USING PATHOGEN INACTIVATION TREATMENT TO STUDY THE STABILITY OF PLATELET MESSENGER RNA

by

Christa Klein-Bosgoed

M. Sc., University of Groningen, 2003

Pharm. D., University of Groningen, 2005

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Abstract

Pathogen inactivation (PI) techniques are designed to increase the safety of blood products by damaging RNA and DNA of pathogens. Even though platelets are anucleate, they synthesize proteins using RNA and the ribosomal machinery derived from megakaryocytes. The role of protein synthesis in platelets, however, is still poorly understood. PI-treated platelets show signs of accelerated storage lesion, but the effect of PI on platelet mRNA and subsequent protein synthesis remains unclear. In this dissertation we investigated to what extend platelet mRNA is affected by PI using Mirasol as a representative PI. In **chapter 3** we demonstrated that the Mirasol treatment affected platelet mRNA negatively in a target specific manner, but prolonged the mRNA half-life. The long mRNA half-life suggested the presence of a mechanism, protecting platelet mRNA from degradation. We investigated the role of p38 as a potential regulator of platelet mRNA and if stress granule (SG) formation could be involved in protecting platelet mRNA. The kinase p38 is a key regulator of a wide range of platelet function. In nucleated cells, UV-induced stress activates the p38 and ultimately leads to increased mRNA stability through the regulation of RNA binding proteins. UV illumination is a key feature of all PI technique, and p38 is activated in Mirasol-treated platelets. In **chapter 4** we demonstrated that inhibition of p38 in Mirasol-treated platelets increased the mRNA half-life, but not through the RNA binding proteins HuR or TTP. SG formation is a protective response in nucleated cells to temporarily store mRNA and consists of mRNAs and specific SG proteins. **Chapter 5** is a description of the preliminary experiments performed to test the effect of a known SG inducer (arsenite) and inhibitor (nocodazole) in platelets, especially in the context of the Mirasol treatment. Nocodazole did not impact the platelet mRNA levels, but affected the mean platelet

volume and count in Mirasol-treated platelets. Arsenite exposure did not affect platelet total RNA or GAPDH mRNA, but showed a reduction in extent of shape change. Our experiments showed that platelets possessed the potential to form stress granules, but a definitive mechanism was not demonstrated.

Lay summary

Blood platelets are cells fragments in the blood responsible for clotting. Like other cells, platelets make proteins using mRNA which needs to be protected throughout their lifespan. This dissertation describes how a technology based on UV light and vitamin B2, designed to kill infectious agents in blood products, might affect how platelets protect their mRNA. The research showed that this treatment reduced some but not all kinds of platelet mRNA. Seeking to understand how this works, we found a protein, p38 MAP kinase, that has a similar function in other cells types and another protein known to protect mRNA. However, the presence of these proteins could only partially explain the observations. This research shows us that treatments designed to make blood transfusions safer do not damage all the platelet functions and that we need to learn more about how these mRNA sparing processes work in order to improve platelet transfusions.

Preface

All the work presented in this dissertation was approved by the University of British Columbia's Clinical Ethics Research Board (certificate number: H12-03515) and the Canadian Blood Services Research Ethics Board (Protocol reference # 2008.02 - NetCAD Reference-X007). The research described in **chapter 3** was selected for oral presentation at the American Association of Blood Banks Annual Meeting (24 – 27 October 2015, Anaheim CA) and is published with minor adjustments as manuscript entitled "Riboflavin and ultraviolet illumination affects selected platelet mRNA transcript amounts differently". Klein-Bosgoed C, Schubert P, and Devine DV. *Transfusion* 2016, Sep 56(9):2286-95. doi: 10.1111/trf.13715 The research described in **chapter 4** was also selected for oral presentation at the American Association of Blood Banks Annual Meeting (21 – 25 October 2016, Orlando FL) and a manuscript is under review. I performed all the data analysis presented in this dissertation. I performed all the experiments unless otherwise acknowledged in the figure caption. Permission for reprint was obtained where applicable.

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List of Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
ARE	AU-rich element
CalDAG-GEF	Calcium- and diacylglycerol-binding guanine nucleotide exchange factor
CASC	Cancer susceptibility candidate 3
CCI	Corrected count increment
CPEB	Cytoplasmic polyadenylation element-binding protein
Ct	Treshold cycle
DAG	Diacyl glycerol
Dcp	Decapping
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	1,4 Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid
eIF	Eukaryote initiation factor
ESC	Extent of shape change
g	Gravity

G3BP	RasGTPase-activating protein SH3-domain-binding protein
GCN2	General control nonderepressible 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GP	Glycoprotein
GTP	Guanosine triphosphate
HLA	Human leukocyte antigen
HRI	heme-regulated initiation factor 2α kinase
HuR	Human antigen R
IL	Interleukin
IP_3	1,4,5-Inositol triphosphate
J	Joule
LPS	Lipopolysaccharide
M	Molar
MAP	Mitogen-activated protein
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NetCAD	Network Centre for Applied Development
OCS	Open canalicular system
PABP	Poly(A) binding protein
PAGE	Polyacrylamide gel electrophoresis

PARN	Polyadenylated ribonuclease
P-bodies	Processing bodies
PBS	Phosphate buffer saline
PC	Platelet concentrate
PCR	Polymerase chain reaction
PERK	PKR-like endoplasmic reticulum kinase
PF4	Platelet factor 4
PI	Pathogen inactivation
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PKR	Protein kinase R
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPP	Platelet poor plasma
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNAseq	RNA sequencing
RNP	Ribonucleic protein
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulfate
SG	Stress granule
snRNA	Small nuclear ribonucleic acid
SPARC	Secreted protein, acidic, cysteine-rich

STAU	Staufen
TF	Tissue factor
TIA-1	T-cell internal antigen 1
TIAR	TIA-1-related
TIMP	Tissue inhibitor of metalloproteinase
TSP	Thrombospondin
TTP	Tristetraprolin
TxA_2	Tromboxane A_2
UV	Ultraviolet
vWF	von Willebrand factor

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And last, but most certainly not least, I want to thank my husband Theo and sons Hugo and Lucas. They have been my anchor and inspiration. Their unconditional love and support helped me keep my sanity and made this journey that much more memorable.

Dedication

To my loving husband Theo and my amazing sons Hugo and Lucas

Chapter 1: Introduction

1.1 The human blood platelet

1.1.1 Origin

Platelets are small (2 – 5 µm) discoid shaped components of blood and have a life span of 10 to 11 days. They are shed from megakaryocytes after a complex process of branching out pod-like extensions, called proplatelets, which are covered with platelet-sized swellings [1]. Before shedding thousands of platelets, megakaryocytes increase in size and produce platelet-specific proteins [2]. The microtubular network within the megakaryocyte facilitates proplatelet elongation as well as transport of the platelet specific components. The release of platelets by megakaryocytes is seen as the final step of megakaryocyte maturation.

1.1.2 General structure

The platelet outer membrane is a lipid bilayer decorated with receptors to facilitate platelet activation (see paragraph 1.1.3). The outer membrane folds inwards, creating the open canalicular system (OCS). The OCS is identical to the surface membrane and allows for additional surface area for uptake and release of products. In addition, the OCS enables the formation of filopodia and platelet spreading in response to external stimuli. Below the outer surface membrane an internal spectrin-based membrane skeleton is connected to long actin filaments that stretch into the cytoplasm creating a rigid internal structure. In the cytosol, but along the outer edge of the platelet sits a rigid microtubule coil, creating the platelets characteristic discoid shape.

In the cytosol, platelets primarily compose of granule-like structures. The most abundant organelles are the α-granules. Alpha-granules contain adhesion molecules (von Willebrand factor (vWF), P-selectin, integrin $\alpha_{IIb}\beta_3$, among others), procoagulant (factor V, factor VIII) and fibrinolytic factors (plasminogen, plasminogen activator inhibitor 1), chemokines (like platelet factor (PF) 4) and many other proteins crucial for regular platelet function. Dense granules are much less abundant and contain primarily small molecules like ions (Ca^{2+}, Mg^{2+}), nucleotides (adenosine and guanosine nucleotides) and serotonin. Platelets contain only a few lysosomes with hydrolases, cathepsins and lysosomal membrane proteins. Mitochondria are present to supply the energy need.

Platelets have no nucleus, but do contain mRNA and pre-mRNA together with components of the translation machinery like ribosomes and all known eukaryote initiation factors [3]. These components originate from platelet precursors, the megakaryocytes, and are actively transported into the proplatelets during platelet biogenesis [4]. The mRNA and ribosomes are associated with the cytoskeletal fraction of the platelets [5]. Platelets use the mRNA and ribosomes to synthesize proteins. A more detailed description of the origin of platelet mRNA and the events involved in protein synthesis in platelets is described in paragraph 1.4.

1.1.3 **Platelet activation**

Activation of platelets is the cascade of processes in which an agonist triggers a platelet surface receptor to initiate a rapid internal reorganization of the actin filaments to achieve a morphological shape change and secretion of granular contents (see Figure 1.1). Under

physiological conditions this process will result in platelet adhesion, aggregation and platelet plug formation.

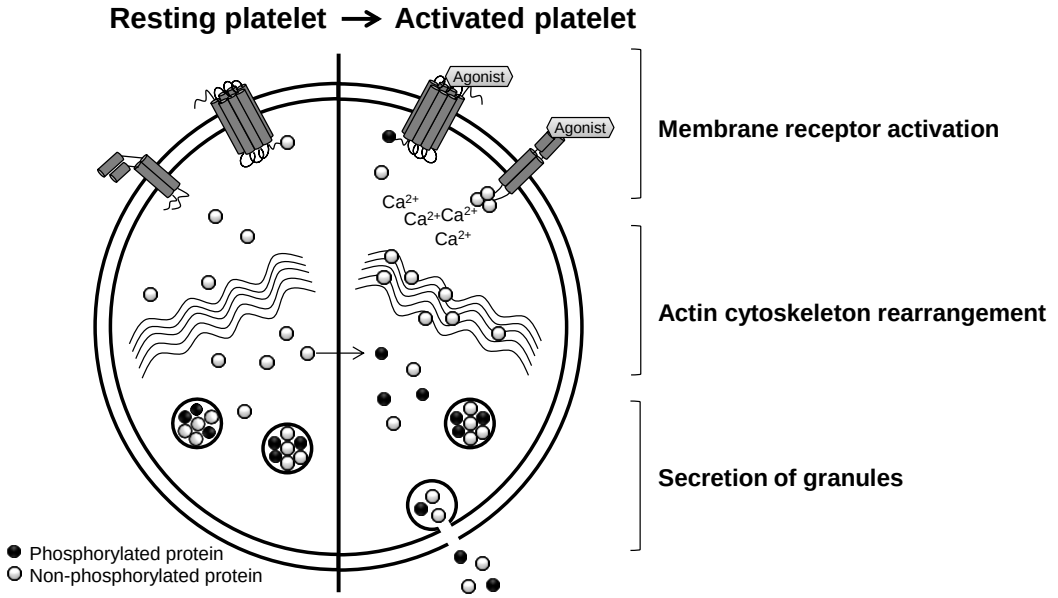


Figure 1.1: Simplified schematic representation of the events occurring during platelet activation.

The activation of membrane receptors by agonists initiate phosphorylation events and triggers an increase in cytosolic Ca^{2+} that drives cytoskeletal rearrangement and the secretion of alpha and dense granules.

Platelets survey blood vessel walls for damaged endothelial cells, marked by the exposure of collagen. Under high shear vWF binds to collagen, which allows platelets to adhere to, primarily via the glycoprotein (GP) Ib-IX-V complex and secondly via the integrin $\alpha_{IIb}\beta_3$ receptor (also known as GPIIb-IIIa). This process is followed by the binding of platelets immediately to collagen via the GPVI/γ-chain complex and integrin α2β1 to form a monolayer on top of the injured area. The ligand-bound platelet receptors trigger downstream phosphorylation events that allow subsequent granule secretion, platelet aggregation and thrombus formation.

Binding of GPIb to vWF or activation of the GPVI receptor, both leads to activation of Syk kinase and phospholipase C (PLC)γ. PLCγ converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into 1,4,5-inositol triphosphate (IP_3) and diacyl glycerol (DAG). IP_3 binds to IP_3 receptors on the endoplasmatic reticulum, releasing Ca^{2+} to increase the Ca^{2+} concentration in the cytosol [6]. DG activates protein kinase C (PKC) and leads to the secretion of alpha and dense granules [7]. The rise in cytosolic Ca^{2+} activates many downstream effects. Amongst others, the calcium influx activates the calcium- and diacylglycerol-binding guanine nucleotide exchange factor (CalDAG-GEF) to convert the GTPase Rap1B-GDP into Rap1B-GTP, initiating the inside-out signaling to activate integrin $\alpha_{IIb}\beta_3$ [8]. The increased Ca^{2+} concentration also activates phospholipase A_2 (PLA_2) that initiates the synthesis of the prostanoid thromboxane A_2 (TxA_2) from arachidonate. TxA_2 is secreted from platelets by diffusion and acts as agonist to amplify the activation signal and recruit more platelets into a growing thrombus [9]. The rise in cytosolic Ca^{2+} activates myosin light chain and cofilin via Rho-activated kinase and LIM-kinase resulting in platelet shape change.

1.1.4 **History and current practice of platelet transfusion**

Transfusing blood to patients has been used as a treatment since the seventeenth century. This risky practice was mostly unsuccessful, until the discovery of the blood type system A, B, AB, and O by Karl Landsteiner in 1901 [10]. He was the first to demonstrate red cell agglutination in some, but not all cases of cross-matching blood. He was able to identify that a blood recipient can get an immunological reaction when exposed to donor blood that carries antibodies against the recipients' blood antigens. This difference originates from specific oligosaccharide antigens found on the surface of red cells. The basic oligosaccharide, the O antigen, is found in all blood

types. In blood type A an additional *N*-acetylgalactosamine is bound to the basic O antigen and in blood type B an additional galatose is bound to the basic O antigen. Red cells with both A and B types of oligosaccharides on the surface are referred to as blood type AB.

The science of blood transfusion made remarkable improvements and allowed for a new type of medical procedure to be introduced. Cross-matching donor and recipient blood became a new routine practice, increasing the success rate of blood transfusion significantly, ultimately recognized in awarding the Nobel Prize in Physiology of Medicine to Landsteiner in 1930.

Another milestone in transfusion medicine, that opened the door to blood platelets as a therapeutic treatment, occured in the 1950s. A plastic blood collection system was developed to replace the glass bottles and rubber tubes, until then the materials commonly used for transfusion of whole blood [11, 12]. This new type of storage container allowed for whole blood to be separated into its components, red cells and plasma, post collection. One donation could be used to treat several patients and physicians had more specific treatment options. The fractionation of whole blood also allowed for different storage conditions for each component to maintain optimal quality. The major obstacle to successful storage of platelets was the rapid deterioration of the viability of refrigerated platelets. In 1969 Murphy *et al* [13] reported for the first time a method to store platelets at 22 °C and under continuous agitation that demonstrated prolonged platelet viability. This remains the optimal storage conditions for PCs till this day. However, platelet transfusion did not become routine medical practice until later in the seventies.

As early as 1910, platelets were mentioned as a component of whole blood capable of reducing the bleeding time [14]. However, it was not until fifty years later that studies demonstrated a correlation between platelet count and the risk of hemorrhage in acute leukemia. Transfusion of platelets was demonstrated to be beneficial and decreased the occurrence of hemorrhage and mortality in patients suffering from leukemia [15-17]. Therapeutic transfusions also increased in popularity following the development of apheresis instruments in the 1960's, which used in-line differential centrifugation to separate specific cells from whole blood. During a donation platelets are being separated and collected, while the red cells and plasma are returned to the donor. Initially these instruments were used to collect plasma, both for therapeutic use and for the fractionation of plasma proteins. It wasn't until 1972 that apheresis of platelets for transfusions became more readily available as a prophylactic treatment for leukemia patients.

In current practice, platelet transfusions are used to treat bleeding in patients with a decreased platelet production or platelet malfunction. In addition, platelet transfusions are used as a prophylaxis in patients undergoing chemotherapy. Platelet concentrates are either prepared via the buffy coat method (pooled platelets) or collected via apheresis. Buffy coat platelets refer to the platelets that are present in the buff-coloured layer of cells (the buffy coat) that, after whole blood centrifugation, remains after removal of the red cells and plasma. Both methods result in PCs with a shelf life of five to seven days from the time of collection. In general platelet transfusions are not required to be ABO group matched, but the plasma of the PC should be ABO compatible with the recipients' red blood cells [18]. Apheresis platelets can be favored over pooled platelets in patients with a known anti-HLA (human leukocyte antigen or

transplantation antigen) to reduce the risk of enhanced clearance of platelets due to the presence of unmatched HLA antibodies.

1.1.5 **Platelet storage lesion**

A platelet population collected during donation is a mixture of young and old platelets. Platelets have a life span in circulation of about ten days and their lifespan is determined by loss due to platelet consumption and senescence [19]. Platelets are being cleared from the blood stream by the spleen and liver [20]. After donation, platelet products have a shelf life of five to seven days depending on the jurisdiction [18, 21]. A reason for the short shelf life is the cellular change platelets undergo during storage *ex vivo*. This cellular change results in loss of *in vitro* quality and is referred to as platelet storage lesion [22]. A wide range of assays have been developed to assess different aspects of platelet quality *in vitro* and are described in Table 1.1. All tests are performed to evaluate different aspects of platelet function *in vitro*. So far, no single assay has been able to predict the behavior and efficacy of a platelet concentrate post-transfusion.

A combination of several assays, like swirl, extent of shape change, hypotonic shock response and pH are used to a certain extent as indicators of *in vivo* viability [22]. Platelets can be monitored *in vivo* using well defined radio-labeling assays [23]. An aliquot of fresh platelets is labeled with either indium-111 or chromium-51 and a stored or treated platelet aliquot from the same donor is labeled with the other radiolabel. Both aliquots are transfused at the same time. The amount of radioactivity detected after transfusion of the labeled platelets is a measure of the recovery of platelets. The duration of detectable radioactivity in the blood is a measure of the survival of the platelets. The platelet recovery and survival are calculated using suitable

analytical software that compensates for degradation and other variables. However, the degree of correlation of the *in vitro* assays with platelet recovery and survival varies greatly [22, 24-26].

Table 1.1: Selection of assays available to evaluate platelet *in vitro* quality

Quality parameter	Assay	Reference
Discoid shape	Swirl, morphology score	[27, 28]
Metabolism	Glucose, lactate, pH	[29, 30]
Platelet activation	Flow cytometric measurement of p-selectin (CD62P)	[31]
Membrane integrity	Hypotonic shock response	[32]
Apoptosis	Mitochondria disruption measured by membrane potential	[33]
	Flow cytometric measurement of phosphatidyl expression	[34]
Responsiveness to	Extent of shape change	[32]
agonist	Aggregometry	[35]
	Flow cytometric measurement of platelet surface receptor expression, including conformational changes	[36, 37]
Microvesicle release	Flow cytometric measurement of platelet-derived membrane receptors	[36]

1.2 The development of pathogen inactivation techniques

1.2.1 Risk of bacterial contamination

The platelet storage lesion is not the only reason for the short shelf life of platelet products. Their storage conditions (at room temperature under continuous agitation) could potentially allow growth of certain pathogens. Contamination of a blood product can occur through insufficient

decontamination of the puncture site, transfer of the skin plug from the needle into the blood product, an equipment defect, or mishandling resulting in a breach of the closed system [38]. The occurrence of an adverse reaction as result of a transfusion-transmitted infection is much smaller compared to non-infectious transfusion reactions, like transfusion-related circulatory overload or an anaphylactic or hemolytic reaction. Between 2009 and 2013 the frequency of a bacterial infection as a result of a blood transfusion in Canada was less than 1% [39]. Still bacterial contamination of blood products is a major safety concern in transfusion medicine. With the implementation of donor screening before donation, diversion of the initial blood into a sampling pouch and leukoreduction of PCs, the rate of contamination of platelet products have substantially been reduced [38, 40]. Serological, nucleic acid and bacterial testing is being performed on all products to further screen for the presence of known pathogens [41]. In situations of identified pathogens that newly emerge as potential threats, however, the lack of proper testing methods limits the ability of blood suppliers to implement safety measures by adjusting their testing regime [42]. Changing the donor questionnaire and introduce (temporarily) travel deferrals are a way to react to these kind of threats. This is not possible for pathogens, still unknown, that could pose a risk to the blood supply in the future.

The outbreak of the Zika virus in South America, that rapidly spread north, reaching southern USA, caused an increase in public awareness and concern for the safety of the blood supply. This outbreak has accelerated the acceptance of the new techniques referred to pathogen reduction technology (PRT) or pathogen inactivation (PI) and propelled their implementation [43, 44]. The PI techniques have been developed to further increase the safety of platelet transfusions by reducing potential bacterial growth [42]. It has demonstrated to be successful in the prevention of

graft-versus-host disease and can be used as an alternative to γ-irradiation [45]. Unlike the reactive approach of donor screening and the testing of blood products, the current development implements a more pro-active approach.

PI treatments target both viral and bacterial contamination of platelets and use illumination with ultraviolet (UV) light with or without the presence of a photosensitizing agent. UV light is able to damage nucleic acids necessary for pathogen replication by the formation of crosslinks between pyrimidine derivatives (see Figure 1.2). Efficacy of nucleic acid damage can be enhanced by the use of photosensitive agents.

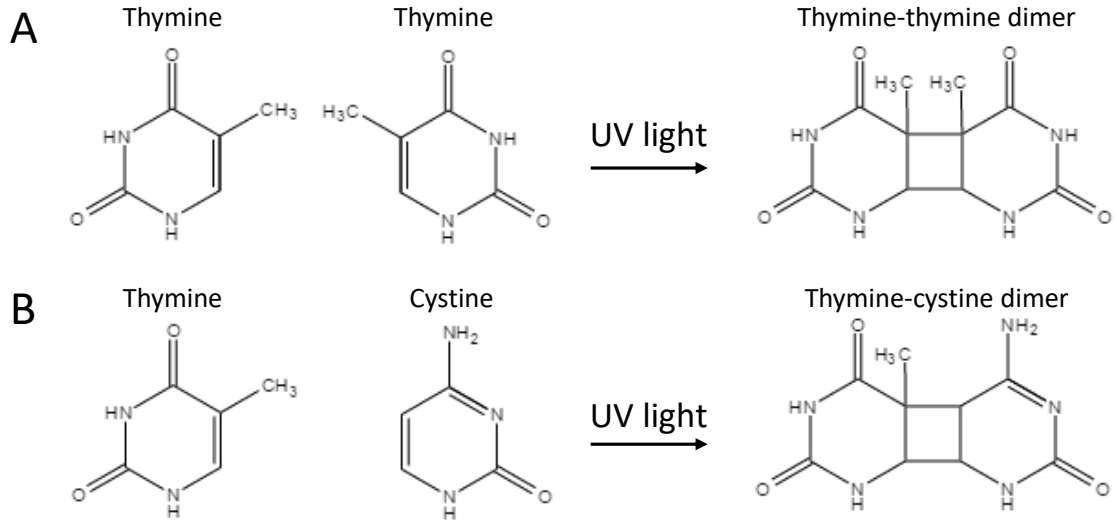


Figure 1.2: Chemical crosslinking in DNA as a result of UV exposure.

Adjacent pyrimidine derivatives create lesion in DNA strand through the formation cyclobutane pyrimidine dimers like A) Thymine-thymine dimer and B) Thymine-cystine dimer. The formation of pyrimidine dimers block DNA transcription and replication. Images were generated using ChemDraw Direct 1.5 (Perkin Elmer, Waltham, MA)

1.2.2 **Riboflavin/ UV illumination**

The Mirasol PRT system (TerumoBCT, Lakewood CO) is developed for the production of pathogen reduced plasma and platelets in plasma or a combination of plasma and additive solution [46]. A method to produce pathogen reduced whole blood prior to component production is still under development [47]. The Mirasol system uses the natural component riboflavin (vitamin B2) as photosensitizer. Riboflavin is an antioxidant and, as its active form flavin adenosine dinucleotide, a coenzyme for several metabolic pathways like the Kreb's cycle and the oxidation of fatty acids [48, 49]. Riboflavin and its photo-product lumichrome are not toxic, so can remain part of the blood product after the pathogen reduction process. The majority of the riboflavin is free in solution and only a small fraction is bound to the substrate. A concentration of 50 μM riboflavin illuminated with 6.24 J/mL has demonstrated to be effective for pathogen inactivation, while maintaining cell quality [50, 51]. The mechanism of action of Mirasol is based on an oxygen-dependent electron transfer caused by the association of riboflavin with the nucleic acid, after exposure to UV light (280 – 400 nm, see Figure 1.3). The modifications and lesions as a result of the electron transfer primarily affect guanine bases in an average frequency of 1 in 350 base pairs. The irreversible damage to the nucleic acids causes an impairment of the repair and replication of viral, bacterial, parasitic, or cellular RNA and DNA [46].

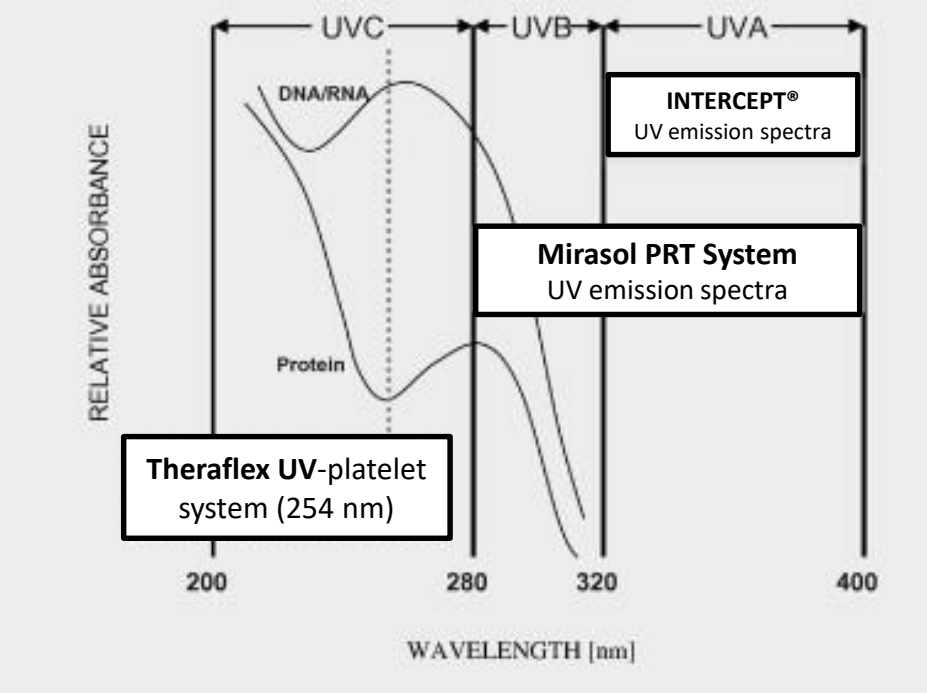


Figure 1.3: Absorption spectra of DNA, RNA and proteins in relation to the emission spectra used in the three described PI techniques. Reprinted with minor edits with permission from Seghatchian *et al.* [52].

1.2.3 **Other PI techniques**

INTERCEPT® Blood System (Cerus Corporation, Concord, CA) uses the photoactive characteristics of the synthetic psoralen compound amotosalen to, in the presence of UVA light, irreversibly cross-link nucleic acids (see Figure 1.3). Amotosalen is a small molecule, able to intercalate into the helical region of RNA and DNA. Upon UVA illumination amotosalen covalently binds to pyrimidine bases in a frequency of about every 80 base pairs [53].

The THERAFLEX UV-platelet system (Macopharma, Tourcoing, France) is the only technique that uses UVC illumination alone to inactivate pathogen proliferation without the presence of any photosensitizing agent (see Figure 1.3). UVC (200-280 nm) is strongly quenched in plasma,

so requires high intensity illumination to allow efficient cross-linking throughout a PC. In the THERAFLEX UV-platelet system this obstacle is circumvented through strong agitation of the PC storage container. The UVC penetrates only a thin layer of PC, but the agitation homogenizes the blood product [54]. The UVC exposure generates primarily in cyclobutane pyrimidine and pyrimidine pyrimidine dimers (see Figure 1.2).

1.2.4 **Function of pathogen-reduced platelets *in vivo* and *in vitro***

All PI techniques have been examined *in vivo* studies to test the dosing, kinetics and safety of the blood products. The Biomedical Excellence for Safer Transfusion collaborative has defined a standardized method to determine platelet recovery and survival [23]. The performance of stored platelets is defined as a percentage of the performance of freshly isolated autologous platelets. The recovery and survival of pathogen-reduced platelets are lower than untreated control platelets independent of the method. Five day old amotosalen / UVA-treated platelets demonstrated a recovery of 43% versus 50% for untreated control platelets and had a survival time of 4.8 days versus 6.0 for untreated control platelets [55]. After five days of storage, riboflavin / UV-treated platelets had a recovery of 50% compared to 67% for untreated control platelets. The platelet survival was reduced from 5.9 days for untreated control platelets to 4.3 days for pathogen-reduced platelets [56]. The UVC – treated platelets demonstrated a similar trend. The UVC-treated platelets showed a significant reduction in recovery of 51% for pathogen-reduced platelets compared to 64% for untreated control platelets. The survival of UVC-treated platelets was also reduced to 5.2 days versus 7.3 days for untreated control platelets [57], presumably due to damage caused the irradiation.

The safety and efficacy of pathogen-reduced platelets have been tested on a larger scale in clinical trials. In general, transfusion with pathogen reduced-platelets trends towards a lower corrected count increment (CCI) compared to untreated control platelets [58]. This can in part be explained by a loss in absolute number of platelets of a pathogen-reduced PC. The reduced platelet yield per unit might also be a contributing factor to the increase in number of transfusions required in patients receiving pathogen-reduced platelets compared untreated standard platelets [59-64]. Several studies have reported an increase in bleeding events [59, 63]. In some studies the occurrence of adverse transfusion reactions is comparable between untreated control platelets and pathogen-reduced platelets [61, 64]. Differences are observed between the different techniques, but also may arise in part due to differences in trial design, patient population and the definition of the primary and secondary outcomes.

The overall impact of pathogen inactivation on the platelet *in vitro* quality is summarized in Table 1.2. Pathogen-reduced platelets show signs of accelerated storage lesion. The development of the storage lesion depends on the platelet additive solution. The negative impact of the PI treatment is more mitigated in platelets suspended in plasma and additive solution compared to platelets suspended in plasma only [65-70].

Table 1.2: Summary of the impact of the different PIs on standard platelet quality parameters.

Quality parameter (method)	Mirasol	Intercept	Theraflex
Metabolism (glucose consumption, lactate production, and pH)	↑ [65, 71]	↑ [72, 73]	↑ [74, 75]
Platelet activation (CD62p expression)	↑ [65, 71]	↑ [73]	↑ [74]
Apoptosis (phosphatidyl expression)	↑ [65, 72]	↑ [72]	↑ [75]
Responsiveness to agonist (aggregation)	↓ [71]	↓ [73]	↓ [74]

1.3 **Messenger RNA metabolism in nucleated cells**

1.3.1 **mRNA processing**

In nucleated cells, DNA localizes in the cell nucleus and contains all the genetic information a cell needs for survival and proliferation. The transcription of nuclear RNA from DNA involves the formation of a pre-initiation complex, followed by transcription initiation, elongation and termination. After transcription pre-mRNA undergoes a number of modifications in the nucleus to give rise to cytosolic mRNA [76]. The modifications involve the addition of 7-methyl guanosine to the 5’ end (capping), the addition of a poly(A) tail to the 3’ end (polyadenylation), and the removal of non-coding introns (splicing).

The 5’ end of a pre-mRNA transcript is modified while transcription is still ongoing. An extra guanosine is added, without an encoding template, followed by the methylation of the 7-position by methyltransferase. The terminal 5’ cap group is known as the 7-methylguanosine (m7G) cap. The addition of the 5’ cap counteracts premature degradation of the nascent pre-mRNA by 5’

exonucleases. The 5’ cap also binds to the cap-binding complex (CBC), a mediator of the export of transcripts from the nucleus to the cytosol, and protects the cap from decapping enzymes [77].

The modifications at the 3’ end of a pre-mRNA transcript can only be performed when the transcription is completed. The process involves active cleavage of the 3’ end followed by the adenylation of the free end by polyadenylate polymerase. The adenylation process starts relatively slow, but the affinity of polyadenylate polymerase increases once the initial adenyl-groups are bound to the RNA binding protein PAB2. The polyadenylation of the 3’ end protects the pre-mRNA transcript from degradation by 3’ exonucleases and promotes nuclear export and translation [78].

In addition to the modifications at the far ends of a pre-mRNA transcript, non-coding RNA sequences called introns, are removed from the transcript by a splicing. This process of exciting intron from a transcript and linking the remaining constitutive exons occurs both during transcription and post-transcription. The latter case is referred to as splice site commitment, where splicing factors bind to pre-mRNA to guide spliceosomes once transcription is completed and splicing is required [78].

Once processing of a transcript is finalizing, it is exported from the nucleus into the cytosol so it can be translated (see Figure 1.4A).

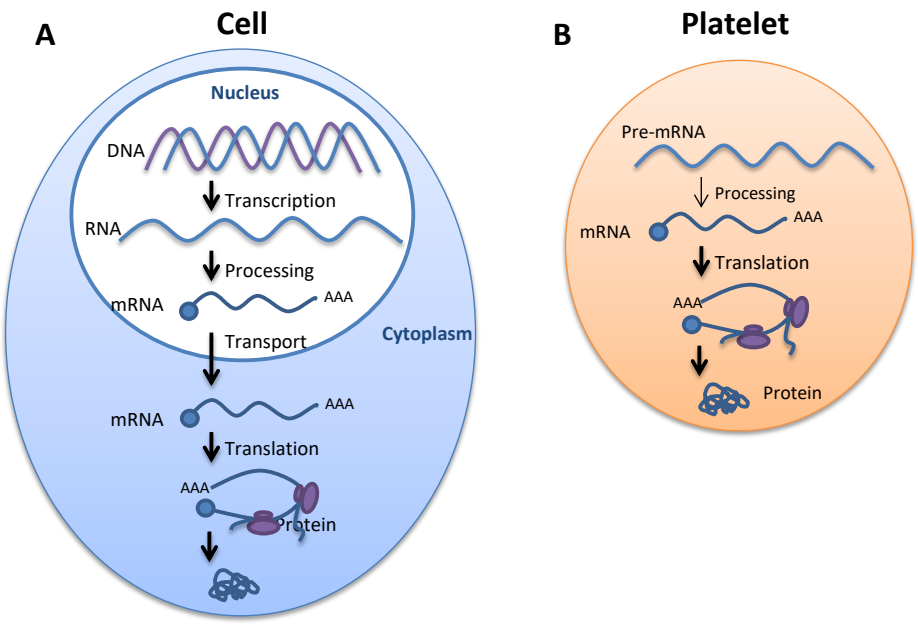


Figure 1.4: Comparison between protein translation in a generalized cell and in platelets.

A) Generalized description of protein translation. The nucleus contains the DNA that can be transcribed in RNA and processed into messenger RNA. The mRNA is transported from the nucleus into the cytosol, where ribosomes have access and synthesize the proteins. B) Processes involved in protein synthesis in the anucleate platelet. The majority of transcripts is present as mature mRNA, however platelets can processes pre-mRNA into mature mRNA.

1.3.2 Ribonucleoprotein complexes

Messenger RNA is transported from the nucleus to the cytosol in RNA granules or ribonucleoprotein (RNP) complexes to be translated. The RNP complexes move along actin filaments (facilitated by myosin) or microtubules (facilitated by dyneins and kinesins) [79]. The composition of the RNP complexes is diverse and orchestrated by *cis*-acting elements present on the mRNA transcript that interact with *trans*-acting factors like RNA binding proteins. The main function of the RNP complexes during transport is to keep the mRNA heading towards the appropriate destination and translationally repressed until after arrival translation can start [80].

The localization and composition of the RNP complexes play an important role in post-transcriptional control.

RNPs are sorted by a highly active and dynamic exchange between the different aggregates [81-83]. When ribosomes associate with mRNA they form polysomes. Environmental stresscan cause an overall inhibition of translation initiation through the phosphorylation of eIF2α by eIF2α kinases like protein kinase R (PKR; activated by UV illumination) or heme-regulated IF2α kinase (activated by arsenite-induced oxidative stress) (see paragraph 1.3.2.2) [84, 85]. Other eIF2α kinases are general control nonderepressible 2 (GCN2; activated by amino acid deprivation), PKR-like endoplasmic reticulum kinase (PERK; activated by unfolded protein accumulation in the ER lumen), and Z-DNA kinase (activated as part of a host antiviral response) [86, 87]. The polysomes disassemble and mRNPs assemble more in other types of granules like stress granules and processing (P)-bodies [83, 88]. Even though stress granules and P-bodies are two distinct types of granules and spatially separated, they share many proteins and are functionally linked to each other [83] (See Figure 1.5). In the following paragraph each type of RNP complex will be described in more detail.

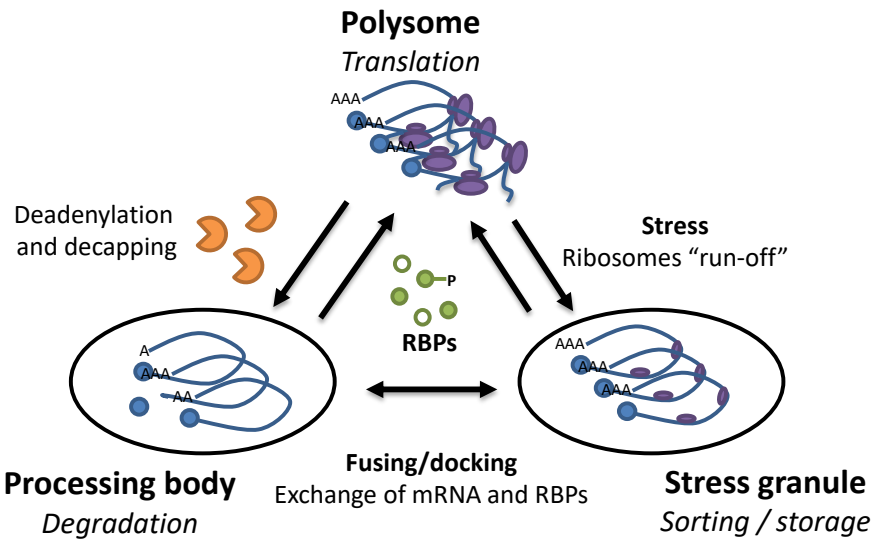


Figure 1.5: Dynamics between polysomes, stress granules and processing bodies.

The dynamic interplay between polysomes, processing bodies and stress granules. When polysomes are exposed to stress, ribosomes run off the transcript and a change in RBP associating allow the formation of stress granules. Deadenylation and / or decapping enzymes are the first step in targeting a transcript to degradation in processing bodies. Stress granules and processing bodies physically interact with each other to allow exchange of components [81, 89].

1.3.2.1 Protein synthesis in polysomes

Protein synthesis occurs in three specific phases; initiation, elongation, and termination. Translation initiation is the rate determining step where most regulation occurs. Multiple ribosomes can associate with a single mRNA transcript to form polyribosomes or polysomes. These polysomes can be found free in the cytosol, membrane-bound to the endoplasmic reticulum or associated with the actin cytoskeleton [90]. Synthesis starts with the formation the eukaryotic initiation factor (eIF)2-GTP-tRNA^{Met} ternary complex, which together with eIF1, 1A, 3, 5, and the 40S ribosomal subunit form the pre-initiation complex. Poly(A) binding protein (PABP) associates with the 3' end poly(A) tail and binds to eIF4G. Together with eIF4A and 4E,

eIF4G compose the eIF4F complex of which eIF4E is anchored to the 5' end cap [91]. The binding of PABP to the eIF4F complex creates a closed loop conformation to allow binding of the pre-initiation complex to enhance translation efficiency [92, 93]. Once the pre-initiation complex scans the 5'-UTR until it locates the start codon through recognition of the anticodon of the $tRNA^{Met}$, after which the 60S ribosomal subunit is sequestered to form the 80S initiation complex and protein synthesis starts. What follows is the elongation of the peptide that ends when the ribosomes dissociates from the transcript and releases the newly synthesized protein. Disruption of the closed loop conformation during translation by drugs or stresses causes disassembly of the polysomes and stimulates the formation of stress granules and P-bodies [94]. For more details about the events involved in the regulation of translation initiation, see references [91, 95, 96].

1.3.2.2 RNA response to stress

In nucleated cells SGs are formed when translation initiation is impaired as a response to environmental stresses like heat shock, oxidative stress, viral infection, UV radiation, nutrient deprivation, or exposure to drugs and toxins [82, 89, 95]. Global protein translation is reduced in almost all types of cellular stress, however at the same time the translation of proteins required for damage repair is enhanced. This process of conserving energy by recruiting 'housekeeping' genes into SGs and reprogramming the protein expression prevents synthesis of unwanted proteins and enhances cell survival [95]. However, studies using fluorescence recovery after photobleaching have demonstrated that major SG components shuttle rapidly in and out, suggesting that SGs have a sorting role [81]. Dependent on the association with the SG component mRNA can be directed to decay, storage or translation initiation [81, 82].

The first step in SG formation involves silencing of protein translation and is triggered by phosphorylation of eIF2α by certain kinases, like PKR, HRI, or PERK. Phosphorylation of the α-subunit of eIF2 stabilizes the eIF2-GDP-eIF2B complex. This prevents the exchange of GDP for GTP by the guanine nucleotide exchange factor eIF2B and thereby preventing the formation of the eIF2-GTP-$tRNA^{Met}$ ternary complex [82, 95, 97]. The inhibition of translation initiation causes ribosomes to run off. The polysomal disassembly releases mRNA and the pre-initiation complex, and enables the accumulation in SGs. Exposure to oxidative stress by means of arsenite treatment induces SG formation by disassembly of polysomes via activation of HRI kinase. Polysome stabilizing translation inhibitors, like emetine or cycloheximide, inhibit the formation of arsenite-induced SG by blocking translation elongation and preventing the release of the ribosomes from a transcript. Translation inhibitors that destabilize polysomes like puromycin, which causes premature chain termination and ribosomal run-off, can induce the formation of stress granules [81, 82]. The primary aggregation of free mRNA and pre-initiation complex into SGs is initiated by RNA binding proteins with RNA recognition motifs like T-cell internal antigen 1 (TIA-1), TIA-1-related (TIAR), and cytoplasmic polyadenylation element-binding protein (CPEB). While SGs grow, the mRNPs will aggregate and fuse into larger granules. As a final step non mRNA binding proteins are recruited to the SGs via protein-protein interactions [82, 89].

The composition of stress granules is extensive and diverse. mRNA transcripts are probably selectively recruited into stress granules. Kedersha *et al* [81] demonstrated that SG growth is the result of shuttling of TIA-1 and PABP-1 in and out of SGs. In addition a study performed by

Kawai *et al* [98] demonstrated that a subset of mRNA is translationally repressed, while another subset (including heat-shock proteins) is translationally enhanced. SGs also comprises of components related to stalled initiation complexes, originating from disassembled polysomes, like mRNA transcripts, PABP, eIF3, 4B, 4F and the small ribosomal units. These components are universal markers for SGs [81, 82, 99]. Other components of SGs are RNA binding proteins linked to mRNA silencing and stability, like TIA-1, TIAR, and CPEB or linked to RNA decay like tristetraprolin (TTP) and Brf1. Proteins involved in other parts of mRNA metabolism have also been identified to be SG components, like RasGTPase-activating protein SH3-domain-binding protein (G3BP) and Staufen.

1.3.2.3 mRNA decay in processing bodies

mRNA is very susceptible to degradation. Especially the 5' end m7G cap and the 3' end poly(A) tail are the regions where decapping and deadenylating can occur. These processes lead to the exposure of the 5' and 3' ends to exonucleases [100]. A complex of decapping protein (Dcp) 1 and 2 is responsible for removing the 5' cap and the enzyme polyadenylated ribonuclease (PARN) is responsible for removing the poly(A) tail [101]. The susceptibility of transcripts for degrading enzymes determines the mRNA stability. In general, transcripts in translation are protected from degradation by their closed loop confirmation [101]. Another mechanism by which mRNA regulates its stability is the presence of AU-rich elements (AREs) in the 3' untranslated region (3'-UTR), so called *cis*-acting elements. These specific sequences are recognized by RNA binding proteins that either stabilized or destabilize a transcript, so called *trans*-acting elements. An example is TTP which binds to AREs and causes destabilization by recruiting the target transcript towards the degradation machinery [102, 103]. Phosphorylation of

TTP reduces the affinity of TTP for the transcripts and allows binding to chaperone protein 14-3-3, which indirectly increases mRNA stability [104, 105]. Human antigen R (HuR) is an RNA binding protein with the opposite effect. Association of HuR recruits transcripts towards stress granules and stabilizes transcripts, and is probably in competition with destabilizing proteins for the similar binding sites [106].

Degradation of mRNA occurs primarily in P-bodies. These cytoplasmic RNP complexes contain components of the 5'-3' decay machinery, but no components from the RNA exosome complex that regulates 3'-5' degradation [107]. P-bodies are involved in RNA processing through silencing, degradation, transport and stabilization [108-112] and are constitutively expressed. Inhibition of mRNA turnover leads to a decrease in P-bodies indicating their role as site of active RNA decay [107, 113]. Under normal conditions nucleated cells contain a small number of P-bodies, but the number and size of P-bodies increase after exposure to stress [109]. However, stress-induced P-body formation occurs independently of SG formation and does not require eIF2α phosphorylation [89, 114]. When ribosomes run off and the mRNA is no longer associated in polysomes it can be recruited into P-bodies [89, 115]. Stabilizing mRNA in polysomes decreases P-body formation as it does for SG formation [109]. This suggests that there is a dynamic equilibrium between the RNA granules and polysomes. (See Figure 1.5) In addition P-bodies and SGs are dynamically linked to each other, enabling transcript interaction and exchange [83]. This supports the theory that RNA granules are a place of RNA sorting. The exact protein composition of P-bodies has not been elucidated, however known components are part of the mRNA decay pathway and proteins that play a role in RNA silencing. Core constituents are the proteins of the decapping complex and exonucleases. P-bodies are also

enriched with components of miRNA-induced silencing complexes (miRISCs), like Ago and GW182. Association of miRISC with mRNA represses translation or targets the mRNA for deadenylation and subsequent degradation [112, 116, 117].

1.4 **Protein synthesis in platelets**

1.4.1 **Discovery**

Human blood platelets do not have a nucleus and do not contain any nuclear DNA. Still they are able to synthesize proteins [118-120]. In 1967 Booyse and colleagues [119] described the presence of stable mRNA in anucleate platelets. This discovery was quickly followed by the observation that ^{14}C-labeled amino acids are incorporated into platelet material insoluble in perchloric acid, they identified as thrombostenin, a complex of actin and myosin. The incorporation was inhibited in the presence of the translation inhibitor puromycin [118]. Later that same year Warshaw and colleagues [121] confirmed the potential of protein synthesis in platelets by the incorporation of ^{14}C-L-leucine which could be inhibited by puromycin, but not by the transcriptional inhibitor actinomycin. This was the start for the exploration of the role of platelets beyond hemostasis [122-124].

1.4.2 **The origin of platelet mRNA**

The mRNA pool present in platelets originates from the megakaryocytes. Among the components packed into the newly formed platelets are numerous mRNAs and miRNAs [125, 126]. The mRNA enables platelets to change their proteomic composition in response to external stimuli [127-129]. For a schematic representation, see Figure 1.4B. Studies have shown that

platelet transcriptome does not vary much among individuals [130], but it remains unclear whether platelet transcriptome correlates to the proteome [131-134].

Even though platelets are shed from proplatelets, the nucleotide composition of platelets is not equal to that of the megakaryocyte. Cechetti and colleagues [4] demonstrated that megakaryocytes sort transcripts of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase (TIMP) into platelets. Only a subset of MMPs and TIMPs present in the megakaryocyte are sorted into platelets, suggesting the sorting and transport of RNA from megakaryocytes into platelets is a directed and active process [4]. This is supported by the fact that many of the highly abundant transcripts are coded for proteins that are a crucial part of platelet function like components of the cytoskeleton, integrins and glycoproteins, signal transduction, and receptors [125, 135, 136].

Gene profiling technologies like microarrays, SAGE (Serial Analysis of Gene Expression) and RNA sequencing (RNAseq) have identified 1,600 – 3,000 different mRNA transcripts, and this may be an underestimation [131, 137]. Among the most abundantly expressed transcripts are messages corresponding to clusterin, β2 microglobulin, β4 thymosin, platelet factor 4, and β actin [125, 131, 135, 138]. Platelets contain mitochondrial-derived genes encoding 13 genes and 2 ribosomal RNAs [125, 136, 139]. Platelets also express numerous miRNAs [126, 140-144], which can modulate platelet function through their interaction with target mRNA. Several miRNAs identified in platelets have been demonstrated to play a role in post-transcriptional regulation, either via platelet signaling [145] or via microparticles released from platelets [146]. Differential platelet miRNA expression has been associated with platelet function like

responsiveness [142] and apoptosis [147], and diseases like cardiovascular disease and arteriosclerosis [148, 149]. They have even been proposed as quality markers for stored platelets [150].

1.4.3 **Characteristics of platelet mRNA**

That platelets have mRNA available during their entire lifespan is unexpected for a cell without nucleus. In *E. coli* the half-life of an individual mRNA is typically about twenty minutes, where nucleated cells contain mRNA with a half-life of several hours [76]. The RNA concentration per platelet is much lower than in nucleated cells [151]. Human platelet contain RNA in the range of 0.1 – 0.3 μg per mL of PC (10^9 cells) [139]. In PCs, platelet mRNA has been shown to have a half-life of days [152]. This long half-life suggests there is a protective mechanism in place, ensuring availability of transcripts for translation during the full lifespan of a platelet. This could be achieved by a combination of stabilizing structural elements present in platelet transcripts in combination with RNA binding proteins, a cellular mechanism like stress granule formation, or another yet unidentified option. However, the major contributor to transcript stability in platelets has not yet been reported.

SAGE analysis has demonstrated that the transcripts in platelets are relatively long (average 2,421 kb) compared to nucleated cells (1,756 kb). And even though the length of the 5’ end is comparable to nucleated cells, the length of the 3’-UTR end is significantly longer (1,047 bp in platelets vs. 492 bp in nucleated cells). The 3’-UTR is a transcript region generally known to harbor post-transcriptional regulatory elements. The long platelet 3’-UTR also has been shown to have a high thermodynamic stability [138]. Platelets are also enriched with circular RNA, which

lack a 5' end m^7G cap or the 3' end poly(A) tail [153] which is speculated to be a mechanism to counteract the rapid degradation of linear transcripts. SAGE analysis has also showed that the highly abundant transcripts in the platelet transcriptome contain more regulatory *cis*-acting elements, like CPE and Brd box, compared to other libraries [138]. Recent work by Mills and colleagues used a RNAseq approach to uncover an abundance of stalled ribosomes on the 3'-UTR of platelet transcripts as a result of an imbalance in ribosomal homeostasis and the lack of proteins usually involved in the ribosome rescue pathway [154, 155]. All the above mentioned observations point towards enrichment of mRNA stabilizing elements, allowing for post-transcriptional control of protein translation in platelets.

1.4.4 **Platelet mRNA processing**

In addition to mature mRNA originated from megakaryocytes, platelets have shown to carry pre-mRNA that can be spliced upon activation by thrombin. Denis and colleagues [128] were the first to demonstrate that platelets contain functional spliceosomal proteins and small nuclear ribonucleic acids (snRNAs) necessary for RNA processing, where up until then RNA splicing was assumed to occur only within the boundaries of the nucleus. The spliceosome in platelets originates from the megakaryocyte precursors and is functional and upon activation capable of splicing interleukin-1β (IL-1β) pre-mRNA to produce mature mRNA that can be translated into IL-1β protein. Similar results were found for tissue factor (TF) and cyclooxygenase-2 [129, 156-158]. The observation of spliceosomal proteins in megakaryocyte proplatelet extensions and platelets is unexpected since these processes and their corresponding proteins were assumed to be restricted to the nucleus [101]. Platelets also contain the proteins necessary to convert pre-miRNA into mature miRNA, like Dicer, TRBP, and Ago2. Landry and colleagues [141] did not

only demonstrate this conversion, but also showed that the miRNA complexes that are formed in platelets are functional and capable of RNA silencing and regulation of gene expression.

1.4.5 **Protein synthesis in platelets**

Platelets contain all the prerequisites, like ribosomes and eukaryote initiation factors, to facilitate protein translation [119, 159, 160]. In nucleated cells, mRNA localization is determined by the composition of the RNP complex and resides primarily in the cytoplasm. In resting platelets the majority of mRNA can be found in association with the cytoskeletal core, and only a small fraction is detected in the cytosol. Lindemann and colleagues [5] demonstrated that in resting platelets, eIF4E is located in the membrane skeleton and the cytosol. Upon activation eIF4E translocates from the membrane skeleton to the mRNA-rich cytoskeletal core, enabling translation. Blocking the translocation, by inhibiting actin polymerization using cytochalasin D, or by blocking platelet activation via integrin αIIbβ3, inhibits protein synthesis [5, 161].

In 1987 Kieffer and colleagues [120] were one of the first to identify several glycoproteins and alpha granule proteins to be newly synthesized by platelets using a similar approach as Booyse [118] did two decades earlier. By comparing platelets from healthy subjects with platelets from splenectomized ITP patients they were able to demonstrate that protein synthesis occurs in circulating platelets, but is enhanced in newly formed platelets. Protein synthesis is also greatly enhanced when platelets are stimulated with an agonist like thrombin or LPS [128, 156, 157, 162], but not only activated platelets synthesize proteins. Ongoing protein synthesis has been demonstrated in platelets stored *ex vivo* up until ten days post collection [152]. Comprehensive proteomic analysis has revealed an increase in protein concentration in platelets stored under

blood banking conditions [163]. Among those identified were proteins which are involved in glucose metabolism, like glucose-6-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, and hexokinase. *De novo* synthesis of plasminogen activator inhibitor-1 and GPIII has been confirmed in resting platelets [152, 162].

Using protein translation inhibitors, it has been shown that protein translation in platelets is linked to *in vitro* platelet function. Already in 1966, Warshaw and colleagues [164] observed a reduction in glucose oxidation when platelets were treated with puromycin. Rosenwald and colleagues [160] demonstrated that puromycin and emetine reduced platelet aggregation in response to ADP and epinephrine, but not to collagen. Schwertz and colleagues [129] showed that blocking TF pre-mRNA splicing in activated platelets significantly reduced the TF-dependent procoagulant activity and delayed the onset of clot formation. Puromycin had a similar impact on the TF-dependent procoagulant activity. The same group demonstrated that Bcl-3 is synthesized by platelets [127] and the overexpression of Bcl-3 enhances clot retraction and that this process is inhibited by the mTOR inhibitor, rapamycin [165].

1.4.6 **Evidence of RNA granules in platelets**

Even though direct evidence of the presence of stress granules or P-bodies in human blood platelets has not yet been reported, stress granule components like TIA-1, G3BP and PABP have been demonstrated to be present in platelets [166, 167]. In addition mRNA transcripts for double stranded RNA – binding protein Staufen homolog 1 (STAU1) and STAU2, EIF4A3 and cancer susceptibility candidate 3 (CASC3, also known as MNL51) have been shown to be expressed in human platelets [4]. The corresponding proteins are known to associate in SG [168, 169]. The

presence of the SG components as protein or transcript could allow platelets to use this mechanism to protect and sort their mRNA transcripts.

1.4.7 **Impact of PI on platelet RNA**

The impact of PI on platelet RNA has been studied much less than the impact on platelet function. A study comparing untreated platelets to γ-irradiated platelets, platelets treated with amotosalen / UV, and riboflavin / UV showed that the mRNA copy numbers for Bcl-xl and clusterin, but not bcl2, were significantly reduced after amotosalen / UV treatment compared to untreated control. The impact of the riboflavin / UV treatment was not significantly different from the untreated control [170]. RNA-sequencing analysis confirmed the difference in impact of the amotosalen / UV and riboflavin / UV treatment by showing a change in composition of the most abundant mRNA transcripts in platelets between the two treatment types. Further differential expression analysis revealed a significant impact of amotosalen-based PI on the platelet transcriptome. This change was not observed in riboflavin / UV treated-platelet [171]. These data suggests that the amotosalen-based PI affects platelet mRNA differently than the riboflavin-based PI does. Puromycin-associated nascent chain proteomic analysis has revealed that protein translation still occurred in stored platelets treated with riboflavin and UV illumination [172]. These data could imply that the application of the riboflavin-based PI treatment initiates an ongoing translational stress response. The enhanced energy consumption that would be necessary is in agreement with the accelerated metabolism observed in PI treated platelets.

Studies that focused on miRNAs demonstrated more differences when untreated platelets were compared to γ-irradiated platelets, the amotosalen-based PI, and the riboflavin-based PI. MiRNAs are small (20 - 25 nt), non-coding RNAs, responsible for post-transcriptional regulation of mRNA expression like RNA silencing. Unlike control platelets or riboflavin / UV treated-platelets that showed no change in the miRNA expression during storage, platelets stored in additive solution (part of the amotosalen-based PI) and γ-irradiated platelets reduced miRNA levels of 2 out of 11 analyzed targets slightly and only after 7 days of storage. The amotosalen-based PI significantly reduced the miRNA levels of 6 out of 11 analyzed targets after 1 day of storage [170]. The same study showed that neither the miRNA synthesis, nor the function was affected by any of the treatment arms.

Bakkour *et al.* used a different approach and have developed an assay that can be used as a quality control measure for the performance of a PI technique [173, 174]. Samples were spiked with a known amount of mtDNA. The inhibition of PCR amplification of primers for different product lengths was used to evaluate the nucleic acid damage of the PI technique.

1.5 **Hypotheses**

Previous research performed in our lab has demonstrated that there is full length mRNA of glycoprotein (GP)IIIa, a subunit of the platelet fibrinogen receptor, in stored platelets. This mRNA is translated during the entire life span of the platelets, and has an unexpectedly long half-life of 2.9 days [152]. Protein synthesis has been repeatedly demonstrated to occur in platelets under different conditions although all the details are not understood. Not much is known about the way platelets regulate and maintain their RNA during their lifespan. With the

development of PI techniques that target RNA and DNA, a need emerged to improve the understanding how platelet mRNA is regulated. Pathogen-reduced PCs show features consistent with accelerated development of the platelet storage lesion. Since a key mechanism of action of PI is the destruction of nucleic acids, it may interfere with platelet mRNA, and possibly result in its accelerated degradation.

This has led us to the following hypotheses:

1. Pathogen inactivation techniques, which are designed to target pathogen RNA and DNA, influence platelet mRNA quality and functionality.
2. Some messenger RNA species in platelets are protected against degradation during storage by the formation of stress granules.

During the project a protein kinase, p38 MAP kinase, which could be involved in the regulation of platelet mRNA exposed to UV stress, was identified. An investigation of this potential target was added to the aims of the project, which are as follows:

1. Determine the impact of Mirasol on platelet mRNA amount of a selected panel of transcripts.
2. Determine the impact of inhibition of p38 MAP kinase on mRNA levels in platelets exposed to the Mirasol treatment.
3. A) Evaluate the impact of the Mirasol pathogen inactivation system on platelet mRNA in the presence of the stress granule inhibitor nocodazole.

B) Create an *in vitro* model of platelet stress using a known stress granules inducer (arsenite) and inhibitor (nocodazole) to establish reference in response.

The impact of Mirasol on platelet mRNA was determined immediately after execution of the treatment and during storage of the platelets and is described in **chapter 3**. We observed a prolonged mRNA half-life after UV exposure based on the mRNA levels in stored platelets. This observation offered potential to evaluate the impact of p38, a kinase involved in regulating mRNA after UV exposure in nucleated cells. The results of the inhibitor study targeting this kinase is described in **chapter 4**. **Chapter 5** summarizes our findings with regard to stress granule formation potential in platelets using a stress granule inhibitor. In addition we describe the experiments performed with a stress granule inducer with the purpose to increase the understanding of the effect of these components on platelets *in vitro* quality and mRNA levels in untreated control platelets and platelets exposed to Mirasol.

Chapter 2: Materials and methods

2.1 Chemicals

Chemicals and reagents were purchased from Sigma-Aldrich or Fisher Scientific. Quantitative polymerase chain reaction (qPCR) reagents and disposables were purchased from Applied Biosystems (Life Technologies).

2.2 Production of platelet concentrates

The study was approved by the Clinical Research Ethics Boards of the University of British Columbia (certificate number: H12-03515) and Canadian Blood Services (Protocol reference # 2008.02 - NetCAD Reference-X007) and informed consent was obtained from healthy donors prior to donation. Platelet concentrates were collected via apheresis from healthy volunteer donors according to Canadian Blood Services standard operating procedures using a Trima Accel Automated Blood Collection System (TerumoBCT, Lakewood, CO) at the Network Centre for Applied Development (NetCAD, Vancouver, BC, Canada). All PCs were leukoreduced as per protocol, resulting in a residual leukocyte count of $< 5.10^6$ per unit [18]. Single or double apheresis units were stored under standard blood banking conditions at 20 – 24 °C under continuous agitation. In selected experiments apheresis PCs were split into 60 mL illumination (mini-)bags (TerumoBCT, Lakewood, CO). These mini-bags were stored under the same conditions as regular size apheresis bags. All units were allowed to rest for at least one hour before the application of any post-production modification, if necessary.

2.3 **Post-production modification**

All post-production modifications were done on the day of collection (Day 0) unless mentioned otherwise. For selected experiments apheresis PCs were pooled together and split to create equal units. For all treatment experiments, one PC was kept untreated to serve as control.

2.3.1 **Inhibitor treatment**

For selected experiments inhibitors were used to study the effect of p38 MAP kinase or the role of microtubule formation. SB 203580 (p38 MAP kinase inhibitor), hereafter referred to as SB, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and dissolved in DMSO. The final concentration used in the experiments summarized in **chapter 4** was 10 μM. The efficacy of the inhibitor was monitored indirectly via flow cytometry, see Figure 2.1. Previous research in our group has demonstrated that the presence of SB reduces the increase in platelet activation after Mirasol treatment [175]. This method was used to demonstrate that in the presence of SB the percentage CD62p positive cells, as marker for platelet activation, was reduced to levels as seen in untreated control platelets, confirming the SB efficacy.

Nocodazole (microtubule formation inhibitor) was purchased from Sigma Aldrich and dissolved in DMSO. The final concentration used in the experiments summarized in **chapter 5** was 20 μM. The inhibitors or their respective vehicle control DMSO were added to the PCs at least one hour after collection, but at least 30 minutes prior to riboflavin / UV treatment. Volumes were added in a biosafety cabinet using a needle and syringe to ensure sterility.

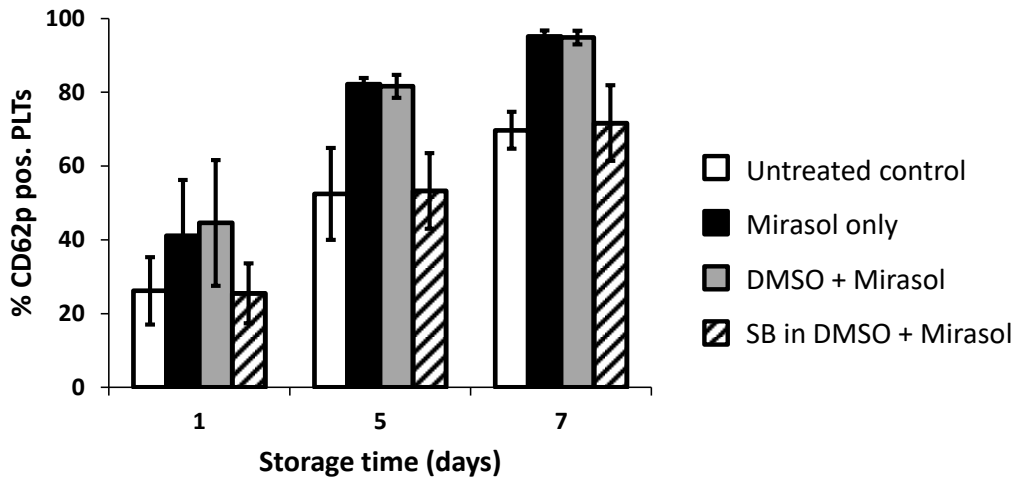


Figure 2.1: Presence of SB inhibitor reduces CD62P expression after Mirasol treatment.

The impact of Mirasol treatment on the activation levels measured by percentage of CD62p expression in untreated PCs, Mirasol-treated PCs, Mirasol-treated PCs spiked with a vehicle control DMSO, and Mirasol-treated PCs in the presence of the SB inhibitor in DMSO. Results were given as mean ± SD for N = 5

2.3.2 **Riboflavin / UV illumination treatment**

For all the experiment described in this dissertation the Mirasol PRT® treatment (TerumoBCT, Lakewood, CO) was used as representative pathogen inactivation treatment. Pathogen inactivation treatment was performed by the addition of riboflavin followed by UV illumination according to the manufacturer's instruction. The PC was sterile docked to an illumination bag system, containing a storage bag and sterile riboflavin solution. The content of the PC was transferred to the storage bag, followed by the addition of 35 mL of riboflavin solution (500 µM riboflavin in 0.9% saline; final concentration was 50 µM riboflavin). The PC was placed in the Mirasol Illuminator and exposed to UV light for 4 – 10 minutes depending on the volume of the PC (6.24 J/mL; 280 – 400 nm). For the experiments performed in 60 mL mini-bags, the riboflavin amount and UV illumination time was reduced to receive an UV dose equivalent to a

full size unit. For selected experiments described in **chapter 3** the illumination dose was adjusted to 50% (3.12 J/mL). After treatment the units were stored under standard blood banking conditions (20 – 24 °C, under continuous agitation). The units were allowed to rest for at least one hour before the sampling.

For all experiments, PI treated platelets are considered platelets that were exposed to both the riboflavin and the UV illumination, as described by the manufacturer. No experiments were performed using either riboflavin alone or UV illumination alone, as this is not an available option for transfusion purposes. Consideration is given to let the experimental outcome be reflective of what is relevant in transfusion medicine. Studies performed with either one of the two components of the PI treatment demonstrate that the addition of riboflavin alone has no impact when evaluated using a PCR inhibition assay [173].

2.4 **RNA related assays**

All disposables used for RNA related assays were free of RNAse and DNAse according to the manufacturer's certificates. Water used for RNA related preparations was treated with diethylpyrocarbonate (DEPC) to remove any RNAse and DNAse contamination. Briefly, 0.1% DEPC was added to the water and thoroughly mixed by hand. The solution was incubated overnight at 37°C followed by autoclaving. Where necessary, non-disposable plastics, glass, and surfaces were cleaned with RNAseZAP (Sigma Aldrich, St. Louis, MO) according to manufacturer's instruction or cleaning in cleaning solution (0.1 M NaOH, 1 mM EDTA in DEPC-treated water).

2.4.1 **Sampling procedure for RNA extraction**

A volume of 10 mL PC was sampled aseptically in the biosafety cabinet and transferred to a 15 mL conical tube. The platelets were sedimented (15 min at max. speed of 2,800 x g (Beckman GS-6R Centrifuge with GH-3.8 rotor)) and washed in three mL ETS (5 mM EDTA, 10 mM Tris base, 150 mM NaCl). A platelet count was obtained from the resuspended platelets using a hematology analyzer (Advia 120 Hematology System, Siemens). The resuspended platelets were split into 3 x 1 mL into bullet tubes and sedimented (15 min at 3,000 x g (Beckman Coulter® 22R Microfuge with S241.5 rotor or Sorvall Legend Micro 21R Centrifuge with 75003424 rotor)). The wash was discarded and each pellet was lysed in one mL TRIzol® Reagent (Invitrogen, Life Technologies, Burlington, ON). The samples were allowed to lyse five minutes at room temperature, followed by storage at -80°C until further use. For small volume experiments the extraction procedure was down-scaled to 5 mL PC starting material and all other volumes were adjusted accordingly.

2.4.2 **RNA purification and quantitation**

Experiments were performed in a fume hood cleaned with RNAseZAP to ensure an RNAse free workspace. RNA was extracted from samples in TRIzol® Reagent according to the manufacturer's instruction. Samples were allowed to thaw at room temperature and were homogenized. Once thawed, 200 µL of chloroform was added to each tube. The tubes were shaken by hand for 15 seconds followed by three minutes incubation at room temperature. The aqueous phase was separated from the organic phase by centrifugation (15 min at 12,000 x g at 4°C). From the aqueous phase, 0.5 mL was transferred to a clean tube. If necessary, three aliquots of a test sample were pooled and split into 2 x 0.75 mL aqueous phase. To each aliquot

an equal amount of 100% isopropanol was added and mixed, followed by 10 minutes incubation at room temperature. The RNA was precipitated by centrifugation (10 min at 12,000 x g at 4°C) and washed in 70% ethanol. The sample was centrifugated (5 min at 7,500 x g at 4°C) to take off the wash. The RNA pellet was air-dried for 10 min before resuspension in DEPC-treated water based on the platelets count to compensate for the change in platelet count during storage (one µL per 100 x 10^6 cells) followed by 10 minutes incubation at 57°C. Normalization to platelet count was preferred over normalization to total RNA since the experiments involved treatments that changed the platelet RNA. Validation of the extraction methods' within-sample repeatability was determined to have a CV ≤ 15. Total RNA content was measured by absorbance at 260 nm using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific). The purity of the RNA was evaluated by the ratio for 260 nm / 280 nm ≥ 1.8. Samples were stored at -80°C until further use. Ribosomal RNA was not analyzed separately.

2.4.3 **DNase treatment**

All reagents for DNAse treatment were purchased from Invitrogen (Burlington, ON). The DNAse treatment was performed according to the manufacturer's instruction with the following adjustments for the low amount of RNA present in platelets. (For effect of the DNAse treatment, see Figure 2.2) Fifty µL of total RNA was treated with 1 µL DNase I in presence of DNAse reaction buffer. After 15 minutes incubation at room temperature 1 µL EDTA (25 mM) was added followed by 10 minutes incubation at 65°C. Samples immediately cooled to 4°C for reverse transcription or stored at -80°C until further use.

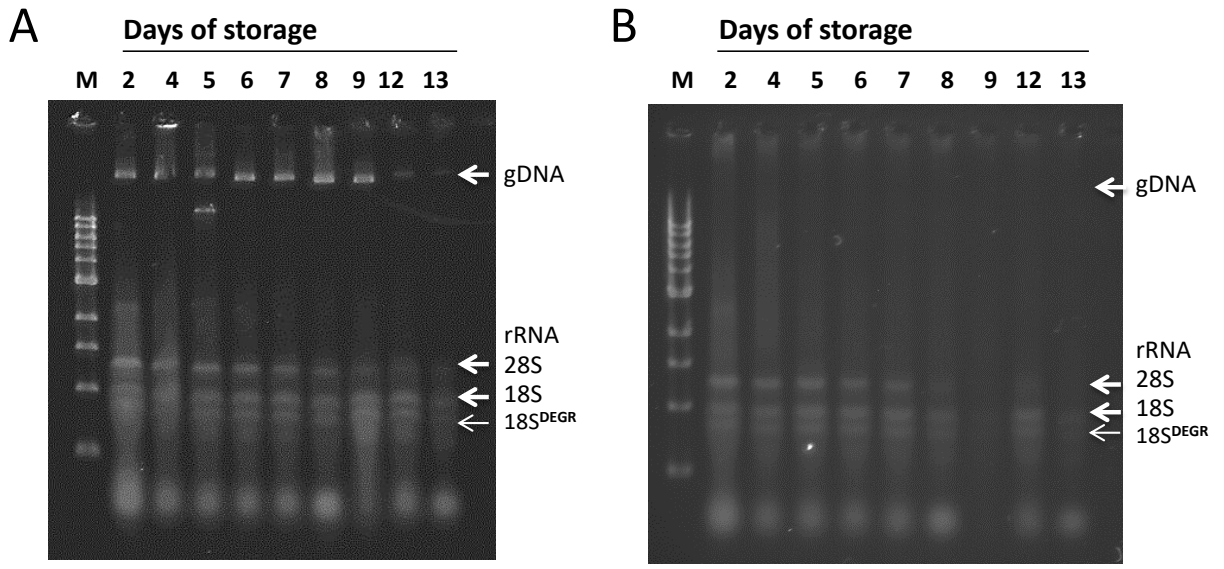


Figure 2.2: Effect of DNAse treatment on total RNA.

A PC was followed for 13 days and RNA was extracted using 10 mL starting material in TRIzol reagent. To allow all the samples to be split into two equal volumes after RNA extraction, the starting volume was corrected for platelet count. The extracted RNA was resuspended in 40 µL DEPC-treated water. Samples were split into 2x20 µL and treated with DNase, according to manufacturer's protocol, or were left untreated. The extracted samples before (A) and after (B) DNase treatment were visualized using an non-reducing agarose gel (1%) post-stained with ethidium bromide (0.5 µg / mL) on an AlphaImager (Innotech). Top arrow marks the gDNA, bottom arrows mark the ribosomal RNA bands. The lowest band showing up after 5 days of storage is most likely degraded 18S rRNA (18S^{DEGR}). M = 1kb DNA ladder (New England Biolabs).

2.4.4 First strand cDNA synthesis

All reagents for first strand cDNA synthesis were purchased from Invitrogen (Burlington, ON) unless mentioned otherwise. For reverse transcription, 5 µL sample was used in 20 µL reaction volume. One µL of oligo(dT)$_{12-18}$, 1 µL dNTP mix (10 mM each) and 6 µL H_2O was added to the sample according to the manufacturer's protocol. The mixture was heated for 5 min at 65°C followed by a minute on ice. After a brief spin to collect the sample 4 µL First Strand buffer

(5X), 1 µL Dithiothreitol (0.1 M), 1 µL RNase inhibitor, and 1 µL Superscript III was added. The reaction mixture was incubated at 50°C for 60 minutes, followed by 15 min at 70°C in a DNA thermal cycler (Perkin Elmer). Samples were cooled to 4°C for polymerase chain reaction or stored at -80°C until further use.

2.4.5 **Primer design**

Primers were designed using primer design software (Primer3 software version 4.0.0 [176] and NCBI/Primer-BLAST [177]) to be specific for the selected targets (see Table 2.1) and synthesized by Integrated DNA Technologies (Coralville, IA). Primers were resuspended in TE buffer (10 mM Tris Base, 1 mM EDTA, pH 8.0) to a stock concentration of 100 µM, aliqoted and stored at -20°C.

2.4.6 **Quantitative real-time PCR**

Relative mRNA amount were determined using quantitative PCR. One µL cDNA was amplified in the presence of specific primers (see Table 2.1) using SYBR® Green Mastermix (Standard or Fast) according to the manufacturer's protocol on an OneStepPlus^{TM} Real-time PCR System (Applied Biosystems, Life Technologies). When Fast SYBR® Green Mastermix was used, primers with melting temperatures ~ 60 °C were added to allow two-phase cycling. The primer pairs were chosen based on the temperature requirements (standard or fast) for the qPCR polymerase used. Results were analyzed using qPCR Software (ExpressionSuite, Applied Biosystems). Samples were run in triplicate and the mean Ct value was used for calculation of the mRNA fold change compared to the untreated control. A melt curve analysis was performed to ensure amplification of a single PCR product. The mRNA amounts were measured during

storage and the mRNA half-life was calculated using the trend line for each individual experiment of specific mRNA target using the equation:

$$T_{1/2} = \frac{\ln 0.5}{slope}$$ Equation (1)

The primer efficiency was evaluated using the trend line of a 5-point 1 in 10 dilution standard curve from the untreated control sample. Efficiency was calculated using the equation:

$$Efficiency = (10^{-1/slope}) - 1$$ Equation (2)

Primers efficiency requirements were set to 0.9 – 1.1 with an $R^2 > 0.98$.

Table 2.1 Primer sequences and corresponding amplicon sizes used in this dissertation.

Target	NCBI Reference Sequence	Primer sequence			Amplicon size (bp)
Glycoprotein IIIa (GPIIIa) [178]	NM_000212.2	F: 5'-	GTGGAAGCTCATGCCTGTAA	-3'	56
		R: 5'-	GAGCCACCACACCTGTCTTA	-3'	
		F: 5'-	GCG TAG GAG GGC CCA ACA TCT	-3'	195*
		R: 5'-	CTC GGG CCT CAC TCA CTG GGA ACT	-3'	
Glycoprotein IIb (GPIIb)	NM_000419.3	F: 5'-	CCACTACATGCGGGCCCTAA	-3'	156
		R: 5'-	TTCCCCACGCTCACCAACAT	-3'	
Glycoprotein Ib (GPIb)[179]	NM_000173.5	F: 5'-	GGTGCGTGCCACAAGGACTGT	-3'	282
		R: 5'-	TTTGGGGCGGGCTCCGGGACG	-3'	
Glyceraldehyde-3-phosphate	NM_002046.5	F: 5'-	TCAACGACCACTTTGTCAAGC	-3'	110
dehydrogenase (GAPDH) [180]		R: 5'-	CCAGGGGTCTTACTCCTTGG	-3'	
Platelet Factor 4 (PF4)	NM_002619.3	F: 5'-	CGAGTTTCCCATCGCACTGA	-3'	381
		R: 5'-	CACACACGTAGGCAGCTAGT	-3'	
		F: 5'-	CTGCCCCACTGCCCAACTG	-3'	134*
		R: 5'-	ATGCACACACGTAGGCAGCT	-3'	

Table 2.1 Primer sequences and corresponding amplicon sizes used in this dissertation. (Continued)

Target	NCBI Reference Sequence	Primer sequence		Amplicon size (bp)
Secreted protein, acidic, cysteine-rich (SPARC, osteonectin)	NM_003118.3	F: 5'-	ATGGTGCAGAGGAAACCGAA -3'	311
		R: 5'-	CAGCTCAGAGTCCAGGCAAG -3'	
		F: 5'-	GGCGAGTTTGAGGTGTGC -3'	126**
		R: 5'-	AGGCCCGATGTAGTCCAGGT -3'	
Thrombospondin (TSP)	NM_003246.2	F: 5'-	CACAACCCAGATCAGGCAGA -3'	479
		R: 5'-	GGCACACTGTGGTCAAAATC -3'	
		F: 5'-	CAGCATCCGCAAAGTGACTG -3'	178
		R: 5'-	GATGGGGCAGGACACCTTTT -3'	
		F: 5'-	AGACGCCTGCCCCATCAATG -3’	115**
		R: 5'-	GGGTGTGGGGTTGTTGCAAGA -3’	

* Primer pairs used to analyze the samples for Figure 3.4. ** Primer pairs used to analyze the samples for Figure 4.1.

2.4.7 **Agarose gel electrophoresis**

Agarose gel electrophoresis was performed as described by Rio *et al* [181]. To visualize the total RNA as shown in Figure 2.2, 0.3 g agarose (Life Technologies) was dissolved in 30 mL TBE buffer (1 M Trise base, 1 M Boric Acid, 20 mM EDTA in DEPC-treated water) by heating in a microwave. The agarose solution was allowed to cool for 5 minutes before poured into the gel tray. The gel tray was placed in the electrophoresis apparatus (GNA-100 Mini Submarine, Amersham Biosciences) filled with TBE buffer. Five μL samples mixed with 1 μL loading dye was loaded onto the gel and the gel was run at 75 V until the marker was 2/3 of the way down the gel. The gel was then placed in a box for staining with ethidium bromide (0.5 μg / mL) for 10 minutes, followed by destaining with DEPC-treated water for 15 minutes. The gel was imaged on an imaging system (AlphaImager^{TM} Gel Imaging System, Alpha Innotech)

2.5 **Protein related assays**

2.5.1 **Sample preparation**

Samples were drawn aseptically to ensure product sterility. A platelet count was obtained using the Hematology Analyzer (Advia 120, Siemens). PLTs were sedimented gently (10 min at 200 x g) and washed with CGS (10 mM sodium citrate, 30 mM D-glucose, 120 mM NaCl, pH 6.5). The washed platelets were sedimented (10 min at 200 x g) and the supernatant removed. Lysis buffer (1% Triton-X 100, 2 μM EGTA, in PBS enriched with protease and phosphate inhibitors (cOmplete, EDTA-free and PhosSTOP respectively, Roche Applied Science)) was added based on the platelet count. Platelet lysates were stored at -80°C.

2.5.2 **Protein quantitation**

For each lysate the total protein concentration was determined by Bradford assay (Bio-Rad Protein Assay, Bio-Rad). Protein lysates were diluted 1:40 in deionized water. A standard curve from 0.5 mg / mL bovine serum albumin was prepared by serial dilution (1:10) and measured alongside the diluted protein lysates. A volume of 10 µL sample was added onto a 96 well plate in triplicate. The Dye Reagent Concentrate was diluted (1:5) and 200 µL was added to each well followed by 5 min incubation at room temperature. The absorbance was measured on a plate reader (SpectraMax 190, Molecular Devices) and the protein quantity determined using spectrophotometer software (SoftMax Pro 5.2).

2.5.3 **Immunoblotting**

For all experiments an equal total protein amount, based on total protein concentration, was loaded onto a 1.5 mm thick sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) gel and separated using a Mini-PROTEAN gel chamber (BioRad) filled with protein running buffer (25 mM Tris base, 192 mM glycine, 0.2% SDS). The proteins were transferred from the gel onto a nitrocellulose membrane (BioRad) by semi-dry transfer (Trans-blot® Turbo™ Transfer System, Biorad) using blotting pads soaked in Towbin transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol). Proteins were transferred for 35 min (single gel) or 45 min (two gels) at a constant voltage of 25 V. After transfer, membranes were blocked in 5% skimmed milk (Safeway Canada) or 5% bovine serum albumin (BSA) (Sigma-Aldrich) in phosphate buffered saline (PBS) with 0.1% Tween®20 (Fisher Scientific) (PBS / Tween) for a minimum of 2 hours. After blocking, the membranes were probed with primary antibodies in PBS / Tween (1:1,000 to 1:5,000) of 1.5 hours to overnight. For a list of the primary antibodies

used in this dissertation, see Table 2.2. Membranes were washed three times for 5 minutes in PBS/Tween followed by 1.5 hour incubation with the respective secondary in PBS / Tween (1:10,000) (IRDye® 680RD Goat anti-Rb or IRDye® 800CW Goat anti-Ms, Mandel Scientific). Membranes were again washed three times for 5 minutes in PBS / Tween prior to imaging on a imaging system (LI-COR Biosciences). Band intensities were quantified by densitometry (Odyssey Imaging Software).

Table 2.2: List of primary antibodies used in this dissertation

Name	**Secondary Ab**	**Manufacturer**	**Catalogue #**
TTP	Mouse	Abcam	119779
Phospho-TTP (ser178)	Rabbit	Santa Cruz Biotechnology	133299
HuR	Mouse	Santa Cruz Biotechnology	5261
Phospho-HuR (ser221)	Rabbit	EMB Millipore	ABE265
TIA/TIAR	Rabbit	Santa Cruze Biotechnology	28237
PABP	Rabbit	Cell Signaling	4992
G3BP	Mouse	Abcam	56574
GAPDH	Rabbit	Abcam	9485
Actin	Rabbit	Sigma Aldrich	A2066

2.6 *In vitro* assays

2.6.1 Platelet parameters

Platelet count, mean platelet volume, platelet distribution width, and "platelet crit" were measured on a hematology analyzer (Advia 120, Siemens).

2.6.2 Platelet immunofluorescence

For confocal microscopy imaging, coverslips were placed in a 24 well plate and incubated with poly-L-Lysine (1 mg / mL) for 20 minutes followed by a wash with ultra-pure water. Platelets were fixed in 4% paraformaldehyde, added into the wells and incubated for 5 min at room temperature. The plate was centrifuged (5 min at 1,000 x g) (Eppendorf) and the supernatant was removed. The wells were washed with PBS followed by permeabilisation of the platelets with 0.1% Triton X-100 for 20 min. Samples were blocked overnight at 4°C in 5% goat serum in PBS. After blocking the samples were stained. Samples were incubated at room temperature for 1 hour with anti-G3BP (1:500) (BD Sciences) and washed three times for 5 minutes in PBS. After the staining with primary antibody, the samples were stained for 1 hour with Brilliant Violet 421 goat anti-mouse secondary (240 ng / mL). Samples were washed three times for 5 minutes in PBS and block for 2.5 hours in 5% goat serum in PBS followed by incubation with anti-PABP (7:200) (Cell Signaling) for 1 hour and Texas Red goat anti-rabbit (4 μg / mL) for 1 hour. Samples were washed three times for 5 minutes in PBS and the platelets were visualized using a 60 x objective on a Nikon Eclipse TI, using imaging software (NIS-Elements AR software).

2.6.3 **Extent of shape change**

The extent of shape change was measured as described by Holme *et al.* [32] by comparing the optical density from platelet poor plasma (PPP) to diluted PC exposed to 10 μM ADP (Chronolog Corp.) in the presence of 1 mM EDTA. The optical density will increase with the increasing number of platelet that will change their shape from discoid to spherical with filopodia. Briefly, cuvettes with stir bars were preheated (37°C for 30 minutes) (Thermomixer, Eppendorf). PPP was prepared by centrifugation (20 min at 2,400 x g). Platelets were diluted to 300.10^9 cells / L using PPP and the diluted platelets were incubated for 30 minutes at 37°C. After the incubation cuvettes were filled with 500 μL diluted samples and the instructions on the aggregometer (SPA2000 aggregometer (Chronolog Corp)) were followed. When prompted a cuvette with PPP was placed in the instrument, followed by the cuvette with the diluted platelets. A volume of 10 μL of EDTA (0.1 M) was added, followed by 10 μL of ADP (500 μM). Samples were run in duplicate.

2.6.4 **Flow cytometry**

The activation marker CD62P was determined by flow cytometry. Platelets were diluted to 200.10^9 cells/L using PPP. A volume of 5 μL sample was incubated for 30 min with 5 μL PE-conjugated anti-CD62P or its respective PE-conjugated IgG control (IO system, Beckman-Coulter) in PBS in a total volume of 55 μL. ADP stimulated platelets were prepared by incubating 5 μL diluted platelets for 15 min with 6 μL ADP (10 μM final concentration) in 39 μL PBS prior to incubation for 15 min with 5 μL PE-labeled anti-CD62P. Immediately before analysis, 700 μL PBS was added and samples were vortexed. CD62P expression was determined

as percentage positive cells compared to the IgG control value of 0.4 by flow cytometry (FACS Canto II, BD Sciences).

2.7 Data analysis

2.7.1 *In silico* analysis of RNA binding proteins

The database of RNA-binding protein specificities (RBPDB) v1.3.1 (21-11-2012), accessed through http://rbpdb.ccbr.utoronto.ca/[182] was used to generate a collection of RNA-binding proteins (RBPs) with published observations of RNA binding. The mRNA transcript sequences for TSP, SPARC, and GAPDH were extracted from the NCBI database (see Table 2.1 for accession numbers). The sequence of their 3'-UTR was uploaded to RBPDB and scanned to predict binding sites. The generated list of RBPs was cross-referenced with the proteomics database PlateletWeb | Systems Biology Workbench (Department of Bioinformatics, University Wuerzburg, Germany [183]) to evaluate the level of detection of the RBPs in platelets specified as proteome, transcriptome, both or none.

2.7.2 Statistical Analysis

Results are given as mean ± standard deviation with N as the number of biological replicates. All statistical analyses were performed using statistical software (Minitab 16.2.4.3, Minitab Inc., State College PA). Datasets were tested for normality (Anderson-Darling) for each parameter. Normally distributed data were analyzed using appropriate parametric tests. Storage data were analyzed using a two-way ANOVA with a Bonferroni correction for repeated measures. Half-life data were analyzed using one-way ANOVA. Difference between study arms were calculated using Student's T-test. Where possible, not normally distributed data were transformed to

normally distributed data. Not normally distributed data were analyzed using appropriate non-parametric tests using the Kruskall-Wallis test with Bonferroni correction. Difference between study arms were calculated using Mann-Whitney tests. P-value < 0.05 was considered significant.

Chapter 3: Riboflavin and ultraviolet illumination affects selected platelet mRNA transcript amounts differently

3.1 Introduction

Human blood platelets contain RNA and undergo protein synthesis upon activation or during storage [126, 127, 152, 161, 184]. Platelets synthesize proteins using the mRNA derived from their megakaryocyte progenitors without the presence of a nucleus, and process pre-mRNA into full length mRNA by post-transcriptional regulation [126, 128, 129]. Over 2000 different transcripts have been identified in platelets [125, 131, 135, 137, 138]. However, the role of protein synthesis in human blood platelets is poorly understood [3, 131, 185, 186].

Bacterial contamination of PCs is a major safety concern in transfusion medicine. The platelet safety profile increases with the application of PI techniques after production. Using UV illumination with or without a photosensitizing agent, these systems target RNA and DNA by cross-linking, or by oxidation of bases resulting in single strand breaks [42].

The effects of PI treatment on *in vitro* platelet quality have been extensively investigated [65-67, 71, 175, 187-191]. PCs exposed to PI treatment demonstrate an accelerated loss of *in vitro* quality. PI treated platelets show increased metabolism and platelet activation, and a decreased agonist response. However, the impact of PI treatment on platelet RNA and subsequent platelet function is still elusive as is how PI illumination affects platelet mRNA stability and protein translation. In this study we investigated the impact of PI on platelet RNA using a PI technology

that combines UV light with riboflavin (vitamin B_2) as a photosensitizer [42, 46]. UV illumination is known to cause photochemical reactions, crosslinking and oxidative damage to the components of RNA and DNA [192-195].

Riboflavin targets nucleic acids by associating with RNA and DNA bases without binding to proteins and subsequently modifies nucleic acids, preferably guanine bases, via electron transfer [194, 196, 197]. Activation of riboflavin with UV light causes breaks in the nucleic acids strands, preventing further repair and replication, and reducing virulence and pathogenicity. This PI system is applied in blood banking to increase the safety of plasma, platelet concentrates or whole blood [46].

In this study we analyzed the effect of PI on total platelet RNA and on specific platelet mRNA transcripts including glycoprotein GPIIIa mRNA, previously reported in stored platelets [152]. Both GPIIIa and the α-granule protein PF4 mRNAs are among the most abundant transcripts present in platelets [131, 135]. Platelet mRNA transcripts of glycoproteins GPIIb and GPIb, the alpha-granule proteins osteonectin (also known as SPARC) and TSP, and GAPDH were also analyzed. Our findings show that PI treatment of PCs immediately decreases the measurable platelet mRNA, an effect that impacts the mRNA levels during the storage period but varies depending on the specific transcript.

3.2 **Results**

3.2.1 **Mirasol treatment resulted in an immediate reduction of platelet mRNA amount**

Quantitative PCR was used to determine the effect of Mirasol treatment on selected mRNA transcripts. Double apheresis PCs were pooled and split immediately after production (Day 0) to create paired samples (see Figure 3.1). After a 1-hour resting period, PI treatment (Mirasol Pathogen Reduction Technology System, TerumoBCT) was applied according to the manufacturer's protocol to one of the units while the other one was kept as an untreated control.

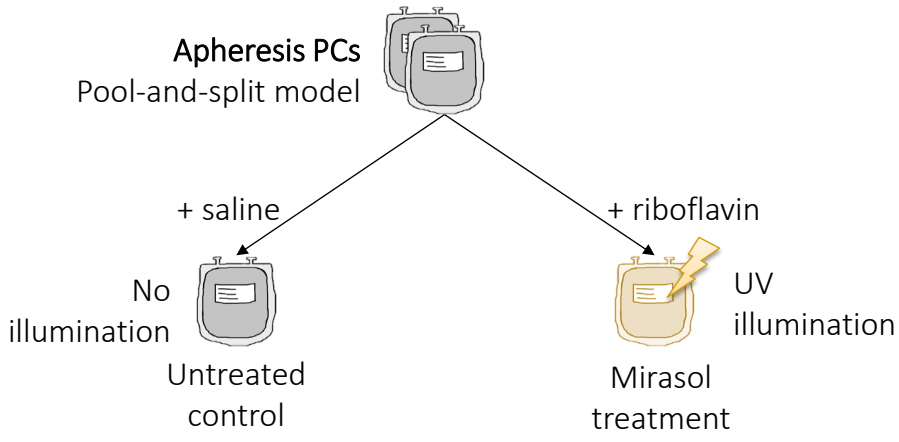


Figure 3.1: Graphic representation of the experimental set-up.

Two apheresis PCs were pooled and split to create paired units. One unit receive saline as a volume control without UV illumination (untreated control), while the second unit was treated with the Mirasol PRT system according to the manufacturers' instruction (see paragraph 2.3.2).

The mRNA amount, obtained one hour and 24 hours after treatment, was compared to the amount of mRNA present in the untreated sample (Figure 3.2). For all targets, a decrease in mRNA was observed one hour after treatment and levels were further reduced 24 hours after

treatment. For all transcripts, except GPIb mRNA, the decrease one hour after illumination was statistically significant ($p < 0.05$).

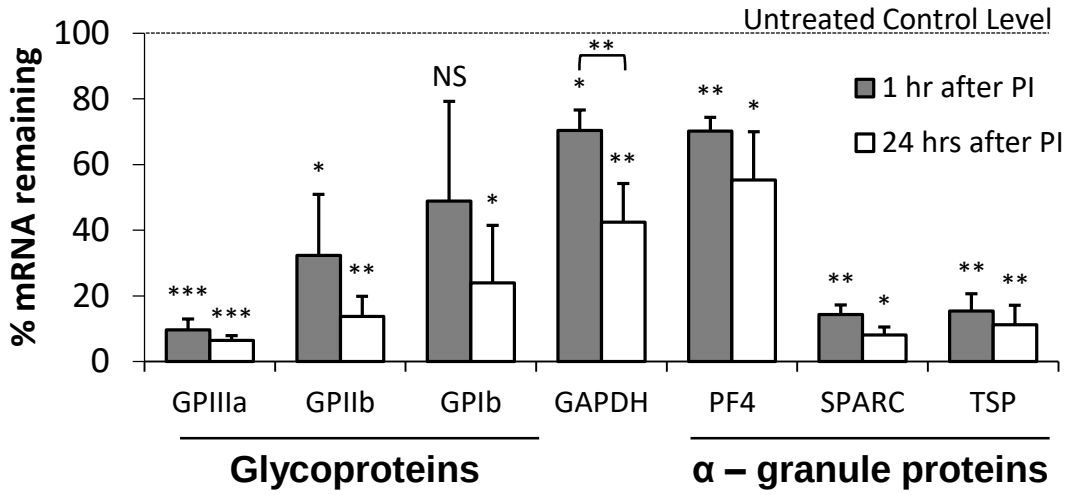


Figure 3.2: The impact of Mirasol treatment on the mRNA amounts of selected PLT mRNA transcripts.

The mRNA of selected targets was measured 1 hour (grey bars) or 24 hours (white bars) after Mirasol treatment. Total RNA was used for cDNA synthesis followed by qPCR for specific targets. The amounts of mRNA detectable after treatment were compared to the initial amount of transcript present before treatment. Results are given as mean ± SD for n ≥ 3. * p < 0.05, ** p < 0.01, *** p < 0.001, corrected for multiple comparison using a Bonferroni correction. Results were compared to the amount present before treatment. Reprinted with permission Klein-Bosgoed [198].

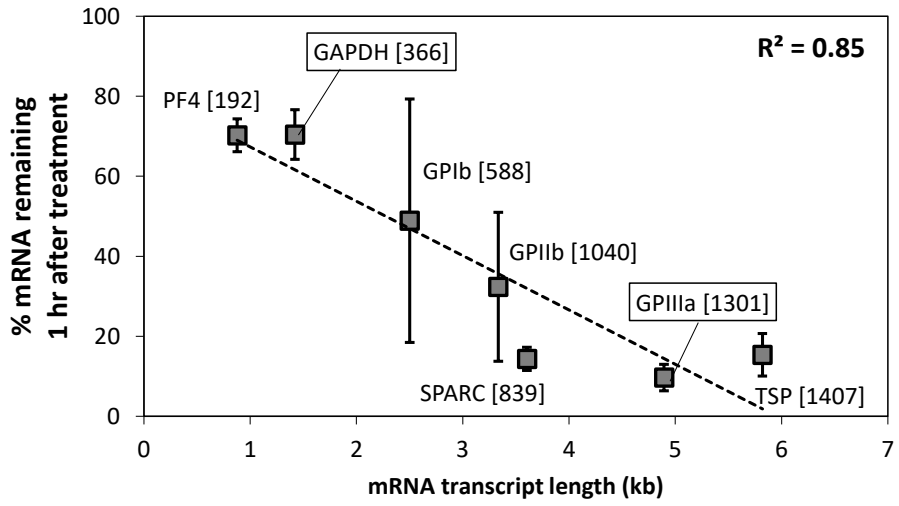
The mRNAs of GPIIIa, SPARC and TSP were affected most, with reductions one hour after illumination to 9.7%, 16% and 15% of the pre-Mirasol treatment level, respectively. However, GAPDH and PF4 were least affected, retaining a relative mRNA amount of 70% one hour after treatment. The mRNAs of GPIIb and GPIb were affected moderately, showing reductions one hour after treatment to 32% and 48% of the untreated levels, respectively.

3.2.2 **Longer transcripts were affected more by Mirasol treatment than shorter transcripts**

Riboflavin as a photosensitizer in combination with UV illumination is known to preferably target guanine bases. The transcript lengths, with their respective guanine base numbers, were plotted against the percentage mRNA remaining one hour after Mirasol treatment (Figure 3.3A). The correlation coefficient (R^2) of the trend line was 0.85. This relationship between mRNA amount remaining one hour after treatment and transcript length was statistically significant (p - value < 0.01). The targets with longer mRNA transcripts (GPIIIa and TSP) were affected more than the targets with shorter mRNA transcripts (PF4 and GAPDH). From the graph it was estimated that with every kb increase in transcript length, the mRNA recovery after Mirasol treatment was reduced by about 14%. A similar trend was observed for the percentage mRNA remaining 24 hours post treatment (Figure 3.3B).

The length of the 5' UTR and 3' UTR of the mRNA transcript were plotted against the impact of the Mirasol treatment in a similar manner as the total mRNA length (see Figure 3.4). No correlation was found between the 5' UTR length and the impact of the Mirasol treatment (R^2 = 0.098). The correlation between the 3' UTR length and the impact of the Mirasol treatment was not statistical significant (R^2 = 0.67).

3.3A



3.3B

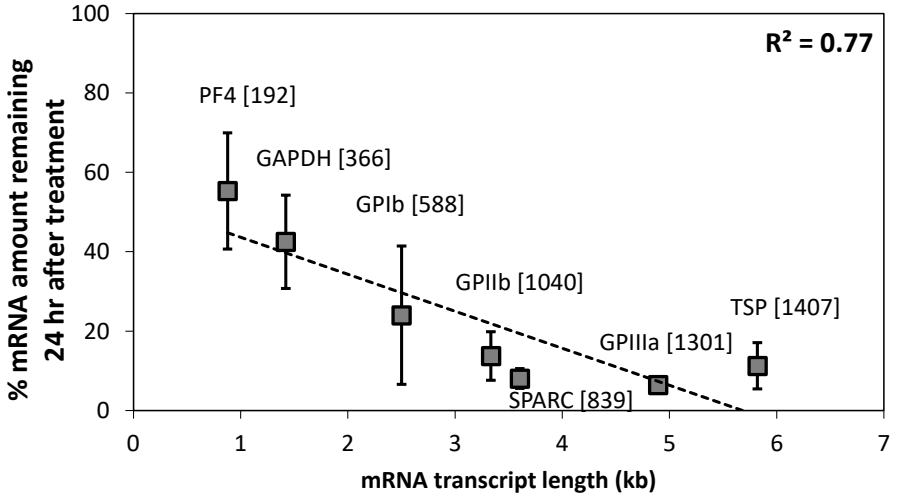
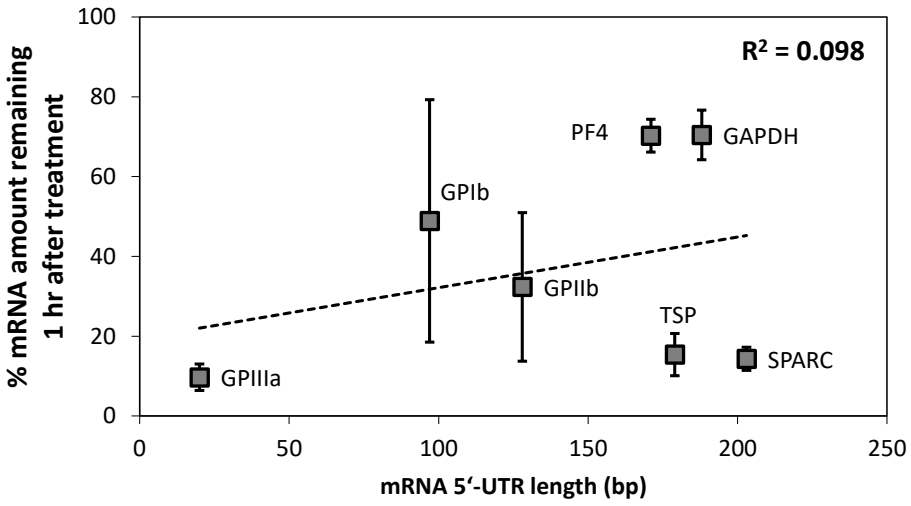


Figure 3.3: Relationship between the total length of the mRNA transcript and the percentage of mRNA remaining.

For the selected targets the total length of the mRNA was plotted against the percentage of mRNA detected 2A) 1 hour after Mirasol treatment and B) 24 hours after Mirasol treatment. The dotted line is the linear trend line for the data, with correlation coefficient R2 given in the figure ($p < 0.01$). In brackets is stated the number of guanine bases present in the transcript. Modified with permission from Klein-Bosgoed [198].

3.4A



3.4B

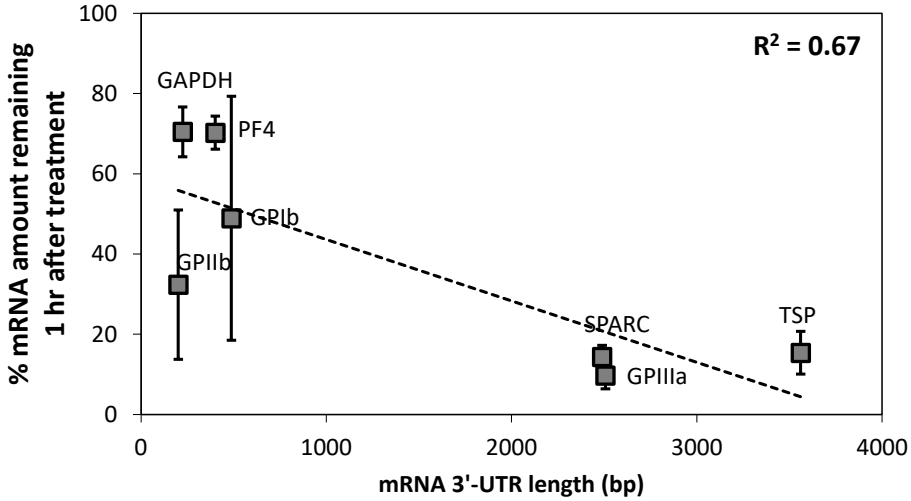


Figure 3.4: Relationship between the length of the 5' UTR and 3' UTR of the mRNA transcript and the percentage of mRNA remaining

For the selected targets the length of the A) 5' UTR and B) 3' UTR was plotted against the percentage of mRNA detected 1 hour after Mirasol treatment. The dotted line is the linear trend line for the data, with correlation coefficient R2 given in the figure

3.2.3 **Mirasol treatment changed the platelet mRNA half-life during storage of PC**

We used qPCR to determine the relative amount mRNA transcripts during storage. At least three independent replicates were analyzed in order to calculate the mRNA half-lives. For each

individual replicate, the mRNA half-life was calculated in untreated platelets and PI treated platelets using an exponential trend line. All tested mRNA transcripts showed a first-order decrease during storage. The mean percentage mRNA remaining in the samples at the different time points was plotted on a semi logarithmic scale relative to the amount present on day 0 in the untreated study arm. Figure 3.5A - C demonstrate the decrease in mRNA of the glycoproteins during storage in untreated platelets and Mirasol treated platelets. The half-life of the three tested glycoprotein mRNAs in untreated platelets was more than one day. Exposing platelets to Mirasol treatment resulted in an immediate reduction in mRNA amounts, up to 90% for GPIIIa. The graph of the residual mRNA post-treatment showed a less steep slope, indicating a slower decrease of the mRNA. This suggests an increase in half-life of the glycoprotein mRNAs remaining after the treatment. The difference in mRNA half-life between Mirasol treated platelets and untreated platelets was statistically significant for GPIIIa only (Figure 3.5A). Figure 3.5D-F show the decrease in mRNA of the alpha granule proteins during storage. The half-life of the three tested alpha granule proteins was similar to the half-lives of the tested glycoproteins, except that PF4 mRNA had a longer half-life of almost three days (Figure 3.5D). PF4 mRNA was less affected by the Mirasol treatment than the other transcripts, with 70% remaining compared to untreated platelets (Figure 3.2). Unlike the other tested transcripts, the graph of the residual PF4 mRNA post-treatment showed a steeper slope compared to PF4 mRNA without treatment. This indicated an accelerated PF4 mRNA reduction after Mirasol treatment, and a shorter PF4 mRNA half-life of 1.9 days. The observed difference in mRNA half-life between Mirasol treated platelets and untreated platelets, however, was not statistically significant. SPARC mRNA amounts decreased during storage similar to the other transcripts (Figure 3.5E). Post-treatment, the 14% of the mRNA that remained had a slightly longer half-life (1.3 days

without treatment versus 1.4 days with treatment), but the difference was statistically significant ($p < 0.05$). Thrombospondin, the longest transcript tested, behaved similarly to the second longest transcript, GPIIIa. TSP mRNA also had a half-life of 1.2 days in untreated platelets (Figure 3.5F). Again the graph of the residual mRNA post-treatment showed a less steep slope, indicating a slower decrease of the mRNA. The half-life of TSP mRNA in Mirasol treated platelets was prolonged to 2.2 days; however, this difference in half-life was not statistically significant. In the case of GAPDH mRNA, the initial impact of the Mirasol treatment was comparable to PF4, with 70% mRNA remaining compared to platelets without treatment (Figure 3.2). However, the behavior of the mRNA during subsequent storage (Figure 3.5G) was more comparable with the glycoprotein transcripts or TSP. The GAPDH mRNA half-life in untreated platelets was 1.7 days, which was prolonged to 2.6 days in the 70% remaining mRNA post-treatment. This difference was not statistically significant.

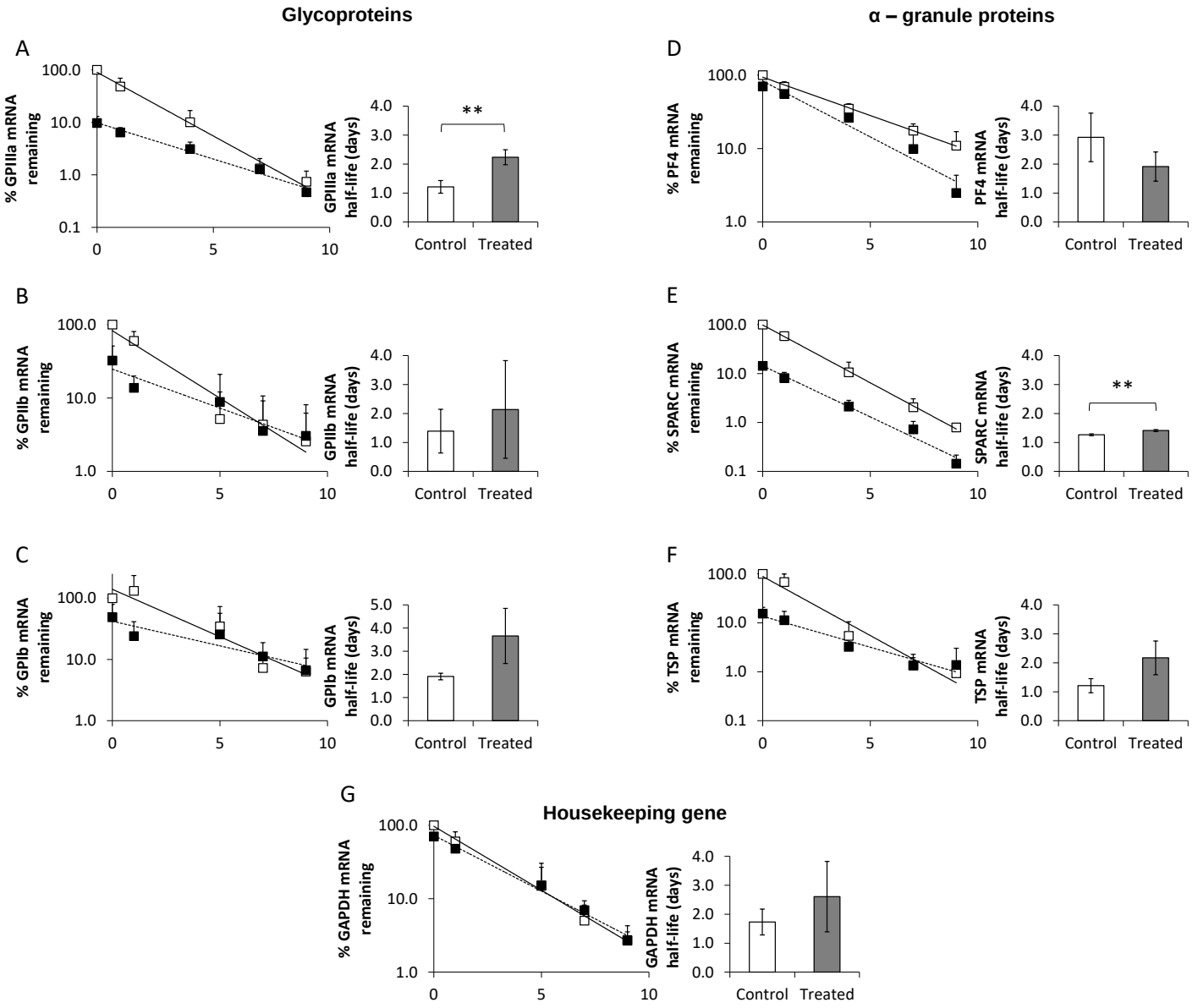

Glycoproteins
α – granule proteins
A
% GPIIIa mRNA remaining
GPIIIa mRNA half-life (days)
**
Control
Treated
B
% GPIIb mRNA remaining
GPIIb mRNA half-life (days)
C
% GPIb mRNA remaining
GPIb mRNA half-life (days)
D
% PF4 mRNA remaining
PF4 mRNA half-life (days)
E
% SPARC mRNA remaining
SPARC mRNA half-life (days)
F
% TSP mRNA remaining
TSP mRNA half-life (days)
G
Housekeeping gene
% GAPDH mRNA remaining
GAPDH mRNA half-life (days)

Figure 3.5: The effect of storage of platelets on the amount of specific mRNA transcripts.

In three independent experiments a double-apheresis PC was pooled and split immediately after production (Day 0) to create a paired sample. One PC was treated with the Mirasol PRT system, while the other was left as untreated control. The PCs were stored under blood banking conditions and sampled during storage for RNA extraction. mRNA amounts were measured using qPCR and data were normalized to the mRNA amount present on Day 0 of storage. □ Untreated PCs and ■ Mirasol-treated PCs. For every individual replicate the mRNA half-life was determined using the exponential trend line and Eq. (1) in untreated control PCs (white bars) or Mirasol-treated PCs (gray bars). Results are given as mean ± SD for n ≥ 3. ** p < 0.01 corrected for multiple comparison using a Bonferroni correction. Reprinted with permission from Klein-Bosgoed [198].

3.2.4 Modulation in illumination intensity correlated to a change in mRNA degradation

The dose of illumination energy was reduced to 50% (3.12 J/mL) and the mRNA amounts for GPIIIa and PF4 transcripts were compared to those from untreated PCs and full-dose illuminated PCs (100% = 6.24 J/mL). After 24 hours of storage, the mRNA amount of both GPIIIa and PF4 was reduced to 87% of the initial mRNA amount (Figure 3.6). In agreement with Figure 3.1, the full UV illumination dose reduced the GPIIIa mRNA amount to 13%. Changing the UV energy dose to half the intensity resulted in a reduction of GPIIIa mRNA to 23% of the untreated level.

The PF4 transcript was the least affected by the PI treatment (Figure 3.2). With a normal illumination dose, 51% of the mRNA was still detectable by qPCR 24 hours after Mirasol treatment. Lowering the UV illumination dose to 50% resulted in mRNA reduction to 67% of the initial amount after 24 hours of storage. This analysis demonstrates a dose-dependent effect on the mRNA amount that differs between the two transcripts.

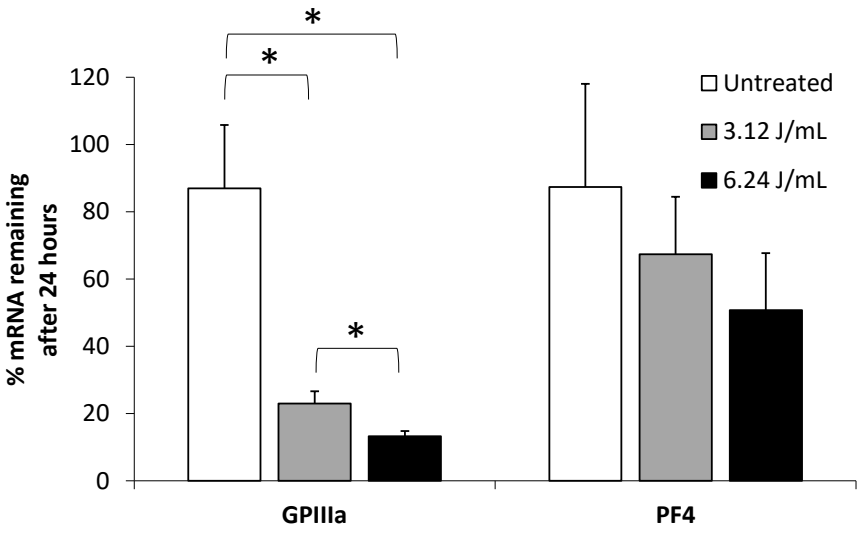


Figure 3.6: Effect of modulation of the illumination dose on specific mRNAs.

In three independent experiments a double apheresis unit was pooled and split into three equal units. Two units received riboflavin (50 µM final concentration) and were illuminated with 50% of the UV dose (3.12 J/mL; grey bar) and 100% UV dose (6.24 J/mL; black bar) respectively. The third unit remained untreated (white bar). The PC bags were sampled after 24 hours and mRNA amounts were measured using qPCR. The mRNA amount was compared to the amount present in the untreated PC on the day of collection (day 0). Results are given as mean + standard deviation. * $p < 0.05$ corrected for multiple comparison using a Bonferroni correction. Reprinted with permission from Klein-Bosgoed [198].

3.2.5 **Total platelet RNA amounts decreased during storage**

Absorbance at 260 nm was used to measure the total amount of RNA present in platelets. All data were normalized to the amount of RNA measured in the untreated platelets on day 1 (Figure 3.7). There was an immediate reduction in total RNA in the treated platelets on day 1 of 23% ± 13% compared to untreated platelets. During storage both untreated platelets and Mirasol treated platelets displayed a significant decrease in total amount of RNA. After 9 days of storage 20% ± 13% of the original amount of total RNA was still detectable in the untreated platelet versus 11%

± 8% in Mirasol treated platelets. Extrapolation suggests a loss of total RNA after 10 days for Mirasol treated platelets and 11 days untreated platelets.

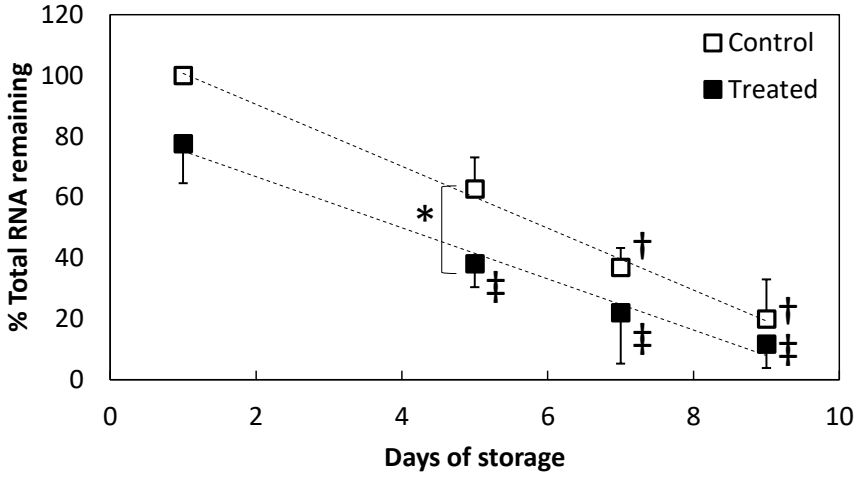


Figure 3.7: The effect of *ex vivo* storage of human blood platelet on the amount of total RNA in untreated and Mirasol treated PCs.

In three independent experiments a double apheresis PC was pooled and split immediately after production (day 0) to create a paired sample. The PCs were stored under blood banking conditions and sampled on day 1, 5, 7 and 9 and total RNA was extracted. RNA amounts were normalized to the amount present in untreated PCs on day 1. Results are given as mean ± standard deviation with a linear trend line. Statistical analysis resulted in a significant difference in total RNA between the untreated platelets and Mirasol treated platelets at day 5 (* $p < 0.05$). There was a significant decrease during storage in untreated platelets († $p < 0.05$ versus day 1 – untreated) and the same trend was visible in Mirasol treated platelets (‡ $p < 0.05$ versus day 1 – Mirasol treated). Modified with permission from Klein-Bosgoed [198].

3.3 **Discussion**

This study examined the effect of the Mirasol Pathogen Reduction Technology system on selected platelet RNA, showing that platelet RNA levels are affected by the treatment in a target-specific manner. The observation that platelet mRNAs were impacted differentially upon Mirasol treatment is a novel finding that refines our thinking on the impact of PI technologies on platelet nucleic acids.

The data demonstrate that UV illumination in the presence of the photosensitizer riboflavin results in a rapid decrease in mRNA amount. This reduction in mRNA was expected since the Mirasol treatment is designed to destroy the RNA and DNA of potential pathogens in blood products. As demonstrated here, Mirasol treatment destroyed a large proportion of mRNA transcripts; however, the remaining transcripts decayed more slowly. Even though a limited number of transcripts were tested, these results suggest that all mRNAs present in human blood platelets could be affected by Mirasol treatment.

Notably, the impact of the treatment was different among the tested transcripts. Even though all transcripts were reduced post-treatment, PF4 and GAPDH were less affected than GPIIIa and TSP. All analyzed mRNA transcripts are among the 50 most abundant transcripts in platelets, with the exception of TSP [125, 130, 131, 135, 170]. For that reason, mRNA abundance is unlikely to account for the observed change in mRNA stability/amount. Riboflavin is a photosensitizer that associates with nucleic acids and accelerates the damaging effect of UV light on RNA and DNA. The frequency of lesions within RNA or DNA upon Mirasol treatment is estimated to be one in every 350 bp [46]. The longer transcripts were expected to sustain more

treatment induced lesions than shorter transcripts. Upon illumination with UV light the riboflavin converts to lumichrome, which primarily modifies guanine bases [46]. The number of guanine bases in a transcript depends on its length, thus it was expected that the total number of base pairs would predict the impact of the treatment. Here we demonstrated a strong correlation between the impact of the riboflavin/ UV treatment on platelet mRNA and the length of the transcript. The longer transcripts GPIIIa and TSP show a greater reduction in mRNA than the shorter transcripts PF4 and GAPDH. This result concurs with the observation by Osman *et al* [170] that microRNAs were not affected significantly by the Mirasol treatment. MicroRNAs are very short, only about 22 nucleotides in length, and likely too short to be affected by this specific PI. The lesions arising in longer transcripts might result in active degradation by platelets in p-bodies, however these complexes have yet to be found in platelets.

Our experiments demonstrated that under normal blood banking storage conditions the mRNAs of all targets decayed more rapidly than PF4 mRNA. However, the calculated mRNA half-lives are considerably longer than mRNA in nucleated cells [100]. In Mirasol treated platelets, GPIIIa mRNA showed a different degradation pattern during storage compared to untreated platelets. The treatment immediately reduced the GPIIIa mRNA amount significantly, but was followed by a slower degradation of the remaining mRNA. This observation suggests the presence of a mechanism to protect the GPIIIa mRNA from further degradation after Mirasol exposure. All transcripts, except PF4 mRNA, demonstrated an increase in mRNA half-life post-treatment. The mRNA in nucleated cells has a rapid turnover and exists only for several minutes to hours [100]. Messenger RNA stability is determined by structural elements of the mRNA transcript itself (*cis*-acting elements) and additionally supported by the interaction with RNA-binding proteins (*trans*-

acting elements). For example, the presence of AREs in the 3'-UTR region of a transcript is considered a destabilizing factor, because most ARE binding-proteins recruit mRNA degradation proteins. In opposition, association of mRNA with PABP or eIF4E protects the 3' end poly(A) tail and 5' end cap, respectively, from degradation by exoribonucleases [100, 106, 199-201]. In nucleated cells UV light can increase the half-life of mRNA through up-regulation of mRNA transcription [202], association with the ARE binding-protein HuR [203-205], or inhibition of deadenylation of the poly(A) tail [206]. The prolonged mRNA half-life in platelets after exposure to PI treatment might be explained by the presence of a transcription-independent protection mechanism, like modulation of RNA-binding protein association or inhibition of deadenylation; however, neither of these mechanisms has been reported in platelets.

The applied energy dose in the Mirasol PI treatment is 6.24 J/mL of PC which is efficient in reducing infectivity of several pathogens [207-210]. The impact of UV illumination on DNA and RNA depends on the dose intensity [211, 212]. Bakkour *et al* [173] demonstrated that increasing the UV energy dose of the PI treatment decreased the mtDNA amount available for PCR amplification. Here we demonstrate that reducing the energy dose improves the recovery of the mRNA transcripts for both PF4 and GPIIIa. Twenty-four hours post PI treatment with 50% of the usual energy dose, the recovery of PF4 mRNA was halfway between the recovery of mRNA in untreated platelets and PI treated platelets with regular energy dose. Compared to PF4 mRNA, GPIIIa mRNA was reduced to about 20% of its mRNA level when only a 50% energy dose was used. The level of mRNA was different between these two transcripts at 24 hours post-illumination, demonstrating that the kinetics of the mRNA reduction is target-specific. This suggests that the decrease in GPIIIa mRNA is not caused by riboflavin / UV light alone.

Another intriguing finding was that total RNA reduced during the platelet's life span, but the decrease occurs much slower than in other cell types. Total RNA is composed primarily of ribosomal RNA (rRNA) (80%) and transfer RNA (tRNA) (16%) [213]. Dependent on the cell type, rRNA has a life span up to 7.5 days [214]. Here we demonstrated that the amount of total RNA in human blood platelets slowly decreased during storage following an almost linear decrease. Extrapolation of this trend line results in the depletion of the pool of RNA after 10 to 11 days (Figure 3.7). This observation is intriguing as the life span of rRNA and tRNA seems to coincide with the life span of the platelet. The importance of this relationship has been demonstrated in yeast, where rDNA determines the life span of a cell and unstable rDNA acts as inducer of cellular senescence [215, 216]. Both ribosomal RNA and transfer RNA are considered stable RNA types, but are susceptible to UV-induced damage. (Reviewed in Wurtmann *et al* [195]) With a length of 76 – 90 bp tRNA is most likely to small to be severely affected by the riboflavin / UV treatment, much like microRNA [170]. Ribosomal RNA is larger, especially the 18S and 28S subunits (1869 bp and 5070 bp, respectively), but as major constituent of total RNA, it showed no significant reduction after treatment. With this in mind, other non-coding RNA species, like long non-coding RNA with a length > 200 bp, could be affected when long enough. However, how this would affect protein translation and post-translational modifications in platelets remains unexplored.

Our study focused on Mirasol treatment of platelet concentrates that uses riboflavin in combination with UV light. Although all PIs are designed to target and destroy RNA and DNA, PI techniques that use other methods (e.g., UV alone or a different photosensitive additive) might

induce a different response in platelet mRNA [170, 171]. In this study seven transcripts were analyzed and these might not be representative of all platelet mRNAs. However, a common pattern could be elucidated showing an immediate reduction in mRNA levels dependent on the transcript. Half-lives of the remaining mRNAs increased post-treatment similar to what is observed in UV exposed nucleated cells. Further analyses are necessary to test which mechanisms influence the mRNA levels upon Mirasol treatment.

The results obtained from this study need to be placed in context of the individual protein levels. Recent proteomic studies revealed that the protein concentration in Mirasol treated PCs are not strongly impacted [217]. The observed decrease in mRNA post-treatment does not necessarily result in a decrease in protein levels, as the translational rate impacts the protein expression level independent of mRNA amount.

In this study selected platelet mRNA levels were reduced after treatment; whether these changes directly result in reported proteomic changes or reduction in platelet function was not demonstrated. This type of indirect evidence reflects the current state of tools to study this question. For example, Angenieux *et al.* [218] have shown for murine platelets that a reduction in mRNA coincides with a strong decrease in total protein translation. Similarly, translation inhibitors affect human platelet function including clot retraction [165] and extent of shape change [219]. These results suggest a role for protein synthesis in normal platelet function but cannot conclusively demonstrate it.

There are several studies demonstrating the impact of Mirasol treatment on platelet recovery and survival [56, 220]. In nucleated cells, UV damage induces cell arrest to allow transcriptional repair or, when the damage is too severe, apoptosis [195, 221]. In Mirasol treated platelets replacement of lost messages is not possible due to the lack of a nucleus. For the majority of our tested transcripts mRNA half-life of the remaining messages increased, however, it is unknown whether retained messages would compensate for the lost mRNA with additional time in storage, another physiologically stressful process for platelets. Additionally, Mirasol treated platelets show signs of increased apoptosis [222, 223] suggesting that recovery facilitated by remaining mRNA is unlikely.

In summary, we have demonstrated that the mRNA transcripts and total RNA in human blood platelets show a degradation pattern that is much slower than that seen in nucleated cells. Mirasol treatment, designed to target RNA and DNA in pathogens, clearly also impacts platelet mRNA. Differences in the stability of selected mRNA transcripts were observed, and while transcript length and UV energy dose correlate with mRNA recovery, these cannot fully explain the impact of the treatment. Our findings suggest mRNA regulatory mechanisms are in place in platelets. Further studies are needed to determine whether the impact of the Mirasol treatment on platelet mRNA transcripts and RNA levels is directly linked with the loss of platelet quality that has been associated with PI treatment [190, 191].

Chapter 4: p38 MAP kinase is involved in regulation of mRNA stability in platelets treated with riboflavin / UV illumination

4.1 Introduction

Messenger RNA serves as the template for protein synthesis, a characteristic crucial to cell survival. Platelets lack a nucleus, so have no innate transcription of DNA into RNA outside of the mitochondrial genome. Platelets circumvent this obstacle by using the RNA supplied by their progenitor cells for their protein synthesis. Similar to other cells types, cell function of platelets correlates to the occurrence of protein transcription. It has been shown that the use of translational inhibitors reduced the response of platelets to agonists [165, 219]. And vice versa, a change in platelet function like platelet activation triggers the *de novo* synthesis of proteins [127, 184]. This suggests that in platelets protein synthesis is a necessary activity to maintain platelet function throughout their lifespan of about 10 days. In general, however, mRNA has only a short half-life, ranging from minutes to hours [100]. Nucleated cells can compensate for the rapid loss by transcribing new RNA, platelets cannot. Still, platelets demonstrate protein synthesis at the end of their lifespan [152]. This can in part be explained by the relative long mRNA half-life of several days platelets have shown to exhibit [152, 198]. For their full lifespan platelets have access to mRNA template to synthesize proteins, however it is unknown what makes mRNA in platelets more stable compared to other nucleated cells.

Currently PI techniques are under development to improve the safety of blood products by targeting viral and bacterial RNA and DNA. This is of extra importance for platelet concentrates,

as their storage conditions at room temperature might allow proliferation of pathogens present under the detection limit or unknown to the current screening assays. Application of PI treatment results in a reduction in platelet *in vitro* quality. This can in part be explained by the activation of the p38 MAP kinase pathway [175]. Inhibition of p38 MAP kinase significantly improves the *in vitro* quality of platelets exposed to riboflavin / UV illumination. As described in **chapter 3** platelet mRNA is negatively affected by PI treatment, resulting in a strong, target-specific reduction of mRNA levels after treatment. However, in pathogen-reduced platelets the half-life of the remaining mRNA is prolonged and these platelets still demonstrate protein synthesis during storage [172]. It is imperative that we elucidate the mechanisms involved in PI-induced mRNA damaged with the objective to mitigate the impact of PI on platelet mRNA and ultimately platelet quality.

To identify potential pathways we looked at nucleated cells, where exposure to UV illumination also leads to an increase of mRNA stability via activation of RNA binding proteins (RBPs) to form ribonucleoprotein (RNP) complexes. The protein composition of the RNP complex determines the fate of the incorporated mRNAs, which include processing, localization, translation, degradation and storage. UV illumination activates p38 MAP kinase and downstream leads to the activation of RNA binding proteins like HuR [205]. Activation of HuR by phosphorylation causes phospho-HuR to translocate from the nucleus into the cytosol to associate with the target mRNA and increase mRNA stability. It is unknown whether platelets have a similar approach to manage the stability of mRNA.

This study investigated the role p38 MAP kinase and RNA binding proteins as modulators for mRNA stability in pathogen-reduced platelets. SB 203580 was used to inhibit the function of p38 MAP kinase to determine the impact of p38 MAP kinase on platelet mRNA. TSP mRNA was selected as model transcript since it is known to be a target for HuR [224]. TSP is a transcript that has shown to be severely affected by pathogen inactivation treatment using riboflavin and UV illumination (see **Chapter 3**). Two other targets were evaluated as well to determine the impact of a transcript that is moderately affected (SPARC (also known as osteonectin)) or slightly affected (GAPDH) by the riboflavin and UV pathogen reduction treatment.

4.2 **Results**

4.2.1 **p38 MAP kinase inhibition did not change the reduction in mRNA levels after Mirasol treatment.**

Three apheresis PCs were pooled and split into four equal volumes, immediately after production (Day 0) to create paired samples. The SB inhibitor in DMSO (final concentration 10 μM), or DMSO as vehicle control, was added 30 minutes prior to PI treatment. An untreated unit was prepared by adding saline to correct for the volume and was not exposed to the UV illumination. The other three units received standard PI treatment (Mirasol Pathogen Reduction Technology System, TerumoBCT) according to the manufacturer's protocol, to generate a standard Mirasol arm, a DMSO arm, and a SB arm (see Figure 4.1). Quantitative PCR was used to determine the relative amount of mRNA during storage in the four different treatment arms. Three targets were analyzed based on the impact of the Mirasol treatment on their mRNA levels as mentioned earlier.

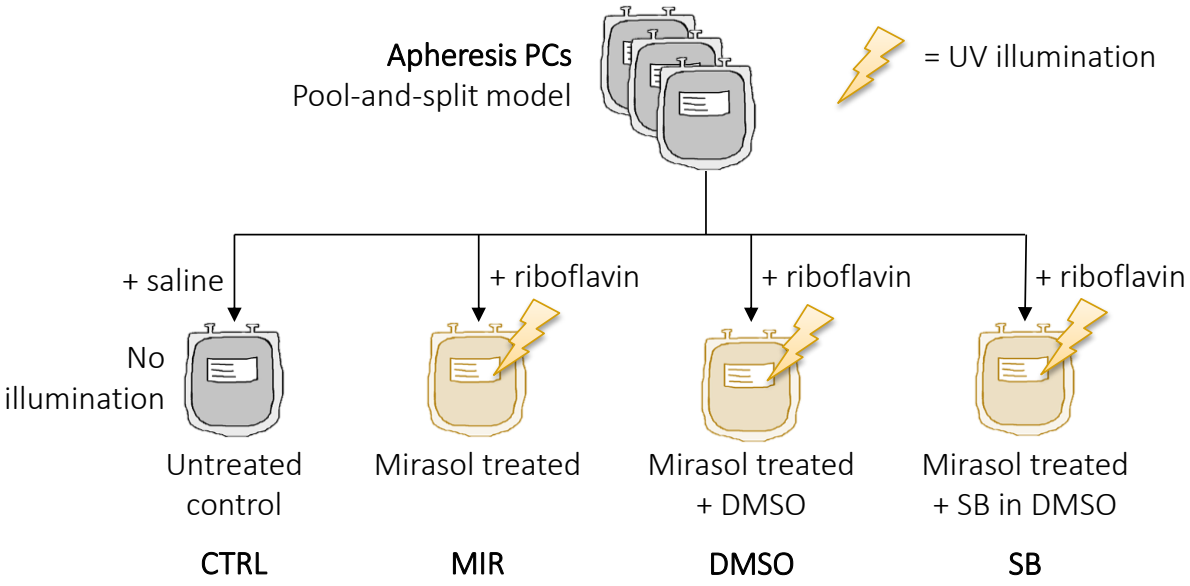


Figure 4.1: Schematic representation of the experimental set-up.

Three apheresis PCs were pooled and split into four equal volumes, immediately after production (Day 0) to create paired samples. An untreated control unit was prepared by adding saline to correct as a volume control and was not exposed to UV illumination (CTRL). The other three units were treated with the standard Mirasol PRT System according to the manufacturer's protocol, either without spiking (MIR), in the presence of DMSO as a vehicle control (DMSO), or in the presence of 10 µM SB (final concentration) in DMSO (SB) (see paragraph 2.3).

The mRNA amount in Mirasol treated PLTs during storage was compared to the amount present in untreated PLTs. For all targets a reduction in mRNA amount was observed after Mirasol treatment and subsequent storage (see Figure 4.2A – C). Platelets pre-incubated with SB were able to maintain slightly higher levels of mRNA towards the end of storage compared to the Mirasol treated platelets with or without DMSO, but this did not reach statistical significance.

4.2.2 **p38 MAP kinase inhibition increased the mRNA half-life in Mirasol-treated PLTs**

The mRNA data collected during storage of the PCs was used to determine the mRNA half-life. Five independent replicates were used to calculate the half-life. Figure 4.2D – F show the change in mRNA half-life for the different treatment arms. No significant difference in mRNA half-life was observed between the untreated platelets and Mirasol-treated platelets either with or without DMSO. However, in the presence of the SB inhibitor the mRNA half-life significantly increased compared to the mRNA half-life in Mirasol-treated PLTs for all three targets ($p < 0.05$). For the transcript TSP, the mRNA half-life in the SB treated PLTs was even significantly increased compared to untreated PLTs ($p < 0.05$).

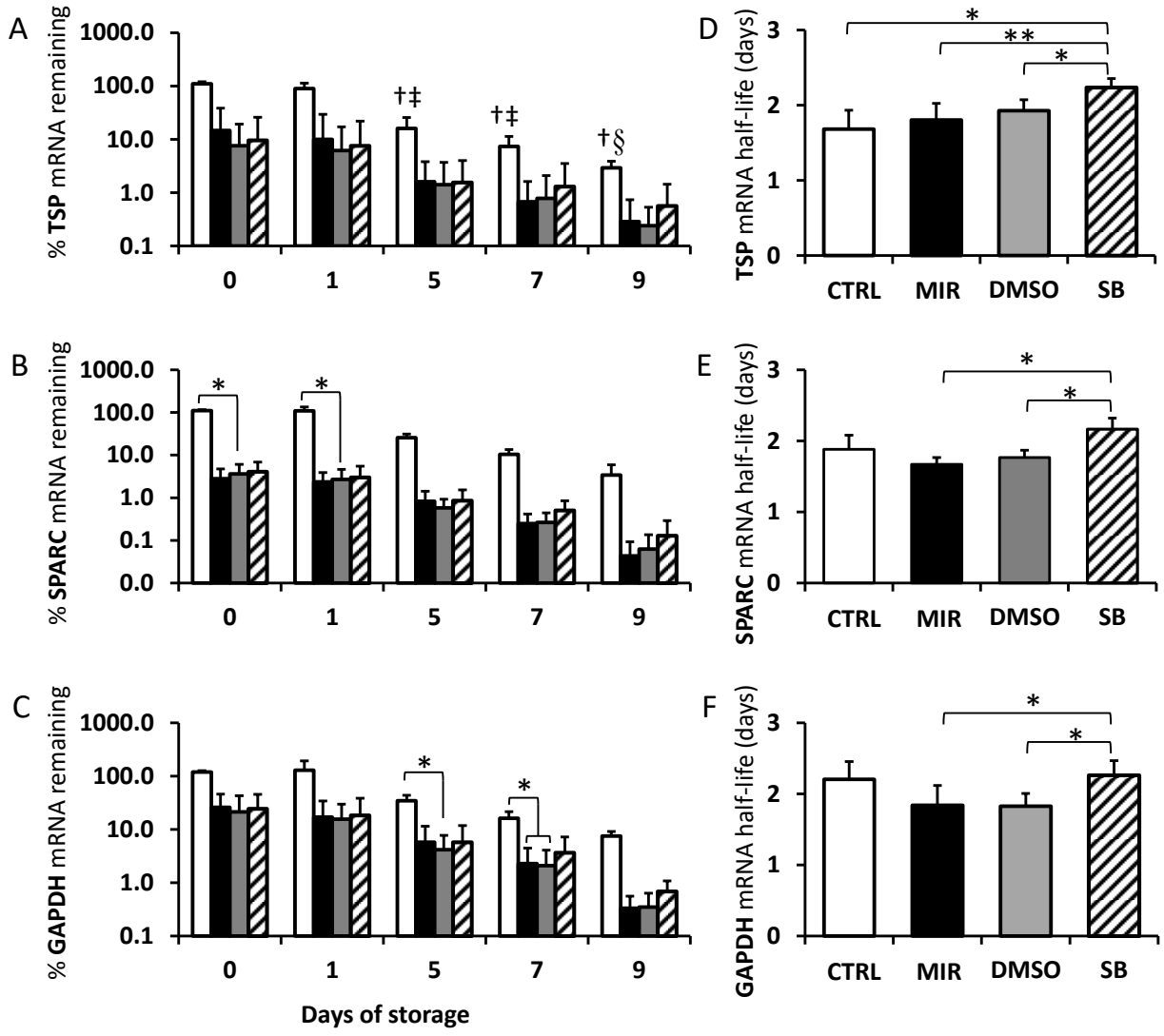


Figure 4.2: Impact of Mirasol PRT in absence and presence of p38 MAP kinase inhibitor SB on platelet mRNA during storage and mRNA half-life.

PCs were prepared as described and stored under blood banking conditions. Samples were drawn one hour after preparation (day 0) and on day 1, 5, 7, and 9 of storage. A) – C) RNA was extracted from the platelets and mRNA levels for TSP, SPARC and GAPDH were determined by qPCR. D) – F) The mRNA half-life was determined for every individual replicate using the exponential trend line. Results are shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$ (A-C) Two-way ANOVA corrected for repeated measures for $N \geq 4$. $p < 0.05$ † versus Day 0, ‡ versus Day 1, § versus Day 5. (D-F) One-way ANOVA for $N \geq 4$

4.2.3 **Platelet mRNA transcripts theoretically contain numerous bindings sites for RBPs**

Messenger RNAs contain specific nucleotide sequences referred to as RNA sequence motifs that can be recognized by RBPs to form RNP complexes.. These RNA sequence motifs can be found primarily in the 3'-UTR and 5'-UTR of a transcript. RBPs contain RNA-binding domains like RNA recognition motifs (RRMs), K homology (KH) domains, or a zinc-finger domain that interact with the RNA sequence motifs to regulate mRNA function [225].

To determine if the 3'-UTR of platelet transcripts harbor potential RNA sequence motifs, the nucleotide sequences of a few selected mRNA transcripts were analyzed using the RBPDB. Based on nucleotide sequence only, the platelet mRNA transcripts contain numerous potential binding sites for RBPs. For the longest transcript TSP, the 3'-UTR consist of almost half of the total transcript length. It has, in theory, 439 binding sites for a total of 28 different RBPs. Of these RBPs, however when cross-referencing the 28 RBPs in PlateletWeb to determine whether they have been identified in platelets, only 12 proteins were confirmed in platelets. See Table 4.1 for the full list of RBPs. Examples of the RBPs with high binding site frequency (> 25) on TSP 3'-UTR, previously identified in platelets, were HuR, eIF4B, and PABP. Other RBPs like SLM, ASF1, and hnRNP G could, in theory also bind to TSP mRNA but have not been identified in platelets. RBPS with only a single potential binding site were hnRNP A1, YB1, YB2A and TTP. The first two have previously been identified in platelets, but the latter two have not. A similar pattern was observed for SPARC (24 different RBPs) and GAPDH (15 different RBPs).

Table 4.1: List of RBPs that could potentially bind selected platelet transcripts.

RBPs and the frequency of their binding site sequence in the 3'-UTRs of thrombospondin, SPARC, and GAPDH mRNA. RBPs are separated based on the level of detection in human platelets.

	Target mRNA	TSP	SPARC	GAPDH
	Total Transcript length	7253	3604	1421
	3'-UTR length	3561	2489	225
RNA binding proteins confirmed in platelets	HuR	60	33	-
	eIF-4B	46	14	2
	PABP-1	29	10	17
	Muscleblind-like protein 1	20	10	2
	HuB	9	-	-
	IRE-BP1	6	2	-
	NonO protein	6	4	1
	Serine/arginine-rich splicing factor 2	3	1	-
	hnRNP A1	1	1	1
	PTB	1	-	-
	Hqk	1	1	-
	Y box-binding protein 1	1	-	-
	# different RBP	*12*	*9*	*5*
	# of potential RNA binding sites	*183*	*76*	*28*
RNA binding proteins **not yet** confirmed in platelets	SLM2	63	26	2
	ASF1	50	19	2
	hnRNP G	37	28	9
	Serine/arginine-rich splicing factor 10	31	5	4
	Pumilio-2	25	14	2
	Serine/arginine-rich splicing factor 9	10	11	1
	FUS	9	10	-
	YTH domain containing protein 1	8	14	1
	hRBMY	6	7	3
	U1 snRNP A	6	2	-
	Fox-1 homolog A	4	8	-
	SAP 49	2	2	-
	TTP	2	1	-
	FUSE-binding protein 2	1	3	2
	Vts1	1	5	1
	Y box-binding protein 2A	1	-	-
	Total number of RBPs	*28*	*24*	*15*
	Total number of binding sites	*439*	*231*	*50*

4.2.4 **Platelets express RNA binding proteins**

To determine how many of RBPs have been detected in platelets, the total list of 416 RBPs was cross-referenced with the platelet proteomics website PlateletWeb [183]. As shown in Figure 4.3, out of the 416 RBPs listed by the RBPDB, 406 had information given on PlateletWeb. For the majority of RBPs (290) the level of detection in platelet is unknown (see Figure 4.3A).

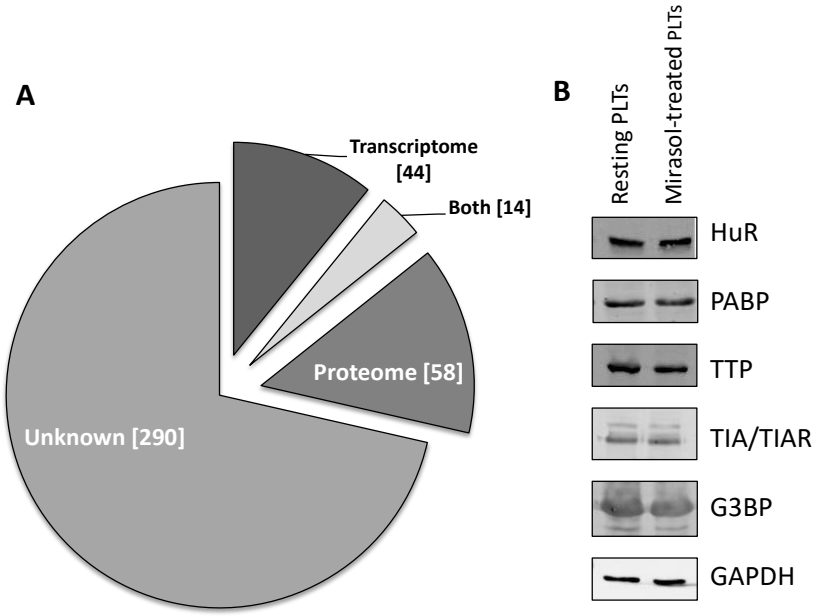


Figure 4.3: Platelet express RNA binding proteins.

A) Pie chart with in brackets the number of RNA binding proteins detected in platelets at the level of transcriptome, proteome, both or neither. B) Validation of presence of a selected panel of RNA binding proteins in platelets by western blot analysis.

The remaining 116 RBPs had been detected in platelets on a proteomic level, transcriptome level or both. A selected panel of RBPs, within binding affinity for TSP as mentioned earlier, and others predicted to have proteomic levels of detection in platelets, were validated by western blot

analysis (see Figure 4.3B). RBP expression was observed in both resting and Mirasol-treated platelets.

4.2.5 **Impact of p38 inhibition on the RBPs HuR and TTP**

Among the RBP with the highest binding site frequency was HuR and in nucleated cells HuR is regulated through p38 MAP kinase. We confirmed the presence of HuR protein in platelets by immunoblot (see Figure 4.3B). To monitor the HuR protein expression during storage PCs were sampled on day 1, 5 and 7. Band intensities were compared to the amount present of day 1 of storage (see Figure 4.4). The platelets showed expression of the HuR protein throughout storage. At the end of storage the protein expression of HuR was significantly reduced ($p < 0.05$) in the Mirasol treated arm (alone or with vehicle control) compared to the untreated control arm, but not in the presence of the inhibitor. We were not able to visualize detectable phosphorylation levels of HuR by immunoblot analysis.

Among the RBP with the lowest binding site frequency was TTP, a RBP in nucleated cells also regulated through p38 MAP kinase. Even though the expression level of TTP in platelets is none according to PlateletWeb, we were able to validate the presence of TTP by immunoblot analysis (see Figure 4.3B). During storage of the PCs under blood banking conditions the TTP expression significantly decreased ($P < 0.05$) in the Mirasol treated arm compared to the untreated control arm. At the end of storage this difference was not statistically significant for the arm with the SB inhibitor present (see Figure 4.4B). The phosphorylation levels of TTP did not change significantly during storage (see Figure 4.4C).

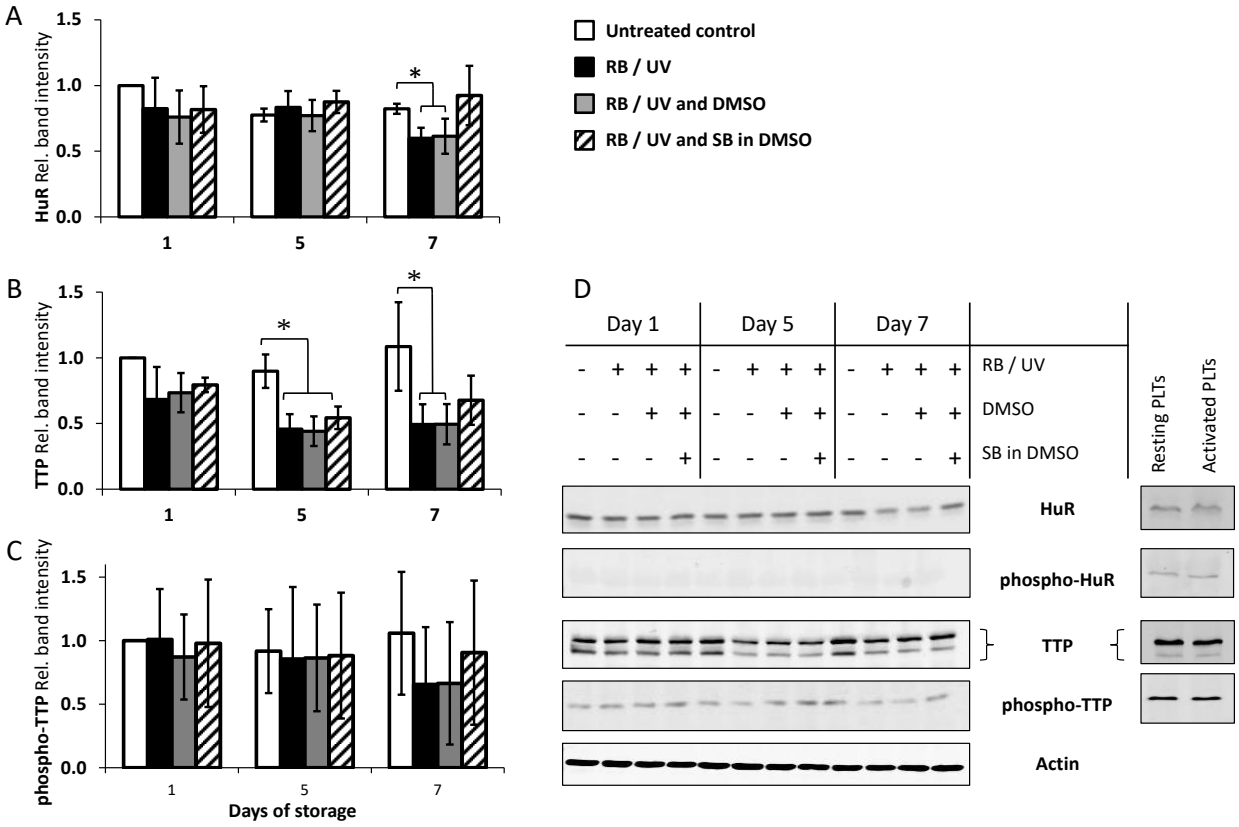


Figure 4.4: The effect of Mirasol treatment and subsequent storage with and without SB inhibitor on the protein expression of the RNA binding proteins HuR and TTP.

Three apheresis PCs were pooled and split into four equal volumes immediately after production (day 0). PCs were prepared as described and stored under blood banking conditions. Whole cell lysate was prepared on day 1, 5 and 7 and 100 µg of total protein was separated by SDS-PAGE. A) – C) Relative band intensities for the proteins HuR, TTP and phosphorylated (phospho-) TTP during storage. The results are expressed as the mean ± SD for N = 5. * $p < 0.05$ using a two-way ANOVA with repeated measures using a bonferroni correction. D) Representative immunoblots for the protein expression of the RBPs during storage and the confirmation of the antibody in normal resting platelets and platelets exposed to 5 µM TRAP for 30 min.

4.3 **Discussion**

This study examined the role of p38 MAP kinase and the downstream RBPs HuR and TTP in the expression of selected platelet mRNA transcripts after Mirasol treatment. In nucleated cells, p38 MAP kinase and its downstream RBPs HuR and TTP are known to be involved in the regulation of RNA stability. We were able to demonstrate that inhibition of p38 MAP kinase using the inhibitor SB 203580 significantly increased the mRNA half-life, suggesting a role for the p38 in regulation of mRNA stability as it does in nucleated cells.

In nucleated cells p38 MAP kinase is best known for its role in inflammation, cell growth and cell differentiation, but is involved in a diverse array of biological processes [226]. Activation of p38 MAP kinase results in an increase in mRNA stability of transcripts like COX-2, IL-1β, IL-6 and TNFα [227]. Several proteins downstream of p38 MAP kinase are identified to regulate mRNA stability. The proteins HuR, TTP, BRF1, FB1, FB3, hnRNPA0, hnRNPA1, HSBP1, KSRP, PABP, and PARN have demonstrated evidence to be phosphorylated either directly by p38 MAP kinase or indirectly via MK2 [228]. All these proteins are RNA binding proteins that target the 3'-UTR region of an mRNA transcript. The change in phosphorylation status and subsequent change in binding affinity is the basic principle behind the regulation of p38 MAP kinase. HuR is an RBP, regulated among others through p38 MAP kinase, and is associated with increase in mRNA stability. Activation of p38 MAP kinase increases the phosphorylation levels of HuR, which causes HuR to translocate from the nucleus into the cytosol. Accumulation in the cytosol of phospho-HuR enhances the association with mRNA transcripts to increase the mRNA stability.

A well characterized p38 MAP kinase regulated RBPs that is associated with a reduction in mRNA stability is TTP. When associated with a mRNA transcript TTP recruits enzymes involved in degradation, like deadenylases and decapping proteins [229]. In nucleated cells, activation of p38 MAP kinase can phosphorylate TTP directly or through MK2. Phosphorylation of TTP inhibits the recruitment of the degradation-related proteins and allows binding of TTP with chaperone protein 14-3-3. The phospho-TTP/14-3-3 complex stabilizes the mRNA transcript through its dissociation [104, 105].

It has been previously demonstrated by our group that p38 MAP kinase is activated upon Mirasol treatment [222]. Inhibition of p38 MAP kinase prior to Mirasol treatment prevents the reduction in *in vitro* quality as commonly observed with standard pathogen inactivation treatment. The presence of the p38 MAP kinase inhibitor did not prevent the reduction in platelet mRNA that occurs immediately after Mirasol treatment, however was able to maintain higher levels of mRNA towards the end of storage. This observation concurs with the effect of p38 MAP kinase inhibition on *in vitro* quality, however is opposite to the effect seen in nucleated cells. In nucleated cells, activation and not the inhibition of the p38 MAP kinase pathway increases mRNA stability [230, 231].

Even though the total length of platelet mRNA is not significantly different than in other cell types, the 3'-UTR is significantly longer [138]. This can allow for more regulatory potential, as the 3'-UTR contains nucleotide sequences (*cis*-acting elements) that can interact with RBPs or other RNAs (*trans*-acting elements). We have analyzed the 3'-UTR of the transcripts used in this study to investigate the potential of platelet transcripts to bind RBPs. Here we demonstrate that

platelet transcripts contain nucleotide sequences that can potentially interact with several RBPs. The 424 RBPs included in the human RBPDB were analyzed and the 3'-UTR of TSP could associate with up to 28 different RBPs. The platelet proteome, however, might not express all RBPs listed as we found only around 40% of the RBPs were previously identified in platelets (see Figure 4.3A). The binding site frequency is calculated based on the occurrence of a nucleotide sequence with a cut-off value set to a sequence match of 80% or higher. This could lead to an overestimation of the frequency, but does allow for mismatches in nucleotide positions where the RBP have less specific affinity.

As mentioned earlier, the interaction between RBP and a transcript requires structural elements of the mRNA transcript itself (*cis*-acting elements) supported by the interaction with RNA recognition motifs of the RNA-binding proteins (*trans*-acting elements). To evaluate whether platelets contain the necessary *trans*-acting elements to establish the interaction with mRNA to form ribonucleoprotein complexes, we cross-referenced the human RBPs listed in the database with their expression level according to PlateletWeb. Bases on these *in silico* analysis platelets express less than 30% of the RBPs summarized in the database, of which only 18% in their proteome. When compared with the three transcripts we analyzed, TSP, SPARC, and GAPDH, around 40% of the RBPs that could bind were identified in platelets, with 29% of the RBPs being expressed on a proteomic level. This could indicate that platelets selectively express the RBPs which could interact with their transcriptome.

With 60 potential binding sites on the TSP transcript, we hypothesized HuR to be the the RBP most likely to be involved in its regulation. Mazan-Mamczarz and colleagues [224] have

demonstrated that in the breast cancer cell line MCF7 HuR can bind to TSP. Here we confirm that platelets express HuR during seven days of storage. HuR is known to increase mRNA stability when it is associated with a transcript by protecting the transcript from degradation for example after exposure to UV light [204, 205], which is one of the steps of Mirasol treatment. Our lab has previously demonstrated that mRNAs exposed to Mirasol treatment have a prolonged half-life [198]. Mirasol-treated platelets demonstrate sign of accelerated storage lesion; amongst others through the activation of the p38 MAP kinase pathway [175, 222]. This has led us to investigate whether p38 MAP kinase is involved in regulating mRNA stability in platelets exposed to riboflavin and UV illumination. The Mirasol treatment changes the HuR protein expression, which was not observed in the presence of the SB inhibitor. If p38 MAP kinase regulates HuR in platelets in a similar manner as in nucleated cells, than phosphorylation levels HuR should increase upon Mirasol treatment and be inhibited in the presence of SB. However, phosphorylation levels of HuR were below the level of detection in our samples, so no difference in expression could be observed. Further investigation will be required to determine whether phosphorylation of HuR occurs in platelets upon Mirasol treatment and if this is regulated through p38 MAP kinase.

Tiedje and colleagues [230] describe the interaction between HuR and TTP in the regulation of TNF mRNA, which is driven by p38 MAP kinase and its downstream effector MK2. TTP is associated with reduction in mRNA stability by targeting the associated transcript to the degradation machinery [103]. Even though no level of expression of TTP was registered in the PlateletWeb database, we have been able to demonstrate the presence of TTP by immunoblot analysis during a seven day storage period. The protein expression of TTP followed a similar

pattern as HuR. In Mirasol-treated platelets the protein expression decreased at the end of storage, which was not observed in the presence of the SB inhibitor. The activation of p38 MAP kinase as a result of the Mirasol treatment did not increase the phosphorylation levels of TTP. The expression of phosphorylated TTP demonstrated a pattern similar to the total TTP protein levels, however no statistically significant differences was reached. These observations suggest that even though the Mirasol treatment increases the phosphorylation levels of p38 MAP kinase, this does not result in an increase in phosphorylation levels of TTP.

In summary, HuR and TTP are among the best studied RBPs regulated through p38 MAP kinase upon UV illumination [229]. Blocking the p38 MAP kinase pathway using the SB inhibitor has a significant impact on the *in vitro* quality of platelets [175, 222]. In our experiment we were able to demonstrate that blocking p38 MAP kinase had a significant impact on the platelet mRNA half-life of TSP, SPARC and GAPDH. However, within our experimental set-up we were not able to demonstrate that HuR was phosphorylated or that the phosphorylation levels of TTP changed significantly upon Mirasol treatment. We were unable to demonstrate that HuR or TTP contributed to the change in mRNA half-life. However, it is possible that other RBPs are activated through the p38 MAP kinase to interact with platelet transcripts. Platelets are able to synthesis proteins during their entire lifespan, even after Mirasol treatment [152, 172]. This suggests that even under stress conditions platelets are able to protect the integrity of their mRNA transcripts to be able to function as translational templates after 7 days of storage.

Chapter 5: Are stress granules in human blood platelets responsible for protection of mRNA species?

5.1 Introduction

As demonstrated in **chapter 3**, not all mRNA transcripts in platelets react equally to the stress induced by PI treatment. A potential explanation of the difference observed in mRNA amount and mRNA half-life, could be a distribution of the mRNA transcripts in different pools within the platelet. A transcript could either be in a pool that that is susceptible to the external stress (labile) or in a pool that is protected from the stress (non-labile). A similar mechanism is present in nucleated cells, where stress like heat shock, UV illumination, and oxidative stress can lead to the formation of specific RNA aggregates within the cell. These aggregates are referred to as stress granules [232].

Stress granules are very dynamic and diverse conglomerates of mRNA, translation initiation factors, RNA binding proteins, and non-RNA binding proteins [94, 233]. They are formed in situations that translation initiation is impaired, either by stress, or translational inhibitors [94]. Stress granule formation plays a role in the mRNA stability by regulation of their localization, and availability of a transcript for translation or degradation [82].

So far it is unknown how platelets protect their mRNA transcripts from rapid degradation, as happens in other nucleated cells. UV illumination is a type of stress that can induce stress granule formation in nucleated cells [82, 234]. In transfusion medicine, UV illumination is a

crucial part of any PI treatment. We hypothesize that exposure to PI treatment initiates the formation of stress granules and helps to protect platelet mRNA from degradation.

To test this hypothesis we performed an inhibitor study using nocodazole, a well-known stress granule inhibitor, and arsenite, a well-known stress granule inducer. Co-localization of stress granule marker proteins after exposure to arsenite was evaluated by immunofluorescence. The amount of mRNA was analyzed before and after Mirasol treatment with or without nocodazole. For comparison, the impact of arsenite alone on platelets was evaluated using several platelet quality *in vitro* assays.

5.2 **Results**

5.2.1 **Platelets express stress granule marker proteins, but arsenite exposure does not induce co-localization**

The formation of stress granules requires the presence of certain core proteins in addition to mRNA transcripts and eukaryote initiation factors. Typical stress granule marker proteins are G3BP, PABP, HuR, TIA-1 and TIAR. In **Chapter 4** we have shown that both resting platelets and Mirasol-treated platelets express these proteins. See Figure 4.3A.

Co-localization of the core stress granule marker proteins is necessary to form stress granules. To investigate if this would occur in platelets we performed immunofluorescent staining of the marker proteins G3BP and PABP. Platelets were exposed to 0.5 mM arsenite for 30 minutes with and without pre-incubation with nocodazole to determine if arsenite would initiate co-

localization of G3BP and PABP in a specific manner and whether the presence of nocodazole would impact the localization pattern. Images were compared to untreated platelets.

As seen in Figure 5.1, in untreated control platelets we observed a diffuse localization signal for PABP, indicating its presence throughout the platelet, whereas G3BP was seen in specific locations in the platelets. When exposed to arsenite only, the localization pattern for PABP remained the same, however G3BP changed its localization pattern from specific loci more towards a diffuse signal throughout the cytosol. By this technique, no notable co-localization in specific aggregates, a requirement for stress granule formation, was seen. Interestingly, pre-incubation with nocodazole prevented the change in G3BP localization pattern caused by arsenite.

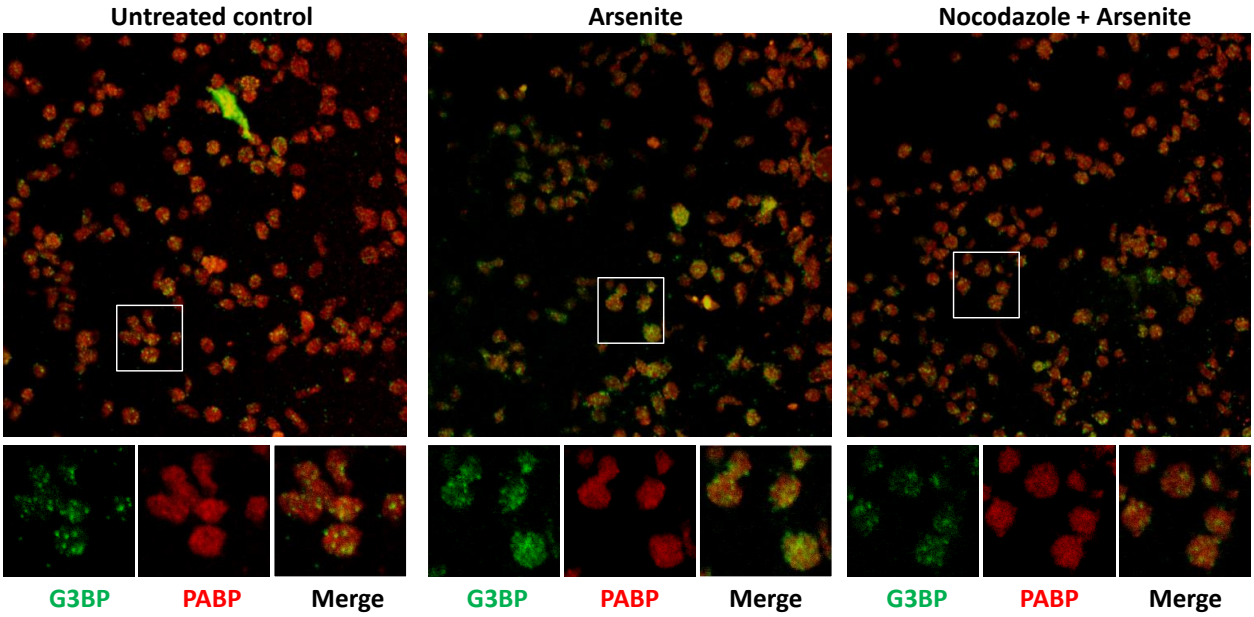


Figure 5.1: Immunofluorescence image of G3BP and PABP in untreated control platelets, and platelets exposed to arsenite in the presence and absence of nocodazole.

Apheresis platelets were sampled the day after collection. Nocodazole (20 µM) was added 30 min prior to arsenite exposure. Samples were incubated with 0.5 mM arsenite for 60 min before imaging. Images were compared to platelets not exposed arsenite or nocodazole (untreated control). (N = 2). Experiment performed in collaboration with Stefanie Novakowski.

5.2.2 **Long-term exposure of nocodazole did not change platelet mRNA amounts**

Nocodazole was used to test whether inhibition of potential stress granules during platelet storage would change the total RNA amount and the amount of highly abundant transcripts. An apheresis PC was split into 60 mL mini-bags and spiked with nocodazole in DMSO, or DMSO alone, followed by Mirasol treatment according to manufacturers' protocol. Two mini-bags without nocodazole or DMSO served as the untreated control (no PI treatment) or the Mirasol PI treatment control (see Figure 5.2). The mini-bags were stored for 7 days under blood banking conditions to determine the long term effect of this inhibitor on platelet RNA. The relative mRNA expression in the different treatment arms was determined using a relative standard curve. The three mRNA targets were selected based on their abundance in the platelet transcriptome [131, 135]. GAPDH, a housekeeping gene, is known to be sequestered into stress granules [82, 235]. The α-granule protein PF4 is synthesized in stimulated platelets and upon Mirasol treatment [120, 172]. The mRNA encoding another α-granule protein, SPARC, is present in platelets in high abundance, however has not been demonstrated to be synthesized. As demonstrated in **chapter 3**, the impact of Mirasol treatment on these mRNA transcripts was the least of the 7 analyzed targets (see Figure 3.1).

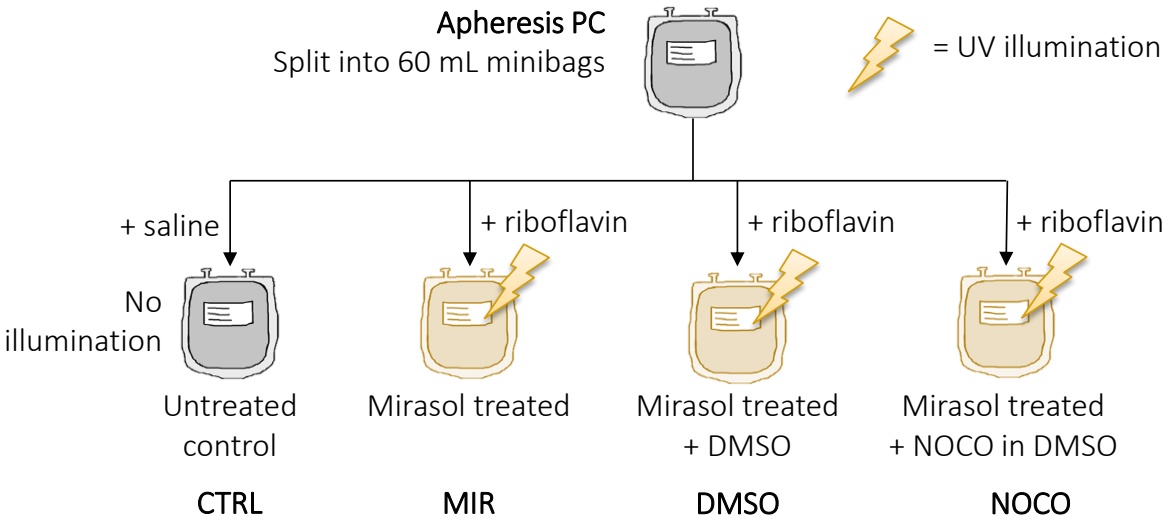


Figure 5.2: Schematic representation of the experimental set-up.

The day after collection a single apheresis PC was split into four equal minibags and all proceedings were scaled down accordingly. An untreated control unit was prepared by adding saline to correct as a volume control and was not exposed to UV illumination (CTRL). The other three units were treated with the standard Mirasol PRT System according to the manufacturer's protocol, either without spiking (MIR), in the presence of DMSO as a vehicle control (DMSO), or in the presence of 20 µM nocodazole (final concentration) in DMSO (NOCO) (see paragraph 2.3).

Total RNA and the mRNA levels of the 3 different transcripts after Mirasol treatment with and without nocodazole were compared to the amount present prior to treatment (Day 1), see Figure 5.3. After 7 days of storage, total RNA in all treatment arms was reduced to 60 ng / µL from 90 ng / µL prior to storage. GAPDH, PF4 and SPARC mRNA were reduced to around 10%, 25% and 5% respectively of the mRNA amounts present on Day 1. The overall mRNA amount for all three transcripts decreased significantly after 7 days of storage ($p < 0.05$); however, the addition of nocodazole had no further impact on the mRNA levels and no difference between treatment arms was detected.

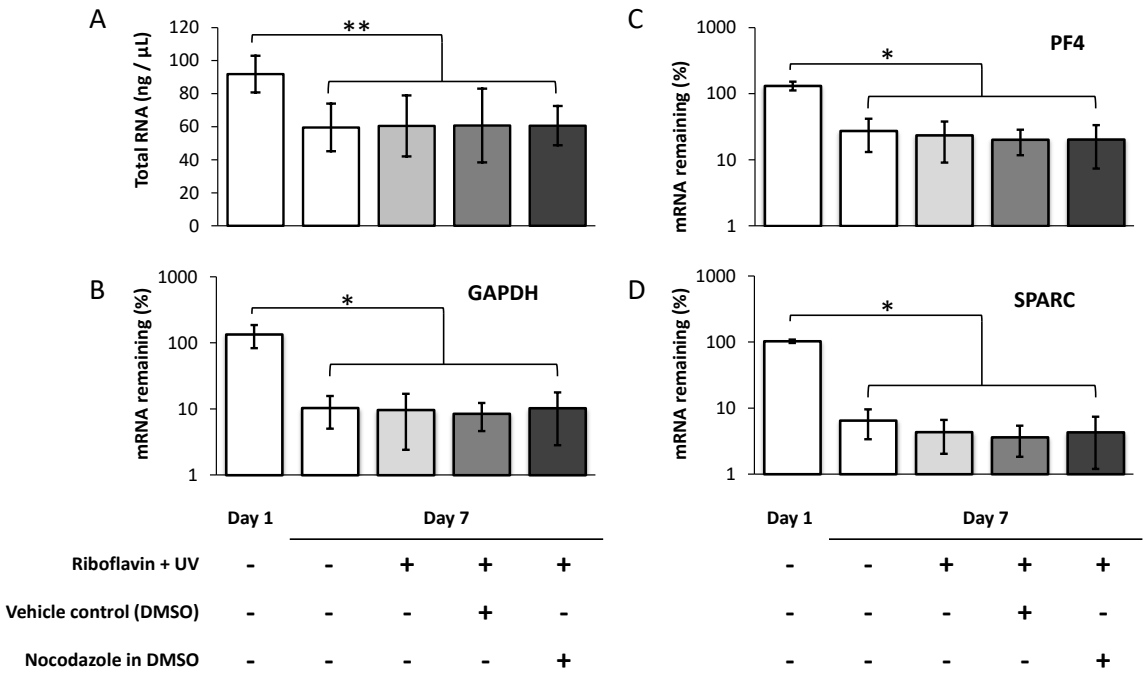


Figure 5.3: RNA expression in stored PCs after Mirasol treatment in the absence or presence of nocodazole. A sample was drawn from an apheresis PC the day after production (Day 1) for RNA extraction. The remainder of the PC was split into four equal volumes in mini-bags to create paired samples and treated according to the schedule below the figure. Samples were stored for 7 days under blood banking conditions prior to RNA extraction. A) Extracted RNA was dissolved in DEPC-treated water based on the platelet count. Total RNA concentration was determined by absorbance at 260 nm. Results are expressed as mean ± SD for N = 6. ** p < 0.01 between day 1 and 7 (ANOVA). B) – D) mRNA levels for GAPDH, PF4 and SPARC were determined by qPCR. Results are expressed as mean ± SD for N = 5. * p < 0.05 between day 1 and 7 (Kruskal-Wallis)

5.2.3 **Nocodazole impacted platelet indices**

The PC indices platelet count, mean platelet volume (MPV), platelet distribution width (PDW), and plateletcrit (PCT) are platelet parameters describing the platelet morphology. The PDW is an

indicator for the variability in platelet size and PCT is the volume of the sample occupied by platelets [236]. These parameters were measured using the hematology analyzer before Mirasol treatment and after 7 days of storage in the different treatment arms. The results are described in Table 5.1.

After 7 days the platelet count in Mirasol-treated PCs with nocodazole was significantly higher ($p < 0.05$) and the MPV was significantly lower ($p < 0.001$) compared to the other treatment arms. The PDW was reduced in the samples pre-treated with nocodazole, but this did not reach statistical significance ($p = 0.06$). The PCT did not change among the treatment arms ($p = 0.81$).

Table 5.1: Platelet indices values in stored PCs after Mirasol treatment in the absence or presence of nocodazole.

	Platelet count ($x10^9$ cells/L)	**MPV (fL)**	**PDW (%)**	**PCT (%)**
Pre- treatment	1347 ± 196	8.4 ± 0.8	52.4 ± 0.8	1.12 ± 0.08
Control	1298 ± 130	7.8 ± 0.3	53.4 ± 2.0	1.01 ± 0.09
Mirasol	1332 ± 110	7.8 ± 0.4	53.1 ± 3.0	1.03 ± 0.08
DMSO + Mirasol	1298 ± 119	7.8 ± 0.5	53.1 ± 2.8	1.00 ± 0.09
Nocodazole in DMSO + Mirasol	1481 ± 70 *	6.8 ± 0.3 ***	47.8 ± 5.2	1.01 ± 0.06

*** p < 0.05, *** p < 0.001 between nocodazole treated samples and all other treatments at day 7 (One way ANOVA for N = 6).**

5.2.4 **Exposure to nocodazole did not significantly change the platelet activation levels or the activation response to ADP.**

The use of nocodazole as a stress granule inhibitor has been well established in nucleated cells [237], however little is known about the effects of nocodazole in platelets. Nocodazole is an inhibitor of microtubule formation. Microtubuli play an important role in regular platelet function, including platelet activation and aggregation. As shown in Table 5.1 we observed a change in platelet characteristics like MPV. We performed additional flow cytometric tests to determine if exposure to nocodazole impacted the level of activation in resting and Mirasol treated platelets and the responsiveness to ADP. Results are summarized in Figure 5.4.

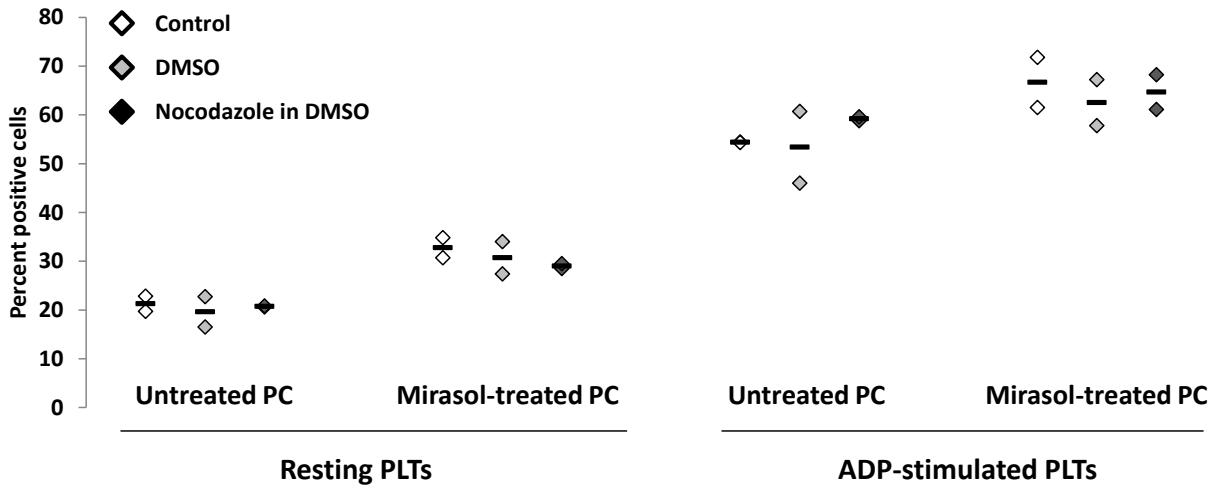


Figure 5.4: The impact of nocodazole on the CD62P expression in resting and ADP-stimulated platelets.

The day after production an apheresis PC was split into 6 mini-bags with each 60 mL PC. In pairs the mini-bags were spiked with nocodazole (20 µM in DMSO) or DMSO alone, or platelets remained untreated (control). Mirasol treatment was performed according to manufacturer's protocol on a set of three bags (Control, DMSO and Noco) 30 minutes after spiking. After a one hour resting period, samples were drawn to determine the levels of platelets activation by measuring the CD62P expression by flow cytometry.

Responsiveness was evaluated by the increase in activation in the presence of 10 µM ADP. Horizontal bar = mean for N = 2.

In resting platelets the CD62P expression was around 20% and did not change in the samples pre-incubated with nocodazole. Pathogen-reduced platelets are known have higher levels of platelet activation [65, 71]. This concurs with our observation that in Mirasol-treated platelets the CD62P expression in resting platelets was about 10% higher than in untreated platelets at 30%. There was no difference in CD62P expression in the different treatment arms.

In the presence of 10 µM ADP, the CD62P expression in untreated platelets increased from ~35% to ~55%, and there was no difference in CD62P expression between control platelets and platelets pre-incubated with nocodazole. After addition of ADP to the Mirasol-treated platelets the CD62P expression also increased from ~35% to ~65% (see Figure 5.4). The presence of nocodazole had no impact on the response of platelets to either the Mirasol treatment, the stimulation with ADP, or a combination of both.

5.2.5 **Arsenite exposure reduced platelet aggregation**

The impact of exposure to arsenite on platelet aggregation was evaluated using the ESC assay. Platelets were exposed to increasing concentrations of arsenite for 15 minutes or 60 minutes (see Figure 5.5). A clear trend of a reduction in aggregation was observed with increasing concentration of arsenite; this was more pronounced after exposure for 60 minutes compared to 15 minutes.

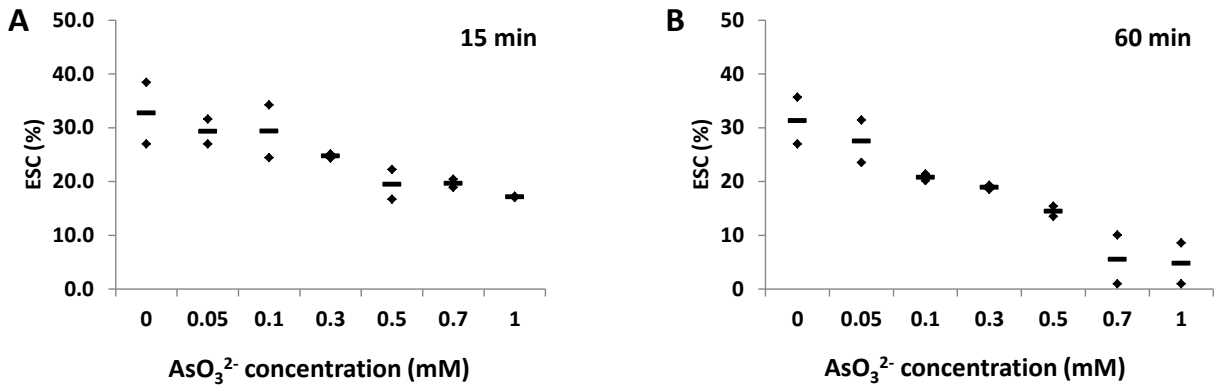


Figure 5.5 Impact of arsenite exposure on ESC assay.

Platelets were exposed to increasing concentration of arsenite and the ESC was determined after A) 15 min and B) 60 min incubation time. Horizontal bar = mean for N = 2. Data courtesy of Peter Schubert.

5.2.6 **Exposure to arsenite did not reduce the total RNA or GAPDH mRNA amounts.**

To test whether arsenite treatment affects platelet RNA like Mirasol treatment, in three independent replicates platelets were exposed to 0.5 mM or in two independent replicates platelets were exposed to 1.0 mM arsenite for different periods of time. Total RNA was extracted and measured by absorbance. RNA concentration was normalized to the amount present before the treatment. Real-time qPCR was used to determine the relative amounts of GAPDH remaining in the platelets after exposure to 0.5 mM arsenite and compared to pre-treatment.

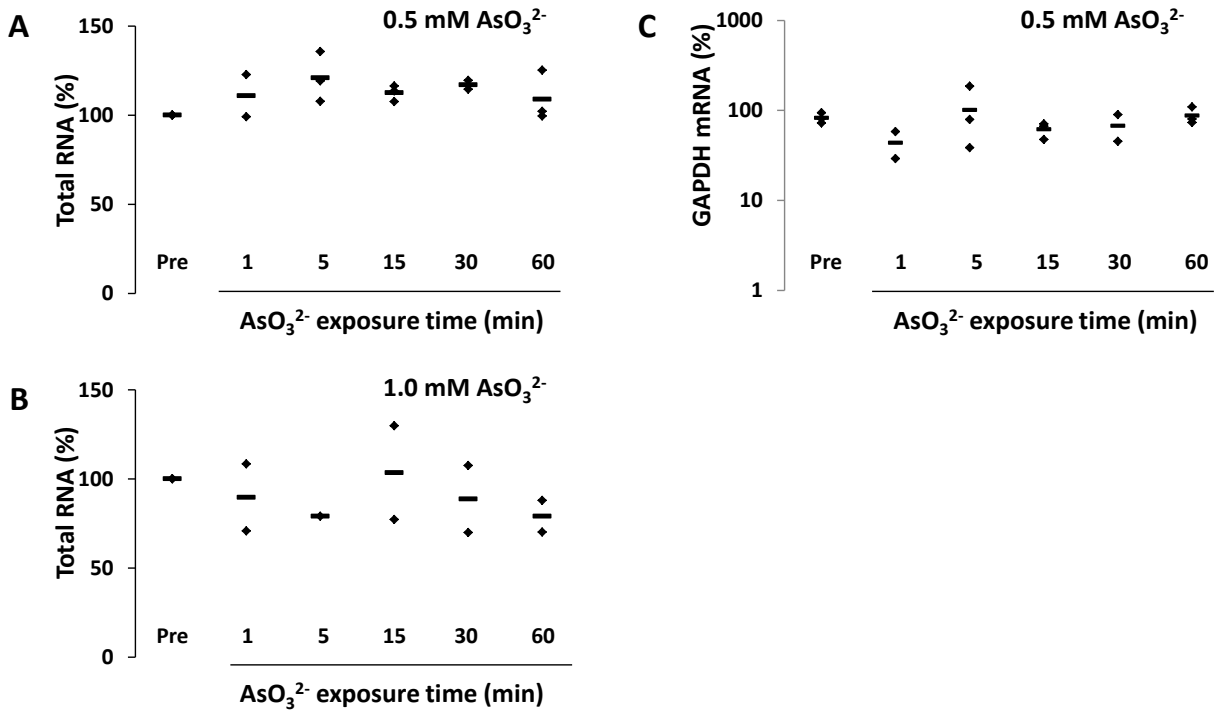


Figure 5.6 Impact of arsenite exposure on total RNA and GAPDH mRNA

Platelets were exposed to 0.5 mM (A) or 1.0 mM (B) arsenite. Samples were drawn at different time points and total RNA was extracted and determined by absorbance. RNA concentration was normalized to the concentration prior to arsenite exposure. C) Platelets were exposed to 0.5 mM arsenite. Expression of GAPDH mRNA was determined by real-time qPCR by relative standard curve. Horizontal bar = mean. A) and C) N = 3, except for time = 1 min and 30 min where N = 2. B) N = 2, except for time = 5 min where N = 1

Figure 5.6 demonstrated that there was no significant change in total RNA concentration in response exposure to 0.5 mM arsenite or 1.0 mM arsenite for up to one hour. As seen in Figure 5.6C, no significant change in GAPDH mRNA amount was seen after exposure to 0.5 mM arsenite.

5.3 **Discussion**

This study examined the potential of stress granule formation in platelets as way to protect platelet mRNA from degradation. In nucleated cells, stress granules are formed in response to exposure to external stress as regulation mechanism of mRNA localization and stability [82]. Even though platelets possessed several stress granule marker proteins, we found no evidence of the formation of stress granules, either via microscopy or by the use of a stress granule inhibitor and inducer on platelet mRNA.

In silico analysis demonstrated that platelets possess proteins which are considered a stress granule marker. The eukaryotic initiation factors, known to be core components of stress granules, are present in platelets and regulate the synthesis of proteins [5]. A second group of proteins which are considered stress granule core components are a subset of RNA binding proteins. In **Chapter 4** we have confirmed by immunoblot analysis that platelets possess G3BP and PABP, two hallmark proteins necessary for the formation of stress granules. Via microscopy we have confirmed the presence of these two proteins in platelets. Exposure to arsenite changed the localization pattern of G3BP from specific loci to a more diffuse presence in the platelet (see Figure 5.1), however co-localization of the two marker proteins under the influence of arsenite has not been demonstrated.

The use of nocodazole as inhibitor of stress granules in nucleated cells has been well described [238], but only limited research has been performed in platelets. In megakaryocytes, nocodazole disrupts the microtubule polymerization which leads to blocking of the proplatelet extension and platelet release [239, 240]. In platelets, high concentrations of nocodazole (30 – 300 μM) inhibit

platelet aggregation induced by collagen, but not ADP [241]. In nucleated cells, nocodazole can disrupt microtubuli in concentrations as low as 6.6 μM for 30 minutes and reduce the percentage of cells displaying stress granules from 75% to less than 10% of cells displaying stress granules [238]. In our experiments we used a nocodazole concentration of 20 μM which had impact on the microtubule dynamics (≥ 10 μM) [242], but no impact on the platelet aggregation (≤ 30 μM) [241].

We tested whether PI-induced stress followed by long-term exposure to nocodazole would impact platelet mRNA levels of transcripts expected to be recruited into stress granules. No impact on the total RNA levels or mRNA levels of the genes encoding for GAPDH, PF4 and SPARC was observed 7 days after Mirasol treatment with or without nocodazole. We found no evidence that mRNA was affected more by the Mirasol treatment in the presence of the stress granule inhibitor.

In PCs stored for 7 days after Mirasol treatment the presence of 20 μM nocodazole we observed a significant increase in platelet count and a significant decrease in MPV, while the PDW and PCT did not significantly change. The PCT is the volume occupied by platelets and can be calculated using the equation [236]:

$$PCT = \frac{platelet\ count \times MPV}{10{,}000} \qquad \text{Equation (3)}$$

The increase in platelet count during storage and after Mirasol treatment has previously been described by our group and others [188, 243]. Other studies reported an increase in MPV after Mirasol treatment [65, 244] which is assumed to be the result of the increased platelet activation. In our study storage alone or storage and Mirasol treatment did not affect any of the measured

platelet indices, suggesting the presence of nocodazole initiated the change to more, but smaller platelets. The reduction in MPV concurred with reports that nocodazole impacts the proteins involved in platelets shape including tubulin and actin [242] and supported our decision that at the used concentration nocodazole interfered with the microtubule dynamic responsible for platelet shape. Whether nocodazole has an effect on processes involved in the maturation of platelets or some other aspect of platelet formation in storage remains to be determined.

The *in vitro* test showed that nocodazole, at the concentration used in our experiments, had no impact on the level of platelet activation measured by the CD62P expression in resting platelets or in response to ADP. This is in agreement with studies looking at other aspects of *in vitro* platelet quality like aggregation [241]

Arsenite-induced stress can result in a reduction in mRNA levels in nucleated cells at concentrations as low as 1 μM when exposed for 24 hours [245, 246]. At higher concentrations (0.5 – 1.0 mM) short exposure is enough to modify the post-transcription modification of mRNAs and stimulate the formation of stress granules. In our experiments we used the dose and exposure time which, in nucleated cells, induces of stress granules. We did not observe a change in total RNA or GAPDH mRNA. Total RNA is a heterogeneous group of RNA, consistent primarily of ribosomal RNA (80%) and transfer RNA (15%). Even though ribosomal RNA is much more stable than mRNA and less susceptible for degradation, in yeast cells Guerra-Moreno and colleagues [247] observed ribosome reduction as a proteotoxic stress response via a down-regulation in the majority of protein component of ribosomal subunits one hour after arsenite exposure (1 mM). The ribosome reduction could serve as a method to limit newly synthesized

misfolded proteins and allow protein degradation pathways to focus on existing misfolded proteins. We did not observe a decrease in total RNA after exposure to arsenite, suggesting platelets might regulate their response to this type of proteotoxic stress differently.

Our data suggest that in the concentration range from 0.5 mM – 1.0 mM, arsenite exposure for 15 min reduces the number of platelets with discoid shape as measured by the ESC. When the incubation time was longer an even lower concentration of arsenite was able to achieve the same result. This concurred with observations that even at low concentrations (15 μM) arsenite reduces aggregation induced by collagen and ADP, and thrombin [248, 249]. Interestingly, Lin et al [248] have been able to link the arsenite-induced reduction in platelet aggregation to inhibition of the PLCγ2-PKC-p38 MAP kinase cascade. This observation adds to the importance of p38 MAP kinase in platelet function, as we investigated in **chapter 4**.

We hypothesized that platelets have the ability to form stress granules and that platelets use this mechanism to protect their mRNA from degradation caused by PI-induced stress. The proposed role of stress granules in nucleated cells is to be a site where mRNA is temporarily stored during cellular stress and indirectly protect mRNA from degradation. Similar to arsenite, UV illumination is an inducer of stress granules [82, 234]. The damage to RNA and DNA caused by UV activates PKR, which downstream causes an inhibition of translation initiation and redirects mRNA transcripts from polysomes into stress granules. UV illumination is a key element of any PI technique. Exposure to PI treatment causes a reduction in ESC change similar to arsenite-incubated platelets [74, 250]. However arsenite exposure did not cause a rapid reduction in platelet total RNA or mRNA like we have shown to occur after Mirasol exposure (**see chapter**

3). Arsenite generates reactive oxygen species, which have a different effect on platelets compared to UV-induced damage. In summary we were able to demonstrate that platelets possess the necessary tools, like stress granule marker proteins, to from stress granules, but the formation of the stress granules was not demonstrated.

Chapter 6: Overall discussion and conclusion

6.1 Hypotheses and aims

The research presented in this dissertation was sparked by developments in transfusion medicine related to both the increased understanding of the biology of stored platelets and the appearance of the first direct treatment technologies used to improve blood safety of fresh components. Pathogen inactivation techniques have been increasingly adopted into the production process of blood components to increase the safety of blood products. These techniques use UV illumination with or without a photosensitizing agent to target viral and bacterial RNA and DNA in platelet concentrates. Extensive research has demonstrated a decrease in *in vitro* quality of platelets exposed to these techniques, however little data were published on the impact of these techniques on platelet mRNA. Our goal was to elucidate the impact of pathogen inactivation treatment on platelet mRNA. Our research and findings are summarized in Figure 6.1.

Our **first hypothesis was that pathogen inactivation techniques, which are designed to target viral and bacterial RNA and DNA, influence platelet mRNA quality and functionality.** We have used the Mirasol PRT technique as a representative PI treatment to test this hypothesis. Our **aim** was to determine the impact of Mirasol on platelet mRNA amount of a selected panel of transcripts. We have been able to demonstrate that platelet mRNA is damaged by Mirasol PRT. Within one hour after treatment, platelet mRNA was significantly reduced compared to mRNA levels in untreated control platelets (see Figure 3.2). Interestingly, the degree to which transcripts were affected varied among the analyzed targets. The longer transcripts were affected more severely than shorter transcripts.

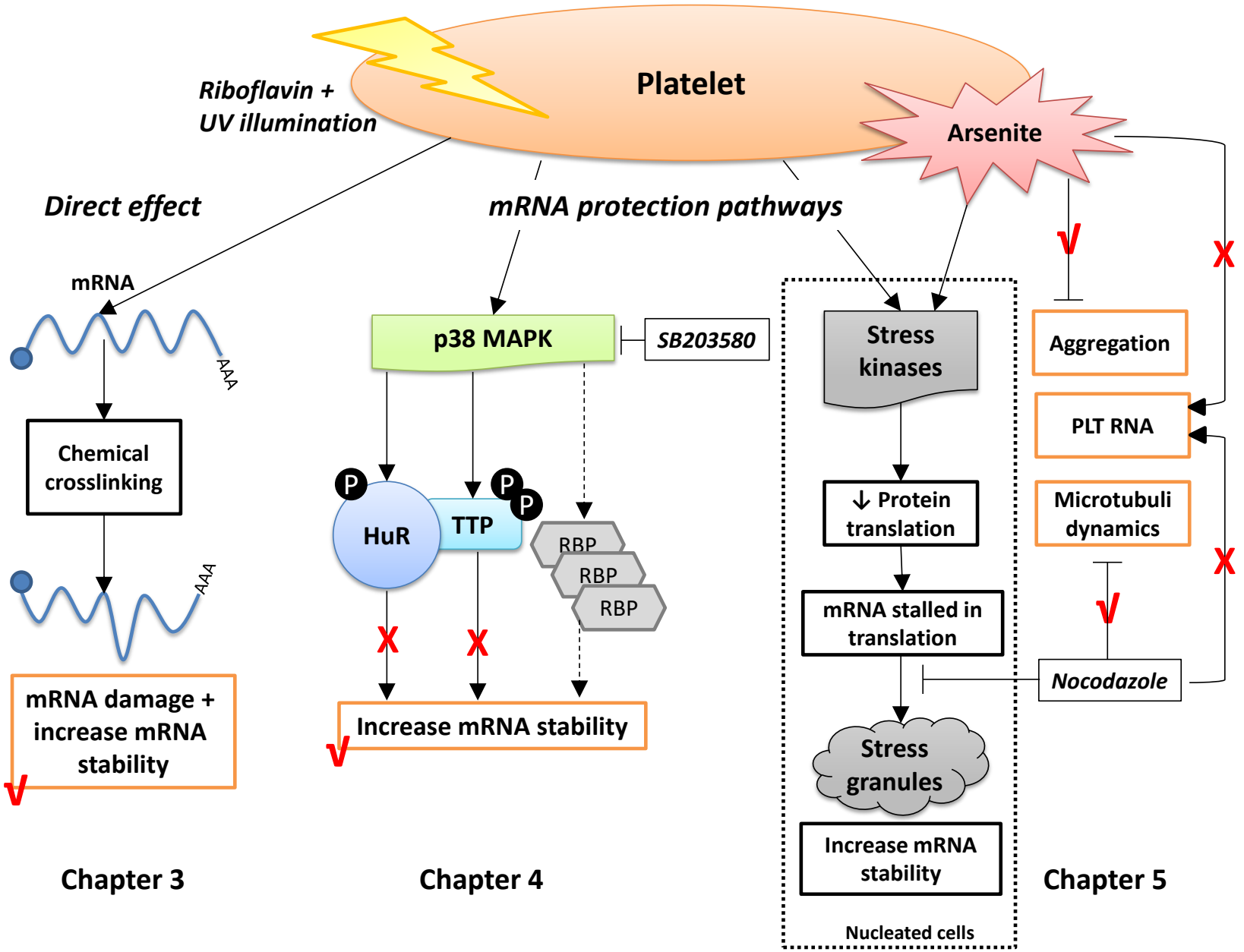

Platelet
Riboflavin +
UV illumination
Arsenite
Direct effect
mRNA protection pathways
mRNA
AAA
Chemical
crosslinking
AAA
mRNA damage +
increase mRNA
stability
√
p38 MAPK
SB203580
P
HuR
P
P
TTP
RBP
RBP
RBP
X
X
Increase mRNA stability
√
Stress
kinases
↓ Protein
translation
mRNA stalled in
translation
Stress
granules
Increase mRNA
stability
Nucleated cells
√
X
Aggregation
PLT RNA
Microtubuli
dynamics
X
√
Nocodazole
Chapter 3
Chapter 4
Chapter 5

Figure 6.1: Overall model of the impact of pathogen inactivation treatment on platelet mRNA and the investigated mRNA protection mechanisms.

Chapter 3 described the direct impact of the Mirasol PI treatment on platelet mRNA. The riboflavin / UV illumination damaged platelet mRNA, but resulted in an increase in platelet mRNA. Chapter 4 summarized the inhibitor study executed to determine the role of p38 MAP kinase in the impact of Mirasol on platelet mRNA, with additional focus on the RNA binding proteins HuR and TTP. In chapter 5 we investigated whether stress granules were formed in platelets upon Mirasol treatment similarly to UV-exposed nucleated cells using nocodazole. √ = observation confirmed, X = observation not confirmed, dotted outlined box = mechanism as it occurs in nucleated cells

This agrees with the observations made by others who have shown PCR inhibition by Mirasol treatment correlates with amplicon length and that this specific PI treatment has no significant impact on small miRNAs [170, 173]. Consistent with the higher susceptibility of guanine bases for the UV-induced damage generated by this specific PI treatment, we observed a correlation with number of guanine bases present in the transcript and percentage of mRNA remaining after treatment (see Figure 3.3).

Previous work in our lab had already demonstrated that protein synthesis in platelets is an ongoing activity, even during storage under blood banking conditions [152, 163]. We also found that platelet total RNA, which consists primarily of ribosomal RNA, has a lifespan of 10 – 11 days, in both untreated and Mirasol treated PCs. This observation supported the already existing data that platelets synthesize proteins during their entire lifespan [152, 251] even after Mirasol treatment [172]. These observations led to our second hypothesis that mRNA in platelets is protected against degradation during storage by the formation of stress granules.

To identify potential pathways we looked at nucleated cells, where exposure to UV illumination can activate p38 MAP kinase which ultimately leads to an increase of mRNA stability. P38 MAP kinase was a target of interest due to the fact that it has already shown to be involved in other platelet functions, like aggregation and adhesion [252], even though the p38 MAP kinase signaling pathway has not fully been characterized. In addition our lab has already shown that blocking p38 MAP kinase reduced the negative impact of Mirasol PRT on platelet *in vitro* quality [175, 222].

In nucleated cells, UV illumination can activate p38 MAP kinase to phosphorylate downstream substrates like the RNA binding proteins HuR and TTP [231, 253, 254]. Phosphorylation of these proteins changes their affinity for mRNA transcripts resulting in an increase in mRNA stabilization. In **chapter 4** we explored this possibility and our **aim** was to determine the impact of inhibition of p38 MAP kinase on mRNA levels in platelets exposed to the Mirasol treatment.

Using the p38 MAP kinase inhibitor, SB 203580, we were able to show that inhibition of p38 MAP kinase significantly increased the half-life of TSP, SPARC, and GAPDH mRNA in Mirasol-treated platelets (see Figure 4.2). The improvement of the mRNA half-life after blocking of p38 MAP kinase is in agreement with other work showing that p38 MAP kinase inhibition prevents the loss in platelet function usually observed after Mirasol treatment [175, 222]. However, this observation opposes the speculated stabilization of mRNA through p38 MAP kinase activation. This suggests the downstream effects of p38 MAP kinase in platelets might be different than in nucleated cells.

To further investigate this we evaluated whether platelet mRNA has the potential to be regulated through RNA binding proteins like p38 MAP kinase as it happens in nucleated cells. As an example we analyzed thrombospondin mRNA for its potential to bind RNA binding proteins. The 3'-UTR of this transcript is over 3500 nucleotides long and theoretically contained 439 binding sites for a total of 28 different RNA binding proteins (see Table 4.1) Even though platelets only express 12 of the 28 proteins, this confirms platelet mRNA has potential to bind RNA binding proteins. By means of an *in silico* analysis of two publicly available databases [182, 183] we were able to determine that platelets express 216 out of the 416 RNA binding proteins listed by the database in their proteome, transcriptome, or both (see Figure 4.3A). These data support the theory that RNA binding proteins could potentially be involved in the regulation of platelet mRNA.

In nucleated cells UV-induced p38 MAP kinase activation can lead to the phosphorylation of RNA binding protein HuR [203, 204]. In our experiments we were able to confirm the presence of HuR in platelets (see Figure 4.4A). The overall expression of HuR was influenced by the Mirasol treatment during storage, and the p38 MAP kinase inhibitor reduced the negative impact. However, the phosphorylation levels of HuR were below the level of the detection of the methods available to us. We were not able to draw a conclusion whether Mirasol changes the phosphorylation levels of HuR and whether p38 MAP kinase had an impact on that. A second RNA binding protein regulated through p38 MAP kinase that we evaluated was TTP. Even though *in silico* analysis resulted in no expression of TTP in platelets, we were able to confirm the presence of the RNA binding protein by immunoblot analysis (see Figure 4.4B). Similar to

HuR, the overall expression of TTP was influenced by the Mirasol treatment during storage, and the p38 MAP kinase inhibitor reduced the negative impact. The phospho-TTP did not show an impact either during storage or upon treatment. These studies yielded no evidence that Mirasol treatment or p38 MAP kinase inhibition impacted the expression levels of phospho-TTP in platelets (see Figure 4.4C). Again this may suggest that the signaling pathway of p38 MAP kinase in platelets regulates other downstream substrates than it does in nucleated cells.

With the investigation of the role of p38 MAP kinase in platelet mRNA stability after Mirasol treatment we attempted to identify a pathway involved in the protection of platelet mRNA. Even though we were able to show that p38 MAP kinase is involved in the mRNA stability, this pathway did not fully explain how platelet mRNA is protected from degradation. Investigation of more mechanisms was necessary to be able to identify the way platelets are able to protect their mRNA from degradation, resulting in mRNA half-life of days rather than minutes. Again we explored parallels in nucleated cells and **our second hypothesis was that some messenger RNA species in platelets are protected against degradation during storage by the formation of stress granules.**

We explored the possibility that platelets form stress granules to temporarily store mRNA and protect it from degradation, especially under stressful conditions like PI treatment. Our **aims** were to evaluate the impact of the Mirasol pathogen inactivation system on platelet mRNA in the presence of the stress granule inhibitor nocodazole and to create an *in vitro* model of platelet stress using a known stress granules inducer (arsenite) and inhibitor (nocodazole) to establish reference in response.

In **chapter 4** we were able to demonstrate by immunoblot analysis the presence of the stress granule marker proteins G3BP, PABP, TIA/TIAR and HuR in both resting and Mirasol-treated platelets (see Figure 4.3B). Especially G3BP and PABP together with eIFs and mRNA transcripts are considered core elements of stress granules. This suggests platelets have the potential to form stress granules, based on their protein expression. Microscopy images confirmed the presence of these two marker proteins in platelets, however no obvious co-localization of the proteins after arsenite-induced stress was observed (see Figure 5.1).

We were unable to test the use of nocodazole as a stress granule inhibitor in platelets, because we did not have conclusive evidence yet that platelets form stress granules. With the assumption Mirasol treatment would induce stress granules we tested the impact of nocodazole on the total RNA amount and mRNA of selected targets. Pre-incubation with nocodazole in concentration that in nucleated cells inhibits the formation of stress granules, did not change the total RNA levels or mRNA levels of the targets TSP, SPARC and GAPDH of platelet exposed to Mirasol treatment (see Figure 5.3). Nocodazole did affect the mean platelet volume, suggesting an impact of nocodazole on proteins involved in shape change like tubulin or actin (see Table 5.1).

We performed preliminary tests to determine the feasibility of the use of a well-known stress granule inducer arsenite. Exposure of platelets to arsenite in a concentration generally used to induce stress granules in nucleated cells did not change the total RNA amount of mRNA expression levels of GAPDH (see Figure 5.6) This result showed that arsenite generated a different type of stress than Mirasol, where after an hour a significant reduction in mRNA was

seen. Arsenite did affect platelet function measured by the ESC (see Figure 5.5). This agreed with previous research done by others that arsenite reduces platelet aggregation [248, 249]. Even though no undisputable evidence for the formation of stress granules in platelets was found, a better understanding about the impact of arsenite and nocodazole was generated.

6.2 **Significance**

How platelets secure the availability of enough mRNA to enable protein synthesis during their entire lifespan is a question that has not been elucidated yet. There is clear evidence platelets initiate protein synthesis in response to stress by external stimuli or *ex vivo* storage [3, 128, 156, 162, 184, 255]. Additional structural research demonstrated platelet mRNA is significantly more stable compared to other cell types, in part by a significantly longer 3'-UTR [138]. This is confirmed by the measurement of the platelet mRNA *in vitro*, showing mRNA half-life of days [152]. The introduction of pathogen inactivation techniques in transfusion medicine offers an extra challenge to the platelets and leaves us with the question how platelets protect their mRNA from degradation. The methods used to pathogen inactivate fresh blood components are specifically designed to damage RNA and DNA and are non-specific [42, 46, 192, 197, 211]. It has already been shown that the application of PI treatment has a negative impact on the *in vitro* platelet quality [65, 67, 71, 72, 189, 256]. Unexpectedly, Mirasol-treated platelets show protein synthesis after seven days of storage [172]. This observation adds to the importance of the protection of platelet mRNA from degradation. So far, no mechanism has conclusively been demonstrated to regulate platelet mRNA stability.

The research presented in this dissertation is an extrapolation from the recent development in transfusion medicine with respect to ensuring blood safety. By using apheresis PCs, collected and stored under blood banking conditions following PI used according to the manufacturer's instruction, the materials used in this study are equivalent to the blood products transfused into patients. The safety and quality of blood products are of primary concern of blood suppliers. This dissertation describes studies that enhance the current understanding of the impact of riboflavin / UV illumination PI technique on platelet mRNA. Several avenues have been pursued to elucidate signaling pathways and molecular mechanism, to find answers to these questions and offers suggestions for further research.

6.3 **Future directions**

The global increase in use of pathogen-reduced PCs increases the importance to enhance our understanding of the pathways or mechanisms involved in the regulation of platelet mRNA. Several studies have linked protein translation to platelet function [165, 219], but a better understanding is required to fully understand the role of protein synthesis in platelets. One aspect that needs clarification is the selection of transcripts used for translation and the translational rate of platelet mRNA. Studies have shown that platelet transcriptome correlates among individuals [130], but it remains unclear whether platelet transcriptome correlates to the proteome [131-134]. Transcripts present in low abundance, but translated at a high rate, can generate high protein amounts and vice versa. Another point is the correlation between the ability of platelets to synthesize proteins and platelet function. Impairment of protein synthesis has shown to correlate to a reduction in response to agonists [219, 257]. In view of the implementation of pathogen inactivation, a reduction in *in vitro* quality is observed after treatment. This dissertation has

demonstrated that PI treatment significantly reduces platelet mRNA levels (see Figure 3.2). The p38 MAP kinase pathways has already shown to be activated through the Mirasol treatment [175, 222] and influences platelet mRNA half-life (see Figure 4.2), but not through RNA binding proteins HuR or TTP. Still this offers opportunity to further investigate the role of p38 MAP kinase in mRNA stability focusing on other proteins regulated via this pathway. The protective response of stress granule formation, which is well described in nucleated cells, although not confirmed in this dissertation, remains an attractive hypothesis. To fully confirm or disprove this hypothesis, however, a substantial amount of research will have to be conducted. The challenge starts with imaging of the stress granules proteins in platelets, which is not easy to perform. Another challenge is finding the proper stressor for this mechanism. Platelets respond differently to different agonist, and this could complicate the screen for a stress granule inducer. Arsenite has given some results suggesting it could fulfill this role, assuming this mechanism occurs in platelets as it does in nucleated cells through the inhibition of translation initiation. However exposure to arsenite at these concentrations is not physiologically relevant, but could serve as positive control. Other more physiological agonists should be evaluated to be able to translate the research observation to a physiological platelet response.

Research presented in this dissertation has added to our understanding of how platelet mRNA is affected by pathogen inactivation, but many more avenues in relation to the regulation of mRNA stability remain to be explored. The presence of RNA binding proteins, as demonstrated in **chapter 4**, suggests platelets harbor pathways using these RNA binding proteins to regulate mRNA stability. Aside from p38 MAP kinase other stress kinases, like PI_3 kinase or the mammalian target of rapamycin, could be interesting targets for additional research. Both kinases

have shown to be involved in the regulation of mRNA stability in other cell types [229, 258], and play a role in platelet biology as well [165, 252, 259, 260]. Inhibitor studies could give clues how these kinases are involved in maintaining mRNA levels in platelets during storage. Studying the role of deadenylases, decapping enzymes or other parts of the RNA degradation machinery in platelets might also provide insights in how platelets control that part of RNA metabolism. Processing bodies are the type of RNA granules involved in RNA degradation and have demonstrated to be in close relationship with polysomes and stress granules [82]. It has already been demonstrated that platelets possess several proteins which are associated with the RNA degradation machinery like Auf1 [261], Ago2 [126], and TTP (see Figure 4.2). The presence of Ago2 also offers another angle. An approach not pursued in this dissertation, but offers additional research objectives is the role of miRNAs in the regulation of mRNA in platelets. Landry and colleagues [141] have demonstrated the presence of functional miRNA silencing complexes in platelets. MicroRNAs in platelets have already been linked to platelet function [142], the development of platelet storage lesions [150] and, in murine platelets, regulation of the mRNA profile [262].

Finding the mechanism or pathway that determines platelet mRNA stability might allow us to ameliorate the damage done by pathogen inactivation treatments and prevent the loss of *in vitro* quality, which could ultimately lead to a safer and better blood product for the treatment of patients.

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