Pathogen inactivation in platelet concentrate storage: Effects on quality and utilization

by

Níels Árni Árnason
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Thesis Committee:

Dr. Ólafur Eysteinn Sigurjónsson, Supervisor,
Professor, Reykjavík University, Iceland

Dr. Óttar Rolfsson, Co-supervisor,
Professor, University of Iceland, Iceland

Dr. Per Sandgren, Co-supervisor Associated
Professor, Karolinska Institutet, Huddinge, Sweeden

Dr. Sisse Ostrowski, Co-supervisor,
Professor, University Copenhagen, Denmark

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Abstract

In transfusion medicine and blood banking, product quality and safety of patients are both essential. Blood transfusion is, in many instances, a lifesaving procedure; however, is not without risk. Blood products contain biological response modifiers (BRMs) that can induce febrile and allergic reactions and there is risk of donor/patient incompatibility, resulting in hemolytic transfusion reaction. Pathogen contamination of donor origin or due to collection and processing is another risk. The implementation of efficient viral screening has made blood transfusions safer, despite not addressing the risks from emerging pathogens or from bacterial contamination. For platelet concentrates (PCs) in particular, the standard storge conditions (room temperature) present an elevated risk of bacterial contamination and transfusion transmitted bacterial infection (TTBI) compared to other blood components, which are stored at subzero or refrigerated temperatures. Though the risk of TTBI can be minimized via the use of various screening assays, TTBI resulting in sepsis still occurs, with a high mortality rate. Therefore, methods have been developed to inactivate pathogens in blood products; such methods include photo or photochemical techniques, which influence the nucleic acids of pathogens and disable transcription. These methods have proven highly efficient in reducing the pathogenic load in blood products, namely PCs and plasma. As these methods have been approved

through clinical trials and then implemented in routine use, indications of negative effects on blood products have emerged, specifically effects on platelet quality have been of concern.

In response to the concern about reduced platelet quality, we investigated effect of pathogen inactivation (PI) with amotosalen and ultraviolet A (UVA) on the quality of stored platelets using a pool and split strategy and whole blood collected buffy coat (BC) platelet concentrates, with the aim of adding to the existing information.

Multiple reports have suggested that micro RNA (miRNA) are important post transcription regulators in platelets, and there have been indications of altered miRNA profile due to pathogen inactivation (PI) methods. Therefore, we examined PI effects on 25 pre-selected miRNAs. Minimal influence was observed, with only 1 out of the 25 showing PI treatment-related down regulation.

The release of BRMs from platelets into the storage media presents a potential risk of adverse events, as well as BRMs being indicators of platelet activation during storge. Monitoring the concentration of 36 proteins, we observed both reduction and increase of BRMs related to PI treatment.

Additionally, PC utilization in national blood transfusion services (at the Blood Bank of Iceland) was analyzed pre- and post-PI implementation. We observed several PI treatment-related effects on both miRNA profiles and protein concentrations in the storage media, as well as elevated expression of markers of platelets storge lesion (PSL), though these effects did not translate to increased utilization or adverse events. We also observed increased product availability and more efficient stock management due to increased storge time, without an increase in outdated stock.

Smithreinsun meðferð á blóðflöguþykkni: Áhrif á gæði og notkun

Níels Árni Árnason

August 2020

Útdráttur

Í blóðbankastarfsemi og við blóðinngjöf skipta gæði afurðar og öryggi sjúklings öllu máli. Í mörgum tilfellum er blóðinngjöf lífsbjargandi meðferð, en ekki laus við áhættu. Blóð inniheldur lífvirka þætti sem geta stuðlað að aukaverkunum eins og hækkun á líkamshita og ofnæmi, að auki er áhætta á blóðgjafa og blóðþega misræmi sem getur valdið niðurbroti á blóðfrumum. Sýking í blóðhluta sem getur átt uppruna frá blóðgjafa eða við vinnslu á blóðhlutanum er annar áhættuþáttur. Innleiðing veiru skimunar í blóðhlutum hefur aukið mikið á öryggi við blóðinngjöf, án þess þó koma í veg fyrir sýkingar vegna óþekktra sýkla eða bakteríu smits. Almennt er blóðflögu þykkni (BÞ) geymt á vöggu og við stofuhita sem eru kjöraðstæður fyrir vöxt baktería, og þess vegna er áhætta á slíku smiti margföld í tilfelli BÞ borið saman við aðra blóðhluta sem eru kældir eða frystir við geymslu. Hægt er að lágmarka áhættu á bakteríu mengun með margvíslegum skimunar aðferðum, en þrátt fyrir slíkar aðferðir eru tilfelli þar sem bakteríu mengað BÞ veldur alvarlegri blóðsýkingu með hárri tíðni dauðsfalla. Til að draga enn frekar úr og jafnvel koma alveg í veg fyrir bakteríu mengun i BÞ hafa verið þróaðar smit-hreinsunar (SH) aðferðir sem byggja ljósa eða ljósa og efnatækni sem hafa áhrif á kjarnsýrur í sýklum og koma í veg fyrir umritun. Þessar aðferðir hafa sannað sig í að draga úr magni sýkla í blóðhlutum, þá sérstaklega BÞ og blóðvökva. Á sama tíma og þessar aðferðir fengu samþykki byggt á klínískum tilraunum og voru innleiddar inn i almenna blóðbanka starfsemi, komu fram vísbendingar um neikvæð áhrif á gæði blóðhluta sérstaklega BÞ.

Til að rannsaka hugsanleg áhrif SH tækni sem byggir á amotosalen og útfjólublá ljósi A á gæði BÞ í geymslu beittum við blöndunar og uppskipti aðferð á BÞ unnið úr heilblóðsgjöfum.

Fjöldi birtra rannsóknarniðurstaða hafa gefið í skyn að stuttar RNA sameindir (miRNA) hafi hlutverk í stýringu á prótein tjáningu í blóðflögum og vísbendingar um neikvæð áhrif SH. Til að rannsaka frekar þessi áhrif völdum við 25 miRNA til að meta áhrif SH. Áhrif SH á þessi 25 miRNA var takmörkuð þar sem aðeins 1 af 25 sýndi breytingu tengda SH meðferð.

Losun lífvirka þátta eins ýmissa próteina frá blóðflögum út í geymsluvökva er áhættuþáttur sem hugsanlega getur valdið aukaverkun og að auki er magn þessara þátta í geymsluvökva vísir fyrir virkjun blóðflaga í geymslu. Eftirlit með magni 36 próteina í geymsluvökva sýndi bæði aukningu og minnkun tengda SH meðferð á BÞ

Notkun BÞ á Íslandi var skoðuð fyrir og eftir innleiðingu á SH í Blóðbankanum. Okkar niðurstöður sýndu áhrif á bæði miRNA og prótein losun sem og aukna tjáningu á vísum fyrir blóðflögu geymslu skemmdum, án þess að greina þessi áhrif í aukinn notkun eða fjölda aukverkanna. Við greindum aukið framboð á BÞ og skilvirkari lager stjórnun.

The undersigned here by certify that they recommend to the Department of Engineering, School of Technology, Reykjavík University, that this dissertation entitled **Effects of pathogen inactivation on quality and utilization of stored platelets** submitted by **Níels Árni Árnason** be accepted as partial fulfillment of the requirements for the degree of **Doctor of Philosophy(Ph.D.) in Applied Sciences**

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date

.....

Dr. Ólafur Eysteinn Sigurjónsson,
Supervisor Professor,
Reykjavík University, Iceland

.....

Dr. Óttar Rolfsson,
Co-Supervisor Professor,
University of Iceland, Iceland

.....

Dr. Per Sandgren,
Co-Supervisor Associated Professor,
Karolinska Institutet, Sweeden

.....

Dr. Sisse Ostrowski,
Co-Supervisor Professor,
University of Copenhagen, Denmark

.....

Dr. Larry Dumont, Examiner
Clinical Professor, University of Colorado School of Medicine, USA
Affiliated Investigator, Vitalant Research Institute, USA

....._____....._____

date

....._____.....

Níels Árni Árnason
Doctor of Philosophy

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Contents

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List of Abbreviations

Argonaut 2 (Ago2)
biological response modifiers (BRMs)
buffy coat (BC)
Centers for Disease Control (CDC)
compound absorption device (CAD)
corrected count increment (CCI)
dense tubular system (DTS)
endoplasmic reticulum (ER)
extracellular vesicles (EV's)
febrile nonhemolytic transfusion reaction (FNHTR)
Food and Drug Administration (FDA)
glycoprotein (GP)
good manufacturing practice (GMP)
horizontal information transfer (HIT)
Human leukocyte antigen (HLA)
Human platelet antigen (HPA)
immune deficiency syndrome (AIDS)
immunodeficiency virus (HIV)
inositol triphosphate (IP3)
invaginated membrane system (IMS)
long non-coding RNA (lncRNA)
micro RNA (miRNAs)
microparticles (MP)
mitochondrial DNA (mtDNA)
mitogen-activated protein kinases (MAPK)
National Institute of Health (NIH)
neutrophil external net (NET)
nitric oxide (NO)
nucleic acids test (NAT)
nucleic amplification test (NAT)
open canalicular system (OCS)
Pan Genera Detection (PGD)
pathogen inactivation (PI)
pathogen reduction (PR)
pathogen-associated molecular patterns (PAMPs)
pattern recognition receptors (PRR)
phosphatidylinositol-tris-kinase (PI3K)
phosphatidylserine (PS)
platelet additive solutions (PAS)
platelet concentrate (PC)
platelet factor 4 (PF4)
platelet rich plasma (PRP)
platelet storage lesion (PSL)
polymer chain reaction (PCR)
primary-miRNA (pri-miRNA)
Principal component analysis (PCA)
prostacyclin 2 (PGl_2)
protein disulfide isomerase (PDI)
P-selectin glycoprotein ligand-1 (PSGL-1)
reactive oxygen species (ROS)
red blood cells (RBCs)
serial analysis of gene expression (SAGE)

soluble CD40L (sCD40L)
thrombin, adenosine diphosphate (ADP)
thromboelastography (TEG)
thromboelastometry (TEM)
tissue factor (TF)
toll-like receptors (TLRs)
transfusion transmitted bacterial infection (TTBI)
Transfusion transmitted infection (TTI)
transfusion-related acute lung injury (TRALI)
transfusion-related adverse events (TRAE)
ultraviolet (UV)
untranslated region (UTR)
vesicle-associated membrane protein 8 (VAMP8)
von Willebrand Factor (vWF)
whole blood (WB)

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their

Roman numerals.

I. **Niels Arni Arnason**, Freyr Johannson, Ragna Landrö, Björn Hardarsson, Johannes Irsch , Sveinn Gudmundsson, Ottar Rolfsson, Olafur E Sigurjonsson. “Pathogen inactivation with amotosalen plus UVA illumination minimally impacts microRNA expression in platelets during storage under standard blood banking conditions.” Transfusion 2019 doi: 10.1111/trf.15575.

II. **Niels Arni Arnason**, Freyr Johannson, Ragna Landrö, Björn Hardarsson, Sveinn Gudmundsson, Aina-Mari Lian, Janne Reseland, Ottar Rolfsson, Olafur E Sigurjonsson. “Protein Concentrations in Stored Pooled Platelet Concentrates Treated with Pathogen Inactivation by Amotosalen Plus Ultraviolet a Illumination.” Pathogens 2022 doi: 10.3390/pathogens11030350

III. **Niels Arni Arnason**, Ragna Landrö, Björn Hardarsson, Sveinn Gudmundsson, Olafur E Sigurjonsson. “Implementation of pathogen reduction by amotosalen plus ultraviolet A illumination for platelets in a national blood service.” Manuscript .. (in publication) (?)

Other publications.

I. Freyr Johannson, **Niels Arni Arnason** Ragna Landrö, Sveinn Gudmundsson, Olafur E Sigurjonsson, Ottar Rolfsson. “Metabolomics study of platelet concentrates photochemically treated with amotosalen and UVA light for pathogen inactivation.” Transfusion 2020 doi: 10.1111/trf.15610.

II. **Niels Arni Arnason**, Olafur E Sigurjonsson . “New strategies to understand platelet storage lesion ISBT Science Sereies” 2017 doi.org/10.1111/voxs.1239

1 Introduction

1.1 Brief History of platelets in transfusion medicine

1.1.1 Discovery of platelets

Suggestions of additional elements in the blood other than white and red cells came as early as 1770s by William Hewson, a British surgeon, anatomist, and physiologist. Hewson, often referred to as the father of hematology, is credited with discovering white blood cells, lymphatic system functions, fibrinogen, and the fundamentals of blood coagulation [1]. However, because they are the smallest recognized cell in the human body, the detection and visualization of platelets was impossible until advances in microscopy in 1830. The first illustration of platelets by British anatomist George Gulliver and a platelet fibrin clot by British physician William Addison were published in 1841 and 1842, respectively [2]. An 1864 publication by British pathologist Lionel Beale included a drawing of blood that clearly showed platelets. Beale, however speculated that these small capsules were precursors of white or red blood cells and that their rapid cell death produced fibrin [3].

William Osler, a physician born in Canada and one of four founders of Johns Hopkins Hospital, described platelets in an 1874 publication. Osler reported on small disc-shaped corpuscles circulating in the blood and their instant aggregation in samples of blood removed from a vessel. In this early published description of platelets, Osler did not confirm whether platelets were normal elements of blood or exogenous organisms [4].

Using *in vivo* microscopy and a primitive flow chamber, conformation that platelets are a part of normal human physiology and a description of their role in hemostasis and thrombosis was published in 1882 by Giulio Bizzozero, an Italian doctor and biomedical scientist [5]. Bizzozero had previously, in 1868, recognized the role of bone marrow in hematopoiesis and observed leukocyte recruitment to platelet aggregates, thus establishing leukocyte-platelet interaction [6]. Bizzozero also recognized megakaryocytes in the bone marrow, but not their role in platelet production. Platelet connection to megakaryocytes was

established in 1906 by James H Wright, an American pathologist who recognized the same type of granules in both cell types [7].

1.1.2 Transfusion, collection, and storage

William Duke, an American medical doctor and a student of Wright, published the first results on the *in vivo* function of platelet in 1910 by analyzing bleeding time in thrombocytopenic patients pre and post whole blood transfusion [8]. Although Dukes results clearly showed the benefits of blood transfusions for thrombocytopenic patient, his findings did not receive proper attention. The field of blood transfusion was in its infancy, with the major ABO blood groups discovered only ten years prior [9] to Duke's landmark experiment and knowledge of the serological risks of blood transfusion were limited [10]. During World War II thrombocytopenia was recognized as symptom of radiation exposure from atomic weapon testing. The USA government started funding research into platelet transfusion medicine [11]. William P. Murphy Jr. a medical doctor and inventor, alongside Carl W. Walter, a surgeon, inventor, and founder of one of the world's first blood banks, introduced plastic containers for collecting and storage of blood in 1950. The implementation of plastic containers and advances in centrifuging technology with the development of temperature controlled centrifuges paved the road to modern blood component processing [12,13].

With the development of more robust chemotherapy drugs, thrombocytopenia became a common side effect and major cause of mortality in cancer patients receiving chemotherapy and 1961 the beneficial role of platelet transfusion for these patients was reported [11,12] [14].

In a 1962 publication the investigators concluded that a platelet count lower than $20x10^9$/L should be the trigger for platelet transfusion to prevent spontaneous bleeding, and prophylaxis platelet transfusion was recommended to prevent bleeding [15]. To date there is no universal consensus on triggers for prophylaxis platelet transfusion due to lack of objective data to make evidence based recommendations [16].

Murphy and Frank H. Gardner, both medical doctors, published their research on platelet storage in 1969, reporting on the shortening of *in vivo* life span of radiolabeled refrigerated platelets and the feasibility of storing platelets at room temperatures for up to four days [17]. Murphy and Gardner subsequently recognized the importance of agitation and of the gas permeability of plastic containers in preserving platelet quality [18]. They concluded that platelet storage beyond four days resulted in unexpectable *in vivo* recovery and increased risk of bacterial contamination [19].

1.1.2 Transfusion transmitted infection

Following the Murphy and Gardner publication recommending a maximum 4-day storage period, there were publications which indicated a minimal risk of bacterial growth in PCs stored at room temperatures [20–22]. Further research using more sensitive culture techniques, however, indicated that up to 6.3% of PCs were contaminated with bacteria. In this study, contamination was detected at all timepoints during storage of the platelets, from day one to day four. A retrospective analysis done in the same study did not show a similar frequency of septic reaction. The authors concluded that in most cases bacterial contaminated PCs did not contain a high enough number of bacteria to be clinically relevant [23].

Even during the early days of room temperature storage of PCs, bacterial screening with an overnight holding period was being suggested [24]. Advances in platelet storage containers using plasticizers with increased gas permeability resulted in increased viability and recovery, leading to the potential for prolonged storage for up to five or seven days [25,26]. In 1983, with the aim of increasing the availability of platelet products for transfusion and based on evidence of low risk of transfusion transmitted bacterial infection (TTBI), the FDA approved 7-day storage of platelets in the USA. Only two years later, however, based on increased reporting of TTBI related to platelet products, the FDA reversed its decision, allowing a maximum of 5-day storage [27,28].

Although the risks of TTBI were known, viral screening of blood products would be

the focus of the transfusion community for the next two decades. The first case of acquired immune deficiency syndrome (AIDS) was reported in 1981 and in 1984 the human immunodeficiency virus (HIV) was identified by Gallo R and colleagues as the cause of AIDS. Gallo's group also provided an antibody test to identify positive donors [29]. The following year the first screening test for HIV antibodies was approved by the FDA and in 1992 blood donor screening for both HIV-1 and HIV-2 antibodies was implemented. In 1996, HIV p24 antigen tests were developed, shortening the window period of undetected new infection [12][30].

The first screening test for hepatitis B was antigen based, recognizing the surface antigen of the virus (HBsAg). This test was approved and mandatory by the FDA in 1972 and in 1986 the hepatitis B core antigen test (HBV-c), was developed further lowering the risk of transfusion related hepatitis B infection [31]. The first screening tests developed for hepatitis C in the 1970's were based on exclusion of serological markers for hepatitis B and A termed (NANB) hepatitis, as well as detection of elevated levels of the liver enzyme alanine amino transferase (ALT). These screening methods had low predictive value with high false negative and positives levels resulting in unnecessary exclusion of valuable donors [31]. In collaboration of scientists from the Centers for Disease Control (CDC), National Institute of Health (NIH) and Houghton M and colleagues the hepatitis C virus was discovered in 1989 and in the following year screening tests for hepatitis C virus antibodies were available [32].

More sensitive tests with specific amplification of viral nucleic acids (NAT) were developed in the mid to late 1990's and first adopted by plasma fractionation industry alongside their pathogen inactivation technology to further reduce the risk of viral contamination in their products [33]. Due to highly sensitive antigen tests available for HBV and vaccination programs, NAT testing for HBV was not implemented in the US until 2009 after a single NAT test for HCV,HIV and HBV was developed. In the beginning of NAT testing the residual risk for transfusion related HBV infection was estimated to be 1 in

500.000 units, but recent data 10 years after NAT implementation show the residual risk as low as 1 in 2 million. NAT testing for HCV and HIV started as early as 1999 in the US and current residual risk for of transfusion related HCV infection is 1 in 2 million units [31,34]. In 1997 Germany was the first country to start using in house developed NAT for HCV,HBV and HIV[33]. Research published in 1999 showed the feasibility of implementing NAT in the blood banks setting, especially for HCV as 2 out 374.000 samples tested were NAT only positive and undetected by serological tests [35]. This ratio was close to the estimated residual risk for transfusion related HCV infection of 2 in every 200.000 units in Germany at the time. This and other cumulative data led to NAT HCV being mandated in Germany in 1999. Fallowing the commercialization of NAT more countries have implemented NAT with over 60 million tests being run annually worldwide [33]. In a survey conducted by the International Society of Blood Transfusions (ISBT) on NAT testing including 33 countries during a 10 year period from 1999 to 2009 and covering 30 million donations, 2808 were identified as only NAT positive and not detected by serological testing [36]

All these developments greatly improved transfusion safety and with improved surveillance and reporting of serious adverse events related to blood transfusion, TTBI gained more focus [37–40]. It was established that the major source of bacterial contamination is the skin flora at the site of the venipuncture during donation, with donor bacteremia or contamination due to processing occurring less frequently [41,42]. Improved phlebotomy techniques, firm donor deferrals and diversion of the first 10 to 30 ml during donation, although reducing the risk, did not prevent bacterial contamination in platelet components. To minimize the risk of TTBI and sepsis, implementation of additional methods including bacterial detection or reduction were needed [30,37–40].

1.2 Platelet biology

1.2.1 Platelet production

The normal platelet count of a healthy adult ranges from 150 to 450 x 10^9 per liter, with an average of 100 x 10^9 platelets produced and cleared from circulation every day. The spleen stores up to one third of platelets, and there is a constant consumption of platelets maintaining vascular integrity, as well as senescent and apoptotic platelets removed from circulation. The platelet lifespan of, on average, ten days in circulation, is determined by the internal proteolytic clock, governed by pro-apoptotic proteins Bax and Bak and their interplay with pro-survival protein Bcl-X_L. Changes in platelet surface glycoprotein receptors, like the loss of sialic acid (desialylation), can also be signals that trigger rapid clearance by hepatocytes and macrophages [43,44].

Platelet precursor megakaryocytes are the largest and rarest cells in bone marrow and produce 5,000 to 10,000 platelets per cell. These large cells are primarily localized in bone marrow, forming elongated structures called pro-platelets that develop into mature platelets that then break off into circulating blood via bone sinusoids [45–47]. Platelets may also be produced in the lungs from migrating megakaryocytes [48]. Under the control of the liver-produced cytokine thrombopoietin [49,50], hematopoietic stem cells in the bone marrow differentiate into common myeloid precursor cells that further differentiate in to megakaryoblasts, which are a precursor of the pro-megakaryocyte that forms into the fully differentiated megakaryocyte [51,52].

To produce their platelet progeny, megakaryocytes multiply their DNA content without dividing, in a process called endomitosis. During this process, the nuclear envelope breaks down and is reassembled again. Multiple cycles of endomitosis give the appearance of one enlarged lobed nucleus with multiple chromosome copies 4 N – 128 N [53]. Polyploidy enables megakaryocytes to up-regulate proteins and lipids in large quantities to assemble the invaginated membrane system (IMS) in the cytoplasm, creating extra surface area for the extension and formation of pro-platelets [54,55]. Formation of pro-platelets and

the eventual production of mature platelets requires the upregulation of multiple platelet-specific proteins, organelles and other factors that occupy the cytoplasm of mature megakaryocytes. Proteins, organelles, and RNA are packaged into newly forming platelets prior to their entry into blood circulation [53,56].

1.2.2 Platelet structure

Platelets are small in size, ranging from 2 to 3 μm in diameter and having a thickness of approximately 0.5 μm. If platelets were recognized as cells instead of as megakaryocyte cell fragments, they would be categorized as the smallest cell in the human body. Platelets are irregularly shaped, and in their inactivated state display a wrinkled discoid shape. These wrinkles are tiny folds that provide the platelet with extra surfaces for activation-induced shape change [57].

Each platelet is organized in to four zones: the peripheral zone, the membrane system, the structural (sol-gel) zone and the organelle zone, as shown in Figure 1. The peripheral zone consists of a lipid bilayer membrane covered with glycoproteins and glycolipids, referred to as the glycocalyx or pericellular matrix. Within the glycocalyx are multiple receptors that are important in the platelet's role in hemostasis: for example, glycoprotein (GP)Ib-V-IX complex, which binds to vWF on exposed subendothelial collagen; GP-VI, with direct collagen binding affinity; and integrin αIIb/β3 complex for fibrinogen binding and subsequent platelet aggregation. The glycocalyx has a high negative charge that provides a repulsive force, preventing spontaneous platelet aggregation and attachment to other components in blood or to the endothelial cell lining of the circulatory system [58].

On the platelet surface are randomly distributed openings of the open canalicular system (OCS). The OCS is an internalized cell membrane providing more extra surface and cell membrane for platelet shape change [59]. These open canals are also a route for platelet secretion via de-granulation and uptake of plasma components like fibrinogen [60,61]. The

OCS is a part of the platelet internal membrane system, which also includes the dense tubular system (DTS) and, in less than 1% of normal platelets, Golgi complex residues from megakaryocyte precursors [62]. DTS is a smooth endoplasmic reticulum (ER) system that serves as the main storage pool of Ca^{2+} and plays a key role in Ca^{2+}-regulated platelet activation [63,64].

In a resting platelet, the cytosolic Ca^{2+} concentrations are maintained at 0.1 μM and upon activation there can be tenfold or more upregulation in concentration. Thrombin, adenosine diphosphate (ADP) and thromboxane A2 (TXA_2) all bind to different platelet receptors to activate phospholipase C, which generates inositol triphosphate (IP3), a key signal in Ca^{2+} release from DTS. Depletion of Ca^{2+} storage in DTS activates the Ca^{2+} sensor protein STIM1, which then triggers an influx of extracellular Ca^{2+} through Orai1, a calcium channel in the plasma membrane [58,65]. The concentration of free Ca^{2+} in the platelet cytosol regulates various proteins that affect the function of the platelet cytoskeleton [62].

The platelet structural zone includes the cytoskeleton, which is mainly constructed of actin filaments and tubulin in microtubules. Platelet microtubules are assembled from α and β tubulin and are arranged in circumferential coils near the cell wall and support the contractile element of the cytoskeleton during platelet shape change. A number of reports suggest that assembled microtubules are important for the platelet to be able to retain its discoid shape [66–68]. The membrane-based skeleton in platelets contains spectrin and interacts with membrane glycoproteins and lipids as well as cytoskeletal proteins. The spectrin skeleton also has a role in proplatelet formation, regulating the size of the forming platelets [69]. The motor protein myosin is also a part of the structural zone responsible for initiating shape change and plays a role in platelet internal contraction, moving dense- and α-granules to the center of the platelet; this may ultimately cause degranulation via the OCS [57,70,71].

Platelets lack a nucleus, but contain various other organelles like granules, lysosomes, and mitochondria. Platelet α-granules are the most abundant organelles, with 50

to 80 copies on average. These α-granules contain various types of proteins secreted or expressed on the platelet surface with multiple functions like cell adhesion, cell recruitment, cell growth, coagulation, inflammation, tumor metastasis, host defense and immune modulation [57,72]. Many membrane-based α-granule proteins are already expressed on the surface of resting platelets, including integrins αIIbβ3 and glycoprotein GPVI, while others such as P-selectin are specifically relocated from α-granules to the surface during activation [73,74].

Dense granules are less abundant, with 3 to 8 copies on average per platelet, and contain both proteins and platelet agonists in the form of nucleotides and neurotransmitters. Dense granules and their contents play a role in hemostasis and contribute to thrombus formations with endo- and autocrine effects [75].

Not all platelets contain lysosomes, but those platelets that do contain these organelles have, on average, 1 to 2 copies. Lysosomes contain protein- and carbohydrate-degrading enzymes. The role of lysosomes in platelets has yet to be fully elucidated, though it is possible that these organelles play a role in endosomal digestion [75].

There have also been reports on platelet organelles named T-granules. These granules were first described in 2012 [76] and contain TLR9, protein disulfide isomerase (PDI) and the SNARE family protein vesicle-associated membrane protein 8 (VAMP8). The prefix letter T stands for the tubular structure of these organelles. Toll-like receptors (TLRs) 2,4 and 9 have been detected on platelets, and their expression is elevated during platelet activation [77,78]. Thon et al. proposed a pathway where T-granules are recruited to the platelet surface during activation and release TLR9 via VAMP8 and contribute to platelet secretion [76]. Others have noted that PDI is specifically located in the ER and thus the DTS of platelets and that of T-granules are more a compartment of DTS and not a specific organelle [79].

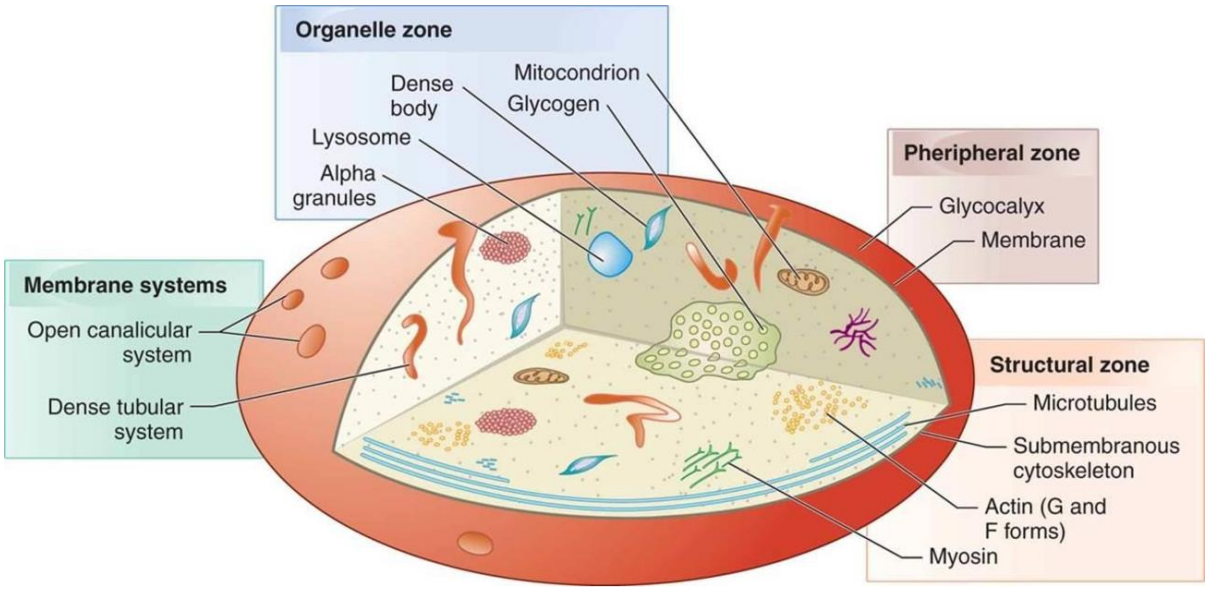

Figure 1. The ultrastructure and content of a resting discoid shaped platelet and its organization into four zones: the membrane system, the organelle zone, the peripheral zone, and the structural zone. [80]

1.2.3 Platelet function

1.2.3.1 Platelets promote hemostasis

Platelets have multiple functions in the human body, the most notable being their role in hemostasis, ensuring the integrity of the endothelial cell linings of the vessel walls in the circulatory system. The platelets' function as immune response modulators has been increasingly studied in recent years, as well as their pathogenic role in thrombosis, cancer immune evasion and metastasis.

Because high shear rates in contracted vessels can promote platelet aggregation and activation, endothelial cells effectively promote the resting state of platelets by secreting vasodilators nitric oxide (NO) and prostacyclin 2 (PGI_2), thus limiting the platelet shear stress exposure (Figure 2A) [81–83]. In addition, during steady state, endothelial cells express on their surface ATPase CD39, which has the ability to hydrolyze circulating ADP, preventing ADP platelet stimulation [84]. NO and PGI_2 have a direct effect on platelet activation by suppressing Ca^{2+} release from the DTS into the cytosol and pumping cytosolic Ca^{2+} out of the platelet [85,86]. This brake on Ca^{2+} efflux from the DTS into the cytosol is, in part, released by autocrine effects of dense granule secretion of ADP binding to the $P2Y_{12}$

receptor, which inhibits the downstream signal of PGl_2 binding [87,88]. PGl_2 binds to prostacyclin receptor IP in the platelet membrane, while NO is diffused through the membrane; through their regulation of low cytosolic Ca^{2+}, they inhibit aggregation, de-granulation and cytoskeletal rearrangement [89].

The actual formation of a platelet thrombus and, eventually, a hemostatic plug to stop bleeding is a complex process with multiple contributing factors and is intertwined with blood coagulation. Novel aspects of this process are still being discovered and debated in the platelet scientific community. As illustrated in Figures 2A and 2B, an injury to the endothelial cell lining, the interior of a vessel, exposes subendothelial collagen with a high binding affinity for von Willebrand Factor (vWF), which is expressed and secreted by endothelial cells [90]. The collagen-bound vWF caches nearby platelets by binding to platelet receptor GPIbα, a part of the glycoprotein complex Ib-V-IX. [91–93]. vWF self-association also plays a role in platelet adhesion, whereby circulating vWF can bind to platelet-bound or subendothelial vWF, a process increased by high shear rates [94,95].

Glycoprotein VI and integrin protein complex α2β1 bind directly to the exposed collagen for more stable platelet adhesion and activation [96,97]. All of these receptor and receptor complexes have downstream signals that results in platelet activation and thrombus formation by upregulation of cytosolic Ca^{2+} from the DTS and extracellular space, release of ADP and thromboxane via de-granulation, and increased binding affinity of the integrin complex αIIbβ3 for fibrinogen [98–102]. In addition to vWF and collagen, the subendothelial extracellular matrix contains laminin, which mainly binds to the α6β1 receptor [103], and fibronectin, which binds to the α5β1, as well as αIIB3 [104] receptors and thrombospondin binding to GPIbα [105].

The mechanism of platelet tethering to the subendothelial matrix is closely related to shear forces, with high shear forces triggering GPIbα and vWF binding [106], while low shear forces relate to platelet binding to collagen, fibronectin and laminin [89]. These first responders spread out and form a monolayer, secreting agonists to activate additional

platelets, which are recruited to the injury site by P-selectin binding and rolling on activated endothelial cells. The rise in cytosolic Ca^{2+} in activated platelets increases phosphatidylserine (PS) exposure on the platelet surface, making it more procoagulant. PS on the surface of platelets and released microparticles provides binding sites for clotting factors that accelerate the coagulation cascade and the release of tissue factor (TF) (Figures 2B and 2C) [107–109].

The formation of a hemostatic plug is initiated via the extrinsic pathway when blood reaches TF in the outermost layer of the vessel, triggering the coagulation cascade that includes converting numerous clotting factors to their activated state. Both the intrinsic and extrinsic pathways come together in the common coagulation pathway to generate thrombin from prothrombin for the conversion of fibrin from fibrinogen to achieve a stable plug (Figure 2D).

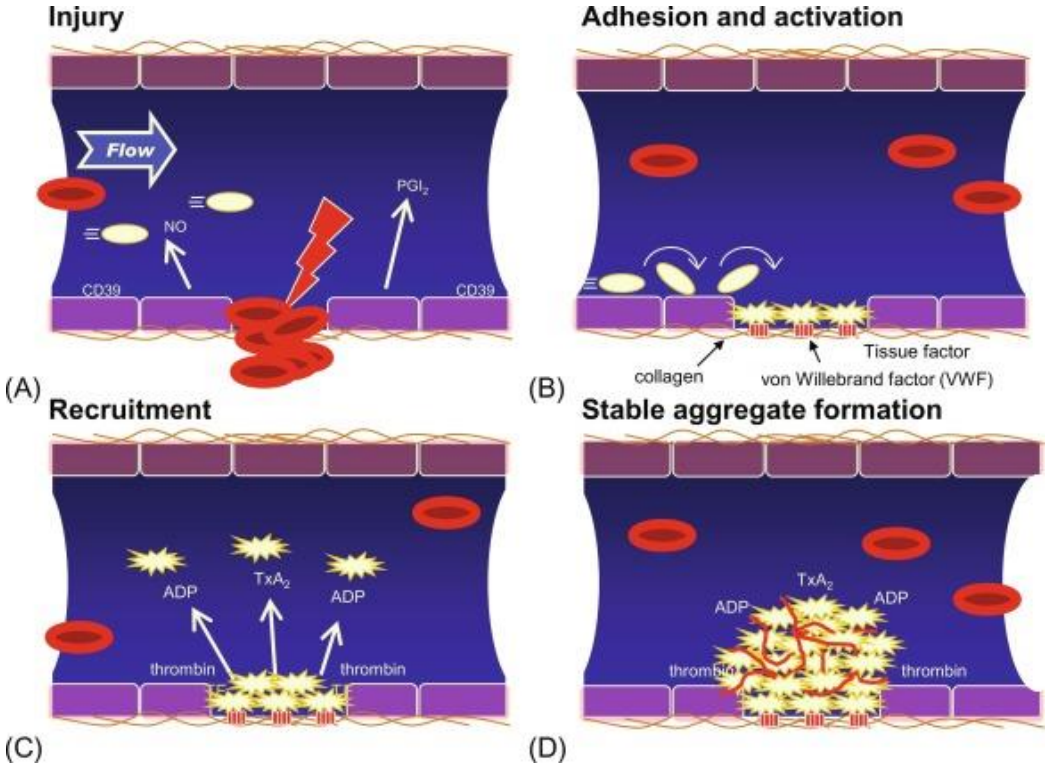

Figure 2. Hemostatic thrombus formation in flowing blood. (A) An injury to the endothelial lining with blood loss. (B) The binding of platelets at the site of injury. (C) Activation and recruitment of additional platelets to the site of injury. (D) Formation of a stable hemostatic plug of platelets and fibrinogen, is shown in red, stopping the blood loss [84].

Thrombin has dual counteracting roles in the coagulation and thrombus process. Thrombin activates platelets via the PAR 1 and PAR 4 receptors and generates fibrin, but it

also activates anticoagulant protein C, which, along with its cofactor protein S, inactivates clotting factors, thus acting as a brake on the coagulation cascade [84,110]. Eventually, plasmin is formed from plasminogen leading to fibrinolysis and clot degradation [111].

Recently, with improvements in microscopic technology and the use of intravital imaging, it has been shown that there is an activation gradient in the hemostatic plug, with the core platelets close to the injury being procoagulant and highly activated mainly by thrombin, and platelets in the outer shell being P-selectin negative and less activated (mostly by ADP and thromboxane) [112,113]. Numerous platelet functional roles and attributes have been discovered by analyzing inherited and acquired platelet defects that often lead to thrombocytopenia, ineffective thrombus formation and coagulation.

1.2.3.2 Platelets in immunological response

The role of platelets promoting endothelial integrity and hemostasis has been recognized and studied for more than a century. More recently, additional roles of platelets have been recognized and intensely studied, including immune modulation and antimicrobial defense. Platelets are constantly patrolling the vasculature, making them ideal first responders of the immune system. In fact, platelets are equipped with diverse tools that play critical roles in direct pathogen response and leukocyte recruitment. Platelets contain pattern recognition receptors (PRR) including Toll-like receptors (TLR) that recognize pathogen-associated molecular patterns (PAMPs). TLRs are expressed on the surface of platelets and their extended pseudopodia, enabling sensing of foreign antigens [105–107].

Platelets play a further role in innate immunity response by pathogen encapsulation [119,120] and secreting antimicrobial peptides like platelet factor 4 (PF4) and RANTES, which are stored in the platelet granules [121,122]. Platelet granules contain numerous pro- and anti-inflammatory factors that modulate the immune response [123]. Platelets also play a role in adaptive immunity by presenting foreign antigens to other immune cells [124]. Platelets not only interact with T cells by antigen presentation, but also by direct signaling with the CD40 ligand and receptor [125,126]. Platelets can promote neutrophil activation and

neutrophil extracellular trap (NET) action [127].

How platelets inherit their multiple immune functions has not been fully elucidated. It has been proposed that platelets evolved from the hemocyte found in invertebrates that, in addition to immunological roles, promotes clotting of the hemolymph tissue, the invertebrate analog to vertebrate blood [128]. Recent investigations have provided evidence that platelet immunological attributes are obtained from the MK precursor cell.

In a recent publication by Cunin et al., a phenomenon called emperipolesis (where an intact cell is found in the cytoplasm of another cell) was studied *in vivo* and in an *in vitro* inflammation mouse model. Using video and figures generated with confocal and electron microscopy, Cunin et al. elegantly showed neutrophil and MK attachment and the subsequent entry of the neutrophil into the MK cytosol. Once inside, a fusion of the neutrophil membrane and the DMS of the MK occurs, thus a transferring the neutrophil membrane to the membrane of future circulating platelets. The neutrophil attaches to the MK via β-integrin and enters the MK through a vacuole termed emperisome that releases its cargo directly into the cytosol of the host cell [129,130].

Recent data shows evidence of platelets extending their immune modulation role through extracellular vehicles (EVs) that contain proteosome activity, and the ability for antigen presentation via MHC-I in mice. Due to their small size, EVs are able to cross tissue barriers and enter the lymphatic tissue and organs where antigens are presented to cytotoxic CD-8 T-cells [131].

1.2 Platelet miRNA and transcriptomics

1.3.1 The platelet proteome

Megakaryocytes upregulate platelet-specific proteins that are sorted into the forming platelet, providing newly formed platelets with the majority of proteins essential for platelet function, both in resting and activated states [132]. The platelet proteome appears to be about 85% stable between healthy individuals [45,53,133]. The platelet proteome profile can be influenced by the ability of platelets to endocytose plasma proteins from circulation [134,135]

and to degrade proteins via the ubiquitination and proteasome pathway [136,137]. In addition, a large proportion of platelet proteins are represented at the transcriptomic level. Warshaw et al. carried out important studies in the 1960s establishing active protein translation in platelets using ^{14}C-labeled amino acids [138]. The labeled amino acids were taken up by the platelets and incorporated into platelet protein extracts. The authors also showed that this protein synthesis was inhibited by puromycin treatment, which affects protein translation in the ribosome, but not by actinomycin treatment, which inhibits transcription. The authors further speculated that platelet protein translation included mRNA from megakaryocyte precursor cells and not, for example, cytoplasmic DNA [138].

In the late 1960s, other investigators reported on labeled amino acid incorporation into platelet contractile proteins from stable mRNA transcripts and on the existence of ribosomes in platelets [139–141]. Further evidence and analysis of platelet mRNA were established in the late 1980s after the invention of polymerase chain reaction (PCR) technology [142]. Following these investigations, integrin-regulated and signal-dependent protein translation became recognized [143–147], as did continuous continues translation [148], a process also detected in blood bank stored platelets [149].

RNA sequencing data indicate that platelets harbor 9000 to 9500 protein coding mRNA transcripts [69,150]. A recent study using a genome-wide transcripts database for platelets and megakaryocytes, generated by the Blueprint epigenome project and data from the analysis of 6 different cohorts, concluded that platelets contain 14800 protein coding transcripts, with high quantitative similarity between platelets and megakaryocytes [151].

There is some debate on how selective or random the megakaryocyte packaging of mRNA into pro-platelets occurs relating to observations of a weak correlation between platelet transcriptome and proteome [152–154]. Despite the lack of correlation between mRNA transcripts and expressed proteins, the platelet transcriptome has very low inter-individual variability and it has been speculated that the high number of transcripts with no corresponding protein in steady state platelets reflects the dynamics of the platelet proteome

in various platelet functions [155–157].

1.3.2 Post transcriptional control in platelets

Transcriptome translation into proteins can be regulated via various pathways including the mTOR pathway, mRNA splicing, intron retention and micro RNAs (miRNAs). All these post-transcriptional regulation tools exist in platelets [158–160].

Micro RNAs are small, non-coding, 18 to 24 nucleotide post-transcriptional regulators that many cell types utilize for fine tuning of their gene expression [161,162]. These small RNAs bind to complimentary regions of their target mRNA, inhibiting translation into proteins and, in most cases, facilitating degradation of their target mRNA [163]. Micro RNA genes are transcribed by RNA polymerase II, generating single-stranded primary-miRNA (pri-miRNA) transcripts with a double-stranded hairpin loop that contains the mature miRNA sequence. Processing of miRNA within the nucleus is initiated by DGCR8 RNA-binding protein that recognizes pri-miRNA transcripts and directs the ribonuclease III enzyme DORSA cleavage at the single/double strand junction. DORSA enables the release of a double-stranded pre-mRNA hairpin that is exported out of the nucleus by the shuttle protein Exportin 5. In the cytoplasm, pre-mRNA is captured by TRBP RNA-binding protein and is further processed by RNase Dicer, producing a short double-stranded pre-mRNA. The RISC protein complex is the next stop in the miRNA processing chain. A mature single-stranded miRNA is generated and guided by Argonaut 2 (Ago2), which binds to target mRNA, inhibiting translation or facilitating its degradation by endonuclease activity of Ago2 [164–167].

Single miRNA can have multiple mRNA targets. Alternately, a single mRNA can be regulated by different miRNA. Micro RNA and mRNA target pairing is regulated by the seed region, a sequence located between positions 2 and 8 at the 5’ end on the miRNA, and a complementary sequence predominantly in the 3’ untranslated region (UTR) on the mRNA target, although miRNA binding sites in the 5’ UTR have also been reported (Figure 3B). The binding of the miRNA seed region follows the classical Watson-Crick base pairing rule; however, complete homology is not required for miRNA binding and mRNA regulation.

Complete homology is related to mRNA degradation, whereas incomplete homology is more related to temporary translation inhibition where miRNA can attach and detach again, adding to the versatile role of miRNA as post-transcriptional regulators [168–171].

Serial analysis of gene expression (SAGE) has revealed that platelet mRNA transcripts are on average longer than transcripts in nucleated cells and, in addition, have a significantly longer 3' UTR region, a possible indication of the increased role of miRNA posttranscriptional regulation in platelets compared to other nucleated cells [172].

Transcription and miRNA processing within the nucleus is non-existent in platelets. However, platelets contain all the necessary components and machinery for the cytoplasmic part of miRNA maturation and mRNA binding [173]. The generation process of miRNA and mRNA binding is illustrated in Figure 3.

The existence of miRNA and their role in platelet function has been recognized and studied in recent years. According to an miRbase 2019 publication [174], 2654 mature human miRNA have been discovered and around 500 to 800 of them have been detected in platelets [175,176], regulating various platelet processes including platelet activation, reactivity, de-granulation and apoptosis [177–184]. Megakaryocyte maturation and platelet formation are also influenced by miRNA regulation [185–187].

An important study by Rowley et al. analyzed the effects of using conditional deletion of the Dicer enzyme in megakaryocytes and platelets in mice. This inhibition of the miRNA processing process had a reduction effect on most of the platelet miRNA that resulted in a platelet phenotype with increased αIIbβ3 receptor complex on the platelet surface. These platelets were more pro-thrombotic than wild-type platelets, implying that Dicer-processed miRNA regulate platelet reactivity and are important in both normal and pathogenic thrombus formation [188]. Platelet reactivity level is a contributing factor to vascular disease, including atherosclerosis and thrombosis [189,190].

Platelets are a major source of circulating miRNA, making platelet miRNA an attractive biomarker for platelet-related pathological processes [191,192]. A recent study

proposed a q-PCR test panel of specific miRNA as biomarkers of platelet activation and a tool to assess the risk of thrombosis or anti-platelet therapy [193]. As mounting evidence have shown a role of miRNA in platelet function, there is growing interest in miRNA research in platelet storage. Several studies have focused on miRNA in platelet blood banking, revealing active miRNA post-transcriptional regulation and changes in the miRNAome during storage.

Some reports have proposed using specific miRNA as markers of storage lesion or as potential targets in controlling the onset and acceleration of platelet storage lesion (PSL) [194–198]. Although this is an interesting possibility for tackling PSL, the modern complexity, practicality, and cost of implementing such methods in platelet storage practice need to be considered.

In addition to investigating changes in the platelet miRNA profile during storage, the effects of PC processing have also been investigated. Osman et al. reported pathogen inactivation (PI)-related alterations in six out of eleven miRNA included in the analysis of single donor apheresis; the same alterations were not observed in untreated or irradiated PCs. As additional steps in PC processing tend to affect the metabolic activity of platelets and contribute to PSL, and there are implications of miRNA having a role in the activation of platelets, some alteration in the miRNA profile would be expected. Our own analysis on BC PCs, published in paper I, did not confirm Osman's results on miRNA; in our study, only a single miRNA displayed treatment-related effects [199]. These results are discussed in more detail in paper I and in the discussion section of this thesis.

Platelet-derived Microparticles (MPs) size (100 nm to 1μm diameter), are diverse EV´s that contain cytoplasmic components, including proteins and nucleic acids. MPs have been implicated as key players in platelet-related hemostasis, as well as in pathogenesis [200,201]. Over 20 years ago, it was established that platelets release MPs during storage [202]. Different collection and processing methods can influence MP content [203], and it has been determined that PCs with high MP content are pro-coagulant [204,205]. More recently,

it has been shown that MPs released by platelets contain miRNA that can be delivered to other cells, potentially serving as a remote control of neighboring cells' gene expression [206–208].

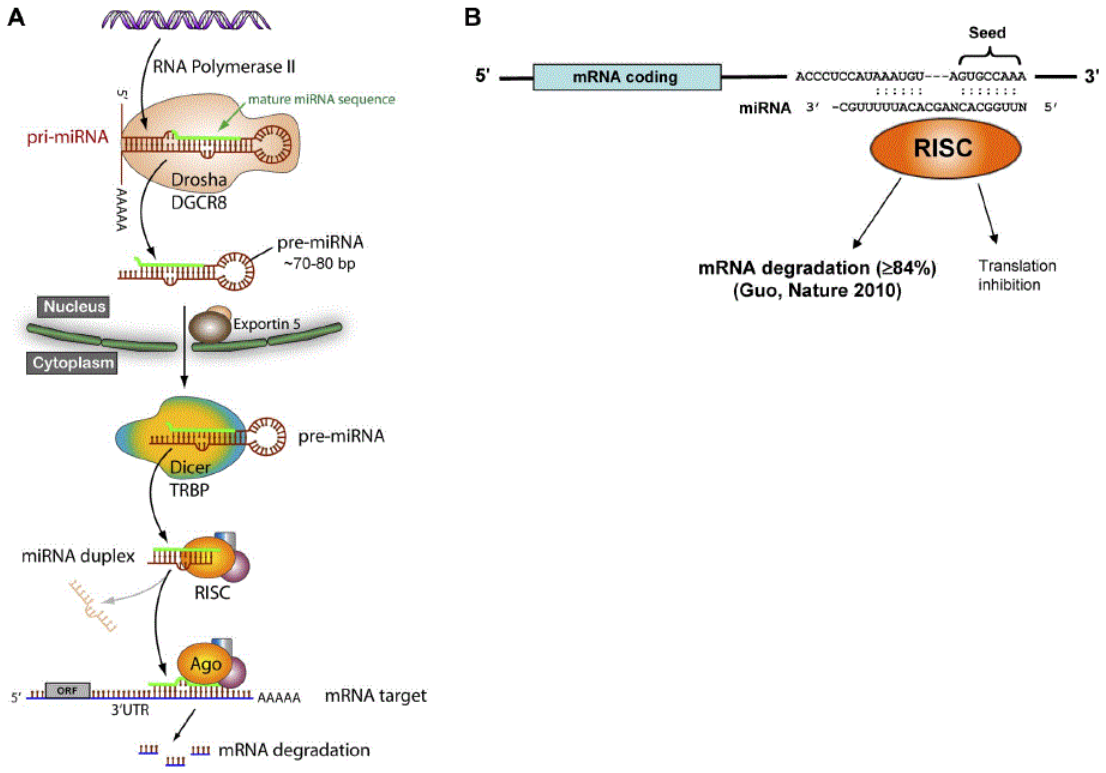

Figure 3. (A) Multiple editing steps in the generation of a mature single-stranded miRNA (B) RISC-bound miRNA binding to the seed region of an mRNA transcript [164]

1.4 Platelet Releasate

Platelets communicate with cells in their environment directly by receptor binding and surface expression of molecules like CD40L and P-selectin glycoprotein ligand-1 (PSGL-1), and by releasing various BRM factors [209–211]. As graphically descripted in Figure 4, during storage platelets are gradually activated and release a variety of factors and extracellular vesicles into the extracellular space. These factors and vesicles are collectively termed platelet releasate, and have possible implications in transfusion-related adverse events (TRAEs) via horizontal information transfer (HIT) [212]. In addition to releasing MPs by outward budding and plasma membrane fission platelets release other EV's like exosomes of endosomal origin from multivesicular bodies by exocytosis [213]. Platelets can shed proteins, referred to as the sheddome, that include the ectodomain of membrane

proteins proteolytically cleaved on the surface of the platelet. Examples of sheddome proteins are glycocalicin (the soluble form of GPIbα), GPVI and soluble CD40L (sCD40L) [214–216]. Platelet degranulation involves secretion of the granule content via exocytosis of the plasma membrane and OCS [214].

Secretomics is a category of platelet proteomics that specifically analyzes the secretion of proteins out of the platelet. Many different proteins have been detected in the platelet secretome, with some inter-individual variation, although some analyses indicate that there is a core set of around 300 proteins with limited inter-individual variation [132,154,217].

As discussed in Chapter 1.2.3, platelets are major players in immunological response and, therefore, the immune modulating effects of allogenic PC transfusions are of research interest. The activation status of platelets, protein surface expression and secretion into the storage media have been investigated in relation to adverse events. TRAEs induced by transfusion of PCs are more frequent than those from plasma or red blood cell (RBC) transfusions [218].

Standard PC storage conditions (room temperature with agitation) keep platelets metabolically active. These storage conditions, as well as exposure to additional stimulants like anticoagulants, preservatives, gases and plastics, gradually activate the stored platelets over time, with release of granule contents into the storage media [219]. Platelet-derived components role in TRAE was recognized when leukocyte reduction of PC units did not have the same effect on reducing the TRAE as observed in leukocyte reduced red cell units [220,221]. It was subsequently established that plasma components in the PCs, rather than the platelets themselves, were the source of these effects. The concentrations of specific biological response modifying cytokines, for example IL-6,IL-8 and TNF-α, have been found to be related to increases in TRAEs, such as febrile nonhemolytic transfusion reaction (FNHTR), and these plasma components increase in concentration in correlation with PC storage time [222–224].

A well-known platelet-released culprit in TRAE is sCD40L [219]. CD40L is in

abundance in α-granules, and upon activation is highly expressed on the surface of platelets. It is subsequently cleaved from the surface and released as soluble sCD40L. Reports on the cellular distribution of CD40L estimate that platelets contain >95% of all CD40L in circulation [219,225]. Different cell types express the CD40 receptor and are potential targets of transfused sCD40L; these include T-cells, endothelial cells, monocytes and their derivatives, macrophages. CD40L binding to endothelial cells can promote leukocyte recruitment and migration in to inflamed tissues.

Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related mortality and, although not without debate, sCD40L has been implicated in pathogenesis of TRALI by neutrophil priming in the lungs [226–228]. It is clear that TRALI is a multifactorial condition and the underlying medical conditions of a patient have an impact on outcomes, as does the patient's own platelets. In animal studies, allogenic platelet lipids have also been implicated in TRALI[229]. A recent study by Tariket et al. using a mouse model of the disease and a neutralizing sCD40L antibody showed reduced pulmonary edema and neutrophil activity [230].

RANTES a α-granule-stored chemokine, has also been detected in relatively high concentrations in stored platelets has been implicated in TRAE-like allergic reactions and FNHTR [231,232]. Many other secreted BRMs have the potential to induce TRAE in transfused patients, especially pro-inflammatory ones like PF-4, OX-40, MIP-α, IL-27 and IL-13 [233–235].

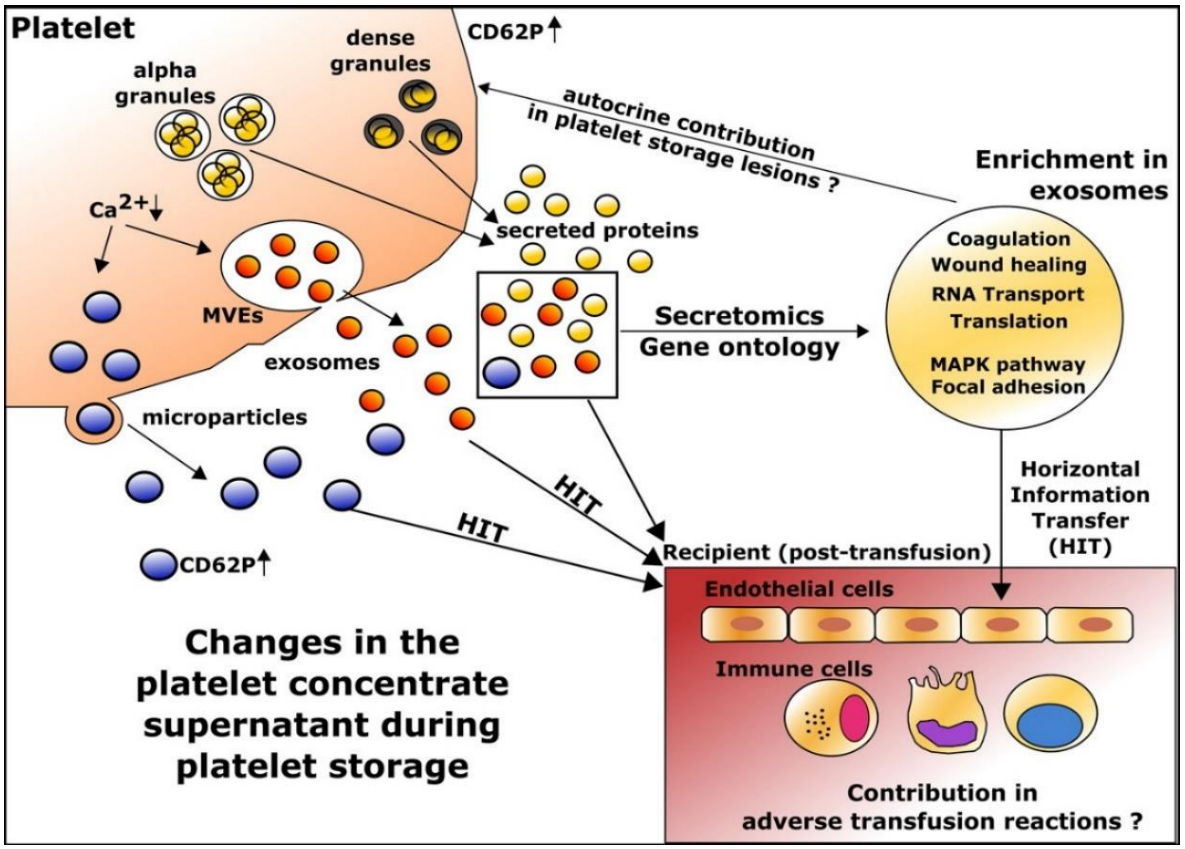

Figure 4. An illustration of various release mechanisms in platelets, including secretion of proteins and release of microparticles and exosomes into the storage media. Also shown are potential effector cells in adverse transfusion reactions [212]

1.6 Platelet blood banking and transfusion

One blood donation can save up to three different patients if the whole blood (WB) is separated into three components: plasma; red blood cells (RBCs); and PC. Modern health care uses blood component therapy, as it reduces unnecessary exposure of patients to components in WB when only specific elements from the WB donation are needed; for example, RBCs can be used to increase hemoglobin, while coagulation factors can be obtained from plasma. In developing countries and on military front lines, WB transfusions are still in practice. There are some reports that leukoreduced WB are preferrable for some indications, namely active bleeding [236,237].

1.6.1 Harvesting platelets

Since blood component processing was introduced, different techniques for the collection and processing of PCs for storage in blood banks have been developed. One such technique is the single donor platelet-rich plasma (PRP) method practiced in most processing centers in the USA, and the second main technique is the pooled BC platelet method, which is preferred in Europe. The key differences between these two methods are the WB centrifugation and separation steps. In the BC method, there is a high g-force hard spin that separates the WB into plasma, a RBC layer and a BC layer containing platelets and leukocytes. The BC layer is removed, and at this point contains a small amount of the RBCs and plasma. Commonly, four to six BC are pooled, along with additive solutions, to create a single dose of PC product. The BC pool is then subjected to a soft spin, separating the platelet concentrate from the leukocytes.

In the PRP, method there is a soft spin with low g-force that separates the platelets and plasma (in one layer) from the RBCs and 30-50% of the leukocytes. A second hard spin separates most of the plasma from the platelets, producing a platelet pellet that is generally resuspended in 50 to 60 mL of residual plasma. In order to avoid multiple transfusion of small PRP units, it is common practice to pool five to ten PRP units to generate a single PC product for transfusion. Both processing methods include leukoreduction steps to reduce incidents of TRAE. Additional processing steps are applied to further deplete platelets of leukocytes for the prevention of transfusion-associated graft vs host disease (TA-GVHD). These methods include irradiation, photochemical inactivation and possibly additional filtering [235,238,239].

Of the two platelet processing techniques, PRP has a longer history; however, there are only few countries still using this method. Comparative analysis has shown that there are higher levels of aggregation and activation of platelets using PRP, resulting in acceleration of PSL compared to the BC method. The close proximity of the platelets in the platelet pellet after the second spin and the low volume, high platelet count storage of PRP likely produces

these difference observed [240]. Additional advantages of the BC method are that: 1) it is more automated process with a streamlined workflow in compliance with good manufacturing practice (GMP); 2) there is higher plasma recovery for fractionation or component production. On the other hand, there is lower RBC recovery using the BC method compared to the PRP method [241].

Apheresis is an additional option for acquiring platelets for medical use. With this technique, which uses built-in centrifuging and automatic addition of anticoagulants and storage solutions, specific components of the blood are collected, while other components are circulated back into the donor. Single donor apheresis PCs minimize donor exposure, lowering the risk of infectious agent transmission and can be antigen- and human leukocyte antigen (HLA)-matched to specific patients.

There have been reports of lower frequencies of bacterial contamination in apheresis compared to PRP platelets. However, these observations have not been confirmed when comparing apheresis to the BC method [242,243]. The apheresis collection technique does have some drawbacks. It is time consuming and requires considerable expertise [241]. During apheresis collection, there is a risk of citrate toxicity due to the potential for citrate to be infused back into the donor, where it can bind Ca^{2+} in donor plasma causing hypercalcemia. Incorrect use of apheresis machine can do harm to the donor.

There are conflicting reports on donor adverse events favouring apheresis [244] or WB [245,246] collections. Regarding transfusion efficacy reports, apheresis and BC are comparable, while PRP inferiority has been documented compared to apheresis [247]. In our own research department of the Blood Bank in Iceland, the metabolomic activity during storage has been analysed and compared for BC and apheresis. In these analyses, two shifts in metabolic activity resulting in three different metabolic phenotypes relating to zero- to three-, four- to six- and seven- to ten-day old PCs were discovered. Apheresis PCs showed a clearer activation phenotype than BC PCs [248,249]. It has been noted in some reviews on this subject that the overall differences between the collection and processing methods are

minimal, and more focus and effort should be on standardizing platelet transfusion and better defining platelet quality and donors from whom high quality platelets can be produced. A mix of WB and apheresis methods for platelet collection is likely to make the most of donated blood and result in stable platelet stocks and availability [250][241].

Along with bacterial contamination risk, a PSL limits the storage time of PC. Due to their storage conditions, platelets are gradually activated and display a metabolic shift during storage [251]. Consequently, granules release their contents into the storage media. Some of these contents have autocrine effects, which promotes further activation, shedding of surface receptors, PS exposure, EV release, apoptosis-like lesions and, ultimately, platelet lysis. PSL affects *in vitro* aggregation and *in vivo* viability and recovery [252–257]. Thus, specific storage solutions, also known as platelet additive solutions (PAS), have been developed to reduce PSL.

1.6.2 Platelet additive solutions

The first generation of PAS were developed in the 1980s, and since that time there have been multiple generations with various combinations of nutrients and buffers. All but one generation of PAS contain citrate anticoagulant, and generally PAS contains acetate as a glucose substitute. Acetate is a substrate for mitochondrial oxidative phosphorylation, promoting less lactate production than glycolysis and results in optimal pH levels. Some generations of PAS contain phosphate to stabilise pH, potassium and magnesium as buffers on the glycolysis rete, gluconate to limit glucose consumption, and glucose, particularly when plasma is highly diluted with PAS [258,259]. PAS were also developed as a substitute for plasma used for fractionations or transfusion. The dilution of plasma by PAS reduces the rate TRAE, as donor plasma may contain immune-modulating components as well as HLA and human platelet antigens (HPA) antibodies [251,260,261].

PSL accelerates with increased storage time, as does risk of bacterial contamination of clinical relevance [26,253]. With improved aseptic protocols during collection, diversion of the first aliquot of the WB collection, donor deferral regulation and rigorous screening, the

rate of transfusion transmission of pathogens has dramatically decreased in recent times. Even with these safety measures, TTBI still occur at an estimated rate of 1:2000 to 1:100,000, resulting in increased morbidity and high mortality rates [262–264]. To decrease the risk of TTBI, some regions limit PC storage time to below five days: for example, maximum storage time is four days in Germany and three days in Japan. Even with such short storage times, TTBI cases are reported in these countries. Limiting storage time results in challenging stock management, with both PC shortages and high discard rates [265–267]. To maximise the storage of safe PCs, bacterial testing or bacterial inactivation treatment can be applied. Using conventional bacterial culture testing, there usually is a minimum holding period of 24 to 48 hours before PCs are released [16].

1.6.3 Bacterial screening

Compared to the short storage time for PCs, the holding time for primary bacterial testing is relatively long. In general, bacterial screening tests require a holding period of 24 up to 72 hours for growth of bacteria in culture or in the PCs themselves for detection. The longer the holding period, the greater the sensitivity and specificity of the test, especially concerning slow-growing bacteria [268]. Culture for up to seven days is common and in many instances a positive result is reported after the PC has already been transfused [269,270].

Rapid tests have been developed that produce results within 4 hours. Rapid methods include nucleic amplification tests (NAT) targeting specific strains of bacterial DNA and global tests targeting bacterial 16s or 23s ribosomal RNA [193,271,272]. Tests applying nucleic acid staining and flow cytometry techniques have been developed but are currently not used in routine practise [193]. Rapid antigen tests that detect bacterial peptidoglycans or lipopolysaccharide and lipoteichoic acids can be used as standalone tests for inventory screening or in combination with primary culture tests to prolong platelet storage for up to seven days [273,274]. To maximise bacterial screening sensitivity, large volume primary culture with secondary culture or rapid detection release tests can be used in combination. Rapid antigen release tests are less sensitive than secondary culture tests, resulting in a

higher risk of false negative results, while secondary culture testing later during storage might detect clinically insignificant bacterial contamination [275]. Some of the rapid detection systems are complex, with a built-in risk of human error.

Pathogen screening involves fewer processing steps and less stress inflicted on platelets compared to the use of pathogen inactivation (PI) treatment. However, there can be some added risk of contamination when sampling PCs for bacterial testing, especially when multiple testing at different time points is required or during retesting when initial test results are inconclusive. As in most biological testing, these tests are not 100% accurate and can produce false positive results, resulting in the potential discarding of uncontaminated products, or, more seriously, false negative results that can lead to TTBI and sepsis in transfusion recipients [276]. With the goal of producing safer PCs with maximum storage time, methods for inactivation of a broad range of pathogens – viral, bacterial and protozoan - have been developed.

1.6.4 Pathogen inactivation technology

There are three pathogen reduction methods available today for PCs, all of which utilize photo or photochemical techniques: the amotosalen-UVA Blood System (Cerus Corporation, Concord, CA, USA); the Riboflavin-UVB PRT (Pathogen Reduction Technology) system (Terumo BCT, Leakwood, CO, USA); and the UVC-Platelet system (Macopharma Mouvaux France). All three methods illuminate the product with UV light of different wavelengths. The Theraflex system uses no photosensitisers and relies solely on UV-C treatment, which causes pyrimidine dimers in nucleic acids and thereby preventing replication of pathogens and leukocytes [277]. The Mirasol system also includes the addition of the photoreactive compound riboflavin, while Intercept uses an amotosalen additive [278]. Riboflavin is vitamin B2. It binds to a range of biomolecules including nucleic acids. With UVA/B light treatment, numerous molecular changes occur, including formation of reactive oxygen species (ROS) that damage and cause breaks in DNA and RNA, preventing replication of pathogens and residual leukocytes [279]. As vitamin B2 is naturally present in

human circulation, there is no need for extra measures to remove leftover riboflavin. Amotosalen is synthetic version of the plant-produced organic compound Psoralen that will intercalate in helical regions of nucleic acids and, upon exposure to ultraviolet A (UVA) light, forms permanent adducts preventing transcription and replication pathogens [280,281]. amotosalen-UVA also includes a PC incubation phase with a compound absorption device (CAD) for the removal of residual amotosalen to avoid toxicity [282].

Of the available technologies, the Intercept system is the most studied and has the longest history of routine use, with clinical approval in Europe and the USA. Mirasol received a CE mark in 2007 and Theraflex in 2009. Intercept is currently in use in more than 40 countries, and Mirasol in 20. Theraflex is still being clinically evaluated and is currently not in routine use [283]. As PI technology effectively inactivates and reduces the viral load of the most common and serious transfusion transmitted infections TTI, including HIV (Intercept, Mirasol), HBV (Intercept) and HCV (Intercept), theoretically by implementing PI, blood collection establishments could replace not only bacterial testing but also expansive serological and nucleic acid testing [284–286]. Many reports on PI efficacy provide results on log reduction in PCs spiked with relevant pathogens. However, these reports have been criticised for lack of standardization and failure to assess the efficacy of PI to prevent infection [287]. A recent summary of the amotosalen-UVA infectivity efficacy of *in-vitro* cell lines and *in-vivo* animal models provides further evidence supporting the safety of replacing some of the blood bank traditional screening with PI technology, although limited effects were observed for some pathogens like HEV [288]. In general, PI treatment does not efficiently reduce prions and some non-enveloped viruses, like HEV [34].

While extremely rare, viral screening can be subject to false negatives due to human error, undetectable early infection window and equipment malfunctions. Also rare, but occurring at a higher rate than false negatives, false positive screens can cause anxiety and discomfort for the donor [284,289–291]. The only true test for the safety of replacing viral screening with PI is real life data, and there is likely to be some reluctance for legislative

change in that direction. An important consideration is that PI methods are likely to also inactivate emerging pathogens for which there are no available tests. The option of protecting the PC inventory and, as a result, patients is a valuable attribute, as was evident in the recent Zika virus epidemic in the Americas and in the global SARS-CoV-2 pandemic [292]. To date during the SARS-CoV-2 pandemic, there have been no reports of transfusion related transmission of the virus, but numerous reports show successful inactivation by PI methods [293–297].

Even with the acknowledgement of PI efficacy in reducing TTI, there are concerns about reduced quality of PI PCs relating to lower platelet counts, platelet damage, increased activation and accelerated PSL. Potentially, these *in vitro*-recorded effects of PI on PC could translate into decreased hemostatic efficacy with increased PC and RBC utilization [298]. Each additional processing step during platelet collection is likely to induce cellular stress, leading to some degree of reversible or irreversible damage or changes to cellular state. To test these effects, a number of molecular tests, including functional tests for clot formation and coagulation, are available. Published *in vitro* results on the effect of PI on platelet quality from different studies sometimes contradict each other, highlighting possible impacts of different storage solution, type of plastic used in collection and storage bags, various collection methods, sample preparation and even donor variation when comparing results from different investigators.

To date there is no global gold standard test for platelet *in vitro* quality to give a decisive answer on the impact of different processing protocols. Compiling the available data from reports on *in vitro* analysis reveals that PI reduces platelet quality to some extent, at least at the laboratory level, albeit to different degrees depending on the type of marker and PI product used in the analysis [299–301]. A recent review by Feys et al. summarizes the different biochemical consequences of the three commercially available PI methods [300]. Effects on nucleic acids, miRNA and mRNA, which thus affect the platelet miRnome and transcriptome, have been attributed to amatosalen UVA treatment in two 2015 publications

by Osman et al., though donor variation and different storage solution effects could not be ruled out [302,303]. In a later publication using samples from their 2015 analysis and small RNA sequencing, the same group of researchers concluded that the miRNA profile of platelets was not affected by amotosalen-UVA or riboflavin-UVB. The investigators recognized specific loading of miRNA in platelet MPs that is hampered specifically by amotosalen-UVA treatment or the PAS (SSP+) additive solution [123]. The interaction of amotosalen and riboflavin with nucleic acids like mRNA, long non-coding RNA (lncRNA) is likely to occur to some degree, although the effect of this interaction on platelet quality or efficacy needs further investigation. The potential transcriptome effects PI treatment seem to only minimally affect the platelet proteome [304,305].

It has been documented that amotosalen intercalates into platelet mitochondrial DNA (mtDNA) without affecting membrane potential, or causing depolarization [306,307]. Amotosalen binding to mtDNA is often used as a quality control marker for successful PI treatment [308]. Amotosalen can bind to lipids in the platelet cell membrane, affecting signal transduction, specifically Akt protein kinase phosphorylation of phosphatidylinositol-tris-kinase (PI3K), which is involved in degranulation, aggregation and thrombus formation [309].

Riboflavin-UVB treatment has been shown to affect the platelet proteome in the form of oxidative damage [310][279]. ROS superoxide anion forms in plasma and PCs treated with Riboflavin-UVB display oxidative damage and significantly carbonylated proteins, a state associated with aging and disease [311]. Several protein modifications relating to Mirasol have been documented and include increased phosphorylation of VASP, a regulator of the cytoskeleton, and p38 mitogen-activated proteins kinases (MAPK) [312,313]. This triggering of p38 MAPK signalling has been implicated in increased apoptosis and altered function of mitochondria [314,315].

The UV-C system has yet to be put to routine use and is still under clinical assessment, so there are fewer reports on *in vitro* effects than there are for the amatosalen UVA and

Riboflavin-UVB systems. There is some data on possible photolysis effects that can dissolve disulphide bonds that, for instance, connect the fibrin receptor complex αIIbβ3, resulting in increased ligand binding, clearance from circulation and platelet exhaustion [316,317].

As noted in the review by Fyes et al. all three PI methods affect the metabolic activity of PI-treated platelets resulting in increased lactate production. Lactate concentration in transfused PCs has consistently been correlated with platelet recovery and survival [318,319]. UV-C and ribioflavin-UVB have been shown to have more severe effects on the metabolic activity of platelets, with increased lactate production compared to amatosalen UVA especially after day 5 of storage. In fact, some blood collection institutions have deemed ribioflavin-UVB treated PCs not to be of acceptable quality beyond 5 days of storge [320,321].

1.6.5 Safety and efficacy?

As has been clearly documented, the increase in safety using PI comes at some cost due to reduced quality, and to date the PI debate is mainly focused on the balance of these two observations as well as the cost of implementation. The amotosalen-UVA system was tested in two large controlled, randomized, double-blinded clinical trials involving thrombocytopenic patients, the SPRINT trial in the USA and euroSPRITE trial in Europe [322–324]. In both trials, hemostatic efficacy in controlling bleeding was comparable for PI and control PCs. In the SPRINT trial analysing single donor apheresis PCs, results showed that for amotosalen-UVA PCs, the 1 hour corrected count increment (CCI) was lower, transfusion interval was shorter, number of PCs per patient was higher and recorded adverse events was lower compared to the control, a difference not recorded in the European trial analysing pooled BC PCs. Data from these trials has been extensively reanalysed and has come under some criticism, specifically with regard to the use of CCI and the World Health Organization grading system for bleeding [325]. CCI analysis alone may not be the best indicator of platelet transfusion efficacy and contribution to patient blood coagulation status [326]. A systematic review on storage duration of PCs transfused for critically ill and

hematology patients observed lower CCI for older PCs; however, there was no effect on clinical outcomes such as bleeding, sepsis or mortality [327]. Thromboelastography (TEG) and thromboelastometry (TEM) have gained increased popularity as point-of-care assays to guide patient blood transfusion management [328][329]. Leitner et al. conducted a prospective observational study that showed a significant improvement TEM parameters after transfusion of PI PCs in patients receiving hematopoietic stem cell transplants. This post-transfusion improvement for the TEM assay did not necessarily correlate with CCI or 1-hour post transfusion increase [330]. The patient population in the SPRINT and euroSPRITE trials was thrombocytopenic, mostly due to myeloablative therapy and receiving prophylactic transfusion, covering about half of the patient population but excluding the other half, which included patients experiencing trauma, circulatory disease and digestive system disease [325].

A number of clinical trials have been conducted on PI PCs, with amotosalen-UVA being the most tested technology. A meta-analysis by Estcourt et al. [283], which included ten amotosalen-UVA trials and three riboflavin UVB, concluded that transfusion of PI-treated platelets does not increase the risk of death, bleeding, or serious side effects, though there was evidence of a reduction in platelet CCI, shorter intervals between transfusions, and increased risk of platelet transfusion refractoriness related to the transfusion of PI-treated platelets. Subgroup analysis between the PI technologies favored the amotosalen-UVA technology for all-cause mortality and transfusion intervals [283]. A recent study, not included in the Estcourt et al. meta-analysis, indicated that amotosalen-UVA treated PCs are non-inferior to standard platelets stored in PAS, but inferior to standard platelets stored in plasma [331]. Most of the existing clinical data is from trials analyzing transfusion efficacy in thrombocytopenic hematology patients requiring prophylactic transfusions. In a clinical study on the transfusion efficacy of amotosalen-UVA treated platelets in actively bleeding and massively transfused patients, PI treated platelets were non-inferior to standard platelets [332].

In addition to clinical studies, hemovigilance studies have also shown positive outcomes from using amotosalen-UVA treated platelets [333,334]. Amotosalen-UVA PI technology has been implemented in over 300 blood centers, with a positive safety profile concerning TTBI. In 2011, Switzerland did a nationwide implementation of amotosalen-UVA PI and in a report comparing rates of TTBI 7 years before and 7 years after implementation there were zero incidents after PI implementation , compared to 16 recorded cases, including three fatalities, in a seven-year period prior to implementation [335]. Similarly, regions in Belgium and France which have implemented amotosalen-UVA PI have recorded significantly lower rates of TTBI compared to prior implementation [336]. There have also been reports of reduced rates of other adverse events such as febrile and allergic reactions, likely resulting from inactivation of leukocytes by PI [337][324].

Regarding cost, there is usually some increase in cost if a new preventive or detection test is simply added to existing ones. With the use of PI, other blood safety measures can be relaxed or discontinued, such as x and γ irradiation, and bacterial and viral screening. Logistics can also be simplified in a standardized one-product-for-all inventory. Reduction or prevention of TTBI and other adverse events should also be included in cost estimates [338].

Over a million amotosalen-UVA treated PCs have been transfused safely worldwide and clinical studies have reported favorable results [283,324,332,339–343]. Nonetheless, there is ongoing debate about the clinical value of implementing amotosalen-UVA PI technology [299,344]. The debate between increased blood safety of PI vs hemostatic efficacy is likely to continue.

The concept of pathogen inactivation was first introduced in the 1980's after the discovery of TTIs of HIV and HCV. At first, PI was only considered for plasma and plasma fractionation products. At that time, there was limited evidence or interest in the complexity of platelets and their role in various biological process other than hemostasis. Platelets were considered cellular "dust", with little or no activity involving nucleic acids. Currently, there

is an abundance of evidence that platelets rely on mRNA, miRNA, and functional mitochondrial DNA for multiple functions. The currently available PI techniques inflict damage on pathogen nucleic acids, preventing replication and proliferation; considering that this is the method of pathogen inactivation, it is likely that nucleic acids of platelets are affected to some extent. The additional processing steps included in amotosalen-UVA technology also inflict more stress on the collected platelets, resulting in increased activation and accelerated storage lesion. Despite these observations, amotosalen-UVA PCs have been shown to have acceptable hemostatic efficacy in many clinical trials. Just as there is no universal *in vitro* marker for PC quality, the method of assessing PC efficacy in clinical trials is also not universal, nor is there a consensus on what platelet counts should trigger treatment with PC transfusion [16]. More direct and productive clinical trials are warranted, as laid out in a recent round table discussion [325]. When predicting potential effects of *in vitro* results on efficacy, the investigator should consider the intended use of the PCs, as it has been suggested that slightly activated platelets have better response times to injury. One major variable in assessing the impact of a new technology in the effectiveness of PC transfusion is the lack of evidence on overall effectiveness of PC transfusions, especially as prophylactic treatment in preventing bleeding [345,346]. Based on the available evidence, it appears that implementation of amotosalen-UVA impacts morbidity and mortality rates due to lowering of TTBI, and there is insufficient evidence showing negative effects on morbidity and mortality relating to decreased quality of amotosalen-UVA -treated PCs.

2 Purpose

The overall purpose of this thesis was to investigate the effect of the amotosalen plus UVA illumination pathogen inactivation (PI) method on the quality of platelets collected and stored under standard blood banking conditions.

The specific aims of the research project can be split into three sections:

- To assess the impact of PI treatment on the miRNA profile of BC PCs and identify if there is any correlation with the onset and acceleration of PSL. The role of small RNA species miRNA in platelet biology has gained interest in the research community. The effect of blood bank processing on the miRNA profile of platelets has implications for platelet quality during storage and possibly efficacy after transfusion;

- To assess the impact of PI treatment on protein concentrations in stored BC PCs. All aspects of collection, processing and storage increase platelet stress and PSL with activation-like properties. One marker of PSL is the release of proteins and other factors into the storage media. This release can have a negative feedback loop with autocrine effects and an increase in PSL, as well as potentially causing adverse events in patients;

- To assess the impact of PI treatment of stored PCs on their utilization in a national blood transfusion service. As there are implication of lower *in vitro* quality of PI PCs, we sought to identify whether these observations translated into different trends in PC utilization during a 5-year period before and after implementing PI.

3 Methods

3.1 Experimental design

A pool and split study was designed using extra BC platelets produced from WB, donated by healthy donors, and not used to produce patient PCs in the Blood Bank (BB), Landspitali - The National University Hospital of Iceland. A pool and split design (Figure 5) was used to exclude donor variation. Standard procedures for BC PC processing in the BB were used. These included pooling of 8 ABO-matched BCs to produce double dose PC units. Three 8-BC pools were further pooled in to one large ABO-matched 24-BC pool, mixed and split up again in to three identical single pools. Two pools were diluted in 65% PAS (SSP+) and one pool in 100% donor plasma. Before separation, BCs rested for 1 hour. The PC separation centrifuging protocol for 65% PAS was split into two steps: a first spin at 40 ×g for 2 min followed by a second spin at 463 ×g for 6.5 min. The 100% plasma unit underwent a harder spin at 987 ×g for 7 min. All PCs were automatically pressed and leukocyte-filtered from the BC pool, generating two types of PCs: one with 65% PAS and one with 100% donor plasma.

One 65% PAS PC received pathogen inactivation treatment (PI-PAS), while a second 65% PAS PC was used as a control (C-PAS). The 100% plasma PC unit was designated as a second type of untreated control (U-PL). The PCs were stored in a platelet incubator under standard blood bank storage conditions of 22 ± 2°C with gentle agitation and sampled on Days 1, 2, 4, and 7. The experimental setup is depicted in figure 5.

The experiment was repeated 8 times (n=8). Day 1 baseline samples for PI-PAS and C-PAS were obtained from the double dose PC. After sampling, the double dose PC unit was split up into single units, with one receiving PI treatment and the other one not (untreated control). A second baseline sample was obtained from the double dose 100% plasma PC unit. After sampling, the double dose 100% plasma unit was split into two single PC units; in this case only one was used for further sampling, while the other was discarded.

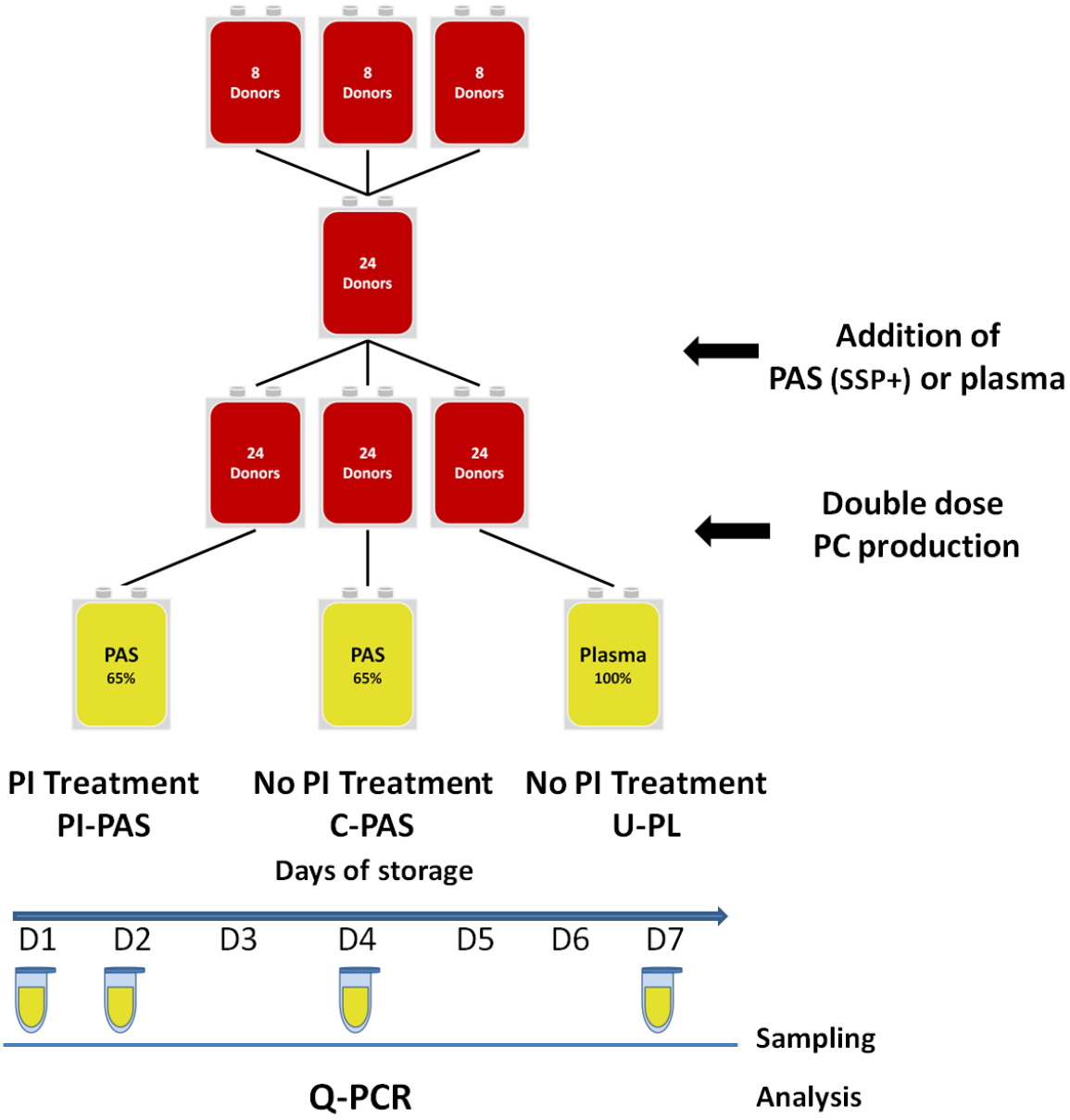

Figure 5. Experimental setup of pool and split design.

3.2 Quality control

In a closed sterile system, a 10 ml sample was collected from a single dose unit. Immediately after sample collection, metabolic activity via glucose, lactate, CO^2, O^2 and pH levels was measured using a blood gas analyzer (ABL90 FLEX). The sample was then split into 3.2 ml and 6.8 ml aliquots. In the 3.2 ml aliquot, platelet count, mean platelet volume (MPV) and platelet distribution width (PDW) were evaluated using hematology analyzer (CELL-DYN Ruby). Remaining platelets were further stained with antibodies for detecting the expression of platelet surface receptors integrin αIIb and glycoprotein GPIbα, membrane protein CD63 and Annexin V binding with flow cytometry (FacsCalibur). The 6.8 ml sample was

leukocyte-depleted using CD45 antibody-labeled dynabeads and subsequently platelets and supernatant were separated by centrifugation. Concentrations of sCD40L, sP-selectin and PF-4 in the supernatant were measured by enzyme-linked immunosorbent assays (ELISAs) on a microplate reader (Multiskan Spectrum). The platelet pellet and extra supernatant were cryopreserved at -80°C for RNA isolation and Luminex cytokine panel analysis.

3.3 miRNA profiling

For RNA extraction, platelets were lysed (TissueLyser) and the RNA precipitated using chloroform, ethanol, and spin columns. Synthetic spike-ins were used to control the isolation. Primers for 25 miRNAs, selected based on their potential role in platelet biology and previous unpublished microarray results, were used for reverse transcription into cDNA. Five control and one spike-in synthetic miRNA were used as controls. The qPCR reaction was performed in 384-well plates in a real-time PCR system (LightCycler 480) using no template controls to detect contamination or primer dimers. The miRNA included in this analysis are listed in Table 1, along with a summary of their relevance.

Table 1. Relevance of miRNA included in this analysis. Adapted from Arnason et al. [199]

miRNA	Relevance	Reference
hsa-miR-223-3p	• P2Y12 receptor binds ADP • Involved in platelet activation	[173]
hsa-miR-96-5p	• VAMP8 (Granule release) • Platelet reactivity	[180] [347]
hsa-miR-126-3p	• SPRED1, PIK3R2, CXCR4 signaling, VEGF pathway and endothelial progenitor cell (EPC) recruitment • Down-regulated in amotosalen-UVA - treated stored platelets	[348–350][303]
hsa-let-7e-5p	• let-7 family highly expressed in platelets • Down-regulated in amotosalen-UVA treated stored platelets	[303][175]
hsa-let-7g-5p	• let-7 family highly expressed in platelets • Down-regulated in amotosalen-UVA treated platelets during storage	[303][175]
hsa-miR-16-5p	• Up-regulated in stored platelets, apoptosis association • Down-regulated in amotosalen-UVA - treated stored platelets	[303][181]

hsa-miR-24-3p	• Down-regulated in stored platelets, apoptosis association • Down-regulated in PI-treated stored platelets	[303][181]
hsa-miR-326	• Up-regulated in stored platelets, apoptosis association	[181]
hsa-miR-320a	• Expression profile can be used to assess platelet quality	[196]
hsa-miR-7-5p	• Down-regulated in stored platelets, apoptosis association	[181]
hsa-miR-127-5p	Expression profile can be used to assess platelet quality	[196]
hsa-miR-376c-3p	• PAR4 expression (differential expression related to race)	[351]
hsa-miR-484	• Regulates mitochondrial fission by suppression of Fis1 translation (apoptosis)	[352]
hsa-miR-20a-5p	• Secreted by platelets • Vascular remodeling	[320][353][354]
hsa-miR-146a-5p	• miR-146a inhibits megakaryocytic production indirectly by suppressing inflammatory cytokine production from innate immune cells	[150][185]
hsa-miR-191-5p	• Highly expressed in platelets • Down-regulated in PI-treated stored platelets	[303][175][355]
hsa-miR-106a-5p	• Extracellular vesicle-packaged miRNA release after short-term exposure to particulate matter is associated with increased coagulation • Released by platelets • Down-regulated in amotosalen-UVA - treated stored platelets	[303][356]
hsa-miR-93-5p	• Based on previous array data	(unpublished)
hsa-miR-17-3p	• Based on previous array data	(unpublished)
hsa-1277-3p	• Based on previous array data	(unpublished)
hsa-miR-1260a	• Based on previous array data	(unpublished)
hsa-miR-1260b	• Based on previous array data	(unpublished)
hsa-miR-134-3p	• Based on previous array data	(unpublished)
hsa-miR-552-3p	• Based on previous array data	(unpublished)
hsa-miR-148a-3p	• Anti-miR-148a regulates platelet FcγRIIA signaling and decreases thrombosis *in vivo* in mice	[357]

3.4 Protein concentration in the storage media

Undiluted platelet supernatant was analyzed using Luminex xMAP Technology to quantify soluble proteins (growth factors, chemokines and cytokines). The Human Cytokine/Chemokine Magnetic Bead Panel (HCYTOMAG-60K) was used; it applies microspheres and fluorescent signaling to quantify 41 pre-selected proteins: EGF, eotaxin, FGF-2, FLT-3L, fractalkine, G-CSF, GM-CSF, IFN-α2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12P40, IL-12P70, IL-13, IL-15, IL-17A, IL-1RA, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGF-α, GRO TNFα, TNFβ, CD40L, and VEGF. Not all 41 proteins were included in the data analysis. RANTES and PDGF AA/BB had very high concentrations and were out of range of the assay, while FLT-3L, IL-2, IL-3, IL-4, and IL-6 were all below the detection limit of the assay. Therefore, the concentrations of 34 proteins were included in the analysis for comparing C-PAS and PI-PAS. Concentration differences were compared on Days 2, 4, and 7. The levels of each protein in the control and treatment groups on Days 2, 4, and 7 were also compared to those in a common baseline Day 1 sample.

3.5 Data presentation

With relatively large data sets with multiple variables, for example, groups of proteins, metabolites or genes responding to treatment at different timepoints, presenting the collected data with large data tables, multiple plots or complicated multidimensional plots is not always applicable. Principal component analysis (PCA) is a dimensional reduction method to preserve as much variance as possible in a lower dimensional output. Data points representing multiple measurements cluster along the x-axis according to the new variable principal component 1 (PC1) and along the y-axis according to PC2. The most important variances, e.g., biological differences, are represented in PC1, and other influencing factors like sampling or donor variation are represented in PC2. PCA plots were generated using R software. Heat map hierarchical clustering was performed using MetaboAnalyst.

3.6 Statistics

For miRNA analysis, all data was normalized to the average of assays detected in all samples. Fold change gene expression $2^{-\Delta\Delta CT}$ method was applied. Differences were

considered significant with a paired T-test p-value <0.05 after applying the Benjamini-Hochberg false discovery rate method. For protein concentrations, the normality of the data distribution was assessed analytically using the Shapiro–Wilks test and graphically with quintile–quintile (Q-Q) plots using DATAtab. Analysis of variance (ANOVA) testing using GraphPad Prism was applied to compare normally distributed data, and the Friedman test using DATAtab was used for data where a normal distribution was rejected. Differences were considered significant if p-values remained below 0.05 after applying the sequential Bonferroni correction method. For PC utilization, distribution of data was assessed using box and whisker charts. Differences between the two time periods (5 years before and 5 years after PI implementation) were assessed using two-sided t tests (with unequal variances) for continuous variables.

4 Results and discussion

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

In 2012, the Blood Bank of Iceland implemented pathogen inactivation technology for all produced PCs, and at the same time irradiation of PCs for selected patients was discontinued. However, reports of deleterious side effects PI treatment on the molecular level were of concern. One reported effect of amotosalen-UVA PI is altered miRNA and mRNA profiles of single donor apheresis PCs [302,303]. Our group used a pool and split strategy to study BC PCs, with the aim of limiting potential donor variation effects during qPCR miRNA analysis. In this our investigation, miRNA that had previously been shown to be affected by amotosalen-UVA PI were included, as well as additional miRNA from an unpublished miRNA array analysis and miRNA with published evidence of having a role in platelet function. In contrast to previous published data from Osman et al. [303], limited effects were observed on the selected miRNA relating to the amotosalen-UVA treatment. One miRNA, miR-96-5p displayed significant PI treatment-related downregulation. In the 100% plasma-stored control PCs, there was also a drop in miR-96-5p levels although they were not as significant as for the PI treatment. Our own unpublished data showed that miR-96-5p is also down-regulated in PCs treated with irradiation (Figure 6).

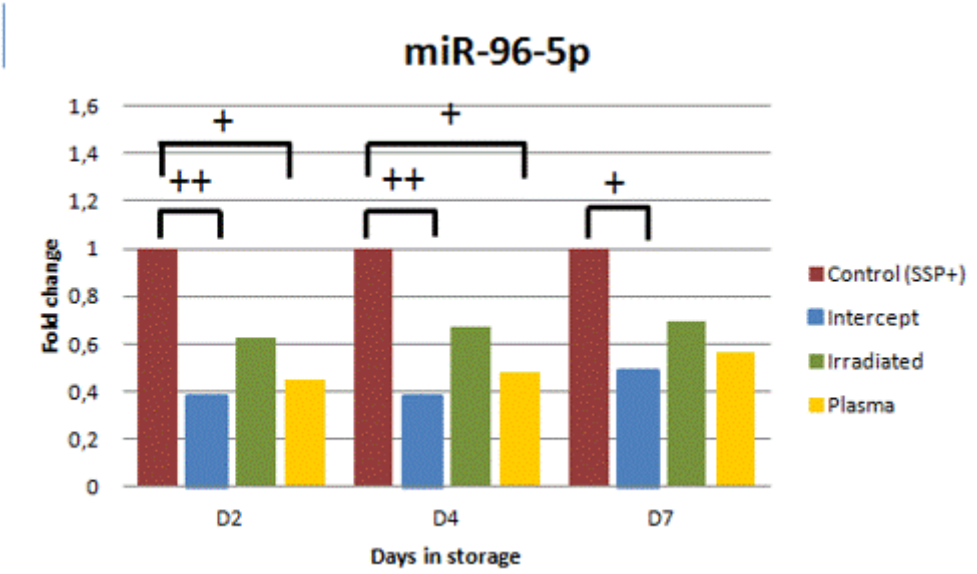

Figure 6. The fold change in miR-96-5p during storage of amotosalen-UVA-treated PCs (blue), Irradiated PCs (green) and PCs in 100% plasma (yellow) in relation to the standard (control) PC (maroon). P-value < 0.05 (+) and < 0.01 (++)

These observations imply that other additional processing, or even the storage media, can impact the miR-96-5p level in stored PCs. Lower levels of a miRNA can point to an accelerated degradation of that specific miRNA or exocytosis via extracellular vesicles. Both are possible effects of increased activation. Vesicle-associated membrane protein 8 (VAMP8) mRNA is one of many targets of miR-96-5p. In platelets, VAMP8 has a role in degranulation, and higher levels of VAMP8 mRNA have been detected in subjects with hyperactive platelets [180]. In our data, we observed significantly higher expression of P-selectin at the end of storage and increased shedding of GPIba in PI-PAS than in C-PAS. We did not do direct correlation analysis of miR-96-5p with markers of activation and PSL, however there are indications that lower levels of miR-96-5p in PI-PAS are related to the acceleration of PSL. Our main observation was that, at least for the 25 miRNA profiles included in this publication, amotosalen-UVA PI treatment did not have a large impact; as visualized in the PCA analysis, only 21% of the variance was related to PC1 and no clear treatment-related clustering was detected. As noted, our results are in contrast to the results

published by Osman et al. where six out of the eleven miRNA included displayed amotosalen-UVA treatment-related downregulation, effects that were not detected in other treatment groups, including irradiation, riboflavin-UVB pathogen inactivation and storage in PAS. These results should be viewed in the light of possible donor variation [351,358,359] and questionable study design. In their analysis, the baseline control was PC stored in 100% plasma, as were the irradiated and riboflavin-UVB treated PCs. They did include a treatment group stored in PAS, the same as the amotosalen-UVA-treated PC, but a direct comparison was not done. Using samples from the Osman et al. study and small RNA sequencing, Diallo et al. looked more closely at MP accumulation and content in relation to PI treatment. Diallo et al. acknowledged the contribution of different storage conditions (100% plasma for control vs 35% plasma and 65% PAS for amotosalen-UVA platelets) and concluded that PI did not affect the miRNA profile of stored platelets. However, they observed that PCs treated with amotosalen-UVA released MPs with an altered miRNA profile compared to control platelets. The authors further purposed that miRNA loading into MPs is a selective process and that amotosalen-UVA treatment somehow deregulates this miRNA selection, bringing on these differences in MP miRNA profiles [123]. Although the same concerns noted earlier in this text apply to these results, platelets stored as PCs in blood banks do release miRNA, harboring MPs that, in theory, could affect the cells of a transfusion recipient. While an interesting point to consider, neither clinical nor retrospective real world data indicate an increase in adverse events related to amotosalen-UVA treatment.

In our unpublished data looking at the miRnome of PCs using microarray analysis with 2200 miRNA probes, we detected on average 850 miRNA in platelet samples from BC PC. We did not confirm if all the detected miRNA were true miRNA. A number of the miRNA had high annotation numbers, meaning that they had only been discovered recently and in some cases validation studies were not available. However, there were a number of miRNA whose abundance changed during storage, showing both up- and down-regulation, as displayed in Figure 7 and Table 2. Even though some of the miRNA detected in this

analysis might be mere products of RNA degradation, these results indicate that the miRNA have roles on platelet processes relating to PSL during storage.

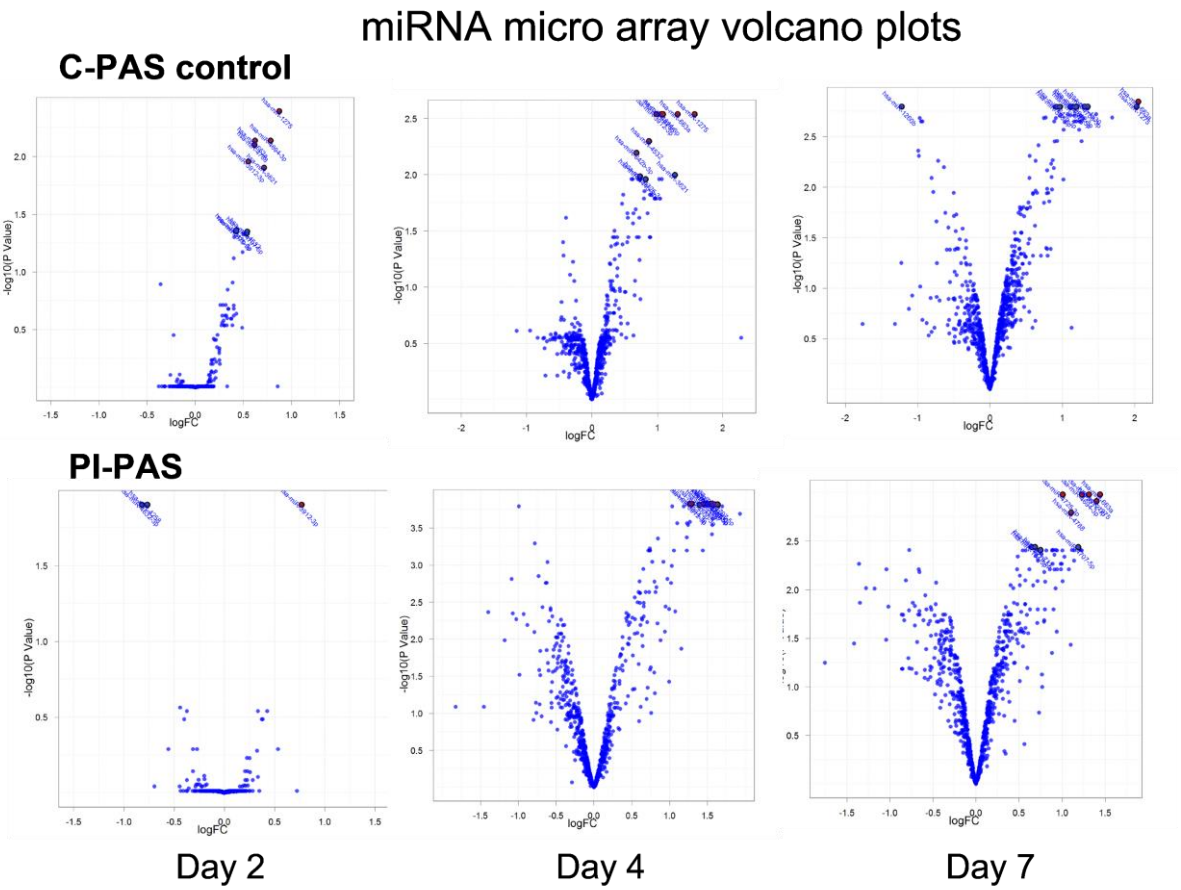

Figure 7. Volcano plots showing signal change of individual miRNA. Fold change compared to the day 1 baseline is represented on the y-axis and changes in signal intensity on the x-axis.

Table 2. Number of miRNA changes during PC storage.

	Number of differently expressed miRNA p-value < 0,05		
	PI-PAS vs. C-PAS	**C-PAS vs. baseline**	**PI-PAS vs. baseline**
Day 2	59	60	40
Day 4	126	155	375
Day 7	127	300	330
	Benjamini Hochberg (FDR) applied		
Day 2	0	12	3
Day 4	0	51	218
Day 7	0	121	195

4.2 Paper II: Protein Concentrations in Stored Pooled Platelet Treated with Pathogen Inactivation by Amotosalen Plus Concentrates Ultraviolet A Illumination

To investigate further effects of amotosalen-UVA PI treatment on the *in vitro* quality of BC PCs, we used Luminex magnetic bead technology to quantify the concentration of 36 proteins in PCs with (PI-PAS) or without (C-PAS) PI treatment. For this analysis, we used a commercially available panel (HCYTIMAG-60K) with 41 pre-selected human cytokines, chemokines and growth factors, and ELISA (Quantikine) for sP-selectin and PF-4 that were not included on the panel. Proteins and their functional classification are listed in Table 3. A total of 7 proteins were out of range for the assay; RANTES and PDGF AA/BB had excessively high concentrations, while FLT-3L, IL-2, IL-3, IL-4, and IL-6 were below the detection limit. The majority of the proteins analyzed gradually increased during the storage period in both arms of the analysis. This would be expected, as degranulation and the release

of multiple factors by platelets into the storage media occurs with increasing activation and PSL. Treatment-related effects were detected at different timepoints for 10 proteins. A subgroup of 6 proteins displayed a drop in concentration on Day 2 of storge after the PI treatment. One protein, Eotaxin, remained at lower concentration compared to C-PAS throughout the storage period, while lower concentrations were observed until Day 7 of storge for IP-10,MCP-1, and MDC. For TNF-α and TGF-α, lower concentrations were only detected on Day 2. There is limited published data on PI-related drops in PC protein concentrations. Thiele et al. reported a decrease in the levels of membrane protein platelet endothelial aggregation receptor 1 precursor (PEAR-1) and protein-tyrosine sulfotransferase 2 (TPST 2). Tauszig et al. reported decreases in levels of RANTES and TGF-β1[360]. Potential causes for these observations are the use of UV light treatment, the compound absorption device (CAD) or even interaction with the photoreactive psoralen compound amotosalen, as psoralens have the ability to bind to both lipids and proteins [361]. In a 2005 publication on possible neoantigen formation, samples from 523 patients participating in seven clinical trials were analyzed with no reports of neoantigenicity. The authors additionally measured the amotosalen interaction in PCs and plasma using high-performance liquid chromatography (HPLC) and found that 15% of the initial amount becomes bound to components in the PC and 15 to 22% is bound in the plasma units. The majority of the residual amotosalen is bound to lipids, and only 1 to 2% is protein-bound [362]. These observations indicate that only a small proportion of the added amotosalen binds to proteins, possibly causing degradation or other modification. Proteins can also absorb UV light, resulting in protein structural changes as well as aggregation, cross-linking and degradation. For the majority of proteins that are unbound to co-factors or prosthetic groups, this absorption occurs at UV wavelengths below 320 nm [363,364]. Using amotosalen-UVA technology, PC are exposed to UV light with wavelengths in the range of 315 to 400 nm (UV-A), with limited absorption by proteins. UV-B and C have wavelengths below this 320 nm limit and are more likely to be absorbed by proteins in the PC. A recent review reported

on the sensitivity of proteins to UV-A and visible light. Under specific conditions, the amino acids tryptophan and tyrosine have a UV absorption spectrum that can extend into the UV-A region [365]. Using a proteomics approach, Prudent et al. have reported limited effects of PI treatment on the global proteome of platelets; however, some proteins showed treatment related-alterations and PI can induce oxidative damage to peptides [305][304][366]. To remove residual amotosalen, PI-treated PCs are incubated for 6-16 hours with a CAD containing immobilized polyester beads. Data generated by our own lab using cell-free solutions and measuring specifically the effects of CAD and UV-A exposure indicated that a number of metabolites were reduced after the CAD incubation, including the hydrophobic amino acids tryptophan and phenylalanine present in many proteins especially linked to the cell membrane [367]. In fact, there was reduction in tryptophan levels after UV-A exposure and CAD incubation (Figure 8).

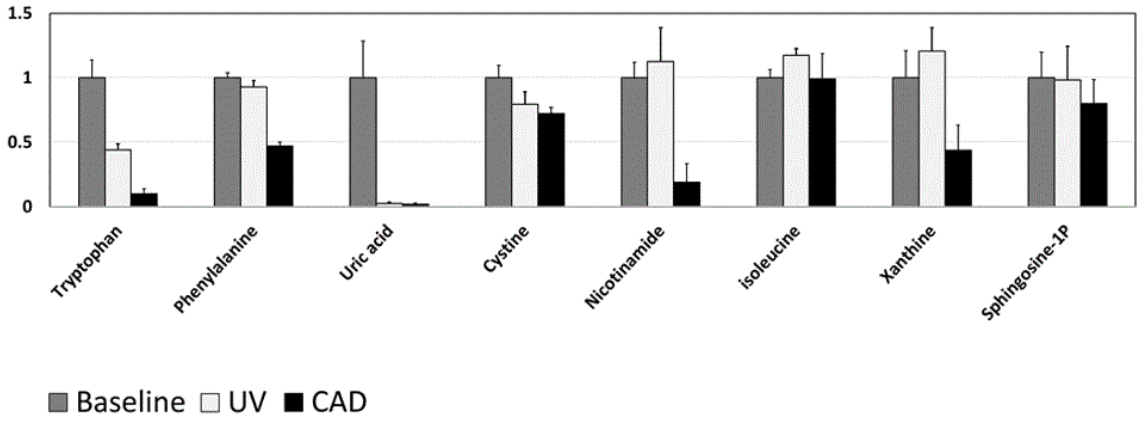

Figure 8. The effects of different parts of amotosalen-UVA processing. The top of each bar is the average of the metabolite levels relative to the baseline, and the error bars depict the standard deviation. The gray column represents level of metabolites in cell free solution before treatment. The white column shows levels after exposure to UV-A light. The black after incubation with CAD [367].

The fact that most of the 6 proteins that experienced a significant decrease in concentration after PI (IP-10, MCP-1, MDC, TNF-α, and TGF-α, but not Eotaxin) have similar or higher

concentrations at the end of the storage period, implies a faster or larger release of these proteins in the PI-PAS group than in C-PAS. Four additional proteins, IL-17A, PF-4, IL-12p70 and G-CSF, all had higher concentrations after Day 2 of storage in the PI-PAS group than in C-PAS, which is a further indication of a more active release in the PI-PAS PCs. Apart from the four proteins with significantly higher concentrations and the 6 with treatment-related decreases, the concentrations of most of the proteins did not display a significant difference between the two research arms. However, in samples from storage Day 7, most of the proteins had higher concentrations in the PI-PAS compared to C-PAS albeit only a few significantly. This observation indicates that the effects of amotosalen-UVA PI treatment is most evident at the end of storage when platelet quality is generally compromised, and PCs are not stored beyond this time point.

The release of pro-inflammatory proteins into the storage media of PCs has been shown to sufficiently prime T-cells and neutrophils *in vitro* with potentially immune modulating effects *in vivo* [368,369]. If certain types of proteins are cleared or reduced in PCs receiving amotosalen-UVA treatment, this could be beneficial in this regard and might explain to some extent the similar or lower transfusion reaction events compared to untreated PCs. Further investigation into possible links to protein structure or interaction for this group of treatment-reduced proteins is of interest.

As displayed in PCA analysis and heatmaps in paper II, the overall effect of treatment on the concentration of this panel of proteins is limited. Most of the variance is likely due to the storage time, as the platelet gradually become activated with increased release of proteins despite PI treatment.

Table 3. Type of proteins analyzed

Cytokines				
TNF-β	**IL-8**	**IL-1β**	**IL-1α**	
TNF-α	**IL-12p70**	**IL-7**	**IFN-γ**	
TGF-α	**IL-12p40**	**IL-5**	**IFN-α2**	
IL-17	**IL-10**	**IL-1ra**	**IL-13**	
IL-15	**IL-9**	**CD40L**		
Chemokines				
MIP-1β	**IP-10**	**PF-4**	**MCP-1**	**Eotaxin**
MIP-1α	**GRO**	**MDC**	**MCP-3**	**Fractalkine**

Growth-factors		
VEGF	**G-CSF**	**FGF-2**
GM-CSF	**PDGF-AA**	**EGF**
Cell adhesion molecule (CAM)		
sP-selectin		

4.3 Paper III: Implementation of pathogen inactivation by amotosalen plus ultraviolet A illumination for platelets in a national blood service

Investigations by our lab revealed minimal effects of amotosalen-UVA treatment on protein concentrations and the miRNA profile of PCs produced and stored in the Landspitali University Hospital Blood Bank in Iceland (the Blood Bank). In line with numerous reports in the literature, our data also indicate an acceleration effect of amotosalen-UVA on PSL. As the Blood Bank is the sole provider of blood components in Iceland, and since all collection, processing, storage, and transfusion takes places within our system, this operation can be categorized as a national transfusion service, although on a global scale our transfusion services are small. We sought out to identify if implementation of PI for all PCs in 2012 affected utilization of the product. Considering there being only, on average, 2000 PC transfusions annually in Iceland, we investigated PC transfusions for a 10-year period to increase the statistical power of the analysis: 5 years before PI and 5 years after PI. In addition, we investigated possible effects on stock management and adverse events. We used a blood bank information system (ProSang) to extract information on PC utilization and stock management. Our own in-house database was used to extract information about platelet content. In agreement with some publications and in contrast to others, we did not detect any change in total utilization of PCs per patient before or after the PI implementation. We further investigated if there was detectible difference in the utilization of specific departments. PC utilization is displayed in Table 4.

Table 4. Number of PC transfusions each year by department. amotosalen-UVA PI treatment

of PCs was introduced in 2012; the blue area indicates data from the 5 years before amotosalen-UVA was introduced, while the red area indicates data from the 5 years following amotosalen-UVA introduction. Red text in a t-test p-value indicates significance.

Table 4 : PC utilization per department

Department	2007	2008	2009	2010	2011	2013	2014	2015	2016	2017	T-test
Emergency	49	61	58	27	40	50	36	42	73	51	0,7102
Intensive care	400	343	494	328	459	370	334	424	281	322	0,1854
Medicine	784	1027	987	881	1237	1348	1031	1128	892	833	0,6105
Obstetrics	34	23	37	12	28	14	21	20	7	18	0,0758
Outside hospital	28	44	77	34	54	91	88	116	175	115	0,0074
Pediatric	69	228	151	271	133	98	116	106	129	246	0,5056
Surgery	114	174	152	123	136	119	172	187	206	195	0,0934
Total	1478	1900	1956	1676	2087	2090	1798	2023	1763	1780	0,5952

The greatest number of transfusions were observed within the medicine department, which includes the hematology and oncology wards with a high number of thrombocytopenic patients. The next largest numbers of transfusions took place in the intensive care and surgery departments. For all the departments included in the analysis, there was only a single department (outside the hospital) with a significant difference showing increased utilization after PI implementation. There are likely other variables in play that explain this difference. Transfusion sites categorized within this department show large fluctuations in their PC utilizations, as displayed in Figure 9. One site (Sel) practiced PC transfusion only in the two year period from 2014 to 2016, but had relatively high utilization numbers within that period. A second site (KEF) discontinued PC transfusion practice in 2007 and reintroduced the practice in 2013.

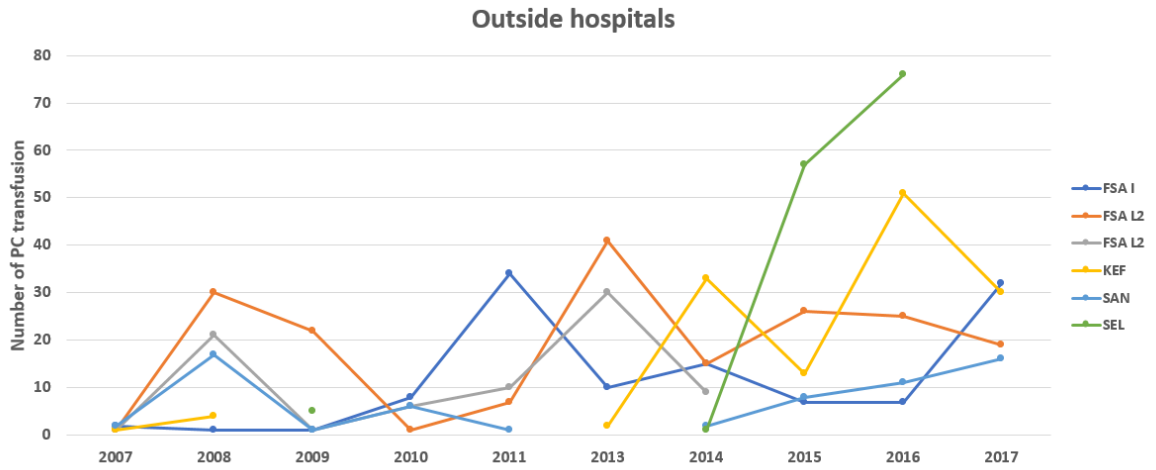

Figure 9. PC utilization at individual transfusion sites categorized as “Outside hospital”.

There was no significant change in the number of recorded adverse events. In our setting there were very few recorded adverse events during this 10 year period. As in many other healthcare systems and hospitals, there is likely a lack of reporting of adverse events and it is difficult to come to any firm conclusions from these results. As it has been a mission of our blood bank to encourage clinicians and other health care personnel to report possible transfusion-related adverse events, one could speculate that the reporting has become better over time.

One of the drivers for implementing PI, secondary to PC safety, was the option to be able to store PCs for 7 days. Prior to implementation, PCs were stored for a maximum of 5 days. With this short storge period, our facility struggled with supply and demand issues. With the implementation of 7-day storage, we were able to maintain a larger stock without increasing outdating. Incidents of PC shortages and delayed delivery were significantly reduced by PI implementation. As a result of PI implementation, the average age of transfused PCs increased from two days to just over four days.

The number of platelets per apheresis unit did not change after PI implementation, though there were lower numbers in BC units after the implementation. These differences can be explained at least in part by modifications in the processing protocol. Before

implementation, we used a five BC pool per single therapeutic PC dose. After implementation, eight BCs were pooled to produce a double dose PC.

5 Conclusions and future directions

5.1 Conclusions

With current standard storage conditions of platelet products (room temperature with agitation), the risk of bacterial contamination is much higher than for other blood components stored at refrigerated or subzero temperatures. At the time of PI implementation, the Blood Bank in Iceland was assessing two options for reducing the risk of TTBI: 1) screening the inventory using a system such as BacT/Alert; or 2) implementing PI. Comparing these two options, the increase in maximum storage time is an obvious advantage of PI that is non-existent with bacterial screening unless secondary or rapid testing is applied. A second advantage of PI is the possibility of inactivating emerging pathogens that are not included in standard screening protocols. Using a bacterial screening system, there is minimal additional manipulation of the PC product, better preserving platelets quality. However, there must be some measures in place to inactivate residual white blood cells that can cause TRAE, particularly in immune compromised patients who are a significant proportion of the patient population receiving PCs. Irradiation of platelets is the common protocol for leukocyte inactivation, with similar or even more negative effects on platelet quality compared to PI. Blood banks using irradiation of PCs thus have a dual stock with different products selected for specific patients. PI processing includes the option for a single PI PC product for all patients. There is also the issue of false negative and positive results using bacterial screening tools. In the final decision making at our facility, the pros of safety and better PC stock management outweighed the possible disadvantages of lowering of the product quality, and PI was implemented for all PC production in 2012. With this implementation, our research and development department had the opportunity to investigate the effects of PI on platelet biology, adding to the data in the literature on this new method.

For decades is has been recognized that platelets contain mRNA and the ability of protein translation. Platelets are enucleated and are not able to control protein expression on the transcriptional level. Since the discovery of miRNA in the early 90s, platelets' role as post-transcriptional regulators has been realized in multiple cellular processes. Recently it was established that platelets contain miRNA and all the components necessary for mature miRNA processing and function. It is intriguing that enucleated cells exploit the miRNA pathway for post-transcriptional regulation. In paper I, we analyzed the effects of PI on the profile of 25 miRNA in BC PCs using qPCR. The effects of PI were minimal, with only a single miRNA showing treatment-related downregulation. This contrasted with other reports on the effect of amotosalen-UVA on the miRNA profile of single donor apheresis PCs. Analysis of proteins in the supernatant of stored PCs showed more pronounced effects, with both treatment-related increases and decreases in the concentrations of certain proteins. In both analyses, storage time affected protein and miRNA profiles.

In conclusion, we did observe amotosalen-UVA PI treatment-related effects on the *in vitro* quality of PCs. These effects did not translate into increased utilization of PCs or adverse events. Over the 10 year period since implementation of this technology, there have been zero reports of TTBI, and with 7 day storage there has been increased security of PC availability.

5.2 Future directions

Donated platelets continue to be an important part of treatment for thrombocytopenic and trauma patients. The storage of PCs has largely stayed the same since the early 1970s, with room temperature and agitation being standard. Multiple PASs have been developed to preserve platelet quality and as substitute for donor plasma, which may be required for fractionation or cryopreserved for later use. Still, the maximum storage time for platelets is only 5 to 7 days, making PC stock management a challenging task. Even with measures like PI in place, there is a 7 day storage limit determined by the lowering of platelet quality

beyond that point. In general, lower storage temperatures for live cell donations are used in order to lower the metabolic activity of the cells and to preserve their quality, resulting in longer storage. In reports from the 1990s where radio labeled platelet aging *in vivo* was compared to the aging of stored *in vitro* platelets at 22°C, the authors observed that 5 days *in vitro* corresponded to 2.1 days *in vivo.* The authors also analyzed ATP turnover in platelets stored at 37 °C compared to 22 °C, and found these measurements correlated with the aging factor. According to these observations and the fact that platelets have normal lifespan of 9-10 days, the storage time of platelets at 22 °C should be 18 to 20 days [370]. A summarized by Gulliksen there are 3 main challenges for longer storage of platelets at 22°C: 1) less activation of platelets during collection and processing; 2) reducing the rate of glucose consumption and lactate production during storage; and 3) and ensuring there is sufficient amount of glucose in the platelet storage environment to last throughout the storage period [371]. To improve and prolong the storage of platelets, there are ongoing multiple enquires including cold storge, cryopreservation, whole blood and *in vitro*-generated platelets stored in bioreactors.

There are also methods being developed and research into making platelet transfusion as safe as possible for the patient. PI is a relatively new technology and undoubtably we will see new versions and approaches to improve upon existing methods. For example, there is now technology available for washing the platelets after storage and prior to transfusion to limit the amount of BRMs that can cause TRAE [372]. Platelet donor genotyping and human platelet antigen screening reduces the rate of platelet refractoriness and TRAE [373–375]. Recently, tools such as next generation sequencing are being applied for donor blood group genotyping [376,377]. Donor variations in platelet quality should receive more attention [378,379]. Currently, it is only a donor's platelet count that is the deciding factor for selecting donors for PC apheresis collection and BC for pooling. However, with a limited pool of donors and increasing deferrals based on a number of platelet quality markers and tests, it could prove difficult to maintain sufficient PC stocks in

the future.

At our lab, there is currently ongoing work in analyzing the quality of cryopreserved and cold-stored platelets with and without PI treatment. Several clinical studies have assessed the quality of cold-stored (CS) or cryopreserved platelets. Trial results have indicated that platelets acquire some damage under such storage conditions and are quickly cleared from circulation after transfusion [380–383]. CS or cryopreserved platelets were also shown to be more activated than room temperature-stored platelets, which could be a positive attribute when treating actively bleeding patients [384–386]. Thus, CS or cryopreserved PCs could prove to be optimal for use in warzones, rural hospitals, or as a reserve stock in blood banks. We are also researching the utilization of outdated PCs for generation of animal-free serum for cell cultures [387–389]. It is of importance to have available animal-free products for the culturing of human cells, especially if intended for therapeutic use. In addition, it is reassuring for the donor that their donation does not go to waste.

As for the next steps regarding this project, we would analyse further the treatment-related reduction of proteins observed in paper II and the generation of EVs for different storage and processing options. At our facility, every PC donation is sampled and analysed by our QC department. It would also be of interest to do correlation studies of donor attributes and platelet quality during storage.

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