asymmetry. *Journal of Lipid Research* 2003; 44: 233-42.

95 Morrissey JH, Tajkhorshid E, Rienstra CM: Nanoscale studies of protein-membrane interactions in blood clotting. *Journal of Thrombosis and Haemostasis* 2011; 9 Suppl 1: 162-67.
96 Clark SR, Thomas CP, Hammond VJ, Aldrovandi M, Wilkinson GW, Hart KW, Murphy RC, Collins PW, O'Donnell VB: Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proceedings of the National Academy of Sciences of the United States of America* 2013; 110: 5875-80.
97 Gerrard JM, Robinson P: Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism* 1989; 1001: 282-85.
98 Engelmann B, Kogl C, Kulschar R, Schaipp B: Transfer of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin from low- and high-density lipoprotein to human platelets. *Biochemical Journal* 1996; 315: 781-9.
99 Engelmann B, Schaipp B, Dobner P, Stoeckelhuber M, Kogl C, Siess W, Hermetter A: Platelet agonists enhance the import of phosphatidylethanolamine into human platelets. *Journal of Biological Chemistry* 1998; 273: 27800-8.
100 Estcourt LJ, Birchall J, Lowe D, Grant-Casey J, Rowley M, Murphy MF: Platelet transfusions in haematology patients: are we using them appropriately? *Vox Sanguinis* 2012; 103: 284-93.
101 McCullough J: Overview of platelet transfusion. *Seminars in Hematology* 2010; 47: 235-42.
102 Holcomb JB, Wade CE, Michalek JE, Chisholm GB, Zarzabal LA, Schreiber MA, Gonzalez EA, Pomper GJ, Perkins JG, Spinella PC, Williams KL, Park MS: Increased plasma and platelet to red blood cell ratios improves outcome in 466 massively transfused civilian trauma patients. *Annals of Surgery* 2008; 248: 447-58.
103 Perkins JG, Cap AP, Spinella PC, Blackbourne LH, Grathwohl KW, Repine TB, Ketchum L, Waterman P, Lee RE, Beekley AC, Sebesta JA, Shorr AF, Wade CE, Holcomb JB: An evaluation of the impact of apheresis platelets used in the setting of massively transfused trauma patients. *The Journal of Trauma* 2009; 66: S77-84; discussion S-5.
104 Perkins JG, Cap AP, Spinella PC, Shorr AF, Beekley AC, Grathwohl KW, Rentas FJ, Wade CE, Holcomb JB: Comparison of platelet transfusion as fresh whole blood versus apheresis platelets for massively transfused combat trauma patients (CME). *Transfusion* 2011; 51: 242-52.
105 Pidcoke HF, Aden JK, Mora AG, Borgman MA, Spinella PC, Dubick MA, Blackbourne LH, Cap AP: Ten-year analysis of transfusion in Operation Iraqi Freedom and Operation Enduring Freedom: increased plasma and platelet use correlates with improved survival. *The Journal of Trauma and Acute Care Surgery* 2012; 73: S445-52.

106 van der Meer PF: Platelet concentrates, from whole blood or collected by apheresis? *Transfusion and Apheresis Science* 2013; 48: 129-31.
107 van der Meer PF: Apheresis versus whole-blood-derived platelets: pros and cons. *ISBT Science Series* 2012; 7: 112-6.
108 Tynngård N: Preparation, storage and quality control of platelet concentrates. *Transfusion and Apheresis Science* 2009; 41: 97-104.
109 Vassallo RR, Murphy S: A critical comparison of platelet preparation methods. *Current Opinion in Hematology* 2006; 13: 323-30.
110 van der Meer PF: PAS or plasma for storage of platelets? A concise review. *Transfusion Medicine* 2016; 26: 339-42.
111 Johnson L, Winter KM, Hartkopf-Theis T, Reid S, Kwok M, Marks DC: Evaluation of the automated collection and extended storage of apheresis platelets in additive solution. *Transfusion* 2012; 52: 503-9.
112 Burnouf T: Modern plasma fractionation. *Transfusion Medicine Reviews* 2007; 21: 101-17.
113 Prowse C: Controlling the blood-borne spread of human prion disease. *ISBT Science Series* 2006; 1: 21-4.
114 Kerkhoffs JL, Eikenboom JC, Schipperus MS, van Wordragen-Vlaswinkel RJ, Brand R, Harvey MS, de Vries RR, Barge R, van Rhenen DJ, Brand A: A multicenter randomized study of the efficacy of transfusions with platelets stored in platelet additive solution II versus plasma. *Blood* 2006; 108: 3210-5.
115 van der Meer PF, de Korte D: Platelet additive solutions: a review of the latest developments and their clinical implications. *Transfusion Medicine and Hemotherapy* 2018; 45: 98-102.
116 Sweeney J, Kouttab N, Holme S, Kurtis J, Cheves T, Nelson E: Storage of platelet-rich plasma-derived platelet concentrate pools in plasma and additive solution. *Transfusion* 2006; 46: 835-40.
117 van der Meer PF, Pietersz RN, Reesink HW: Storage of platelets in additive solution for up to 12 days with maintenance of good in-vitro quality. *Transfusion* 2004; 44: 1204-11.
118 Saunders C, Rowe G, Wilkins K, Holme S, Collins P: In vitro storage characteristics of platelet concentrates suspended in 70% SSP+TM additive solution versus plasma over a 14-day storage period. *Vox Sanguinis* 2011; 101: 112-21.
119 van der Meer PF, Kerkhoffs JL, Curvers J, Scharenberg J, de Korte D, Brand A, de Wildt-Eggen J: In vitro comparison of platelet storage in plasma and in four platelet additive solutions, and the effect of pathogen reduction: a proposal for an in vitro rating system. *Vox Sanguinis* 2010; 98: 517-24.
120 Gulliksson H, AuBuchon JP, Cardigan R, van der Meer PF, Murphy S, Prowse C, Richter E, Ringwald J, Smacchia C, Slichter S, de Wildt-Eggen J: Storage of platelets in additive solutions: a multicentre study of the in vitro effects of potassium and magnesium. *Vox Sanguinis* 2003; 85: 199-205.
121 Gyongyossy-Issa MI, Zhang JG, Culibrk B, Hunter F, Levin E, Scammell K, Weiss S, Holmes DL, Holme S: Novel system for storage

of buffy-coat-derived platelet concentrates in a glucose-based platelet additive solution: parameters and metabolism during storage and comparison to plasma. *Vox Sanguinis* 2009; 97: 102-9.
122 Johnson L, Tan S, Jenkins E, Wood B, Marks DC: Characterization of biologic response modifiers in the supernatant of conventional, refrigerated, and cryopreserved platelets. *Transfusion* 2018; 58: 927-37.
123 Keuren JF, Magdeleyns EJ, Govers-Riemslag JW, Lindhout T, Curvers J: Effects of storage-induced platelet microparticles on the initiation and propagation phase of blood coagulation. *British Journal of Haematology* 2006; 134: 307-13.
124 Cardigan R, Sutherland J, Garwood M, Bashir S, Turner C, Smith K, Hancock V, Wiltshire M, Pergande C, Williamson LM: In vitro function of buffy coat-derived platelet concentrates stored for 9 days in CompoSol, PASII or 100% plasma in three different storage bags. *Vox Sanguinis* 2008; 94: 103-12.
125 Valeri CR, Saleem B, Ragno G: Release of platelet-derived growth factors and proliferation of fibroblasts in the releasates from platelets stored in the liquid state at 22 degrees C after stimulation with agonists. *Transfusion* 2006; 46: 225-9.
126 Schrezenmeier H, Seifried E: Buffy-coat-derived pooled platelet concentrates and apheresis platelet concentrates: which product type should be preferred? *Vox Sanguinis* 2010; 99: 1-15.
127 Pietersz RN: Pooled platelet concentrates: an alternative to single donor apheresis platelets? *Transfusion and Apheresis Science* 2009; 41: 115-9.
128 Vamvakas EC: Relative safety of pooled whole blood-derived versus single-donor (apheresis) platelets in the United States: a systematic review of disparate risks. *Transfusion* 2009; 49: 2743-58.
129 Hod E, Schwartz J: Platelet transfusion refractoriness. *British Journal of Haematology* 2008; 142: 348-60.
130 Verma A, Agarwal P: Platelet utilization in the developing world: strategies to optimize platelet transfusion practices. *Transfusion and Apheresis Science* 2009; 41: 145-9.
131 van der Meer PF, de Korte D: Platelet preservation: agitation and containers. *Transfusion and Apheresis Science* 2011; 44: 297-304.
132 Blajchman MA, Goldman M, Baeza F: Improving the bacteriological safety of platelet transfusions. *Transfusion Medicine Reviews* 2004; 18: 11-24.
133 Ohto H, Nollet KE: Overview on platelet preservation: better controls over storage lesion. *Transfusion and Apheresis Science* 2011; 44: 321-25.
134 Shrivastava M: The platelet storage lesion. *Transfusion and Apheresis Science* 2009; 41: 105-13.
135 Ng MSY, Tung J-P, Fraser JF: Platelet storage lesions: what more do we know now? *Transfusion Medicine Reviews* 2018; 32: 144-54.

136 Seghatchian J, Krailadsiri P: The platelet storage lesion. *Transfusion Medicine Reviews* 1997; 11: 130-44.
137 Rinder HM, Murphy M, Mitchell JG, Stocks J, Ault KA, Hillman RS: Progressive platelet activation with storage: evidence for shortened survival of activated platelets after transfusion. *Transfusion* 1991; 31: 409-14.
138 Caram-Deelder C, Kreuger AL, Jacobse J, van der Bom JG, Middelburg RA: Effect of platelet storage time on platelet measurements: a systematic review and meta-analyses. *Vox Sanguinis* 2016; 111: 374-82.
139 Blumberg N, Gettings KF, Turner C, Heal JM, Phipps RP: An association of soluble CD40 ligand (CD154) with adverse reactions to platelet transfusions. *Transfusion* 2006; 46: 1813-21.
140 Kreuger AL, Caram-Deelder C, Jacobse J, Kerkhoffs JL, van der Bom JG, Middelburg RA: Effect of storage time of platelet products on clinical outcomes after transfusion: a systematic review and meta-analyses. *Vox Sanguinis* 2017; 112: 291-300.
141 Holley A, Marks DC, Johnson L, Reade MC, Badloe JF, Noorman F: Frozen blood products: clinically effective and potentially ideal for remote Australia. *Anaesthesia and Intensive Care* 2013; 41: 10-9.
142 Lelkens CC, Koning JG, de Kort B, Floot IB, Noorman F: Experiences with frozen blood products in the Netherlands military. *Transfusion and Apheresis Science* 2006; 34: 289-98.
143 Woolley T, Badloe J, Bohonek M, Taylor AL, Erik Heier H, Doughty H: NATO Blood Panel perspectives on changes to military prehospital resuscitation policies: current and future practice. *Transfusion* 2016; 56 Suppl 2: S217-23.
144 Jenkins DH, Rappold JF, Badloe JF, Berséus O, Blackbourne L, Brohi KH, Butler FK, Cap AP, Cohen MJ, Davenport R, DePasquale M, Doughty H, Glassberg E, Hervig T, Hooper TJ, Kozar R, Maegele M, Moore EE, Murdock A, Ness PM, Pati S, Rasmussen T, Sailliol A, Schreiber MA, Sunde GA, van de Watering LMG, Ward KR, Weiskopf RB, White NJ, Strandenes G, Spinella PC: Trauma hemostasis and oxygenation research position paper on remote damage control resuscitation: definitions, current practice, and knowledge gaps. *Shock* 2014; 41 Suppl 1: 3-12.
145 Johnson L, Tan S, Wood B, Davis A, Marks DC: Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. *Transfusion* 2016; 56: 1807-18.
146 Becker GA, Tuccelli M, Kunicki T, Chalos MK, Aster RH: Studies of platelet concentrates stored at 22 degrees C and 4 degrees C. *Transfusion* 1973; 13: 61-8.
147 Sandgren P, Shanwell A, Gulliksson H: Storage of buffy coat-derived platelets in additive solutions: in vitro effects of storage at 4 degrees C. *Transfusion* 2006; 46: 828-34.

148 Stolla M, Bailey SL, Fang L, Fitzpatrick L, Gettinger I, Pellham E, Christoffel T: Effects of storage time prolongation on in vivo and in vitro characteristics of 4 degrees C-stored platelets. *Transfusion* 2020; 60: 613-21.
149 Murphy S, Gardner FH: Platelet preservation: effect of storage temperature on maintenance of platelet viability - deleterious effect of refrigerated storage. *The New England Journal of Medicine* 1969; 280: 1094-8.
150 Strandenes G, Sivertsen J, Bjerkvig CK, Fosse TK, Cap AP, Del Junco DJ, Kristoffersen EK, Haaverstad R, Kvalheim V, Braathen H, Lunde THF, Hervig T, Hufthammer KO, Spinella PC, Apelseth TO: A pilot trial of platelets stored cold versus at room temperature for complex cardiothoracic surgery. *Anesthesiology* 2020; 133: 1173-83.
151 Mack JP, Miles J, Stolla M: Cold-stored platelets: review of studies in humans. *Transfusion Medicine Reviews* 2020; 34: 221-6.
152 Miles J, Bailey SL, Fang L, Osborne B, Corson J, Mack JP, Stolla M: Evaluation of efficacy and safety of cold-stored platelets in healthy human subjects treated with dual antiplatelet therapy. *Blood* 2019; 134: 718-.
153 Wood B, Padula MP, Marks DC, Johnson L: Refrigerated storage of platelets initiates changes in platelet surface marker expression and localization of intracellular proteins. *Transfusion* 2016; 56: 2548-59.
154 Reddoch KM, Pidcoke HF, Montgomery RK, Fedyk CG, Aden JK, Ramasubramanian AK, Cap AP: Hemostatic function of apheresis platelets stored at 4 degrees C and 22 degrees C. *Shock* 2014; 41: 54-61.
155 D'Alessandro A, Thomas KA, Stefanoni D, Gamboni F, Shea SM, Reisz JA, Spinella PC: Metabolic phenotypes of standard and cold-stored platelets. *Transfusion* 2020; 60 Suppl 3: S96-s106.
156 Zhao HW, Serrano K, Stefanoni D, D'Alessandro A, Devine DV: In Vitro Characterization and Metabolomic Analysis of Cold-Stored Platelets. *Journal of Proteome Research* 2021; 20: 2251-65.
157 Sandgren P, Hansson M, Gulliksson H, Shanwell A: Storage of buffy-coat-derived platelets in additive solutions at 4 degrees C and 22 degrees C: flow cytometry analysis of platelet glycoprotein expression. *Vox Sanguinis* 2007; 93: 27-36.
158 Montgomery RK, Reddoch KM, Evani SJ, Cap AP, Ramasubramanian AK: Enhanced shear-induced platelet aggregation due to low-temperature storage. *Transfusion* 2013; 53: 1520-30.
159 Getz TM, Montgomery RK, Bynum JA, Aden JK, Pidcoke HF, Cap AP: Storage of platelets at 4 degrees C in platelet additive solutions prevents aggregate formation and preserves platelet functional responses. *Transfusion* 2016; 56: 1320-8.
160 Badlou BA, Ijseldijk MJ, Smid WM, Akkerman JW: Prolonged platelet preservation by transient metabolic suppression. *Transfusion* 2005; 45: 214-22.

161 Johnson L, Tan S, Jenkins E, Wood B, Marks DC: Characterization of biologic response modifiers in the supernatant of conventional, refrigerated, and cryopreserved platelets. *Transfusion* 2018: 927-37.
162 Johnson L, Vekariya S, Wood B, Tan S, Roan C, Marks DC: Refrigeration of apheresis platelets in platelet additive solution (PAS-E) supports in vitro platelet quality to maximize the shelf-life. *Transfusion* 2021; In Press.
163 Cap AP: Platelet storage: a license to chill! *Transfusion* 2016; 56: 13-6.
164 Stubbs JR, Tran SA, Emery RL, Hammel SA, Haugen AL, Zielinski MD, Zietlow SP, Jenkins D: Cold platelets for trauma-associated bleeding: regulatory approval, accreditation approval, and practice implementation-just the "tip of the iceberg". *Transfusion* 2017; 57: 2836-44.
165 Warner MA, Kurian EB, Hammel SA, van Buskirk CM, Kor DJ, Stubbs JR: Transition from room temperature to cold-stored platelets for the preservation of blood inventories during the COVID-19 pandemic. *Transfusion* 2021; 61: 72-7.
166 Cohn CS, Dumont LJ, Lozano M, Marks DC, Johnson L, Ismay S, Bondar N, T'Sas F, Yokoyama APH, Kutner JM, Acker JP, Bohonek M, Sailliol A, Martinaud C, Pogłód R, Antoniewicz-Papis J, Lachert E, Pun PBL, Lu J, Cid J, Guijarro F, Puig L, Gerber B, Alberio L, Schanz U, Buser A, Noorman F, Zoodsma M, Meer PF, Korte D, Wagner S, O'Neill M: Vox Sanguinis international forum on platelet cryopreservation: summary. *Vox Sanguinis* 2017; 112: 684-88.
167 Johnson L, Reid S, Tan S, Vidovic D, Marks DC: PAS-G supports platelet reconstitution after cryopreservation in the absence of plasma. *Transfusion* 2013; 53: 2268-77.
168 Johnson LN, Winter KM, Reid S, Hartkopf-Theis T, Marks DC: Cryopreservation of buffy-coat-derived platelet concentrates in dimethyl sulfoxide and platelet additive solution. *Cryobiology* 2011; 62: 100-6.
169 Valeri CR, MacGregor H, Giorgio A, Ragno G: Circulation and hemostatic function of autologous fresh, liquid-preserved, and cryopreserved baboon platelets transfused to correct an aspirin-induced thrombocytopathy. *Transfusion* 2002; 42: 1206-16.
170 Khuri SF, Healey N, MacGregor H, Barnard MR, Szymanski IO, Birjiniuk V, Michelson AD, Gagnon DR, Valeri CR: Comparison of the effects of transfusions of cryopreserved and liquid-preserved platelets on hemostasis and blood loss after cardiopulmonary bypass. *Journal of Thoracic and Cardiovascular Surgery* 1999; 117: 172-84.
171 Noorman F, van Dongen TTCF, Plat M-CJ, Badloe JF, Hess JR, Hoencamp R: Transfusion: -80°C frozen blood products are safe and effective in military casualty care. *PLoS One* 2016; 11: e0168401.
172 Dumont LJ, Cancelas JA, Dumont DF, Siegel AH, Szczepiorkowski ZM, Rugg N, Pratt PG, Worsham DN, Hartman EL, Dunn SK, O'Leary M, Ransom JH, Michael RA, Macdonald VW: A randomized controlled trial evaluating recovery and survival of 6% dimethyl sulfoxide–frozen

autologous platelets in healthy volunteers. *Transfusion* 2013; 53: 128-37.
173 Reade MC, Marks DC, Bellomo R, Deans R, Faulke DJ, Fraser JF, Gattas DJ, Holley AD, Irving DO, Johnson L, Pearse BL, Royse AG, Wong J, Cryopreserved vs Liquid Platelet Investigators tA, New Zealand College of Anaesthetists Clinical Trials N, the A, New Zealand Intensive Care Society Clinical Trials G: A randomized, controlled pilot clinical trial of cryopreserved platelets for perioperative surgical bleeding: the CLIP-I trial. *Transfusion* 2019; 59: 2794-804.
174 Australian, Centre NZICR, Cross AR: The cryopreserved vs. liquid platelets trial. https://ClinicalTrials.gov/show/NCT03991481, 2021.
175 Johnson L, Reade MC, Hyland RA, Tan S, Marks DC: In vitro comparison of cryopreserved and liquid platelets: potential clinical implications. *Transfusion* 2015; 55: 838-47.
176 Barnard MR, MacGregor H, Ragno G, Pivacek LE, Khuri SF, Michelson AD, Valeri CR: Fresh, liquid-preserved, and cryopreserved platelets: adhesive surface receptors and membrane procoagulant activity. *Transfusion* 1999; 39: 880-8.
177 Six KR, Compernolle V, Feys HB: Platelet biochemistry and morphology after cryopreservation. *International Journal of Molecular Sciences* 2020; 21.
178 Johnson L, Coorey CP, Marks DC: The hemostatic activity of cryopreserved platelets is mediated by phosphatidylserine-expressing platelets and platelet microparticles. *Transfusion* 2014; 54: 1917-26.
179 Waters L, Padula MP, Marks DC, Johnson L: Cryopreserved platelets demonstrate reduced activation responses and impaired signaling after agonist stimulation. *Transfusion* 2017; 57: 2845-57.
180 Raynel S, Padula MP, Marks DC, Johnson L: Cryopreservation alters the membrane and cytoskeletal protein profile of platelet microparticles. *Transfusion* 2015; 55: 2422-32.
181 Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y, Kume S: Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *Journal of Biochemistry* 1997; 121: 969-73.
182 Pienimaeki-Roemer A, Kuhlmann K, Böttcher A, Konovalova T, Black A, Orsó E, Liebisch G, Ahrens M, Eisenacher M, Meyer HE, Schmitz G: Lipidomic and proteomic characterization of platelet extracellular vesicle subfractions from senescent platelets. *Transfusion* 2015; 55: 507-21.
183 Silliman CC, Kelher MR, Khan SY, West FB, McLaughlin NJD, Elzi DJ, England K, Bjornsen J, Kuldanek SA, Banerjee A: Supernatants and lipids from stored red blood cells activate pulmonary microvascular endothelium through the BLT2 receptor and protein kinase C activation. *Transfusion* 2017; 57: 2690-700.
184 Silliman CC, Bjornsen AJ, Wyman TH, Kelher M, Allard J, Bieber S, Voelkel NF: Plasma and lipids from stored platelets cause acute lung injury in an animal model. *Transfusion* 2003; 43: 633-40.

185 Silliman CC, Voelkel NF, Allard JD, Elzi DJ, Tuder RM, Johnson JL, Ambruso DR: Plasma and lipids from stored packed red blood cells cause acute lung injury in an animal model. *Journal of Clinical Investigation* 1998; 101: 1458-67.
186 Peters AL, Vervaart MA, van Bruggen R, de Korte D, Nieuwland R, Kulik W, Vlaar AP: Non-polar lipids accumulate during storage of transfusion products and do not contribute to the onset of transfusion-related acute lung injury. *Vox Sanguinis* 2017; 112: 25-32.
187 Silliman CC, Dickey WO, Paterson AJ, Thurman GW, Clay KL, Johnson CA, Ambruso DR: Analysis of the priming activity of lipids generated during routine storage of platelet concentrates. *Transfusion* 1996; 36: 133-9.
188 Wyman TH, Bjornsen AJ, Elzi DJ, Smith CW, England KM, Kelher M, Silliman CC: A two-insult in vitro model of PMN-mediated pulmonary endothelial damage: requirements for adherence and chemokine release. *American Journal of Physiology - Cell Physiology* 2002; 283: C1592-603.
189 Johnson L, Waters L, Green S, Wood B, Marks DC: Freezing expired platelets does not compromise in vitro quality: an opportunity to maximize inventory potential. *Transfusion* 2020; 60: 454-9.
190 Wood B, Johnson L, Hyland RA, Marks DC: Maximising platelet availability by delaying cold storage. *Vox Sanguinis* 2018; 113: 403-11.
191 Bertolini F, Murphy S: A multicenter inspection of the swirling phenomenon in platelet concentrates prepared in routine practice. Biomedical excellence for safer transfusion (BEST) working party of the international society of blood transfusion. *Transfusion* 1996; 36: 128-32.
192 Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D: Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *Journal of Lipid Research* 2008; 49: 1137-46.
193 Sud M, Fahy E, Cotter D, Brown A, Dennis EA, Glass CK, Merrill AH, Jr., Murphy RC, Raetz CRH, Russell DW, Subramaniam S: LMSD: LIPID MAPS structure database. *Nucleic Acids Research* 2007; 35: D527-D32.
194 Liebisch G, Vizcaino JA, Kofeler H, Trotzmuller M, Griffiths WJ, Schmitz G, Spener F, Wakelam MJ: Shorthand notation for lipid structures derived from mass spectrometry. *Journal of Lipid Research* 2013; 54: 1523-30.
195 Valkonen S, Holopainen M, Colas RA, Impola U, Dalli J, Käkelä R, Siljander PRM, Laitinen S: Lipid mediators in platelet concentrate and extracellular vesicles: molecular mechanisms from membrane glycerophospholipids to bioactive molecules. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 2019: 1168-82.
196 Gaud C, C. Sousa B, Nguyen A, Fedorova M, Ni Z, O'Donnell V, Wakelam M, Andrews S, Lopez-Clavijo A: BioPAN: a web-based tool to explore mammalian lipidome metabolic pathways on LIPID MAPS. *F1000Research* 2021; 10.

197 Reddoch-Cardenas KM, Bynum JA, Meledeo MA, Nair PM, Wu X, Darlington DN, Ramasubramanian AK, Cap AP: Cold-stored platelets: a product with function optimized for hemorrhage control. *Transfusion and Apheresis Science* 2019; 58: 16-22.
198 Stolla M, Fitzpatrick L, Gettinger I, Bailey SL, Pellham E, Christoffel T, Slichter SJ: In vivo viability of extended 4°C-stored autologous apheresis platelets. *Transfusion* 2018; 58: 2407-13.
199 Johnson L, Vekariya S, Tan S, Padula MP, Marks DC: Extended storage of thawed platelets: Refrigeration supports postthaw quality for 10 days. *Transfusion* 2020; 60: 2969-81.
200 Maslanka K, Uhrynowska M, Lopacz P, Wrobel A, Smolenska-Sym G, Guz K, Lachert E, Ostas A, Brojer E: Analysis of leucocyte antibodies, cytokines, lysophospholipids and cell microparticles in blood components implicated in post-transfusion reactions with dyspnoea. *Vox Sanguinis* 2015; 108: 27-36.
201 Silliman CC, Clay KL, Thurman GW, Johnson CA, Ambruso DR: Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. *The Journal of Laboratory and Clinical Medicine* 1994; 124: 684-94.
202 Hannun YA, Obeid LM: Principles of bioactive lipid signalling: lessons from sphingolipids. *Nature Reviews Molecular Cell Biology* 2008; 9: 139.
203 Andrieu-Abadie N, Gouaze V, Salvayre R, Levade T: Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radical Biology & Medicine* 2001; 31: 717-28.
204 Yatomi Y, Yamamura S, Ruan F, Igarashi Y: Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. *Journal of Biological Chemistry* 1997; 272: 5291-7.
205 Green SM, Padula MP, Marks DC, Johnson L: The lipid composition of platelets and the impact of storage: an overview. *Transfusion Medicine Reviews* 2020; 34: 108-16.
206 van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL: The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochimica et Biophysica Acta - Biomembranes* 2017; 1859: 1558-72.
207 Taha TA, Mullen TD, Obeid LM: A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochimica et Biophysica Acta* 2006; 1758: 2027-36.
208 Valeri CR, Macgregor H, Ragno G: Correlation between in vitro aggregation and thromboxane A2 production in fresh, liquid-preserved, and cryopreserved human platelets: effect of agonists, pH, and plasma and saline resuspension. *Transfusion* 2005; 45: 596-603.
209 Silliman CC, Moore EE, Kelher MR, Khan SY, Gellar L, Elzi DJ: Identification of lipids that accumulate during the routine storage of prestorage leukoreduced red blood cells and cause acute lung injury. *Transfusion* 2011; 51: 2549-54.

210 Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP: Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009; 114: 663-6.
211 Winkler MS, Martz KB, Nierhaus A, Daum G, Schwedhelm E, Kluge S, Graler MH: Loss of sphingosine 1-phosphate (S1P) in septic shock is predominantly caused by decreased levels of high-density lipoproteins (HDL). *Journal of Intensive Care* 2019; 7: 23.
212 Guide to the preparation, use and quality assurance of blood components. Strasbourg, France, European Directorate for the Quality of Medicines & HealthCare, 2017.
213 Zwaal RF, Comfurius P, Bevers EM: Lipid-protein interactions in blood coagulation. *Biochimica et Biophysica Acta* 1998; 1376: 433-53.
214 Logue SE, Elgendy M, Martin SJ: Expression, purification and use of recombinant annexin V for the detection of apoptotic cells. *Nature Protocols* 2009; 4: 1383-95.
215 Iwamoto K, Hayakawa T, Murate M, Makino A, Ito K, Fujisawa T, Kobayashi T: Curvature-dependent recognition of ethanolamine phospholipids by duramycin and cinnamycin. *Biophysical Journal* 2007; 93: 1608-19.
216 Larson MC, Woodliff JE, Hillery CA, Kearl TJ, Zhao M: Phosphatidylethanolamine is externalized at the surface of microparticles. *Biochimica et Biophysica Acta* 2012; 1821: 1501-7.
217 Lauber K, Bohn E, Kröber SM, Xiao Y-j, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, Xu Y, Autenrieth IB, Schulze-Osthoff K, Belka C, Stuhler G, Wesselborg S: Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 2003; 113: 717-30.
218 Tsuda T, Yoshimura H, Hamasaki N: Effect of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine on the activated factor X-prothrombin system. *Blood Coagulation & Fibrinolysis* 2006; 17: 465-9.
219 Tsuda T, Yoshimura H, Hamasaki N: Effect of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine on the protein C/protein S anticoagulation system. *Blood Coagulation & Fibrinolysis* 2006; 17.
220 Vlaar AP, Hofstra JJ, Kulik W, van Lenthe H, Nieuwland R, Schultz MJ, Levi MM, Roelofs JJ, Tool AT, de Korte D, Juffermans NP: Supernatant of stored platelets causes lung inflammation and coagulopathy in a novel in vivo transfusion model. *Blood* 2010; 116: 1360-8.
221 Maślanka K, Smoleńska-Sym G, Michur H, Wróbel A, Lachert E, Brojer E: Lysophosphatidylcholines: bioactive lipids generated during storage of blood components. *Archivum Immunologiae et Therapiae Experimentalis* 2012; 60: 55-60.
222 Park KS, Lee HY, Lee SY, Kim M-K, Kim SD, Kim JM, Yun J, Im D-S, Bae Y-S: Lysophosphatidylethanolamine stimulates chemotactic

migration and cellular invasion in SK-OV3 human ovarian cancer cells: involvement of pertussis toxin-sensitive G-protein coupled receptor. *FEBS Letters* 2007; 581: 4411-6.

223 Makide K, Kitamura H, Sato Y, Okutani M, Aoki J: Emerging lysophospholipid mediators, lysophosphatidylserine, lysophosphatidylthreonine, lysophosphatidylethanolamine and lysophosphatidylglycerol. *Prostaglandins & Other Lipid Mediators* 2009; 89: 135-9.

224 Gaits F, Fourcade O, Le Balle F, Gueguen G, Gaigé B, Gassama-Diagne A, Fauvel J, Salles JP, Mauco G, Simon MF, Chap H: Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. *FEBS Letters* 1997; 410: 54-8.

225 Silliman CC, Paterson AJ, Dickey WO, Stroncek DF, Popovsky MA, Caldwell SA, Ambruso DR: The association of biologically active lipids with the development of transfusion-related acute lung injury: a retrospective study. *Transfusion* 1997; 37: 719-26.

226 Nguyen A, Rudge SA, Zhang Q, Wakelam MJO: Using lipidomics analysis to determine signalling and metabolic changes in cells. *Current Opinion in Biotechnology* 2017; 43: 96-103.

227 Murphy S, Gardner F: Platelet storage at 22 degrees C: role of gas transport across plastic containers in maintenance of viability. *Blood* 1975; 46: 209-18.

228 Fijnheer R, Pietersz RN, de Korte D, Gouwerok CW, Dekker WJ, Reesink HW, Roos D: Platelet activation during preparation of platelet concentrates: a comparison of the platelet-rich plasma and the buffy coat methods. *Transfusion* 1990; 30: 634-8.

229 Bock M, Rahrig S, Kunz D, Lutze G, Heim MU: Platelet concentrates derived from buffy coat and apheresis: biochemical and functional differences. *Transfusion medicine* 2002; 12: 317-24.

230 Dobner P, Koller E, Engelmann B: Platelet high affinity low density lipoprotein binding and import of lipoprotein derived phospholipids. *FEBS Letters* 1999; 444: 270-4.

231 Wiesner P, Leidl K, Boettcher A, Schmitz G, Liebisch G: Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry. *Journal of Lipid Research* 2009; 50: 574-85.

232 Engelmann B, Wiedmann MKH: Cellular phospholipid uptake: flexible paths to coregulate the functions of intracellular lipids. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 2010; 1801: 609-16.

233 Urban S, Zieseniss S, Werder M, Hauser H, Budzinski R, Engelmann B: Scavenger receptor BI transfers major lipoprotein-associated phospholipids into the cells. *Journal of Biological Chemistry* 2000; 275: 33409-15.

234 Connelly MA, Klein SM, Azhar S, Abumrad NA, Williams DL: Comparison of class B scavenger receptors, CD36 and scavenger receptor BI (SR-BI), shows that both receptors mediate high density

lipoprotein-cholesteryl ester selective uptake but SR-BI exhibits a unique enhancement of cholesteryl ester uptake. *Journal of Biological Chemistry* 1999; 274: 41-7.

235 Ibrahim S, Calzada C, Pruneta-Deloche V, Lagarde M, Ponsin G: The transfer of VLDL-associated phospholipids to activated platelets depends upon cytosolic phospholipase A2 activity. *Journal of Lipid Research* 2007; 48: 1533-8.

236 Ibrahim S, Djimet-Baboun A, Pruneta-Deloche V, Calzada C, Lagarde M, Ponsin G: Transfer of very low density lipoprotein-associated phospholipids to activated human platelets. *Journal of Lipid Research* 2006; 47: 341-8.

237 Vorland M, Thorsen VA, Holmsen H: Phospholipase D in platelets and other cells. *Platelets* 2008; 19: 582-94.

238 Oliver AE, Tablin F, Walker NJ, Crowe JH: The internal calcium concentration of human platelets increases during chilling. *Biochimica et Biophysica Acta - Biomembranes* 1999; 1416: 349-60.

239 Yung YC, Stoddard NC, Chun J: LPA receptor signaling: pharmacology, physiology, and pathophysiology. *Journal of Lipid Research* 2014; 55: 1192-214.

240 Eichholtz T, Jalink K, Fahrenfort I, Moolenaar WH: The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochemical Journal* 1993; 291 (Pt 3): 677-80.

241 Sano T, Baker D, Virag T, Wada A, Yatomi Y, Kobayashi T, Igarashi Y, Tigyi G: Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood. *Journal of Biological Chemistry* 2002; 277: 21197-206.

242 Leblanc R, Houssin A, Peyruchaud O: Platelets, autotaxin and lysophosphatidic acid signalling: win-win factors for cancer metastasis. *British Journal of Pharmacology* 2018; 175: 3100-10.

243 Vance JE: Thematic review series: glycerolipids, phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids. *Journal of Lipid Research* 2008; 49: 1377-87.

244 Vance JE, Tasseva G: Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochimica et Biophysica Acta* 2013; 1831: 543-54.

245 Stone SJ, Vance JE: Phosphatidylserine synthase-1 and -2 are localized to mitochondria-associated membranes. *Journal of Biological Chemistry* 2000; 275: 34534-40.

246 Shiao YJ, Lupo G, Vance JE: Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine. *Journal of Biological Chemistry* 1995; 270: 11190-8.

247 Tan ST, Ramesh T, Toh XR, Nguyen LN: Emerging roles of lysophospholipids in health and disease. *Progress in Lipid Research* 2020; 80: 101068.

248 Murohara T, Scalia R, Lefer AM: Lysophosphatidylcholine promotes P-selectin expression in platelets and endothelial cells. Possible involvement of protein kinase C activation and its inhibition by nitric oxide donors. *Circulation Research* 1996; 78: 780-9.
249 Murugesan G, Sandhya Rani MR, Gerber CE, Mukhopadhyay C, Ransohoff RM, Chisolm GM, Kottke-Marchant K: Lysophosphatidylcholine regulates human microvascular endothelial cell expression of chemokines. *Journal of Molecular and Cellular Cardiology* 2003; 35: 1375-84.
250 Ojala PJ, Hirvonen TE, Hermansson M, Somerharju P, Parkkinen J: Acyl chain-dependent effect of lysophosphatidylcholine on human neutrophils. *Journal of Leukocyte Biology* 2007; 82: 1501-9.
251 Olofsson KE, Andersson L, Nilsson J, Björkbacka H: Nanomolar concentrations of lysophosphatidylcholine recruit monocytes and induce pro-inflammatory cytokine production in macrophages. *Biochemical and Biophysical Research Communications* 2008; 370: 348-52.
252 Huang LS, Hung ND, Sok DE, Kim MR: Lysophosphatidylcholine containing docosahexaenoic acid at the sn-1 position is anti-inflammatory. *Lipids* 2010; 45: 225-36.
253 Hung ND, Sok D-E, Kim MR: Prevention of 1-palmitoyl lysophosphatidylcholine-induced inflammation by polyunsaturated acyl lysophosphatidylcholine. *Inflammation Research* 2012; 61: 473-83.
254 Tani M, Sano T, Ito M, Igarashi Y: Mechanisms of sphingosine and sphingosine 1-phosphate generation in human platelets. *Journal of Lipid Research* 2005; 46: 2458-67.
255 Taha TA, Hannun YA, Obeid LM: Sphingosine kinase: biochemical and cellular regulation and role in disease. *Journal of Biochemistry and Molecular Biology* 2006; 39: 113-31.
256 Green SM, Padula MP, Dodgen TM, Batarseh A, Marks DC, Johnson L: Alternative storage of platelets alters the abundance of bioactive lipid mediators. *Transfusion* 2020; 60: 7A-260A.
257 Reddoch-Cardenas KM, Peltier GC, Chance TC, Nair PM, Meledeo MA, Ramasubramanian AK, Cap AP, Bynum JA: Cold storage of platelets in platelet additive solution maintains mitochondrial integrity by limiting initiation of apoptosis-mediated pathways. *Transfusion* 2021; 61: 178-90.
258 Siskind LJ, Kolesnick RN, Colombini M: Ceramide channels Increase the permeability of the mitochondrial outer membrane to small proteins. *Journal of Biological Chemistry* 2002; 277: 26796-803.
259 Ganesan V, Perera MN, Colombini D, Datskovskiy D, Chadha K, Colombini M: Ceramide and activated Bax act synergistically to permeabilize the mitochondrial outer membrane. *Apoptosis: An International Journal on Programmed Cell Death* 2010; 15: 553-62.
260 Perera MN, Lin SH, Peterson YK, Bielawska A, Szulc ZM, Bittman R, Colombini M: Bax and Bcl-xL exert their regulation on different sites of the ceramide channel. *Biochemical Journal* 2012; 445: 81-91.

261 Stiban J, Perera M: Very long chain ceramides interfere with C16-ceramide-induced channel formation: a plausible mechanism for regulating the initiation of intrinsic apoptosis. *Biochimica et Biophysica Acta - Biomembranes* 2015; 1848: 561-7.
262 Johnson L, Wood B, Marks DC: Cold-stored platelets: Procoagulant or apoptotic? *Research and Practice in Thrombosis and Haemostasis* 2019; 3: 559.
263 Liu M, Bagdade JD, Subbaiah PV: Specificity of lecithin:cholesterol acyltransferase and atherogenic risk: comparative studies on the plasma composition and in vitro synthesis of cholesteryl esters in 14 vertebrate species. *Journal of Lipid Research* 1995; 36: 1813-24.
264 Silliman CC, Boshkov LK, Mehdizadehkashi Z, Elzi DJ, Dickey WO, Podlosky L, Clarke G, Ambruso DR: Transfusion-related acute lung injury: epidemiology and a prospective analysis of etiologic factors. *Blood* 2003; 101: 454-62.
265 Park S-J, Lee K-P, Im D-S: Action and signaling of lysophosphatidylethanolamine in MDA-MB-231 breast cancer cells. *Biomolecules & Therapeutics* 2014; 22: 129-35.
266 Lee J-M, Park S-J, Im D-S: Calcium signaling of lysophosphatidylethanolamine through LPA(1) in human SH-SY5Y neuroblastoma cells. *Biomolecules & Therapeutics* 2017; 25: 194-201.
267 Silliman CC, Thurman GW, Ambruso DR: Stored blood components contain agents that prime the neutrophil NADPH oxidase through the platelet-activating-factor receptor. *Vox Sanguinis* 1992; 63: 133-6.
268 Silliman CC, Johnson CA, Clay KL, Thurman GW, Ambruso DR: Compounds biologically similar to platelet activating factor are present in stored blood components. *Lipids* 1993; 28: 415-8.
269 Holme PA, Solum NO, Brosstad F, Pedersen T, Kveine M: Microvesicles bind soluble fibrinogen, adhere to immobilized fibrinogen and coaggregate with platelets. *Thrombosis and Haemostasis* 1998; 79: 389-94.
270 Jerez-Dolz D, Torramade-Moix S, Palomo M, Moreno-Castaño A, Lopez-Vilchez I, Hernandez R, Badimon JJ, Zafar MU, Diaz-Ricart M, Escolar G: Internalization of microparticles by platelets is partially mediated by toll-like receptor 4 and enhances platelet thrombogenicity. *Atherosclerosis* 2020; 294: 17-24.
271 Feng W, Chang C, Luo D, Su H, Yu S, Hua W, Chen Z, Hu H, Liu W: Dissection of autophagy in human platelets. *Autophagy* 2014; 10: 642-51.
272 Ouseph MM, Huang Y, Banerjee M, Joshi S, MacDonald L, Zhong Y, Liu H, Li X, Xiang B, Zhang G, Komatsu M, Yue Z, Li Z, Storrie B, Whiteheart SW, Wang QJ: Autophagy is induced upon platelet activation and is essential for hemostasis and thrombosis. *Blood* 2015; 126: 1224-33.
273 Wood B, Padula MP, Marks DC, Johnson L: Cryopreservation alters the immune characteristics of platelets. *Transfusion* 2021; 61: 3432-42.

274 Rinder HM, Ault KA: Platelet activation and its detection during the preparation of platelets for transfusion. *Transfusion Medicine Reviews* 1998; 12: 271-87.
275 Johnson L, Hyland R, Tan S, Tolksdorf F, Sumian C, Seltsam A, Marks D: In vitro quality of platelets with low plasma carryover treated with ultraviolet C light for pathogen inactivation. *Transfusion Medicine and Hemotherapy* 2016; 43: 190-7.
276 Zhang H, Nimmer PM, Tahir SK, Chen J, Fryer RM, Hahn KR, Iciek LA, Morgan SJ, Nasarre MC, Nelson R, Preusser LC, Reinhart GA, Smith ML, Rosenberg SH, Elmore SW, Tse C: Bcl-2 family proteins are essential for platelet survival. *Cell Death & Differentiation* 2007; 14: 943-51.
277 Del Conde I, Shrimpton CN, Thiagarajan P, Lopez JA: Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. *Blood* 2005; 106: 1604-11.
278 Menck K, Sönmezer C, Worst TS, Schulz M, Dihazi GH, Streit F, Erdmann G, Kling S, Boutros M, Binder C, Gross JC: Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane. *Journal of Extracellular Vesicles* 2017; 6: 1378056-.
279 Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brügger B, Simons M: Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 2008; 319: 1244-7.
280 Hoehn RS, Jernigan PL, Japtok L, Chang AL, Midura EF, Caldwell CC, Kleuser B, Lentsch AB, Edwards MJ, Gulbins E, Pritts TA: Acid sphingomyelinase inhibition in stored erythrocytes reduces transfusion-associated lung inflammation. *Annals of Surgery* 2017; 265: 218-26.
281 McVey MJ, Weidenfeld S, Maishan M, Spring C, Kim M, Tabuchi A, Srbely V, Takabe-French A, Simmons S, Arenz C, Semple JW, Kuebler WM: Platelet extracellular vesicles mediate transfusion-related acute lung injury by imbalancing the sphingolipid rheostat. *Blood* 2021; 137: 690-701.
282 Bianco F, Perrotta C, Novellino L, Francolini M, Riganti L, Menna E, Saglietti L, Schuchman EH, Furlan R, Clementi E, Matteoli M, Verderio C: Acid sphingomyelinase activity triggers microparticle release from glial cells. *EMBO Journal* 2009; 28: 1043-54.
283 Egea-Jimenez AL, Zimmermann P: Phospholipase D and phosphatidic acid in the biogenesis and cargo loading of extracellular vesicles. *Journal of Lipid Research* 2018; 59: 1554-60.
284 Laulagnier K, Grand D, Dujardin A, Hamdi S, Vincent-Schneider H, Lankar D, Salles JP, Bonnerot C, Perret B, Record M: PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. *FEBS Letters* 2004; 572: 11-4.
285 Kooijman EE, Chupin V, de Kruijff B, Burger KN: Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic* 2003; 4: 162-74.

286 Losito I, Patruno R, Conte E, Cataldi TR, Megli FM, Palmisano F: Phospholipidomics of human blood microparticles. *Analytical Chemistry* 2013; 85: 6405-13.
287 Losito I, Conte E, Cataldi TRI, Cioffi N, Megli FM, Palmisano F: The phospholipidomic signatures of human blood microparticles, platelets and platelet-derived microparticles: a comparative HILIC-ESI–MS investigation. *Lipids* 2015; 50: 71-84.
288 Diehl P, Nienaber F, Zaldivia MTK, Stamm J, Siegel PM, Mellett NA, Wessinger M, Wang X, McFadyen JD, Bassler N, Puetz G, Htun NM, Braig D, Habersberger J, Helbing T, Eisenhardt SU, Fuller M, Bode C, Meikle PJ, Chen YC, Peter K: Lysophosphatidylcholine is a major component of platelet microvesicles promoting platelet activation and reporting atherosclerotic plaque instability. *Thrombosis and Haemostasis* 2019; 119: 1295-310.
289 Braig D, Nero TL, Koch H-G, Kaiser B, Wang X, Thiele JR, Morton CJ, Zeller J, Kiefer J, Potempa LA, Mellett NA, Miles LA, Du X-J, Meikle PJ, Huber-Lang M, Stark GB, Parker MW, Peter K, Eisenhardt SU: Transitional changes in the CRP structure lead to the exposure of proinflammatory binding sites. *Nature Communications* 2017; 8: 14188.
290 Habersberger J, Strang F, Scheichl A, Htun N, Bassler N, Merivirta RM, Diehl P, Krippner G, Meikle P, Eisenhardt SU, Meredith I, Peter K: Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. *Cardiovascular Research* 2012; 96: 64-72.
291 Bolen AL, Naren AP, Yarlagadda S, Beranova-Giorgianni S, Chen L, Norman D, Baker DL, Rowland MM, Best MD, Sano T, Tsukahara T, Liliom K, Igarashi Y, Tigyi G: The phospholipase A1 activity of lysophospholipase A-I links platelet activation to LPA production during blood coagulation. *Journal of Lipid Research* 2011; 52: 958-70.
292 Aoki J, Taira A, Takanezawa Y, Kishi Y, Hama K, Kishimoto T, Mizuno K, Saku K, Taguchi R, Arai H: Serum lysophosphatidic acid is produced through diverse phospholipase pathways. *Journal of Biological Chemistry* 2002; 277: 48737-44.
293 Mizumura K, Justice MJ, Schweitzer KS, Krishnan S, Bronova I, Berdyshev EV, Hubbard WC, Pewzner-Jung Y, Futerman AH, Choi AMK, Petrache I: Sphingolipid regulation of lung epithelial cell mitophagy and necroptosis during cigarette smoke exposure. *The FASEB journal* 2018; 32: 1880-90.
294 Tynngård N, Wikman A, Uhlin M, Sandgren P: Haemostatic responsiveness and release of biological response modifiers following cryopreservation of platelets treated with amotosalen and ultraviolet A light. *Blood transfusion = Trasfusione del sangue* 2020; 18: 191-9.
295 Silliman CC, Kelher MR, Khan SY, LaSarre M, West FB, Land KJ, Mish B, Ceriano L, Sowemimo-Coker S: Experimental prestorage filtration removes antibodies and decreases lipids in RBC supernatants mitigating TRALI in vivo. *Blood* 2014; 123: 3488-95.

296 Jonnalagadda D, Sunkara M, Morris AJ, Whiteheart SW: Granule-mediated release of sphingosine-1-phosphate by activated platelets. *Biochimica et Biophysica Acta* 2014; 1841: 1581-9.
297 Bohonek M, Kutac D, Landova L, Koranova M, Sladkova E, Staskova E, Voldrich M, Tyll T: The use of cryopreserved platelets in the treatment of polytraumatic patients and patients with massive bleeding. *Transfusion* 2019; 59: 1474-8.
298 Johnson L, Schubert P, Tan S, Devine DV, Marks DC: Extended storage and glucose exhaustion are associated with apoptotic changes in platelets stored in additive solution. *Transfusion* 2016; 56: 360-8.
299 Bali R, Savino L, Ramirez DA, Tsvetkova NM, Bagatolli L, Tablin F, Crowe JH, Leidy C: Macroscopic domain formation during cooling in the platelet plasma membrane: an issue of low cholesterol content. *Biochimica et Biophysica Acta - Biomembranes* 2009; 1788: 1229-37.
300 Gousset K, Wolkers WF, Tsvetkova NM, Oliver AE, Field CL, Walker NJ, Crowe JH, Tablin F: Evidence for a physiological role for membrane rafts in human platelets. *Journal of Cellular Physiology* 2002; 190: 117-28.
301 Gomez-Larrauri A, Presa N, Dominguez-Herrera A, Ouro A, Trueba M, Gomez-Muñoz A: Role of bioactive sphingolipids in physiology and pathology. *Essays in Biochemistry* 2020; 64: 579-89.
302 Ki KK, Johnson L, Faddy HM, Flower RL, Marks DC, Dean MM: Immunomodulatory effect of cryopreserved platelets: altered BDCA3+ dendritic cell maturation and activation in vitro. *Transfusion* 2017; 57: 2878-87.
303 Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J: High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis* 2001; 158: 277-87.