IMPROVEMENT OF QUALITY STANDARDS FOR BLOOD TRANSFUSIONS: USE OF FUNCTIONAL MEASURES TO PREDICT PLATELET TRANSFUSION EFFICACY

by

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Abstract

Transfusions of platelet concentrates (PCs) are given to maintain primary hemostasis in patients with various thrombocytopenic disorders. There is poor correlation between *in vivo* PC transfusion outcome and *in vitro* tests, which typically do not test the functional effectiveness of platelets, but rather measure platelet characteristics. Thus a PC quality assay that would accurately predict transfusion efficacy should test the efficacy of platelet activation and clot formation in a manner that more closely models these same processes in the bloodstream.

The first aim of this thesis is to determine whether Thromoelastography (TEG)/rotational Thromboelastometry (ROTEM) technologies involving global hemostatic analyzers could be used to assess the quality of PCs under a variety of conditions. Due to their procoagulant properties, platelet microvesicles' (PMVs') contribution to the clot signature was assessed.

The second aim was to investigate the effect of pathogen inactivation technology (PI) using riboflavin/UV light (Mirasol) on the hemostatic potential of PCs and plasma in transfusion trauma packages composed of reconstituted whole blood (WB). The packages were composed of red blood cells (RBC), plasma, and platelet, in a ratio of 1:1:1.

As there is an increasing interest by practitioners in returning to the use of WB (2-7 days old) in the civilian setting for the treatment of massively hemorrhaging patients, our third aim was to determine whether ROTEM could be used to assess the impact of PI-treated WB in a trauma model. Due to the reduction in the activity of multiple plasma coagulation proteins following PI-treatment, supplementation of fibrinogen to correct the negative impact was assessed.

Hemostatic analysis showed no significant change in maximum clot formation during the storage of PCs up to Day 10. Hemostatic measurement was sufficiently sensitive to dissect platelet and PMV contributions to clot formation and to detect PCs stored under poor conditions. This study suggests a potential solution to the apparent reduction in the hemostatic capability of blood products as caused by treatments with Mirasol; the use of fibrinogen supplementation appears to largely correct the Mirasol defect.

Lay summary

Blood transfusion is the most common hospital procedure. It is ideal, when blood products are needed, to ensure they function well upon transfusion. Current laboratory tests measure certain product characteristics, but cannot assure that products function properly once transfused. Therefore, it is important to find a test which is effective in determining the functionality and responsiveness of platelet components in a manner resembling our blood stream. This thesis project used a technology that better imitates a patient's blood composition to develop a way to measure the effectiveness of the platelet transfusions routinely used in hospitals. The new method was able to measure the effect of poor storage conditions as well as the effect of treating the platelets with pathogen inactivating procedures. It was also used to develop a model to study the use of pathogen inactivated blood products in trauma victims and may guide better transfusion therapy in these patients.

Preface

The University of British Columbia (UBC) extended ethics approval for this study conducted at the Centre for Blood Research (UBC Ethics approval no.: H12-03694). The Canadian Blood Services (CBS) Research Ethics Board has granted approval to the research study conducted at the Network Centre for Applied Development (netCAD approval reference no.: 2014-004).

This thesis was conducted under the supervision of Dr. Dana V. Devine at the Centre for Blood Research, at UBC, Vancouver. The possibility of adapting thromboelastography for use in investigating platelet concentrates rather than whole blood was proposed by Dr. Devine. Chapter 1 contains the literature review. Chapter 2 is based on a manuscript written by A. Arbaeen, K. Serrano, E. Levin and D. Devine. (2016, *Transfusion*) under the title, "**Platelet concentrate functionality assessed by thromboelastography or rotational thromboelastometry**". I designed this study under the supervision of Drs. Devine, and Serrano. I was also responsible for performing the study, optimizing the technology, and writing the draft of the manuscript.

A version of Chapter 3 was a published paper, written by A. Arbaeen, P. Schubert, K. Serrano, C. Carter, and D. Devine. (2016, *Transfusion*) under the title, "**Pathogen inactivation treatment of plasma and platelet concentrates and their predicted functionality in massive transfusion protocols**". I designed this study under the supervision of Drs. Schubert and Devine. This was to predict the greater degree of risk experienced by trauma patients when receiving pathogen-inactivated components. Dr. Carter assisted me in conducting this project's *in vitro* study in a scenario closely resembling the *in vivo* setting. I conducted all the testing for this research and wrote most of the manuscript.

Chapter 4 was inspired by a work published by Schubert *et al.* to reflect the lesser degree of negative impact in treating WB in preference to conducting individual treatments; I likewise designed this study and applied the identical model to it as that of the above mentioned published article whose contents are recorded in Chapter 3. I was responsible for conducting the study on the impact of pathogen inactivation on WB, in which I employed fibrinogen to decrease the negative impact on the coagulation factors. All of the pathogen inactivation treatments were performed by Brankica Culibrk, and Dr. Zhongming Chen at netCAD. This manuscript is now ready for submission in 2017 in the journal, *Transfusion*, as written by A. Arbaeen, P. Schubert, and D. Devine, under the title, "**Pathogen inactivated whole blood: Supplementation with fibrinogen partially corrects treatment damage**". Chapter 5 contains the conclusion for the study that I wrote.

Finally, although I am the primary author of the three above mentioned articles, all of my co-authors have greatly contributed towards our achievement of a high quality outcome.

Publications

Arbaeen AF, Serrano K, Levin E, Devine DV. **Platelet concentrate functionality assessed by thromboelastography or rotational thromboelastometry**. *Transfusion* 2016 Aug 16.

Arbaeen A, Schubert P, Serrano K, Carter C, Culibrk B, and Devine DV. **Pathogen inactivation treatment of plasma and platelet concentrates and their predicted functionality in massive transfusion protocols**. (Accepted for publication January 4th, 2017, *Transfusion)*.

Arbaeen AF, Schubert P, Devine DV. **Supplementation of pathogen-reduces whole blood with fibrinogen and their predicted functionality in massive transfusion protocols.** (Ready for submission to *Transfusion*).

Abstract presentations in the annual meeting of the American Association of Blood Banks (AABB):

(AABB) 2014, Abstract Title: The Efficiency of Thromboelastography (TEG) to Discriminate Good vs Poor Quality of Buffy Coat Platelet Concentrates.
Ahmad F. Arbaeen, Katherine Serrano, Elena Levin, and Dana V. Devine

(AABB) 2014, Abstract Title: Fas receptor signaling in platelets is involved in platelet clot fibrinolysis.
Peter Schubert, Brana Culibrk, Ahmad F. Arbaeen, and Dana V. Devine

(AABB) 2015, Abstract Title: Platelet Concentrate Functionality Assessed by Thromboelastography or Rotational Thromboelastometry: Platelet Microvesicle Contribution.
Ahmad F. Arbaeen, Katherine Serrano, Elena Levin, and Dana V. Devine

(AABB) 2016, Abstract Title: Spiking pathogen-reduced whole blood with fibrinogen enhances hemostatic functionality in vitro: a simulation of massive transfusion protocols.
Ahmad F. Arbaeen, Peter Schubert, Brana Culibrk, and Dana V. Devine

(AABB) 2016, Abstract Title: Pathogen inactivation treatment of plasma and platelet concentrates and their predicted functionality in massive transfusion protocols.
Ahmad F. Arbaeen, Peter Schubert, Katherine Serrano, Brana Culibrk, and Dana V. Devine

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List of abbreviations

AA	Arachidonic acid
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
aPTT	Activated partial thromboplastin time
BCPC	Buffy coat platelet concentrate
CCI	Corrected count increment
CFT	Clot forming time (ROTEM)
CT	Clotting time (ROTEM)
EVs	Platelet-derived extracellular vesicles
FFP	Fresh frozen plasma
g-force	Gravitational force
HCT	Hematocrit
K-time	Kinetic time
MA	Maximum amplitude
MCF	Maximum clot formation (ROTEM)
ML	Maximum lysis
nS&A at 30°C	No shaking in air permeable bags at 30°C
nS&nA at 30°C	No shaking and in air impermeable bags at 30°C
PAI-1	Plasminogen activator inhibitor-1

PC	Platelet concentrate
PI	Pathogen inactivation
PMV	Platelet microvesicles
POC	Point of care
PPP	Platelet poor plasma
PROmPT	Platelet responses and outcome from platelet transfusion
PRP	Platelet rich plasma
PRT	Pathogen reduction treatment
PT	Prothrombin time
RBC	Red blood cells
ROTEM	Rotational thromboelastometry
R-time	Reaction time
S&A at 22°C	Shaking and in air permeable bags at 22°C
S&nA at 22°C	Shaking and in air impermeable bags at 22°C
TEG	Thromboelastography
t-PA	Tissue plasminogen activator
TXA2	Thromboxane A2
vWF	von Willebrand factor
WB	Whole blood

Glossary

$MA_{(platelets)}$	Maximum amplitude of clot formation provided by platelet contribution, and measure by TEG/ROTEM $MA_{(platelets)}$= MA of platelet concentrate – $MA_{(inhibited\ platelets)}$ Equation 1
$MA_{(inhibited\ platelets)}$	Maximum amplitude of clot formation provided by fibrinogen contribution using cytochalasin D to inhibit actin polymerization
Trauma transfusion packages	The package prepared for trauma patient with severe bleeding, composed of RBC, plasma, and platelet in a ratio of 1:1:1 as follows: - Control package containing untreated RBC, plasma, and BCPC. - Package containing PI-treated BCPC but untreated RBC and plasma. - Package containing PI-treated plasma but untreated RBC and BCPC. - Package containing both PI-treated BCPC and plasma but untreated RBC.
Blood replacement	The replacement of the hemodiluted blood with different percentage of trauma transfusion packages. It was performed *in vitro* to mimic the transfusion scenario.

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To my parents; for investing in me and enabling me to pursue this passion.

I am so grateful for your generosity.

Chapter 1: Introduction

Hospitalized patients who require blood transfusion should receive blood products that have been specifically prepared to ensure optimal transfusion outcomes. Nowadays, when a clinician orders blood products for a patient, it is necessary to make sure that these products do not contain pathogens that could cause transfusion transmitted diseases. It is also essential to ensure that the blood unit being administered is the one most suitable for the recipient, so that the recipient can attain the greatest possible benefit from the transfusion. The administration of an optimal product minimizes the need for further transfusions.

Successful transfusion therapy presents numerous challenges to blood centres and hospitals. There is a constant demand for improvement and control within the transfusion process. For example, it is vital to maintain the optimum storage and shipment environment for products being transferred to hospitals to guarantee the greatest functionality of the products. A major problem is the limited shelf life of platelet concentrates (PCs). These concentrates are essential to maintain hemostasis in trauma patients, and for patients requiring therapeutic (for active bleeding) or prophylactic transfusions for patients with severe thrombocytopenia.[1,2] Currently, in Canada, PC may be stored for up to 5 days when accompanied by continuous and gentle shaking following their preparation, but can be stored for up to 7 days in many other countries. Storage needs to be maintained at room temperature to avoid platelet activation at colder temperatures.[3]

1.1 Platelets

Platelets, also termed thrombocytes, are the cellular mediators for hemostasis, and as such are crucial to minimizing blood loss in trauma patients. Normal platelet counts range from between

150 and 450 × 10^9 platelets/L, but severe trauma or significant soft tissue contusions can cause a depletion in the number of platelets present. Thrombocytopenia occurs when the platelet count decreases to less than 150×10^9 platelets/L. Slichter and Harker have determined that an average of 7 × 10^9/L of platelets per day is required to support vascular integrity, and that the threshold platelet count of <5 × 10^9/L can result in severe hemorrhage.[4,5] Transfusion guidelines commonly employ platelet counts to guide platelet transfusions.[6]

Clinical studies have concluded that ranges of 10 × 10^9 and 20 × 10^9 platelets/L are considered the threshold for platelet transfusions in adults and pediatric patients, respectively, for routine prophylactic transfusions required to reduce bleeding.[7–9] A count of 20 - 50 × 10^9 platelets/L is the minimum level of platelets that should be maintained during active bleeding, and this is also recommended in cases of non-critical site surgery (e.g. laparotomy) or invasive procedures.[1,7] Likewise, a count of 100 × 10^9 platelets/L is necessary in cases of multiple traumas (such as surgeries involving critical sites, including those of the brain and eye), based on the severity of the hemorrhage and when in combination with other risk factors.[10,11] Following transfusion of the first unit of platelets, an average increment of 15 × 10^9/L is recommended for critically ill patients with thrombocytopenia.[12]

1.1.1 Platelets in bleeding

Platelets are anucleate fragments that reside in the bone marrow. They are released into the blood circulation to maintain vascular integrity and to respond to lesions in blood vessels via the formation of platelet aggregates. [13,14] Platelets promote hemostasis through the adhesion and aggregation of activated platelets and fibrin at the site of an injury.[15,16]

At the site of a vessel injury, collagen is considered to be a strong thrombogenic substrate. The adhesion of platelets to the exposed collagen on endothelial cell surfaces is usually mediated by von Willebrand factor (vWF); platelets attached to this factor become immobilized at the sub-endothelial layer.[17] Collagen likewise binds to the immunoglobulin-like receptor, GPVI, and initiates platelet activation, which is essential for the adhesion and degranulation of activated platelets.[18]Platelet integrins bind in sequence, resulting in the firm binding of the platelets to the collagen found in the extracellular matrix (see Figure 1.1 for platelet shape and receptors).

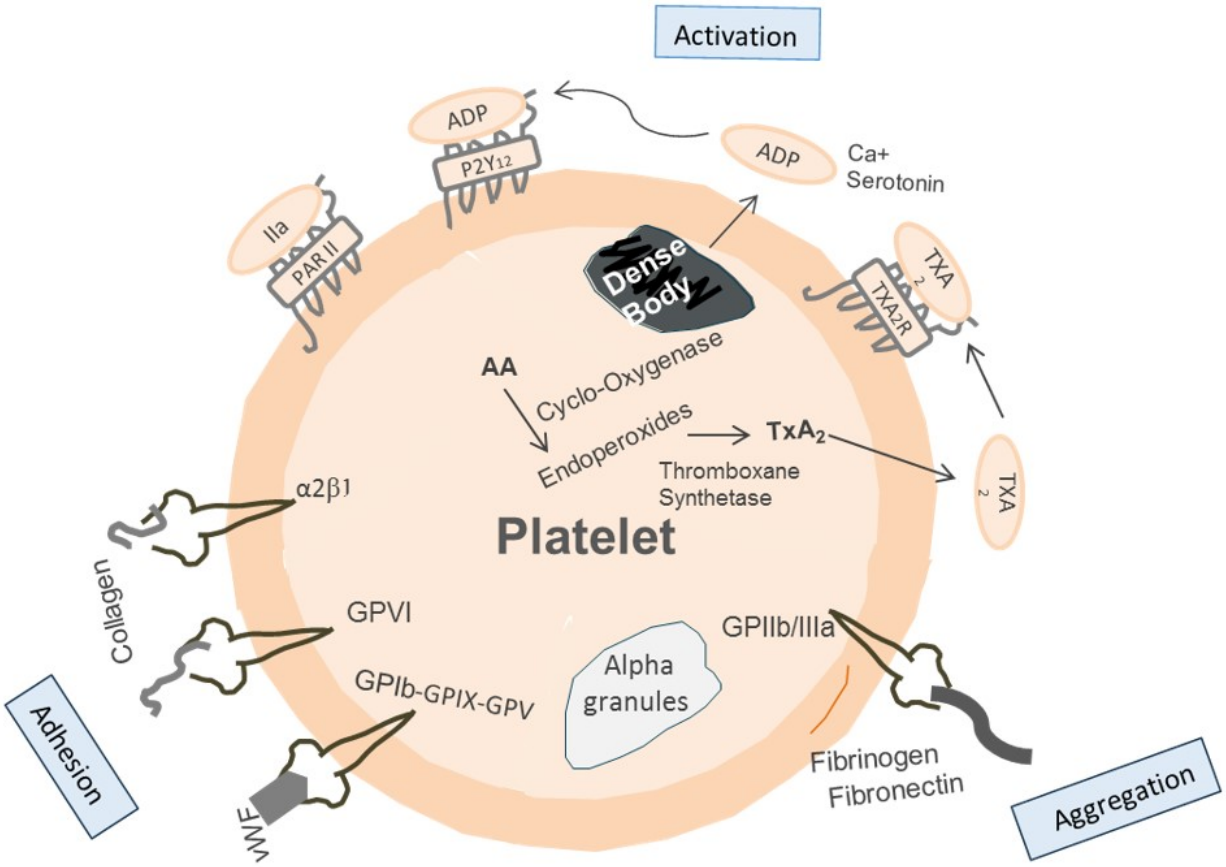

Figure 1-1: Platelet activation by different receptors.

Platelet receptors bind with agonists to facilitate platelet adhesion to the Von Willebrand factor and collagen exposed in the subendothelial cells at the site of damaged blood vessels. Thrombin, ADP, and TXA are agonists for platelet activation. The aggregation of platelets is by the attachment of fibrinogen to GP IIb/IIIa.

Platelets contain α-granules, dense granules, and lysosomes, which can be secreted at the site of platelet activation to promote the recruitment of resting platelets and initiate the coagulation cascade. Alpha granules contain chemokines, adhesion molecules, coagulation and fibrinolytic factors, and other proteins. Dense granules contain ionic calcium, magnesium, phosphate, pyrophosphate, adenosine diphosphate (ADP), adenosine triphosphate (ATP), and other nucleotides. Activated platelets release their granule contents into the plasma.

Granule contents bind to receptors on the platelets surface. ADP has two receptors, P2Y1 and P2Y12. The P2Y1 receptors may mediate platelet shape change and aggregation by enhancing the mobilization of intracellular calcium ions. P2Y12 may contribute toward increasing platelet activation by the suppression of cAMP production. When cAMP is supressed, GPIIb/IIIa can be activated. ADP enhances platelet response to other agonists, such as arachidonic acid (AA), and thrombin. AA is eventually converted to thromboxane A2 (TXA2) through cyclooxygenase. Thrombin is produced on the membranes of stimulated platelets to activate more platelets through protease-activated receptors 1 and 4 (PAR1 and PAR4). Thrombin, also, participates in the release of the secreted molecules, TXA2 and ADP, and leads to more platelet aggregation, joining of additional circulating platelets, and primary thrombus formation.[19]

The activated platelets provide a catalytic membrane surface on which coagulation factors can generate thrombin and stabilize the primary thrombus formation. The clotting cascade occurs through two separate interacting pathways, the intrinsic and extrinsic pathways. In the presence of calcium, tissue factor and factor FVIIa (in the extrinsic pathway) activate Factor X in the common pathway. The activated Factor XI (in the intrinsic pathway) and the cofactors, Factor

VIII (intrinsic) and Factor V (common) promote the amplification of thrombin generation. Factor Xa on the platelet surface, and the cofactor Factor Va generate a thrombin burst that catalyzes the conversion of fibrinogen, a soluble plasma protein, to fibrin, an insoluble plasma protein.[20] The fibrin proteins adhere, forming a clot. This is known as the hemostasis model because it identifies the coagulation process as a series of proteolytic reactions that occur on anionic phospholipid surfaces; mainly phosphatidylserine (PS)-rich surfaces are necessary for the assembly and optimal functioning of the coagulation cascade.

However, there is a critical cell-based model theory of coagulation being proposed in which the coagulation process alone may be not sufficient to provide hemostasis *in vivo*. This assumes that coagulation is controlled by cellular components, and particularly by the platelets. There are three overlapping stages in the cell-based model of coagulation, namely, initiation, amplification, and propagation. The initiation of coagulation begins with the tissue-factor-bearing cells that offer a platform for some of procoagulant stimulus such as Factors IXa and Xa, and thrombin to initiate coagulation.[21]

The amplification phase commences following the adherence of platelets to the exposed tissue factors and the von Willebrand Factor at the site of vascular injury. The coagulation process will move from the injury site to the surface of adhered and activated platelets to accumulate more activated coagulation factors on their surfaces. This will lead to the surface exposure of phosphatidylserine as well as the release of procoagulant molecules from platelet granules. The increase in the activity of coagulation factors on the surfaces of the platelets results in a burst in thrombin generation; this is known as the propagation phase. Thrombin then activates the

platelets and converts fibrinogen to fibrin (via PARs), generating fibrin-platelet aggregates containing trapped RBCs known as blood clots.[22]

Platelet surface receptors are linked to intracellular effectors. There is a network of signaling molecules and regulators including the heterotrimeric G-protein, which is involved in the activation of Phospholipase C and the phosphatidylinositol-3-kinase (PI 3-kinase) dependent pathways; and in the suppression of cyclic adenosine monophosphate (cAMP). cAMP suppression normally inhibits platelet activation through the prevention of Ca+2 mobilization, which leads to increased platelet activation. This results in an inside-out signaling process leading to an active conformation in GPIIb/IIIa, a major glycoprotein on platelet membranes. The activation of GPIIb/IIIa allows vWF and fibrinogen to create cross-links between two GPIIb/IIIa receptors located on adjacent platelets, causing platelet aggregation. Activated platelets in conjunction with the coagulation cascade form firm hemostatic plugs, thus aid in preventing hemorrhage resulting from endothelial damage.[23]

To achieve hemostasis, a balance between coagulation and fibrinolysis is crucial to forming and later degrading fibrin clots. Damaged or stimulated endothelial cells release plasminogen activator inhibitor-1 (PAI-1), which down-regulates fibrinolysis in the circulation; the pro-fibrinolytic factors that initiate clot degradation and cleave platelet aggregates. PAI-1 inhibits tissue plasminogen activator (t-PA), which triggers the conversion of plasminogen to plasmin, which then dissociates the fibrin-mesh.[24] Following this, endothelial cells play a crucial role in inactivating thrombin, and in the fate of thrombin and fibrin, which helps in restoring hemostasis. Clot dissolution occurs when plasmin catalyzes the degradation of fibrin.[25]

1.1.2 Newly identified roles of platelets

Platelet function is not only involved in hemostasis. Platelets have many preformed inflammatory molecules and immune mediators in their α-granules, dense granules, and lysosomal granules that play primary or secondary roles in the immune response. α-Granule constituents have limited thrombotic functions and instead perform as chemokines and cytokines that recruit and activate white blood cells and/or prompt endothelial cell inflammation. Although dense granule constituents facilitate platelet activation, they also have immune cell–modifying effects. Platelets contain messenger RNAs and pre–messenger RNAs, some of which are used to synthesize proteins such as IL-1β, which is a potent inducer of the acute phase response (APR).[26] Activated platelets release platelet microvesicles (PMP), and an increased number of PMPs in the blood circulation correlate with the development of atherosclerosis in patients with diabetes. Through PMPs, platelet RNA and micro RNAs can be functionally transferred to other cell types, and thus mediate vascular inflammatory processes in a transcellular manner.[27]

Platelets have been associated with many pathogen-initiated immune complications, creating an enormous burden on public health worldwide. They have a role in the infectious disease pathogenesis of malaria, sepsis, HIV, and influenza.[28] Bacteria interact with platelets and cause platelet activation or aggregation, releasing their pro-inflammatory mediators, such as α-granules [containing Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES), platelet factor 4 (PF4; also known as CXCL4), sCD40L, soluble P-selectin, platelet-derived growth factor (PDGF)-AB) and dense granules (containing ADP and ATP)].[29] Some varieties of bacteria can interact directly with GPIIb/IIIa receptors, resulting in platelet activation through outside-in

signaling. The rate of increase in these pro-inflammatory markers is associated with the severity of the sepsis.[30]

Platelets can also interact with the complement system by accelerating vascular inflammation and atherosclerosis, triggered by the inflammatory mediators C3a and C5a; on the other side, platelets and PMP can support microbial clearance by activated complements. Platelets also have a role in metastatic cancer. If they interact with tumor cells, this results in the latter's activation, the expression of p-selectin and the formation platelet-tumor microthrombi that might protect the tumor cells from the innate immune system.[31] There is an increase in the clinical use of antiplatelet medications to treat systemic inflammation associated with infections, which arises from investigations of the role of platelets in inflammation and the immune response. [32]Although platelets already have the ability to induce coagulation, a further role for platelets may be indicated.

1.1.3 The preparation of platelet concentrates

It is important to take into consideration the means by which the PC is produced. There are three main production protocols: apheresis PC, the platelet-rich plasma method (PRP-method), and buffy coat PC. In apheresis PC, the entire process of platelet separation runs automatically following the insertion of a needle into the donor's arm. Apheresis technology performs the separation of the PC, while simultaneously returning the remaining components of the whole blood to the donor arm, in a procedure involving a high level of control, and in the absence of manual input.[33]

All other PC products are produced from whole blood units following donation. The difference between buffy coat and PRP is the amount of gravitational force (g-force) applied in the first

centrifugation. In the buffy coat protocol, the whole blood unit is subjected to a hard spin (3500 g-force using the accumulated centrifugal effect) in the first centrifugation resulting in the formation of a buffy coat layer between the red cells and the plasma, which contains white cells and platelets. However, in the PRP-method, the first centrifugation results in the suspension of platelets and plasma from the pelleted red cells. In the subsequent step of PC production, the buffy coat protocol applies a soft spin (1250 g-force for 6 min) to suspend the platelets and pellet from the remaining blood components. Conversely, in the PRP-method, the unit undergoes a hard spin to pellet the platelets and suspension and then remove the plasma, figure 1-2.[34]

One unit of a male donor's plasma is then added to each buffy coat from a pool of four blood group matched donors, this is to lower the risk of transfusion-related acute lung injury (TRALI). In some blood centers, the platelets will be suspended in an additive solution.[35]

The platelets that are prepared for therapeutic or prophylactic transfusions are stored between 20 to 24ºC, for up to five days in North America, and up to seven days in Europe and the UK.[36–38] Platelets require continuous gentle agitation to prevent their aggregation and facilitate gas exchange. Contamination from bacteria introduced from the skin puncture during the phlebotomy is currently the most important infectious risk associated with platelet product transfusion.[39]

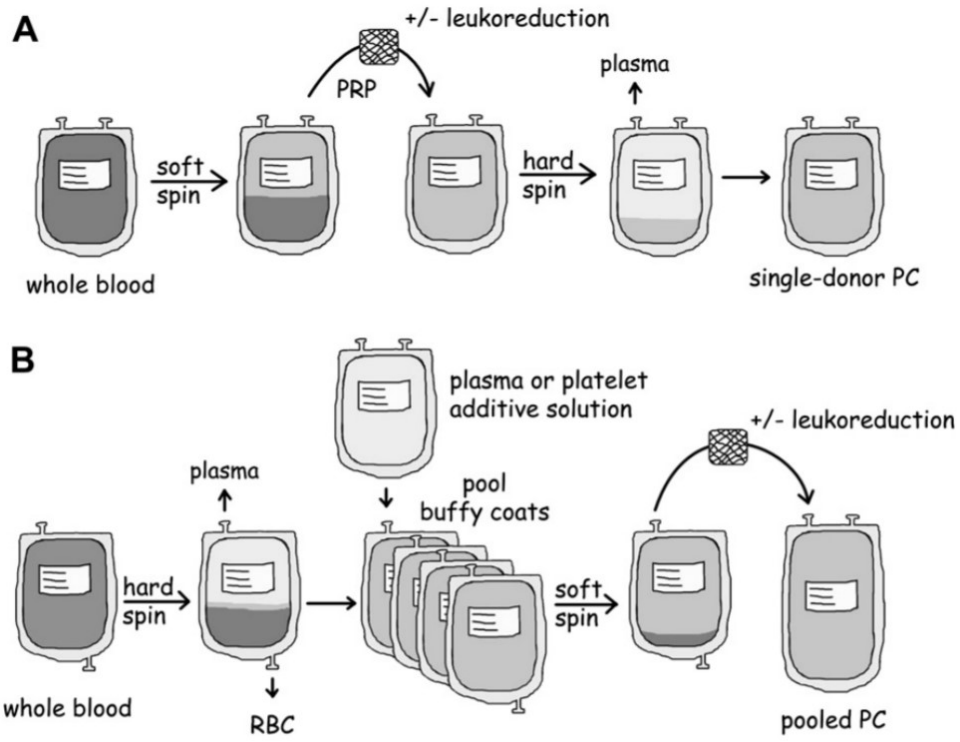

Figure 1-2: Platelet rich plasma and buffy coat (top and bottom) platelet production schemes.

The main difference between platelet rich plasma (PRP) and buffy coat platelet concentrate (BCPC) production methods lies in the amount of force applied in the first centrifugation step. The PRP method uses soft spin (1250 g-force for 6 min) to pellet the red cells and leave the platelets suspended in the plasma. In the buffy coat method, a hard spin (3500 g-force using the accumulated centrifugal effect) is applied causing formation of a buffy coat layer (platelets and WBC) between the red cell and plasma layers. The next step in the PRP method is a hard spin of the PRP to remove most of the plasma from the platelets. In the buffy coat method, a soft spin removes the WBC and the residual RBC, and keeps the platelets suspended in the plasma. 4 buffy coat units are pooled together with one plasma or platelet additive solution[34]. This figure was reprinted with permission. The platelet storage lesion. *Clin Lab Med, 2010;*30(2):475-87.

Buffy coat PC (BCPC) products are always under continuous quality control assessments and process improvement; their production processes have undergone further changes during the course of transfusion history, which include, for example, the lowering of their pH [40], and the introduction of a resting time period for platelet concentrates during production to prevent platelet aggregation.[41] One of the changes was the application of leukoreduction filters to remove

white blood cells in order to reduce the risk of alloimmunization and graft-versus-host disease. Devine *et al* have demonstrated that platelet activation during production can cause irreversible aggregate formation in BCPC units.[42] The key issue considered during the improvement in the production of blood products is patient safety. Will the patient acquire optimum benefit from the products? What is the best way to measure the quality of the blood products? In addition to the functionality of these units in patients following transfusions, referred to as transfusion outcome, it is of importance to determine if clinical efficacy can be predicted by *in vitro* quality tests that are performed prior to transfusion.

1.1.4 PC transfusion and patient outcome: post-transfusion and *in vitro* tests

Approximately 4 million prophylactic or theraputic platelet transfusions occur annually in Canada and the USA, and 2.9 million in Europe, as a treatment of bleeding complications.[43–45] Trauma in patients results in activation of the coagulation system due to exposure and/or upregulation of tissue factor and excess generation of thrombin.[46] This results in an increase in consumption of the coagulation factors and platelets. These patients will often need blood transfusion. Tauma patients with massive bleeding should be transfused with a balanced ratio of blood components (red blood cell (RBC), plasma, and platelet) to replace the blood loss and prevent further bleeding. Studies indicate that the high ratio of platelets in the blood components is associated with better survival. Platelet transfusions, also reduce mortality from hemorrhage in patients with acute leukemia.[45] Prophylactic platelet transfusion are now part of the treatment of a number of diseases, including cancer, hematological malignancies, and marrow failure, as well as for hematopoietic stem cell transplantations.[45]

Ineffective clinical outcomes remain a problem. Post-transfusion testing of platelets following their administration is desirable to assess the level of response in trauma patients. One way to determine the quality of transfused PC is to measure the response of recipients to the transfused platelets, such as quantifying bleeding episodes and platelet count.[4]

1.1.4.1 Evaluating the transfused buffy coat PC components

At present, no *in vitro* tests have been designed to assess the quality of transfusion products prior to transfusion. The process has rather depended on the practitioner's observation after the transfusion to determine the impact that the products had on the patients. It would be beneficial to use *in vitro* platelet measurements to predict the clinical impact of platelet transfusions. Is the optimal transfusion outcome linked to the patients' platelet demand, or to the functionality, storage condition or age of the platelets? It is noted that platelet age is reflected in the number of days after PC production, but not in the median age of the platelets comprising the transfusion units.

Several studies on transfused BCPC and clinical outcome have been performed, and these will be discussed below. Since 1969, it was assumed that the *in vitro* characteristics of stored PC could be correlated with the *in vivo* transfusion outcome;[47] this was mainly determined through an assessment of the platelet life-span in the circulation of the recipients using ^{51}Cr labeled platelets following storage of the PCs at different temperatures. One of the methods to assess a transfused platelet product is to calculate the corrected count increment (CCI) following transfusion. The CCI is estimated by multiplying the body surface area (BSA) of the recipient with the platelet count increment divided by the platelet count of the transfused PC unit.[5]

Some studies have demonstrated that fresh PCs have at least a 67% recovery and 50% survival rate if they are radiolabeled and transfused for the same subject, and that this process can benefit a recipient more than the use of old platelets.[48] Conversely, subsequent to this publication, Au Buchon *et. al.* failed to observe any significant difference in recovery and survival of fresh PC collection, in a single day, or 5 or even 7 days, regardless of whether the PC was collected by apheresis or pooled from buffy coats.[49,50] However, all of these transfusions were performed as autologous transfusions in healthy donors with normal platelet counts. It may not be realistic to consider that the same outcome could occur in recipients receiving prophylactic or therapeutic treatment.

To summarize, many *in vitro* studies conclude that fresh, autologous PCs have superior *in vitro* characteristics to those stored for up to 5 days;[50–53] such as pH, activation level, and metabolic activity, and that this can be correlated with a better outcome when the platelets are transfused. However, other studies still demonstrate that stored PC (buffy coat or apheresis) can function better than fresh PC, with at least 66% of the recovery and 58% of the survival rate of fresh PC.[54–56] Therefore, the aging of the unit might not be an issue, particularly when it has been verified that bacterial contamination has not taken place.

While we cannot depend on the age of the PC unit to recognize the quality of the platelets, it appears that agreement is lacking on whether CCI is the optimum tool to assess the quality of the PC. Even if one depends on CCI, approximately 30% of transfusions are clinically ineffective as measured by 1hour CCI (CCI < 7.5).[57,58] Sigle *et al.* argue against the request of special or fresh platelets for specific patient group storage time.[57] Supporting this, MacLennan *et al*. have demonstrated in their clinical study that neither CCI (≥4.5) or a bleeding score of World Health

Organization (WHO) (≥2) could differentiate between PCs stored from 2 to 5 days from those stored for 6 to 7 days which was then transfused to stable hematological patients.[59] Failure to meet the desired clinical outcome may be primarily attributed to ineffective platelet concentrates rather than age of the platelets.

The previous studies were clinical and their assessments conducted following transfusions; as such, they are only able to identify the need for additional transfusions in ailing patients who had not gained any benefit from transfused PCs, rather than identifying PC effectiveness prior to transfusions. Therefore, the effectiveness of BCPC criteria prior to transfusions should be addressed to ensure that each unit of the PCBC functions well, and to identify the specific criteria that would help blood centers release optimal PC for specific patients.

There is a clear need to develop a credible system for determining the quality of platelets prior to transfusions. Ideally, for the best interest of the patients, stored platelets should be tested prior to transfusions. Instead, platelets are transfused without clear pre-transfusion functional testing, and the patient's outcome is the only indicator of platelet effectiveness. This practice is potentially of significant risk to patients.[60]

1.1.4.2 *In vitro* tests for buffy coat PC

Numerous tests have been performed on BCPCs prior to transfusion, and these reveal a number of biochemical changes that occur during storage that are collectively termed the platelet storage lesion.[34,61] However, these tests are not performed for BCPCs to assess their functionality prior to their release. In general, only visual assessments of PC units are used to evaluate the suitability of the products occurs before they are released to the hospitals or from the blood bank

to the patient.[62] If a product contains visible macroaggregates, fails to exhibit swirling or its color has altered, or if there is any evidence of bacterial growth in the unit, the unit will be discarded.

Slichter *et al.* focused their research on platelet quality tests which they correlated with *in vivo* recovery and survival in cases involving autologous transfusion.[56,63] In general, many *in vitro* tests have been performed that include platelet morphology scores, expression of activation or apoptotic markers on platelet surfaces, the counting of platelet microparticles using flow cytometry methods, response to hypotonic shock, the extent of shape change, the detection of platelet mitochondrial activity.

The dependence on the morphology score has not been entirely successful.[62,64] The score is partially affected by the pH which can transform platelets' shape from discoid to spherical when the pH is below 6.0; this alteration can revert again to discoid should the pH be increased back to physiologic pH. Platelets in these studies were shown to regain their viability post-transfusion. However, this morphology scoring does not assess for the presence of microparticles in the units. One of the studies used flow cytometry to measure platelet activation. P-selectin (CD62P) is a sequestered granular membrane protein expressed on the surface of activated platelets. P-selectin cell surface expression gradually increases during storage time.[61,65,66] Therefore, although these studies demonstrated a simultaneous increase of P-selectin during storage time, P-selectin cannot then be used as a tool to predict patient outcomes. It can nevertheless be used to reflect activation levels post-production for optimization and development purposes.[67]

As a result of the mechanical stress "differential centrifugation" places on platelets and also the effects of their storage, there is a release in plasma-membrane-derived microparticles (PMPs) and platelet-derived extracellular vesicles (EVs). These are generically termed platelet

microvesicles (PMV).[68] The quantification of these microvesicles can be performed via flow cytometry or nanoparticle tracking analyses if they are smaller than 300 nm.[69] These microvesicles have some of the same characteristics as platelets when observed in different processes including hemostasis, the maintenance of vascular health, thrombosis and immunity, and they range from 100 to 1000 nm.[70] Although PMVs have from 50 to 100 times higher procoagulant activity than platelets[71], little attention has been paid to their count in PC, plasma or RBC units prior to transfusions. Two groups of studies refer to these elements, one suggesting that the transfusion of high numbers of PMVs per unit could result in multiple organ failure, or the decreased short- or long-term survival of patients with compromised physiologies;[72,73] while the other suggests that an increase in PMVs as a result of storage or from the transfusion of many units could enhance hemostasis and stop bleeding in trauma patients, due to the tremendous procoagulant activity present on the surface of PMVs[74 75 76]. There is a need for tests to evaluate the effect of PMVs in PCBC on hemostasis. Recently, ThromboLUX is a device that has been developed as a technique to measure the dynamic light scattering of stored PCs replacing the manual swirling method.[60] Its score is combined with the quality of platelets exposed to different temperatures and the presence of PMV, but this requires further optimization since it does not correlate with other *in vitro* tests.[77]

We have also assessed one of the tests for platelet aggregation quality by which aggregation is accomplished via the use of several agonists;[78] however this study found that the rate of these responses decreased during storage. Tests that show the extent of shape change and response to hypotonic shock employ quantitative measurements with a high correlation to the platelet recovery;[79] the tests, however, have limited application and are not routinely used.

When assessing metabolic activity, both anaerobic glycolysis, and oxidative phosphorylation in mitochondria, must continue to take place within the bag during the storage period for ATP production to continue in order for the platelets to retain their function. Activated platelets in PC used for transfusion, release microparticles known as extracellular mitochondria that could lead to inflammatory responses,[80] and could contribute to hemostasis as in the PMVs.[81] Although we can ascertain the degree of metabolic activity occurring during storage time, we still lack a technique that reflects the hemostatic behavior of platelets at specific levels of O_2, CO_2, ATP, glucose, and lactose. In other words, it is essential to establish a technique that can predict transfusion outcomes based on tests that can utilize all of these measurements.

While a number of *in vitro* tests (Table 1-1) have been utilized to study platelet transfusion products, no testing algorithm exists with this selection of tests that accurately predicts platelet transfusion efficacy.[5] There is a poor correlation between *in vivo* transfusion outcome and *in vitro* testing. This could be since current *in vitro* tests do test the hemostatic functionality of platelets but rather determines platelet characteristic as platelet count, pH, and response to agonists.[60]

Thus, there is great need for assays that can be applied to platelet concentrates so as to test the efficiency of clot formation and fibrinolysis in a manner that more closely models these processes in the bloodstream. Newer tests that have been developed to monitor coagulation and platelet function in the near-patient setting (e.g. the operating room) may provide one possible option to fill this gap for the assessment of stored platelets.

Table 1-1*: In vitro* tests for buffy coat PC during storage time.

Visual assessment: macroaggregates or alterations in colour[62]
Platelet morphology scores, mainly affected by the level of the pH[62,64]
Increased expression of activation or apoptotic markers on platelet surfaces during storage[61,65,66]
An increase in platelet microvesicles (PMV) with procoagulant activity during storage[68,70]
A decreased response to hypotonic shock, along with shape change, during storage[78]
Increased platelet mitochondrial activity during storage[82,83]
Variable measures of platelet microparticles using ThromboLUX[60]
Apoptotic changes related to the BCPC storage[84]

In hospitals, clinicians need an indicator that can reveal the hemostatic state of patients with coagulation disorders, since even should clotting factors level not be deficient, the clinician would not be able to determine the clotting kinetics of the patients' blood until the actual time that the platelets' activity is required (when the patient is bleeding or susceptible to bleed). Conventional testing methods (platelet count, thrombin time, activated partial thromboplastin time (aPTT), prothrombin time (PT) can help but do not reflect the full coagulation picture. Particularly in critically ill patients, the conventional tests will not reflect the true hemostatic state of patients susceptible to bleeding. Clinicians require a test that is predictive in terms of risk of bleeding and that integrates all of the steps in formation of a stable clot, because coagulation is not only what the patients need, but as well, the balance between coagulation and fibrinolysis.

Thromboelastography was created in 1940 by Hartert for research purposes to assess global hemostatic function, and it was first applied clinically during the treatment of a patient undergoing a liver transplant surgery.[85] While the technology is more than 50 years old, it has been updated with the application of the modern TEG analyzer 5000 series. In 1996, it acquired

the registered trademark of TEG® from the Hemoscope Corporation (Niles, IL) before the company was incorporated under Haemonetics.[86]

1.2 Thromboelastography and rotational Thromboelastometry

Thromboelastography, or thromboelastogram (TEG; Haemonetics, Niles, IL) measures the physical properties of clots in whole blood samples placed under low shear conditions. Measurements are performed via a pin suspended in a cup containing the blood sample from a torsion wire connected to a mechanical–electrical transducer in the TEG (Figure 1-3). TEG measures the clot strength and fibrinolysis which take place during the viscoelastic changes of the entire clotting process.

The rotation of the cup at an arc of 4.75° around the fixed plastic pin represents sluggish blood flow through a vein and active blood coagulation. The changes in the rotation of the pin as the blood sample clots are converted into electrical signals creating a graphical output representing a TEG hemostasis profile (Figure 1-3). The Y-axis of the profile demonstrates the amplitude of the pin's motion in millimeters, and the X-axis demonstrates the time transpired (Figure 1-4).

The TEG system has been in use for an extended period of time; recently, however, the Rotational Thromboelastometry (ROTEM; TEM Innovations, Munich, Germany) system has been developed from TEG. It has numerous clinical applications besides the evaluation of hypercoaguability.[87] It serves a similar function to the TEG but with some slight modifications. Specifically, the TEG oscillates the cup, whereas the ROTEM oscillates the pin in the centre of the fixed plastic cup at an arc of 4.75°. The specific parameters measured and the nomenclature employed for both are listed, in addition to their reference ranges, in Table 1-2.[88,89]

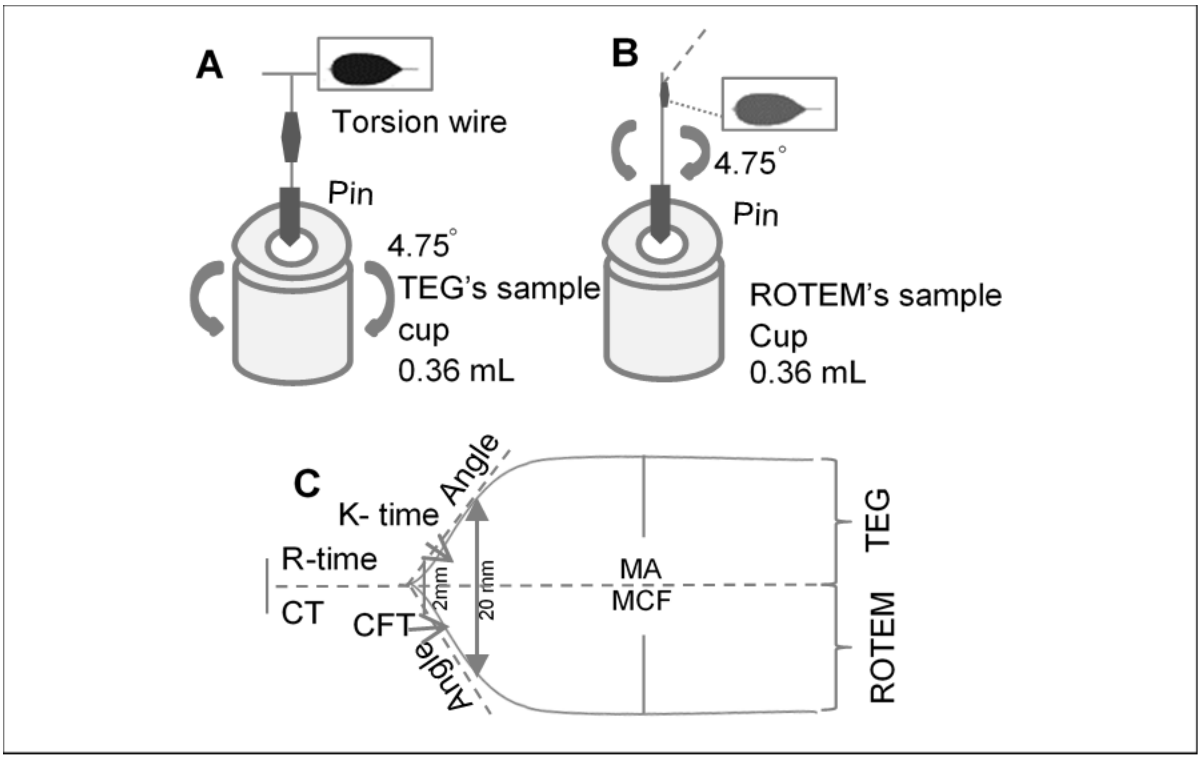

Figure 1-3: Principle of TEG and ROTEM.
(A) In TEG, a pin connected to a torsion wire is immersed in a cup warmed to 37°C. The TEG's cup oscillates whereas the torsion wire is fixed. (B) In the ROTEM, the cup is fixed, whereas the pin oscillates. (C) A typical tracing of TEG (top tracing) and ROTEM (bottom tracing) signatures. In TEG, the change in torque is detected electromechanically while ROTEM uses an optical detector. The latency time from the onset of the test to the point of initial fibrin formation (2 mm) is represented by the reaction (R)-time (TEG) or clotting time (CT; ROTEM). The time necessary to achieve 20 mm, a fixed level of clotting strength, is called the kinetic (K)-time (TEG) or clot forming time (CFT; ROTEM). The angle (a) represents the rate of polymerization of the clot and correlates with fibrin formation and its interaction with platelets. Clot firmness is represented by maximum amplitude (MA; TEG) and maximum clot formation (MCF; ROTEM).[87]

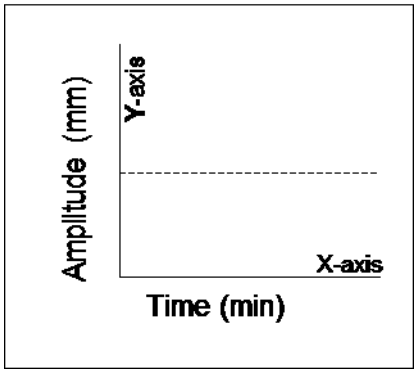

Figure 1-4 The Y- and X-axis of thromboelastographic signature.
The Y-axis of the profile demonstrates the amplitude of the pin's motion in millimeters, and the X-axis demonstrates the time line in minutes.

Table 1-2: The nomenclature systems of TEG and ROTEM.

Measurement	Nomenclature			
	TEG	Normal ranges	ROTEM	Normal ranges
Coagulation time	R (seconds)	180-480	CT (seconds)	100-240
Clot formation time	K (seconds)	60-180	CFT (seconds)	35-110
Angle: rate of clot polymerization	alpha	55-78	alpha	71-82
Maximum clot firmness	MA (mm)	51-69	MCF (mm)	53-72
Maximum lysis	ML	<15	ML	<15

Note:
The TEG and ROTEM both measure the similar parameters, but differ in their nomenclature systems. The normal ranges for citrated WB are established according to the manufacturers for the ROTEM and TEG. The TEG's value is based on that established during the manufacture of citrated WB from unspecified surgical patients, which is re-calcified, after which kaolin is utilized to initiate the intrinsic coagulation pathway.[88] The ROTEM's perimeters are established by multi centers using INTEM (ellagic acid).[89]

1.2.1 Clinical hemostatic signature

1.2.1.1 Coagulation time (thrombin formation)

Thrombin formation time, expressed by reaction time (R-time) in the TEG or clotting time (CT) in the ROTEM, is the time required for a specific concentration of thrombin to be generated in the oscillated TEG or stable ROTEM cup, Figure 1-5. It is crucial that thrombin formation takes place for the TEG pin to initiate oscillating the cup. Clot formation then occurs by the cleavage of fibrinogen, formed between the surface of the pin and the cup, into fibrin. Although coagulation time is in practice the time from the commencement of the test when the pin is fixed until the time the pin oscillates in the TEG, it is more precisely defined as the time until the blood clot reaches 2 mm either in the TEG or the ROTEM. Thus, it is a reflection of the coagulation factors required to create the collection of enzymatic reactions known as the intrinsic, extrinsic, and common coagulation cascade. The normal range of the time needed for

citrated whole blood to clot is 6 to 8 minutes. A more elongated coagulation time is an indication of decreased, inefficient, or inhibited procoagulant clotting factors.

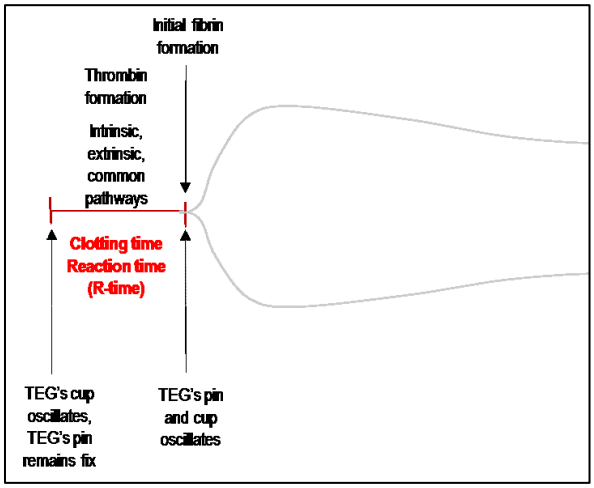

Figure 1-5: Thrombin time and R-time.

1.2.1.2 Clot kinetics (clot formation time and the rate of clot polymerization)

The rate of the interaction taking place between thrombin, fibrin, and plateletss is recognized by clot kinetics performed in the TEG and ROTEM. Clot kinetics is measured by kinetic time or the rate of fibrin-platelet interaction (Figure 1-6). Kinetic time (K-time) is the time taken to achieve a fixed level of clot strength that reaches the 20 mm amplitude (K ≈ 9±3 min in citrated WB); this is the rate, also called the angle or alpha (measured in degrees) of the speed of clot growth which correlates to fibrin build up (alpha ≈ 50-60° in citrated WB).[88]

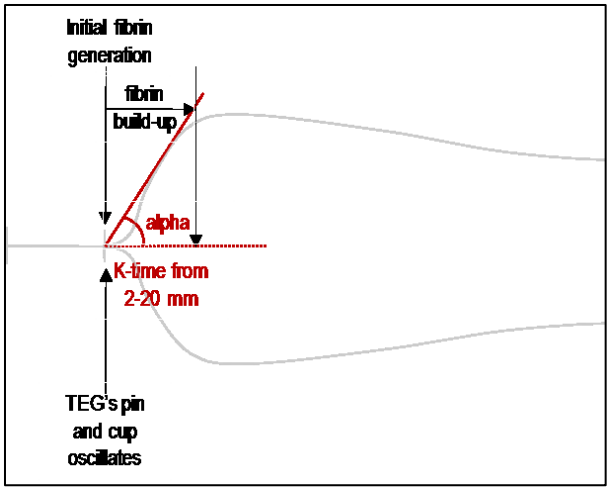

Figure 1-6: Kinetic of clot polymerization.

1.2.1.3 Maximum clot strength

Maximum amplitude (MA) is the maximum strength or stiffness of the developed clot (MA ≈ 50 - 60 mm in citrated WB in the absence of an agonist). MA has diverse categorizations according to its value. It is hypercoagulable in cases of increased platelet counts or activity, and patients can be treated with such antiplatelet agents as Aspirin and Clopidogrel. Alternately, it is hypocoagulable in cases of low fibrin-platelet interactions that results from platelets of minimal or insufficient functioning. Moreover, if the hypocoagulability stated resulted from a lack of interaction between the platelets and fibrin; the patients are required to receive platelet transfusions (Figure 1-7). The Coagulation Index (CI) is a global hemostasis index reflecting the coagulability level of whole blood. This is a linear amalgamation of kinetic parameters and clot strength (R, K, angle, and MA) involved in development of clot. For instance, CI > +3.0 is called hypercoagulable, CI < -3.0 is called hypocoagulable.

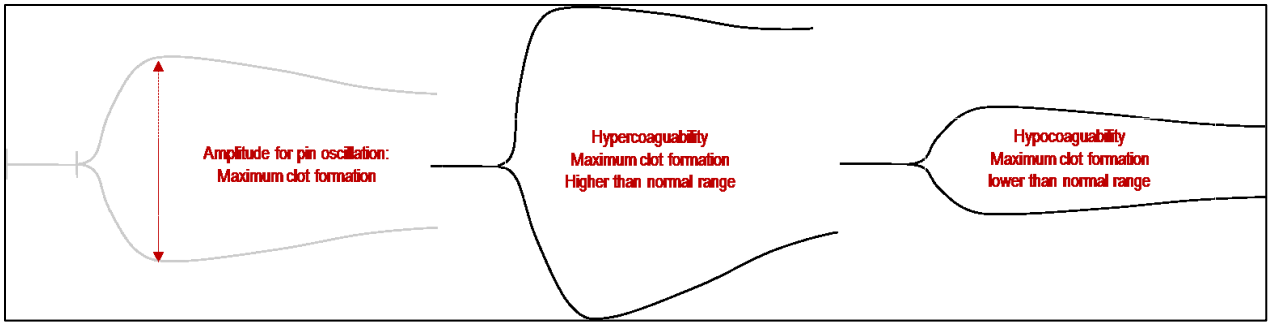

Figure 1-7: Clot formation amplitude: Maximum clot firmness

1.2.1.4 Fibrinolysis

Following the development of maximum clot firmness, coagulation begins to decrease with an increase in fibrinolysis. Maximum lysis is the maximum percent of lysis that the sample can achieve. LYS30 is the percent of decrease in amplitude of pin oscillation 30 minutes following the actualization of MA. Estimated Percent Lysis (EPL) is the estimated rate of change in amplitude following the attainment of MA, Figure 1-8.

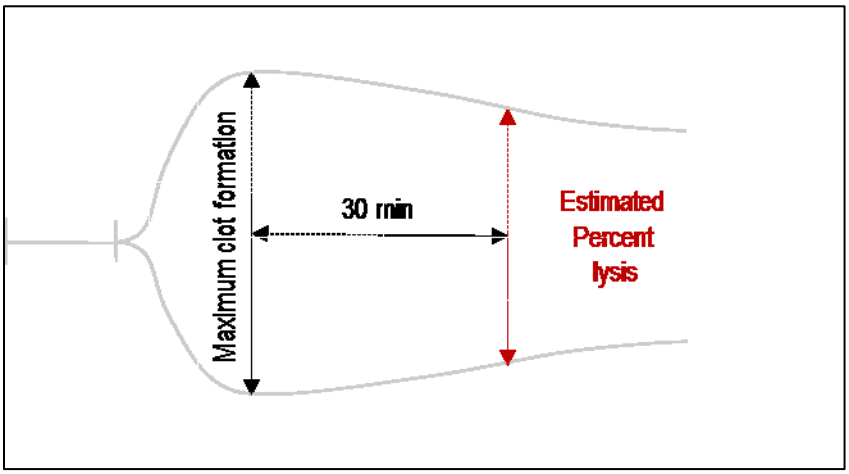

Figure 1-8: Maximum lysis following clot formation.

1.2.2 The relationship between TEG and ROTEM parameters and hemostasis

From the above description, it is observed that the TEG and ROTEM signatures summarize all hemostasis phases, initial fibrin formation during clotting time, and the development of fibrin and fibrin-platelet clots referred to as clot kinetics, maximum clot formation at maximum amplitude, and, finally, fibrinolysis percentage. This may support clinical decisions and provide clinicians with greater confidence to diagnose and treat coagulation defects, since every patient generates his/her own unique hemostasis signature. For instance, patients being treated with anticoagulants or suffering from hemophilia have deficiencies in coagulation factors and show profiles where R- and K-time are prolonged and alpha and MA are decreased; patients with platelet dysfunction have normal R-time, prolonged K-time and decreased MA; patients with fibrinolysis have normal R-time functions, while their MA levels continuously decrease; and, in hypercoagulability cases, R-time and K-time are decreased and alpha, with MA being increased.[90]

1.2.3 TEG and ROTEM integration in transfusion algorithms: the prediction of hemorrhage

Predicting patient susceptibility to bleeding is crucial for the patient and also provides information that can be used by blood centres to ensure adequate blood component availability.[91] It is advantageous for the treating physician to be aware of their surgery patient's hemostasis capacity. This awareness could result in either a rescheduling of the operation or the preparation of essential, patient-specific blood products. The TEG has been shown to be a significantly enhanced predictor (87% accuracy) for the risk of postoperative hemorrhage and the necessity for reoperation compared to the activated clotting time (30%) or the coagulation profile (51%).[92]

Several clinical investigations suggest that ROTEM-based strategies should be applied to test patients' suitability to bleeding prior to surgery, which indicates its significant usefulness in reflecting patient hemostatic levels.

The interest in clinicially applying the TEG/ROTEM as a Point of Care (POC) test has also increased with trauma resuscitation cases, particularly for managing the acute coagulopathy of trauma patients and in supplementing decision making regarding transfusions[93]. For instance, Williams *et al.*, Bolliger *et al*., and others in their investigations into practices in numerous centres have been dependent on ROTEM-guided coagulation management in preoperative or intraoperative transfusions during complex cardiovascular and liver transplantation surgeries.[94–97] They have discovered that utilizing these technologies can limit thrombosis and ischemic events; such discoveries resulted from studies on uncontrolled selection of the correct therapeutic transfusion products to support patient hemostasis, coagulation factor concentrates (fibrinogen concentrates and/or prothrombin complex concentrates), or blood products (RBC, plasma, or PCs). Furthermore, Girdauskas *et al*. and Perry *et al*. likewise discovered that clot strength in TEG- and ROTEM-based transfusion algorithms could aid in minimizing the incidence of massive transfusion packages if used to control hemostasis in patients undergoing aortic surgeries when under circulatory arrest.[90,96]

While TEG and ROTEM are not different in principle, Sankarankutty *et al.* demonstrated that their results are closely related, although they agree that clinical standardization is required.[98,99] Clot firmness (measured by MA in TEG or MCF in ROTEM) may be the parameter sufficiently superior to reflect the impact of platelet counts and fibrinogen concentrations on the techniques.

This implies that high platelet counts and fibrinogen concentrations can support greater clot strength and stiffness.[92,100]

From all of the above, and based on the ability of the TEG and ROTEM to detect platelet dysfunction and hyporcoagulability and prevent inappropriate transfusions of hemostatic blood products to non-coagulopathic patients,[99] these tools may play a major role in improving the quality of blood transfusions.[101] This suggest a role for them in product evaluations at the blood centre.

1.3 Pathogen reduction treatment for blood product

Emerging pathogens and viruses under particular investigation at present include Zika and dengue viruses; other pathogens, with which we are not yet familiar, almost certainly exist. Because PC are stored at room temperature, there is potential for contamination from bacteria infused from the donor skin. These factors place the transfusion scenario at great risk for the incidence of sepsis, alloimmunization, transfusion-associated graft-versus-host disease (ta-GVHD), or other clinical condition.[102]

Blood centers investigate for a number of known pathogens. Blood testing cannot exclude all possibilities.[91] Several pathogen inactivation (PI) technologies have been developed for application to plasma and platelet concentrates (PCs)[103]. One of these systems, the Mirasol treatment, (Mirasol Pathogen Reduction Treatment (PRT) System Terumo BCT, Lakewood, Colorado, USA) utilizes Riboflavin (Vitamin B2) to modify nucleic acids upon their exposure to UV light; it prevents the replication of pathogens and leukocytes by modifying guanine residues through direct electron transfer oxidative damage. Only Mirasol technology has been applied to whole blood (WB) to date to mitigate pathogens in cases of causalities where insufficient time or

the absence of comprehensive tests have been present to screen the blood samples for known pathogen transfusion risks such as those from the Ebola virus[104].

In some cases, to save time, whole blood can be transfused into massively hemorrhaging patients. However, this practice runs the risk of introducing infective agents and pathogens into the patient, jeopardizing blood safety. Presently, using either of the two licensed pathogen reduction treatment technologies (Mirasol or Intercept), platelet and plasma units are pathogen reduced separately; however, it is desirable for PI treatments to be performed one step prior to the production of blood components. The riboflavin and ultraviolet (UV) light process for PIs was first applied to treat WB units,[105] and was reported to be an alternative for gamma irradiation to prevent transfusion-associated graft-versus-host disease[106]. For instance, a study on the clinical and biological efficacy of Mirasol-treated fresh WB revealed that Mirasol decreased *P. falciparum* viability *in vitro* and retained acceptable blood quality during 21 days of cold storage.[107,108] Although our research is concerned with testing the hemostatic state of PCs, nevertheless, as whole blood can be transfused into massively hemorrhaging patients, our interest can feasibly be extended to test WBs following treatment. Furthermore, this treatment will affect the activity of coagulation factors for PCs, plasma, or even whole blood; this is proposed in the following chapters with a novel solution to support the coagulation system subsequent to treatments.

1.4 Objectives

The intent of this research is to determine whether TEG and ROTEM technologies, increasingly employed in operating rooms to assess the hemostatic states of patients susceptible to bleeding, can be applied to the quality assessment of platelet concentrates. Unlike other *in vitro* platelet tests, the TEG and ROTEM offer the opportunity to assess platelet function as part of a more

complete coagulation reaction. The relevant studies have focused on traditional storage conditions and the effects of pathogen inactivation treatment (PI) by means of riboflavin/UV light (Mirasol) on the hemostatic potential of buffy coat platelet concentrates (BCPC) and plasma.

1.4.1 General Objective I: Platelet concentrate functionality assessed by TEG or ROTEM.

On the basis of the information described above, it is reasonable to hypothesize that TEG and/or ROTEM evaluations of the procoagulant activity of stored PCs can predict platelet quality and, ultimately, transfusion outcome. As TEG and ROTEM were designed for use with whole blood samples, the first studies optimized the conditions under which TEG could be used to assess the quality of stored PCs. TEG was then used to characterize the procoagulant activity of PCs under a variety of conditions as a measure of platelet effectiveness. Following the validation and optimization of the technique, the TEG findings were compared with those of ROTEM to determine whether the data from these two techniques were interchangeable.

In order to determine whether TEG and/or ROTEM might potentially be applied for *in vitro* assays of stored PC function, three objectives were addressed:

1.4.1.1 Specific objective 1

The creation of a data set describing the hemostatic profile of the platelets to be transfused using TEG. As TEG and ROTEM were designed to deal with whole blood only, it was essential to determine the ability of the TEG to distinguish between blood components; for this reason, the researcher has have determined the coagulation profile using TEG to diverse blood constituents, namely, WB, PRP, platelet poor plasma (PPP), and fresh frozen plasma (FFP). Following this,

the researcher adapted the TEG/ROTEM to investigate the platelet functionality of PCs (lacking RBCs and WBCs) instead of whole blood.

1.4.1.2 Specific objective 2

To characterize the procoagulant activity of PCs under a variety of conditions. Factors affecting the quality of prepared platelet products were studied independently under specified protocols, such as the effect of storage time on platelet viability. The PCs underwent testing following their production on diverse days of storage. This PC testing continued until the end of a shelf-life period of 5 or 7 days to maximize the possibility for detecting changes in the samples should they exist.

1.4.1.3 Specific objective 3

As part of the protocol development, the PCs were reconstituted with other blood components by decreasing their cellular components and maintaining their coagulation factor levels, then analyzed using TEG. Furthermore, the effects of manipulating the platelet status with agonists or inhibitors were tested, with the results being compared to other hemostatic *in vitro* measurements, to understand the relationships among these tests.

1.4.2 General objective II: pathogen inactivation of plasma and platelet concentrates and their predicted functionality in massive transfusion protocols

As a TEG/ROTEM application to assess the quality of traditionally stored PCs, the technology was used to assess the effect of pathogen reduction treatment by Mirasol. The intent was to assess the effect of pathogen inactivation of plasma and platelet concentrates and model their functionality in the setting of massive transfusion protocols where each trauma transfusion package is composed of RBCs, plasma, and BCPC at a ratio of 1:1:1.

1.4.2.1 Specific objective 1

The application of the TEG/ROTEM technologies to assess the procoagulant activity of the pathogen-inactivated platelets. As the platelets would be expected to undergo biochemical changes from the treatment that include changes in mitochondrial activity and the accelerated formation of platelet storage lesions, the in vitro quality and hemostatic functionality of the pathogen-reduced BCPC were assessed. As well, the technology was used to assess the coagulation profile of pathogen-inactivated plasma.

1.4.2.2 Specific objective 2

The purpose was to discover whether the effect of pathogen inactivation on procoagulant activity or coagulation profile would wane once the treated PCs or plasma units were blended with other units already prepared for the trauma transfusion packages. Therefore, the hemostatic functionality of the trauma transfusion packages containing the PI-treated BCPC and/or plasma was assessed following the creation of the various transfusion packages.

1.4.2.3 Specific objective 3

The impact on the hemostatic function of the transfusion trauma package when diluted with untreated fresh blood was determined by mimicking diverse transfusion scenarios. Dissimilar ratios of blood replacement were applied under this model. This step was made to resemble as closely as possible the clinical scenario.

1.4.3 General objective III: whole blood treated with riboflavin/UV light: a recombination of blood components to modulate the effect of pathogen inactivation on the components' hemostatic function

Once this technique was adapted to be solely used with platelets and plasma, and a distinction was established between PI-treated and non-treated PC or plasma samples, it was advisable for us to test the procoagulant activity of the PI-treated WB. This was due to the trend of hospital practitioners to return WB transfusions for trauma patients. To investigate the efficacy of this former protocol, the hemostatic function of PI-treated WB was determined.

1.4.3.1 Specific objective 1

To evaluate the effects of Mirasol on the hemostatic potential of WB using ROTEM. This attempt was to propose the potential for treating WB rather than treating blood components individually.

1.4.3.2 Specific objective 2

In addition, an *in vitro* simulation of an *in vivo* situation was conducted as the second stage of the inquiry. The recombination of PI-WB with hemodiluted blood was performed *in vitro* to simulate relative contributions toward hemostatic function *in vivo*.

1.4.3.3 Specific objective 3

The potential was determined for applying the Mirasol treatment to WB and supplementing it with coagulation factors to enhance hemostatic functionality *in vitro*. PI-WB was supplemented with RiaSTAP, a lyophilized fibrinogen concentrate, to increase clot firmness.

1.5 Significance

Knowing that platelet transfusion is a vital therapy and that its use will probably increase globally, the pre-testing of platelets prior to their transfusion will reduce the bleeding risk of patient, and thus improve their well-being. Therefore, the main objective of this research was to characterize PCs prior to transfusion rather than depending on outcomes post-transfusion as a measure of platelet effectiveness. The study thus aimed to decrease the potential need for excessive transfusion therapy.

This research is intended to yield insight into understanding platelet quality and coagulation, and transfusion medicine. It introduces a novel approach to prevent excessive bleeding. As the results assessed hyper-, moderate, and non-responsive PCs, the findings will eventually aid in discarding PCs of low responsiveness, as determined by the algorithm of the proposed application. Therefore, TEG/ROTEM might be used at blood centres to assess the quality of PCs and even WB prior to their being issued to hospitals.

Chapter 2: Platelet concentrate functionality assessed by TEG or ROTEM[1]

2.1 Introduction

Transfusions of platelet concentrates (PC) are given to maintain primary hemostasis in patients with various thrombocytopenic disorders. Approximately 4.5 million platelet transfusions occur annually in North America and Europe.[43] However, there is evidence that a significant proportion of them may be ineffective.[57] Currently, the efficacy of transfusions can only be determined post-treatment; PCs are transfused without pre-transfusion characterization, and the transfusion outcome is the only indicator of platelet effectiveness. This practice is time-consuming, costly, and of significant risk to patients, who due to the lack of efficacy may be exposed to multiple PC transfusions unnecessarily.[60] Ideally, stored PCs should be tested prior to transfusion and there is a strong need for the development of an assay that can reliably determine the quality of a PC prior to transfusion.[34]

Compounding this issue, there is poor correlation between in vivo transfusion outcome and in vitro testing. Current in vitro measurements, including standards, do not test the efficacy of platelets, but measure PC characteristics such as count, pH, and response to agonists. No testing algorithm exists to accurately predict platelet transfusion efficacy.[58] In the "Platelet Responses and Outcome from Platelet Transfusion" (PROmPT) study, which measured corrected count increments, bleeding scores, and inter-transfusional intervals in recipients, no difference was

[1] Arbaeen AF, Serrano K, Levin E, Devine DV. Platelet concentrate functionality assessed by thromboelastography or rotational thromboelastometry. Transfusion 2016 Aug 16.

ascertained between patients receiving apheresis PCs from donors with high or low responsiveness to adenosine diphosphate and collagen-related peptide.[82] Thus, the PC quality assay that would accurately predict transfusion efficacy should test the efficacy of platelet activation and clot formation in a manner that more closely models these same processes in the bloodstream.

Recently, tests have been more widely applied to monitor coagulation and platelet function in the near-patient setting and these may provide one possible option to fill the gap in assessment of stored PCs. In this study, we wished to determine whether thromboelastography (TEG) and/or rotational thromboelastometry (ROTEM), both currently used in operating rooms to assess the hemostatic state of patients susceptible to bleeding, could be applied to assess the quality of PCs. Unlike other in vitro platelet tests, TEG and ROTEM assess platelet function in a nearly complete coagulation reaction, minus the endothelium. Coagulation initiation in both assays is similar, however, their outputs use different nomenclature.[87,109] Both TEG and ROTEM rely on an immersed vertical pin in the cup, resulting in torque pressure on the pin as a clot develops. The TEG oscillates the cup whereas the ROTEM oscillates the pin in the cup and the shear elasticity is measured. [87,98,110–112] See Figure 1-3 for TEG and ROTEM signature and principle.

We hypothesized that TEG or ROTEM evaluation of the procoagulant activity of stored PCs can predict platelet quality and, ultimately, transfusion outcome. As TEG and ROTEM were designed for use with whole blood samples, we first optimized the conditions under which TEG could be used to assess the quality of stored PCs. We then used TEG to characterize the procoagulant activity of PCs under a variety of conditions as a measure of platelet effectiveness. We compared the data from TEG and ROTEM and found that they were interchangeable. Our

findings suggest that TEG and/or ROTEM have potential to be applied as an in vitro assay for stored PC function.

2.2 Materials and methods

PC and pooled frozen plasma (FP) collection

Healthy volunteers gave informed consent for the protocol approved by the University of British Columbia and Canadian Blood Services' research ethics boards. Pooled PCs were prepared from 450 mL +/- 10% whole blood donations and stored in 100% plasma using the buffy coat production method.[113–115] PC units were stored in air permeable bags at 22°C inside a platelet shaker (Thermo Forma, Thermo Scientific, Asheville, NC). Four units of FP were pooled from the buffy coat production, called pooled FP, and aliquots frozen at ≤ -80°C within 24 hrs of donation. Frozen aliquots were used later for dilution of PC samples to desired concentration. For some experiments, platelet poor plasma (PPP) from the PC was obtained by centrifugation at 2,950 x g for 10 min. gentle shaking on a platelet agitator in air permeable bags at 22°C

Platelet microvesicle (PMV)-rich and PMV-poor plasma samples

Samples rich in PMV were prepared by 6 cycles of freezing (-80°C, 20 min) and thawing (37°C, 10 min) PC aliquots. On the day of testing, PMV-rich plasma was obtained by centrifugation twice at 2,400 x g for 20 min. PMV-poor plasma was obtained by ultra-centrifugation at 540,000 x g for 20 min.

PC reconstitution with different blood components

PCs were sampled using aseptic technique during their shelf-life (5 days), and beyond that (8-10 days) to maximize the effect of storage time. Platelet count was obtained on a hematology

analyzer (Advia 120, Siemens, Mississauga, ON, Canada). PCs were diluted with pooled FP to platelet counts of 400, 300, 200, 100, 40, and 10 x 10^9/L, on day 1, 5, and 10 of storage. Eight PCs were diluted directly to 100 x 10^9/L with pooled FP, then kaolin was added to enhance the coagulation reaction and increase consistency of the assays. Eight PCs were diluted with PMV-rich plasma or PMV-poor plasma at a platelet count of 100 x 10^9/L, following adjustment to 400 x 10^9/L with the addition of PPP from the same unit.

PCs stored under "non-ideal" conditions

Non-ideal platelet storage conditions were used to intentionally generate PCs with varying degrees of “poor” quality. On the day of production, PCs were split into four bags and were stored under 4 different conditions for 8 days: (1) standard storage conditions, namely gentle shaking on a platelet agitator in air permeable bags at 22°C (S&A at 22°C); (2) no shaking in air permeable bags at 30°C (nS&A at 30°C); (3) shaking and in air impermeable bags at 22°C (S&nA at 22°C); and (4) no shaking and in air impermeable bags at 30°C (nS&nA at 30°C). To generate bags impermeable to air, standard blood bags were wrapped in plastic wrap to prevent gas exchange. Storage conditions in which the bags were not agitated and were kept at 30°C were intended to simulate units that had fallen off the shaker onto the heating element during storage. The 6 poorly stored PCs were analyzed following dilution with pooled FP to platelet counts of 100 x 10^9/L on storage days 2, 5, and 8.

TEG and ROTEM profile generation

A TEG®5000 (Haemonetics Corp., Braintree, MA, USA) and a ROTEM (Tem International GmbH, Munich, Germany) were used to assess the PCs. The specific parameters measured and the nomenclature used for both TEG and ROTEM are listed in Table 1-2. Mechanical and

electronic calibration of each TEG/ROTEM channel was conducted before each study according to the manufacturer's recommendations. Immediately prior to testing, each sample was pre-warmed for 1 min at 37°C, then re-calcified with 30 uL of 0.2 M $CaCl_2$ to a final concentration of 17 mM to initiate coagulation.

For some experiments, kaolin was used to initiate the contact activation pathway of coagulation and thereby speed up the assay. The use of kaolin also acted to decrease the standard deviations of clotting time measurements by standardizing these parameters. Briefly, 1 mL of diluted PC was transferred into a vial containing 40 µL kaolin (Haemonetics Corp., Braintree, MA, USA), and 330 µL of this mixture was transferred into the instrument cup. To understand the contribution of platelets to the TEG profile, we examined PCs treated with cytochalasin D, which blocks platelet function by inhibiting actin polymerizationn[116]. Diluted PC was incubated with cytochalasin D (Sigma-Aldrich, catalog no. C8273) at a final concentration of 1.4 µM, for 10 min. Dimethyl sulfoxide, the vehicle in which cytochalasin D was dissolved, was also tested. The contribution of the platelets to the maximum amplitude (MA) was assessed using equation 1:

$$MA_{(platelets)} = \text{Maximum amplitude of reconstituted PC} - MA_{(inhibited\ platelets)}$$

where $MA_{(platelets)}$ = Maximum amplitude provided by the platelet contribution; $MA_{(inhibited\ platelets)}$ = maximum amplitude of cytochalasin D-treated PC.

Platelet in vitro quality analysis

The pH of PCs was measured within 2 hours of sampling (Orion™ 8115BNUWP ROSS Ultra™ Electrode, Thermo Fisher Scientific Inc., Beverly, MA, USA). Degranulation as a measure of platelet activation was assessed by flow cytometry following staining with anti-CD62P-

phycoerythrin (Beckman Coulter, Marseille, France) and staining a second sample with IgG1-phycoerythrin (Beckman Coulter) as the isotype antibody control. The surface expression of phosphatidylserine (PS) was measured by annexin V binding using FITC-labeled annexin A5 (BD Pharmingen, Mississauga, ON, CA), as previously described.[117]

PMV were enumerated by flow cytometry using a calibrated fluorescent bead standard (Fluoresbrite TM Carboxylate, Polysciences, Inc. Warrington, PA, USA). At the time of testing, PMV-rich or PMV-poor plasma was diluted with filtered PBS, and stained with FITC-conjugated platelet-specific antibody (CD41-PC5, Beckman Coulter). For the negative control, nonspecific FITC-conjugated antibody (IgG1-PC5, Beckman Coulter) was used. After incubation for 40 min in the dark at room temperature, samples were further diluted with PBS (pH 7.4). A known amount of 1 μm beads was added to each sample and 10,000 bead events were acquired at a low flow rate using a FACS Canto II (BD Biosciences) flow cytometer. The assay detects particle sizes of 100 nm - 1 μm.

Statistical analysis

Statistical analysis was performed using a two-way repeated measures ANOVA, with Minitab 16 software (Minitab Inc., 2013, State College, PA, USA). The nonparametric Kruskal-Wallis test was used for PC samples with different platelet counts during storage time, while the Student's t-test was used to assess differences between TEG and ROTEM measurement at specific time points. Significance was accepted at $p\text{-value} < 0.05$. The Bonferroni correction was used to adjust the p-value to account for multiple comparisons. A Spearman's rank correlation coefficient was used as nonparametric measure of rank correlation between TEG MA and in vitro platelet quality measures.

2.3 Results

Assay and sample preparation optimization

The effect of platelet storage on TEG MA

The pooled FP used for the dilution of the PC showed a TEG profile of R-time = 16.1 ± 6.1 min, K-time = 5.5 ± 1.2 min, alpha = 34.8 ± 5.6°, and MA = 28.9 ± 1.2 mm on its own when re-calcified with $CaCl_2$ (n = 4). PCs diluted with pooled FP and re-calcified with $CaCl_2$ showed steady hemostatic function and no significant difference in MA during storage up to day 10 when concentrations of 100–400 x 10^9 platelets/L were used (p = 0.2, Table 2-1). This consistency among all the units tested led to the preparation of poorly stored PCs to challenge the TEG.

Dissecting the contribution of fibrinogen and platelets to TEG MA

Inhibition of platelets from the same units with cytochalasin D resulted in a consistent $MA_{(inhibited\ platelets)}$ of 30.4 ± 2.5 mm for platelet concentrations from 100-400 x 10^9 platelets/L up to day 10 of storage, (p = 0.9; n = 5 PC units). This $MA_{(inhibited\ platelets)}$ reflected only the fibrinogen contribution to the clot and was considered the baseline of the platelet contribution. At lower platelet concentrations (less than 100 x 10^9 platelets/L), standard deviations were large (9.3 – 35.5 mm), and therefore these samples were excluded from the baseline calculation. The alpha of the same inhibited platelets was also not statistically different between on the storage day at any platelet concentration (alpha = 41.7 ± 8.4, p = 0.4).

Platelet contribution to TEG MA

$MA_{(platelets)}$ showed steady dynamic hemostatic potential during storage, even at day 10 (nonparametric Kruskal-Wallis test, p = 0.2). The $MA_{(platelets)}$ for PC reconstituted with pooled FP was 38.5 ± 5 mm, which corresponded to a 56% platelet contribution of total MA. (Table 2-1).

Table 2-1: Buffy coat PCs coagulability measured by TEG at different platelet counts on Days 1, 5, and 10.

	PLT count x 10^9 PLTs/L	Day 1*	Day 5	Day 10
MA of PC†	PLT 400	70.24±2.1	72±4.2	70.9±1.6
	PLT 300	71.56±2.8	73.1±2.9	72.1±1.7
	PLT 200	66.3±2.6§	67.4±1.0	67.1±3.5
	PLT 100	63.0±2.2§	67.8±4.2	65.3±5.1
$MA_{(platelets)}$ ‡	PLT 400	40.6±4.7	42.4±1.7	40.0±4.6
	PLT 300	42.1±1.7	44.0±2.7	42.2±2.8
	PLT 200	34.7±3.6	36.1±1.9§	35.7±4.2
	PLT 100	31.6±3.7§	38.2±5.1	35.0±5.9

*Sample collection and analysis was 1 day after production (Day 1), and after 5 (Day 5) and 10 days (Day 10) of storage. Results are reported as means of five independent replicates ±SD. PLT means platelet.

† MA of PC is the maximum amplitude of PC sample at specified platelet count. For MA of PC there was no significant change during storage time (nonparametric Kruskal-Wallis test, p=0.2).

‡ $MA_{(platelets)}$ is the MA when only platelets contribute to clot formation, calculated by subtracting the MA of platelet inhibited samples (cytochalasin D-treated platelets) from the total MA. § $p<0.01$ compared to respective platelet 400 on the same day of testing. MA: maximum amplitude.

TEG *vs.* ROTEM: Assessing PCs during storage

In the absence of kaolin, TEG and ROTEM showed a significant difference in maximum clot formation at a platelet concentration of 100 x 10^9 platelets/L on day 5 (66.1 ± 5.3 and 59.0 ± 3.5, respectively; $p<0.01$), and day 8 (65.3 ± 1.8 and 59.8 ± 1.6, respectively; $p<0.001$). Measurements of MCF made with ROTEM were 10-15% lower than MA measurements made

with TEG ($p<0.001$). However, the coagulation time was similar (Table 2-2). When kaolin was used to initiate the intrinsic coagulation pathway ($n = 8$, $p < 0.05$), it resulted in significant differences in R-time, K-time, alpha, and MA compared to samples prepared without kaolin. In the presence of kaolin, the differences between ROTEM MCF and TEG MA disappeared (Table 2-2). Due to the interchangeable nature of TEG and ROTEM results in the presence of kaolin, further experiments were performed using only TEG.

Table 2-2: Comparison between TEG and ROTEM measurement of buffy coat PCs as a function of storage time at platelet concentration 100 x 10^9 platelets/L on Days 2, 5, and 8

		Day 2		Day 5		Day 8	
		TEG	ROTEM	TEG	ROTEM	TEG	ROTEM
No Kaolin	R-time/ CT (min)	13.3 ± 4†	13.9 ± 3.4†	14.3 ± 2.4	13 ± 3.2	13.3 ± 2.6	13.1 ± 2
	K-time/ CFT (min)	3.2 ± 1.1	4.1 ± 1	2.9 ± 0.6	4.3 ± 2	2.6 ± 0.8	3.2 ± 0.4
	Alpha	49.5 ± 7.3	51.5 ± 4	55.2 ± 6.9	51 ± 3.8	57.5 ± 8	56.1 ± 2.5
	MA/ MCF	64.9 ± 3.3	59.6 ± 7.2	66.1 ± 5.3*†	59 ± 3.5*†	65.3 ± 1.8*	59.8 ± 1.6*
Kaolin‡	R-time/ CT (min)	6.8 ± 0.9	7.93 ± 0.5	7.5 ± 0.8†	8.3 ± 0.3†	7.7 ± 1.2	8.6 ± 0.4
	K-time/ CFT (min)	0.9 ± 0.2	1.05 ± 0.2	0.9 ± 0.08†	1.08 ± 0.1†	0.9 ± 0.13†	1.2 ± 0.1†
	Alpha	76.5 ± 1.6	77.6 ± 2.5	77.2 ± 2	77 ± 2.1	75.1 ± 3.4†	76.2 ± 1.9†
	MA/ MCF	67.9 ± 1.5	66.4 ± 0.9	66.7 ± 1.7†	66.2 ± 1.1†	64.4 ± 5.3†	65.6 ± 1.5†

* $p < 0.05$ TEG parameters vs ROTEM parameters for the same day of testing when samples were only re-calcified.

† Indicates a positive correlation ($r \geq 0.6$; $p < 0.05$) between respective TEG and ROTEM parameters.

‡ Kaolin was used as an initiator of the coagulation intrinsic pathway prior to recalification with $CaCl_2$. Results are reported as means of eight independent replicates ±SD.

Measurement of poorly-stored PCs using TEG and flow cytometry

The poor quality of these units was confirmed by correlating TEG with *in vitro* quality parameters. There were significant increases in CD62P expression and annexin A5 binding and

decreased pH for platelets stored nS&nA at 30°C, or S&nA at 22 °C, on days 5 and 8 compared to day 2 ($p<0.01$ for all). Graphing the MA results from the standard and poorly stored conditions against the *in vitro* quality parameters (Figure 2-1A-C), we found a negative correlation between MA and CD62P with $r = -0.71$ ($p<0.01$), and between MA and annexin A5 with $r = -0.64$ ($p<0.01$). There was a positive correlation between MA and pH with $r = 0.30$, but this did not reach significance. While not statistically significant, the trend suggested that lack of agitation led to higher CD62P levels and possibly lower pH levels than did preventing air permeability. Figure B1 (appendix B) shows TEG tracings of PCs with good and poor in vitro quality on Day 5.

TEG MA for the PCs stored at nS&nA at 30°C significantly decreased with increasing storage time (day 8 compared to days 2 and 5; $p = 0.001$; Table 2-3). The MA of nS&nA at 30°C on day 8, dramatically decreased to 42.9 ± 6.6, compared to 67.3 ± 5.9 in the S&A at 22°C samples which reflects the low platelet contribution from very poorly stored platelets (Table 2-3). The TEG alpha showed a negative correlation with CD62P ($r = -0.39$, $p < 0.01$), and annexin A5 ($r = -0.51$, $p < 0.01$). The other TEG parameters were quite variable and did not correlate with the *in vitro* tests.

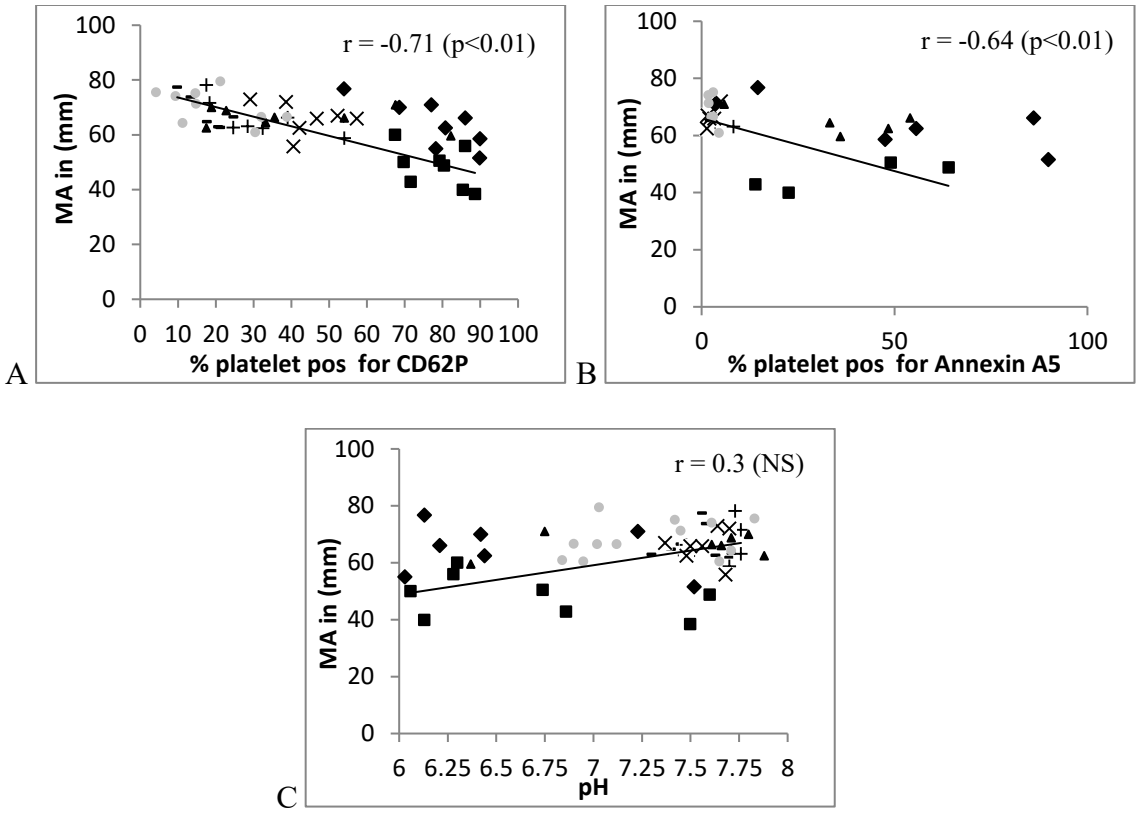

Figure 2-1: Correlation between TEG MA and in vitro platelet quality measures.

Correlation between TEG MA and (A) CD62P expression (n=56), (B) annexin A5 binding (n=36), and (C) pH (n=56) are shown on day 2,5, and 8. The storage conditions are represented as follows: -, +, x = standard storage conditions (S&A at 22 °C) at days 2, 5, and 8 respectively; ■ = nS&nA at 30 °C; ♦ = S&nA at 22 °C; ▲= nS&A at 30 °C (on days 5 and 8). Additionally, data from the three poor storage conditions measured on day 2 were grouped together and are represented on the graph by a single symbol (●). These cluster close to the standard storage conditions as on day 2, less than 24 hours after the samples were generated, the effect of the poor storage conditions are not pronounced. MA has a range of 40 to 80 mm. The vertical dashed/dotted line in panel C represents the lower limit of acceptability for pH (6.4).

Table 2-3: TEG parameters of PCs stored under various storage conditions.

	Day 2				Day 5				Day 8			
	Standard storage condition	nS&A @ 30 °C	S&nA @ 22 °C	nS&nA @ 30 °C	Standard storage condition	nS&A @ 30 °C	S&nA @ 22 °C	nS&nA @ 30 °C	Standard storage condition	nS&A @ 30 °C	S&nA @ 22 °C	nS&nA @ 30 °C
R-time	18.4 ± 5.0	21.1 ± 2.9	19.0 ± 5.9	20.7 ± 4.0	16.2 ± 3.9	24.2 ± 4.2	17.7 ± 3.6	22.2 ± 12.3	18.7 ± 4.4	25.1 ± 6.4	17.1 ± 2.6	21.3 ± 3.7
K-time	4.2 ± 1.0	2.8 ± 1.4	3.2 ± 1.0	3.1 ± 1.8	3.4± 0.8	4.2 ± 1.9	4.2 ± 0.3	3.5 ± 1.2	4.2 ± 1.3	5.9 ± 3.7	3.1 ± 0.5	4.6 ± 1.4
Alpha	53.5 ± 7.2	63.8 ± 18.8	58.2 ± 11.3	59.5 ± 16	52.3 ± 5.7	47.5 ± 13.5	48.2 ± 4.6	44.2 ± 22.4	47.9 ± 9.9	42.2 ± 8.8	54.4 ± 6.7	40.5 ± 9.6
MA	68.9 ± 7.9	67.9 ± 6.8	70.1 ± 8.4	67.3 ± 5.9	67.9 ± 8.7	67.4 ± 3.9	66.1 ± 10.6	59.1 ± 9.5	66.7 ± 7.9	64.8 ± 3.9	62.3 ± 13	***42.9 ± 6.6**
pH22	7.6 ± 0.1	7.7 ± 0.2	7.2 ± 0.3	7.0 ± 0.3	7.7 ± 0.0	7.5 ± 0.5	6.6 ± 0.4	6.8 ± 0.6	7.6 ± 0.1	7.4 ± 0.7	6.3 ± 0.9	6.6 ± 0.7

Standard storage condition: Agitation & air permeable bags at 22 °C, n=6; nS&A @ 30 °C: No agitation & air permeable bags at 30 °C, n=4; S&nA @ 22 °C: Agitation & impermeable bags at 22 °C n=4; nS&nA @ 30 °C: No agitation & impermeable bags at 30 °C. R-time: reaction time (min), K-time: kinetic time (min), MA: maximum amplitude of clot formation (mm).

***** There was a significant difference in MA of poor storage (nS&nA @ 30 °C), measured by TEG on days 8 vs 5, and vs the different storage conditions, $p < 0.01$ (nonparametric Kruskal-Wallis test). Results are reported as means ±SD, n=6.

The effect of PMV-rich PCs on TEG MA

At platelet counts of 100 x 10^9/L, the MA of PMV-rich PCs, averaged from measurements taken on days 2, 5 and 8, was 68 ± 4 mm. This was 8.5% higher than PMV-poor PCs ($p < 0.001$). No significant difference was seen with storage time (Figure 2-2A). Flow cytometry measurements confirmed a significant increase of PMV in PMV-rich compared to PMV-poor PCs ($p<0.001$).

PMV counts in the PCs reconstituted with PMV-poor or PMV-rich plasma show a significant correlation with maximum clot formation ($r = 0.51$, $p < 0.01$; Figure 2-2B). PMV also contributed to a significant increase in the rate of clot formation represented by the TEG alpha (52.3 ± 5.5). This was 17% higher compared to PMV-poor PCs ($p < 0.001$). PMV did not affect the clotting time.

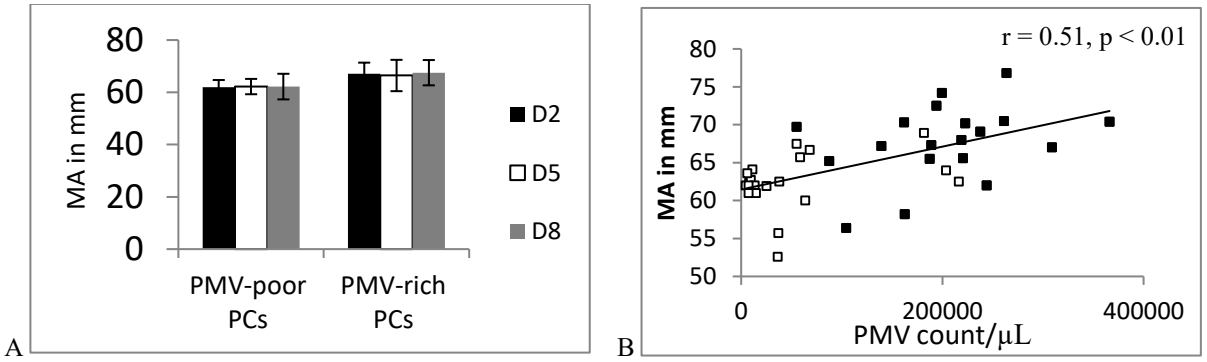

Figure 2-2: The effect of platelet microvesicles (PMV) on maximum clot formation during storage time.
(A) PMV-rich and PMV-poor plasma was used for reconstitution of PCs for TEG analysis to understand the PMV contribution to maximum amplitude (MA) on day 2, 5, and 8. There is a significant difference between the two groups ($p<0.001$), but no significant difference between storage days in each group. (B) The correlation between PMV count and maximum clot formation in PC. The symbol (□) represents the PMV count and MA value in PC reconstituted with PMV-poor plasma (59,888 ± 17,481 PMV/µL), and (■) represents the PMV count and MA value in PC reconstituted with PMV-rich plasma (220,530 ± 12,467 PMV/µL), ($r = 0.51$, $p < 0.01$).

2.4 Discussion

Better understanding the quality of platelet concentrates could lead to better transfusion outcomes.[63,113,118] TEG was developed to test the hemostatic state of whole blood for patients in the operating room. Here we assessed the ability of TEG or ROTEM to detect a loss in platelet quality over storage, with the ultimate aim of adapting the TEG to predict buffy coat PC quality prior to transfusion. As TEG is not designed to test PCs, the assay conditions required optimization; then different PC handling routines were performed to challenge the sensitivity of TEG measurements and to find the most meaningful reflection of platelet quality.

We developed and optimized the TEG assay for use with PCs manufactured by the buffy coat method used at Canadian Blood Services which generates a pooled platelet product stored in plasma. Our preliminary investigation, in which PCs were diluted at various points during their storage time with different fresh blood components (whole blood, platelet poor/rich-plasma, pooled FP) and tested by TEG, gave results that were inconsistent across the diluents used (Appendix A1). Pooled FP was chosen as the preferred diluent to minimize variation that results from mechanical stress and associated coagulation factor activation subsequent to the centrifugation steps[34]. PCs diluted with pooled FP were assayable by TEG as long as the platelet concentration was within normal human physiologic levels.

TEG generates a number of read-outs that report different aspects of clot formation. Examining these, the R and K-time of the PC diluted with pooled FP to different fixed platelet counts were not consistent, and were variable throughout storage. The seeming inconsistency in these TEG parameters may have resulted from not using an agonist (kaolin) to standardize the coagulation

pathway. MA decreases when the platelet counts decrease from 400, 300, 200, to 100 x 10^9/L confirming that platelet count is important for clotting.[119]

Therefore, MA was chosen as the TEG read-out that might be the most meaningful for assessing PC quality and function. At higher platelet concentrations, TEG showed a steady hemostatic capacity of buffy coat PC over the storage time, particularly with the parameter of maximum clot formation, reflecting the ability of platelets to participate in clot formation even 10 days after collection. A concentration of 100 x 10^9 /L was a suitable working concentration that has maximal platelet sensitivity. The MA increased slightly from day 1 to day 2, possibly as a result of the PC being rested after the mechanical stress of their production;[114] therefore, day 2 was chosen rather than day 1 as the early time point to assay.

The TEG assay was further optimized to focus on the platelet contribution to clot formation using cytochalasin D which generates the baseline platelet contribution to clot formation as it inhibits platelet cytoskeletal rearrangement and GPIIb/IIIa interactions with fibrinogen, and eventually prevents the platelet contribution to clot strength such that the TEG measures clot formation from fibrin polymerization only[120].

Results showed that platelet concentrations of less than 100 x 10^9/L are not sensitive to cytochalasin D. Inhibited platelets in cytochalasin D-treated PC showed an $MA_{(inhibited\ platelets)}$ of 30.4 ± 2.5 mm which was set as the baseline of the platelet contribution when PCs are diluted with pooled FP to concentrations of 400, 300, 200, and 100 x 10^9/L. This $MA_{(inhibited\ platelets)}$ was similar to MA of pooled FP alone, which has a platelet count of close to zero, supporting a significant role of the platelet in the thromboelastographic signature.

Overall, our results regarding stored buffy coat PC are in agreement with a recently published paper,[121] where TEG reflected the procoagulant activity of stored apheresis-PC, but with no significant difference according to the number of storage days. In contrast to ours, that study used a microvesicle-free diluent (Octaplas, OctaPharma, Lachen, Switzerland) to dilute the PC. We also applied ROTEM, an alternative clot formation assay, to platelets during storage and similarly saw no significant differences over storage.

Overall, ROTEM parameters correlated well with TEG when the agonist kaolin was applied. It is important to understand the difference between TEG and ROTEM when testing PCs for transfusion. In this study, clot amplitude and the reaction time were not identical between the two instruments, likely because although the test principle is the same for both instruments, the method of testing is slightly different.[122] However, using kaolin resulted in standardizing and accelerating the coagulation reaction, and showed that the key parameters of the TEG and ROTEM are more interchangeable.

Under standard storage conditions, as the platelet storage lesion increased as a function of storage time, the crucial observation was the inability of the TEG to pick up on the slightest change in MA during the storage time. We wished to challenge the TEG and determine its ability to test PCs produced under the standard production conditions but stored under poor storage conditions. We have on occasion experienced a situation of poor storage in the research lab, for example when a PC unit has fallen off the shaker onto the heating element such that it is no longer being agitated. Also, in trying to identify different ways to stress the PC units, we considered that units placed in secondary plastic bags for shipment may experience decreased air exchange. Deviations in storage conditions of PC are not common due to rigorous attention to

standard operating procedures, and we would not expect PC to regularly undergo the stressed storage conditions applied in our study, but we were interested in testing the ability of TEG to identify deviations from standard conditions.

TEG was able to detect poorly stored PC and particularly, extremely poor quality PCs, which had MAs close to the baseline of platelet contribution. In other words, the absence of agitation and air permeability resulted in acceleration of the storage lesion and eventually no platelet contribution towards clot formation. However, TEG's MA reflected that adequate gas exchange in the storage bags affected clotting functionality in the PC units more than did shaking, as previously reported.[123]

Typical of the storage lesion, poorly stored PC also displayed a gradual fall of pH and increased production of lactic acid and increased annexin A5 binding and CD62P expression, all of which correlated with the TEG results. Interestingly, while it might be thought that degranulated platelets that have high negative charge on the surface should lead to an enhanced platelet contribution to coagulation, the TEG showed a decrease in the MA. It is clear that the platelets were already activated, with all granules released upon platelet activation, and no extra cytosolic Ca^{2+} - the main pathway towards platelets aggregation - resulting in an exhausted platelet with hypo-functionality.[124,125] This proved true even if tested in the presence of $CaCl_2$. The decrease in the TEG's alpha parameter in poorly stored PC supports the idea that platelets are not only involved in enhancing coagulation, but also subcellular components are involved in increasing the rate of clot building and maximizing its formation. Further studies are needed to investigate this hypothesis.

Finally, we assessed the contribution of PMVs as increasing PMV release during storage time might contribute to the total MA. The effect of PMV was apparent as a rich background in the PC reconstitution. High quantities of PMV increased the MA and enhanced the coagulation reaction by increasing the alpha, likely by the presence of negatively charged phospholipid surface. This finding may have a clinical use in cases that need PC with highly procoagulant platelets. However, normal levels of PMV released during storage time, which in our lab range around 53,284 ± 3500 PMV/μL on day 8, did not have an effect on TEG parameters, in line with the finding of Bontekoe *et al*.[121]

Overall, this study optimized a potential approach to assess the *in vitro* functional quality of stored buffy coat PCs. TEG correlated with pH, CD62 and annexin A5 in poorly stored PCs; however, the observation that TEG does not reflect the development of the platelet storage lesion over time indicates that it lacks sensitivity. It may also be considered that the platelets tested even at the end of their storage period have adequate hemostatic capacity. The methods are sufficiently sensitive to identify the contribution of platelets and PMV to clot formation. It is important to understand where viscoelastic analyzers stand among other *in vitro* tests and how they may be used.

Our study suggests that with our protocol, when a PC is reconstituted with pooled FP to a concentration of 100 x 10^9 platelet/L and kaolin is used to initiate the intrinsic coagulation pathway, a MA score between 30 and 60 mm indicates a poor quality PC, whereas a score lower than 30 mm indicates non-responsive PC. While traditional platelet *in vitro* quality tests have not reliably correlated with transfusion outcome, our TEG results show the potential of platelets to contribute to immediate hemostasis even after being subjected to poor storage conditions. While

the majority of our tests were performed by TEG, we demonstrated that if kaolin is used, the time of clot formation in PCs is dramatically shortened and ROTEM is comparable to TEG. Ultimately, TEG and ROTEM represent more complete tests than any other assays used to test platelet concentrates and they deserve further investigation as predictors of transfusion outcome.

Chapter 3: Pathogen inactivation treatment of plasma and platelet concentrates and their predicted functionality in massive transfusion protocols[2]

3.1 Introduction

Over five million deaths occur per year worldwide resulting from bleeding in traumatic injuries.[126–128] Damage control resuscitation using a trauma transfusion package is crucial to rescue those patients with severe blood loss. Trauma transfusion packages are a rapid hemorrhage control modality consisting of the common transfusion components, red cells, plasma, and platelets, constituted at a biological ratio of 1:1:1 to treat coagulopathy, acidosis, hypothermia and endothelial permeability,[129] in order to increase hemostasis and decrease hemorrhage-related deaths in patients during the first 24 hours.[130–132]

One important development in blood banking is the introduction of pathogen inactivation (PI) technologies (PITs) which are designed to mitigate transfusion transmitted infections.[103,133,134] PITs are currently on the market or in late stage development for use with whole blood, RBC concentrates, platelet concentrates or plasma. Several randomized clinical trials and hemovigilance data have confirmed the inactivation efficacy of PITs on viruses, bacteria, protozoa, and white blood cells.[135] One of these systems, the Mirasol technology, uses riboflavin

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[2] Arbaeen A, Schubert P, Serrano K, Carter C, Culibrk B, and Devine DV. Pathogen inactivation treatment of plasma and platelet concentrates and their predicted functionality in massive transfusion protocols. *Transfusion*, 2017; 57:1208-1217.

(vitamin B2) to modify nucleic acids upon their exposure to UV light; it prevents the replication of pathogens and leukocytes by modifying guanine residues through direct electron transfer oxidative damage.[104]

Despite improving transfusion safety for transmission of infectious agents, studies of PI have indicated that there is an associated acceleration of the development of platelet storage lesions (PSL) in treated platelet concentrates (PCs),[136,137] and decreased plasma protein activity in PI treated plasma units.[138] A recent study showed only a minimum impact of PI treatment on platelet aggregation and the hemostatic functionality of buffy coat PC in additive solution upon Mirasol treatment.[139] Comparing the safety versus efficacy of PI-treated products, a recent review by J. Hess et al.[140], used predictive mathematical modeling from published reports to calculate a risk of 400 extra trauma deaths annually attributed to the loss of potency of PI-treated PC and plasma. They showed that reduction in blood component potency (30% of platelet potency and 20% of effective coagulation activity) caused by the use of PI could lead to a net loss of life. We were interested in determining whether these mathematical models could be tested experimentally.

Although the importance of proper monitoring and validation of new technologies is well understood,[141] we lack simple tools to predict transfusion outcome. There is poor correlation between common in vitro quality parameters and platelet recovery and survival.[142,143] Recently, new approaches that seek to include most of the elements of hemostasis have appeared. Thromboelastography (TEG) and more recently thromboelastometry (ROTEM) are viscoelastic technologies measuring fibrin polymerization as a reflection of hemostatic functionality of blood in vivo at 37°C.[111] Although most commonly applied to the guidance of blood product use in

surgeries,[144,145] TEG and ROTEM have also been adapted to test blood product function,[146] including PC during storage time.[115,121]

This study aimed to determine whether ROTEM could be used to test mathematical models of the impact of PI treatment on transfusion efficacy. We initially sought to establish whether ROTEM could detect the effect on buffy coat PC of riboflavin/UV light (Mirasol) on the hemostatic potential of buffy coat PC produced in plasma and subsequent reconstitution with fresh frozen plasma (FP). We then investigated the impact of including PI-treated plasma or platelets in a typical trauma transfusion package of RBC, plasma, and buffy coat PC at a ratio of 1:1:1. Finally, to model actual transfusion use, we investigated the impact of the dilution of the transfusion trauma package with untreated fresh blood at various hematocrits on its hemostatic function.

3.2 Materials and methods

Blood components collection and preparation

This study was approved by the research ethics board of Canadian Blood Services (CBS) and healthy volunteers gave informed consent. Donors were asked about medication use in the days prior to donation, including use of aspirin or NSAIDs, as an exclusion criterion. Whole blood was collected at the CBS netCAD facility (Vancouver, BC, Canada) and all units were held overnight on cooling plates for a minimum of 18 hours. Buffy coat PC were prepared from whole blood donations using the buffy coat production method as previously described [113], and were stored at 20-24°C on a platelet shaker (Thermo Forma, Thermo Scientific, Asheville, NC).

Additionally, plasma units and packed red blood cells were produced from the whole blood units and were stored at 4°C for up to 5 days. For some experiments, platelet poor plasma (PPP) was obtained from either the buffy coat PC or the plasma units by centrifugation at 18,000 x g for 40 minutes at 22°C. This PPP was used to evaluate the effect of Mirasol treatment on the coagulation profile in the absence of detectable platelets.

Hemodiluted blood derived from whole blood of healthy donors was prepared by decreasing the hematocrit to 20%, a level chosen to reflect a realistic clinical situation for severe hemorrhage. The blood was collected in citrated vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) directly before running the experiment**,** then diluted with 0.9% saline, pH 5.5 (Baxter Corp., Mississauga, Ontario, Canada).

Pathogen reduction of buffy coat PC (BCPC) and plasma

BCPCs produced in plasma and plasma units were illuminated according to the manufacturer's instructions. PI was achieved with riboflavin and UV (Mirasol system) light in which 35 mL of riboflavin solution (500 μmol/L) was added to the BCPC or plasma units before PI treatment. For studies assessing the direct effect of Mirasol on BCPC, a pooled and split design was used in which one product was treated and the other BCPC was loaded with 35 mL saline and retained as a paired control. For all other studies, BCPC and plasma, either treated or untreated were not derived from pooled samples but used as individual donations to mimic transfusion scenarios.

Buffy coat PC (BCPC) sampling and preparation for hemostatic functionality

BCPCs were sampled aseptically in biosafety cabinets and the platelet count was obtained on a hematology analyzer (Advia 120, Siemens, Mississauga, ON, Canada). For testing the

functionality of an individual BCPC, aliquots of group AB frozen plasma (FP) stored at -80°C were thawed at 37°C and used to dilute the BCPC for testing, as previously described.

BCPC samples were reconstituted with FP to a platelet count of 100 x 10^9/L and tested on day 2 (= the following day of the illumination which was on day 1), days 5, 7, and 9 of the storage period. Eight independent experiments were conducted.

The preparation of transfusion package after illumination

Transfusion packages were prepared using ABO matched packed red cells, plasma, and BCPC. The reconstitution was at a ratio of 1 RBC unit: 1 plasma unit: 1 BCPC unit where a BCPC is composed of the platelets from buffy coats of four whole blood donations and the RBC and plasma units are prepared from a whole blood donation. After the illumination process was completed on day 1, the packages were combined as follows: (a) control package containing untreated RBC, plasma, and BCPC; (b) package containing PI-treated BCPC units but untreated RBC and plasma; (c) package containing PI-treated plasma units but untreated RBC and BCPC; and (d) package containing both PI-treated BCPC and plasma units but untreated RBC (Figure 3-1).

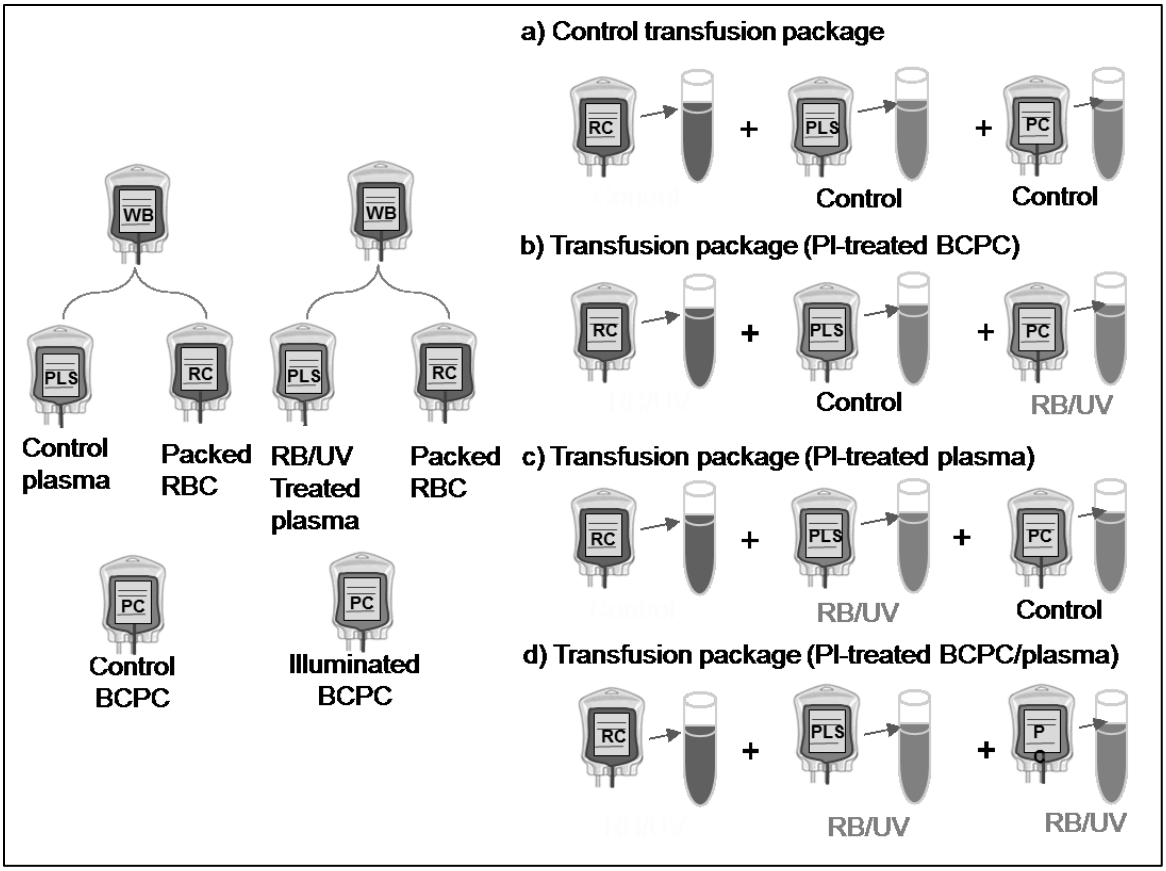


Figure 3-1: An in vitro simulation of trauma pack containing packed RBC, plasma, and BCPC

A simulation of an *in vitro* transfusion package composition that contains packed red cells, plasma, and buffy coat PC (BCPC) at 1:1:1 ratio. The packages represent a) control transfusion package containing non-treated blood components [RBC, plasma, BCPC], b) transfusion package containing treated BCPC component [RBC, plasma, Mirasol-treated BCPC], c) transfusion package containing treated plasma component [RBC, Mirasol-treated plasma, BCPC], and d) transfusion package containing treated BCPC and plasma units [RBC, Mirasol-treated plasma, Mirasol-treated BCPC].

The replacement of the hemodiluted blood with packages A or D (control package or the package containing both treated components) was performed *in vitro* to mimic the transfusion scenario *in vivo* and was performed with different degrees of reconstitution: 30% blood replacement (70% hemodiluted whole blood + 30% transfusion package) "HCT ≈ 27.5%", 50% blood replacement (50% hemodiluted whole blood + 50% transfusion package) "HCT ≈ 33.5%",

and 70% blood replacement (30% hemodiluted whole blood + 70% transfusion package) ‘’HCT ≈ 37.5%” were reconstituted. Eight independent experiments of the entire series were conducted.

The hemostatic profile generation by ROTEM

ROTEM (Tem International GmbH, Munich, Germany) was used to determine the hemostatic profile of the different blood components. Simulated trauma transfusion packages were combined before loading them into the ROTEM cup. Each sample was incubated in a water bath at 37 °C to mimic physiological conditions in the human blood, and the mechanical and electronic calibration of each ROTEM channel was checked before each study according to the manufacturer's recommendations. A volume of 30 µL of 0.2 M $CaCl_2$ was added to re-calcify the samples loaded into each ROTEM cup. Kaolin was used to initiate the contact activation pathway of coagulation as recommended by the manufacturer. Recombinant human tissue plasminogen activator (wild-type tPA) (ANIARA DIAGNOSTICA, West Chester, OH, USA) was used at a final concentration of 2.5 nM to initiate fibrinolysis when indicated.

The key parameters of ROTEM profile, are the clotting time (CT) which is the time to reach 2 mm amplitude from the beginning of the test, the clot forming time (CFT) which is the time to reach 20 mm amplitude from a 2 mm amplitude; alpha, which is the rate of fibrin-platelet interaction; and the maximum clot formation (MCF) which is the maximum amplitude reached (in mm). MCF reflects the ability of platelets and fibrinogen to produce the maximum clot quality and it is influenced by Factor XIII and fibrinolysis.

Platelet *in vitro* quality analysis

In parallel to the ROTEM analyses, the pH of the sampled BCPCs was measured within 2 hours of sampling (Orion™ 8115BNUWP ROSS Ultra™ Electrode, Thermo Fisher Scientific Inc., Beverly, MA, USA) and platelet responsiveness was assessed by staining with fluorescent antibody (anti-CD62P-phycoerythrin, Beckman Coulter, Marseille, France) as previously described.[114,117] ADP was used particularly because it is not a potent agonist like thrombin. Platelet responses to ADP require the coordinate activation of two G protein–coupled receptors, P2Y1 and P2Y12, to stimulate granules secretion. The response of platelets to 10 μM ADP was determined, reported as the delta between platelet positive for CD62P with and without exposure to 10 μM ADP. Six independent experiments (n=6) were performed.

Statistical analysis

First, normality of distribution of the data was tested using GraphPad 6 Prism software (GraphPad Software, Inc., 2016, La Jolla, CA, USA). When not normally distributed, a transformation was applied using Minitab 16 software (Minitab Inc., 2013, State College, PA, USA). A statistical analysis was performed using a two-way ANOVA to determine differences between pathogen-reduced BCPC and control BCPC during storage time and transfusion packages containing combinations of pathogen-reduced components and finally comparing WB, hemodiluted blood, and its replacement with different ratios of transfusion packages.

In case the transformation was not possible, nonparametric analyses were carried out using the Kruskal-Wallis test at different platelet counts during storage time for BCPC reconstitution, and to compare different transfusion packages before and after their dilution with hemodiluted blood. Sample size calculations assumed a power of 80%, and a p-value of less than 0.05 to detect a

potential difference in the *in vitro* quality variables. Data are reported as means and one standard deviation (±SD). The Bonferroni correction was used to adjust the p-value to account for multiple comparisons.

3.3 Results

In vitro tests of treated BCPC

The pH (Figure 3-2A) changed significantly in both groups during storage, revealing a significant drop in the pathogen-reduced BCPC compared to that of the control BCPC ($p < 0.01$). The pH fell below 6.8 in pathogen-reduced BCPC by day 9. The degree of platelet activation was significantly different between the pathogen-reduced BCPC and the control, and increased significantly during the storage time ($p < 0.01$). The addition of ADP to a final concentration of 10 mM resulted in a significant increase in platelet activation, reflected by CD62P surface expression (Figure 3-2B); however, the overall response to ADP decreased significantly with storage. The response to ADP was reduced for treated BCPC in plasma compared to the control.

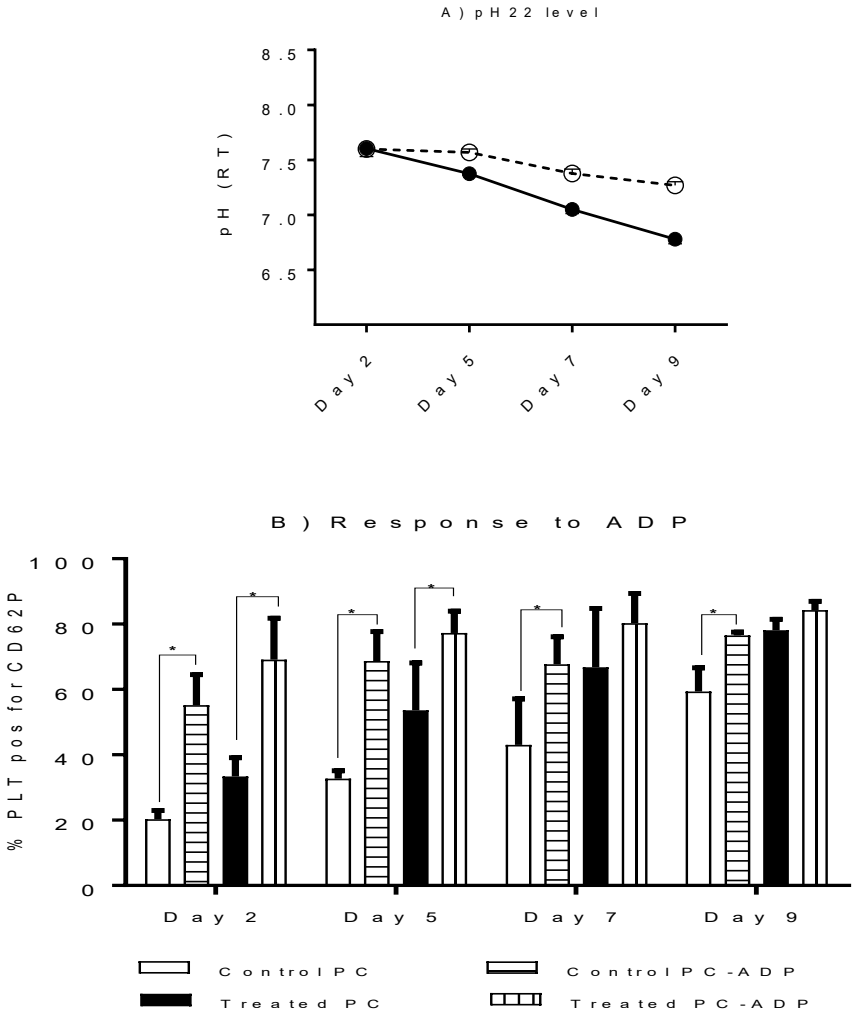


Figure 3-2: pH at RT (22°C) and activation level of BCPC measured as CD62P expression.

A) The pH of Mirasol-treated platelets in plasma for pathogen-reduced BCPC (●) and control BCPC (○). B) Platelets responsiveness to ADP, (*) indicates a significant difference in the response to the addition of ADP of the treated PC when compared to the control PC on the day of testing ($p < 0.001$, nonparametric Kruskal-Wallis test). Results are displayed as the mean ±SD of 6 replicates.

The hemostatic analysis of treated BCPC with ROTEM

The illumination of BCPC did not result in any significant difference in either the clotting time or the clot forming time compared to the untreated samples (Figure 3-3A and B). Following Day 7, a significant decrease in the rate of the fibrin– platelet interaction was observed, as expressed by the alpha value in the pathogen-reduced BCPC (Figure 3-3C). MCF was significantly reduced in the pathogen-reduced BCPC as compared to the control BCPC at all storage days tested ($p \leq 0.01$; Figure 3-3D). The fibrinolysis resistance was slightly decreased in the pathogen-reduced BCPC and was significant after 7 days of storage ($p < 0.05$; Figure 3-3E).

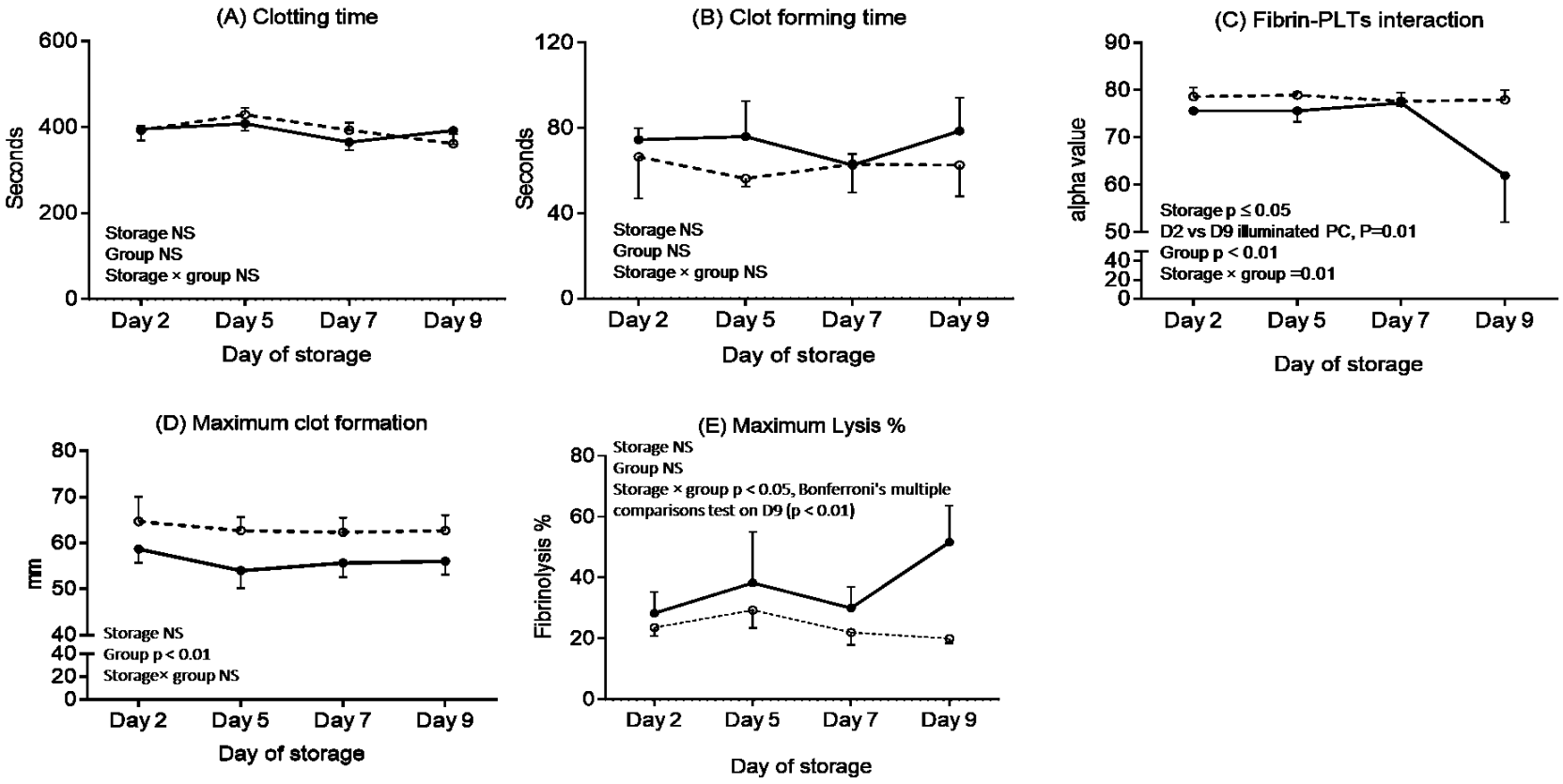


Figure 3-3: Hemostatic functionality of pathogen-reduced BCPC using ROTEM.

Paired BCPC units were pooled-and-split, and one unit was treated. Results are displayed as the mean ±SD of 6 replicates. The solid line represents the PI-treated BCPC, and the dotted line represents the control BCPC during the storage time. Statistical analysis by two-way analysis of variance followed by Bonferroni's multiple comparisons test is reported in each figure.

Impact of PI on plasma

PI treatment resulted in significant delay in the time for the clot to increase (CFT) from 2 to 20 mm in PPP isolated from BCPC units or plasma units ($p < 0.01$) with an approximately five-fold increase in the plasma CFT and three-fold in the CFT with treated BCPC. Furthermore, both the rate of fibrin-platelet interaction (alpha) and maximum clot firmness dropped significantly in the treated BCPC and plasma units compared to their respective controls ($p < 0.05$; Figure 3-4).

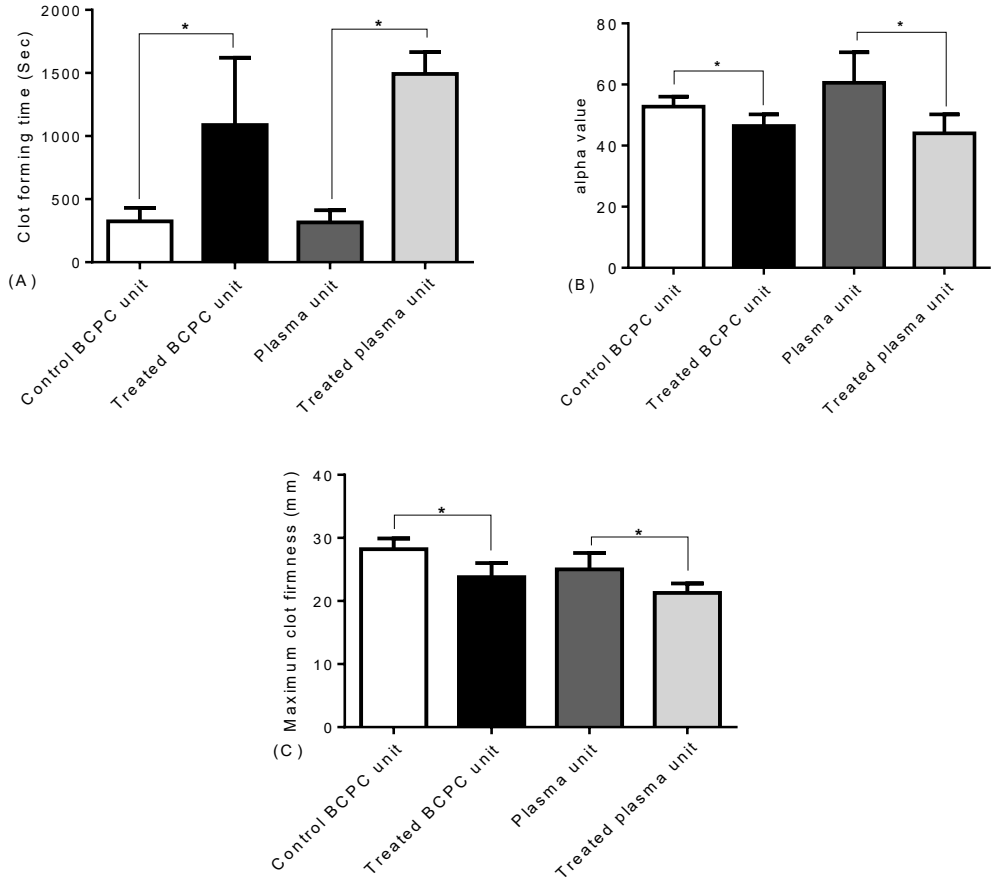


Figure 3-4: The coagulation profile of platelet-poor plasma (PPP) isolated from BCPC or plasma units after Mirasol treatment

Plasma units and BCPC were prepared from different donors. Pathogen inactivation treatment occurred on the day of production. PPP was prepared within 6 hours after the treatment. Results are displayed as means of six replicates ±SD. * Significant difference between the two study arms ($p < 0.001$).

Modeling the use of transfusion packages in the treatment of trauma

The control samples were collected from WB of healthy donors and hemodiluted with 0.9% normal saline to a hematocrit of 20%. The replacement of hemodiluted blood in the test mix with the transfusion packages was at different ratios to create 30%, 50%, and 70% blood replacement. The transfusion packages with or without pathogen reduced components were tested separately (Figure 3-5).

As expected, hemodiluted blood had a significantly altered ROTEM profile consistent with hypocoagulability which provided a model system in which to test the transfusion packages for their ability to return the profile to that of fresh WB [30]. ROTEM traces of the transfusion package with treated components were negatively impacted compared to the control transfusion package as demonstrated by the reduction in the overall readout, $p < 0.01$. CFT was 179.4 ± 42.9 vs 130.1 ± 27.4 sec, rate of platelet-fibrin interaction was 65.4 ± 4.6 vs 56.5 ± 6.0 and MCF was 56.2 ± 2.5 vs 50 ± 2.5 mm (Figure 3-5A, B, and C).

Transfusion packages containing either of pathogen-reduced plasma or pathogen-reduced BCPC had similar hemostatic profiles, CFT = 153.2 ± 29.2 sec and 160 ± 20.1 sec, respectively and MCF = 52.2 ± 2.2 mm and 51.8 ± 2.6 mm, respectively, but their rate of fibrin-platelet interaction (62.5 ± 2.1 and 62.8 ± 1.8, respectively) was superior to transfusion packages containing both pathogen-reduced plasma and pathogen-reduced BCPC (58.5 ± 5.8, $p < 0.05$). To model the worst case scenario for currently licensed pathogen inactivation technologies, we used both treated platelets and plasma in subsequent experiments.

Replacing the hemodiluted blood with the transfusion packages at 30%, 50%, or 70% resulted in an increasing alpha and MCF and shortened CFT. Although the ROTEM profile of 30% blood

replacement was significantly different from the WB ROTEM profile, the overall effect of treatment was less severe than that seen with higher transfusion package ratios ($p \geq 0.05$; Figure 3-5).

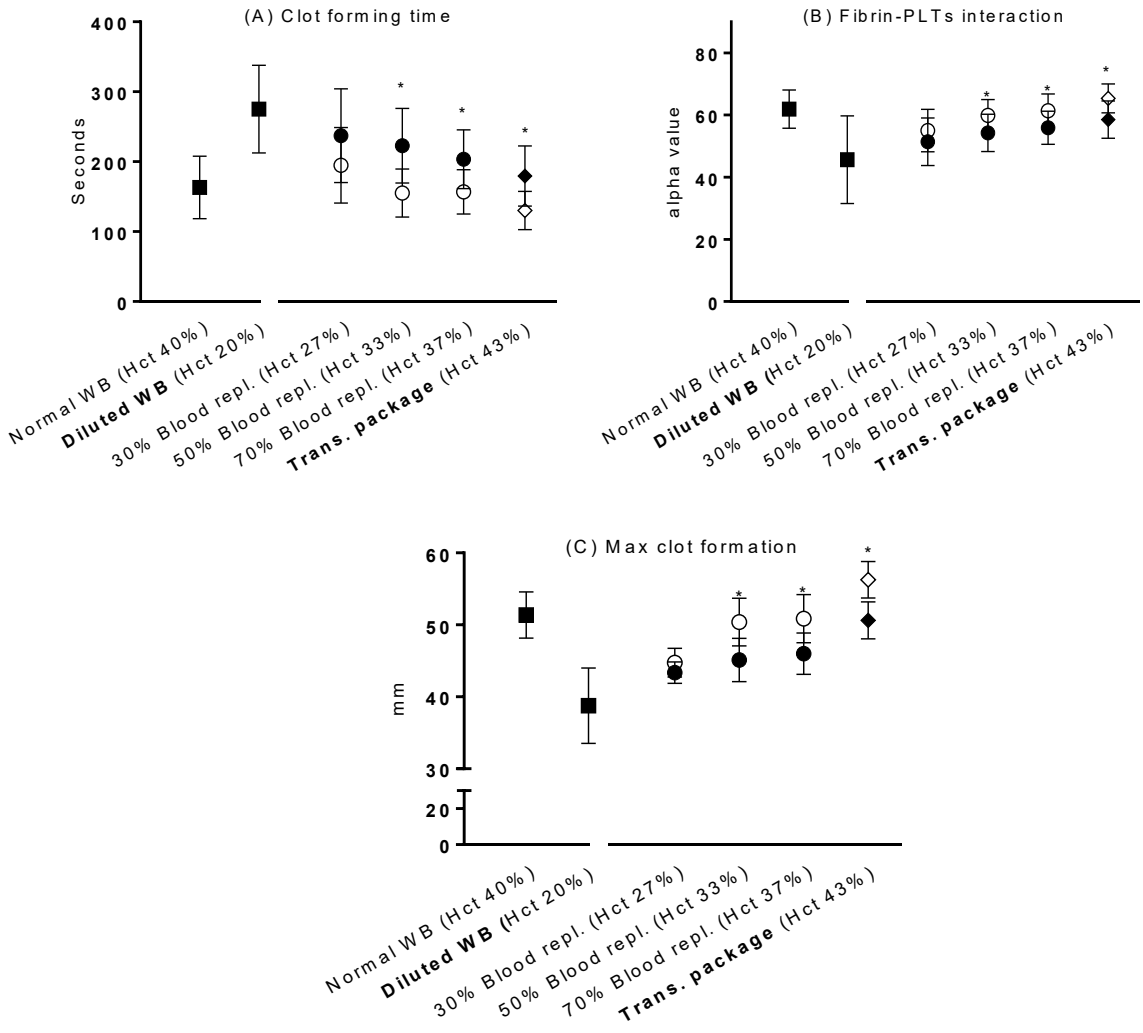


Figure 3-5: In vitro simulation of hemostatic functionality in vivo: Trauma transfusion package.

Clot forming time (a), rate of fibrin-platelet interaction (b), and clot maximum amplitude (c) in hemodiluted blood reconstituted with treated or control transfusion packages. After the pathogen inactivation process was completed, the reconstitution was performed at a ratio of RBC unit: plasma unit: BCPC unit of 1:1:1 of the three blood products. The symbol (■) refers to normal WB before or after the hemodilution with an approximate hematocrit level of 40 or 20% respectively. The symbols (●) and (○) refer to the *in vitro* replacement of the hemodiluted blood with the trauma transfusion package from treated or non-treated plasma and platelets respectively. The replacement was at three different concentrations: 30% blood replacement (70% hemodiluted whole blood + 30% transfusion package) "HCT ≈ 27.5%", 50% blood replacement (50% hemodiluted whole blood + 50% transfusion package) "HCT ≈ 33.5%", 70% blood replacement (30% hemodiluted whole blood + 70% transfusion package) ''HCT ≈ 37.5%". The hemostatic functionality of the transfusion package alone is indicated by (◊), for the control package, and (♦), for the

package containing treated plasma and platelets. * Significant difference between the two study arms ($p < 0.01$). Results are displayed as means of 8 replicates ±SD.

3.4 Discussion

This study investigated the ability of trauma transfusion packages consisting of pathogen-reduced or untreated blood components to correct the hemostatic profile using ROTEM in an *in vitro* model of transfusion in trauma patients. Our data support the concept that the use of blood products treated with pathogen inactivation technologies may reduce transfusion efficacy for patients undergoing massive transfusion.[140]. However, this risk is related to the combinations of products used and the amount of blood volume replaced.

Our studies have focused on the Mirasol pathogen inactivation treatment as applied to platelet concentrates or plasma. We saw that Mirasol treatment lowered the hemostatic profile of PI-treated BCPC but that the product may still be efficacious for transfusion as suggested by clinical assessments of these products.[147,148] While shear-stress-independent *in vitro* tests showed a higher degree of deterioration of the platelets after treatment, the ROTEM profile indicated that Mirasol treated BCPC did not show an effect until Day 7 of storage.

The dramatic decrease in the alpha parameter of the pathogen-reduced BCPC units after Day 7 might have been caused by the decrease in the residual coagulation factor activity, notably fibrinogen which is a significant contributor to the alpha parameter and is known to be affected by Mirasol treatment,[149] along with other coagulation factors such as factor VIII.[150] Impaired platelet function could also contribute to the modified clot signature as intentional impairment of platelet function has been shown to impact the ability to detect fibrinogen activity at the expected level.[151] Another possibility is that some level of conversion of fibrinogen to fibrin during the

treatment was associated with altered ROTEM measurements as has been reported for the inhibition of some coagulation factors in other settings.[152]

An increase in the fibrinolysis of the pathogen-reduced BCPC seen at the end of the storage period may have resulted from an imbalance between tPA and the plasminogen activating inhibitors (PAI-1) present in the platelet. tPA should be neutralized by a certain concentration of PAI-1 from the platelet. Thus, if the final concentration of tPA is higher than that of PAI-1, more fibrinolysis would have occurred; the converse would yield a lower degree of fibrinolysis. This would imply that the activity or level of PAI-1 had already decreased following illumination, resulting in a greater amount of fibrinolysis. Notably, a significant increase in fibrinolysis was not seen until day 9, well after the normal 5 or 7-day allowable storage period.[153,154]

Hemostatic profiles of Mirasol-treated BCPC as well as PPP derived from plasma or BCPC units indicated a decreased activity compared to their respective controls. However, PPP from BCPC was less affected than the PPP from plasma units. This result might be attributed to a protective effect provided by the cellular components in the units whereby the coagulation proteins receive less direct damage from the UV dose.[155,156]

Numerous studies have shown the impact of illumination on the *in vitro* quality parameters of plasma and BCPC. Although clinical trials of both licensed pathogen inactivation technologies show variable reductions in corrected count increments and time between transfusions, neither these changes nor the aforementioned *in vitro* quality changes translated into increased adverse events.[157–159] However, since few studies have been conducted with multiple types of treated products in patients with severe hemorrhage, it is important to determine the impact of the PI on the quality of the transfusion packages used in massive transfusion protocols (MTP). In this

study, we have attempted to mimic as closely as possible the *in vivo* state by performing the replacement of RBC, plasma and platelet to hemodiluted blood with the transfusion packages as prepared for MTP.

Hemodiluted blood shows a prolonged CFT with a lesser degree of MCF at an earlier stage than that observed in standard laboratory-based monitoring.[160] This behavior results from a massive loss of endogenous inhibitors of fibrinolysis.[161,162] Therefore, we chose healthy donors and diluted their blood samples with normal saline to simulate hemorrhage occurring in trauma patients who have been treated for fluid loss but not yet transfused with cellular components or plasma and are left with a 50% loss of red cell mass. However, we did not decrease the hematocrit level to less than 20% such as may be seen with high mortality trauma.

The assessment of these simulated transfusions by ROTEM showed that the parameters measured in the clot signature were affected by the use of Mirasol-treated products in this model. The ratio of hemodiluted blood to the transfusion packages affected the CFT, alpha value and MCF of the clot signature. The addition of Mirasol treatment significantly reduced the efficacy in the two groups with the highest proportion of transfusion packages, 50% and 70% blood replacement. No difference was observed when the treated transfusion packages were used to supplement at 30% of the blood volume.

Although direct translation to human transfusion can only be speculated, the 50% replacement model approximates a 4-unit RBC transfusion, a quantity that has been shown, at least in cardiac surgery, not to be associated with an increase in mortality.[163] The 70% replacement model approximates situations of massive transfusion in which there is already a high mortality rate. In

this setting, these studies suggest hemostasis would be further compromised by the use of PI-treated platelets and plasma in support of concerns raised by others.[140]

As with all models, our study has its limitations. ROTEM lacks the involvement of the endothelium and thus is an imperfect way to measure hemostasis. Whether these ROTEM results are predictive of clinical use of pathogen-reduced blood products remains to be determined by clinical trials. The study reported here suggests that if ROTEM represents a closer assessment of *in vivo* hemostasis than other typical *in vitro* assays performed on single components, the effects of pathogen reduction treatment may be slightly less than suggested by mathematical modeling.[140] Importantly, we did not see a complete failure to form a clot even at the highest volumes of treated products tested. Nevertheless, the use of multiple pathogen-reduced components in transfusion packages used for MTP should be undertaken with caution and with consideration of the use of other means to promote hemostasis. The ongoing determination of the balance between increased blood safety from pathogen transmission and decreased efficacy arising from the treatment itself remains an important consideration.

Chapter 4: Pathogen inactivated whole blood: supplementation with fibrinogen partially corrects treatment damage

4.1 Introduction

Hemorrhage resulting from severe injury is a leading cause of death in developing and underdeveloped countries and has currently been increasing much more significantly.[164,165] Ideally, trauma patients should receive early balanced transfusions of blood components to restore their blood following massive bleeding.[166–170] Practically, whole blood (WB) may have superiority over the combination of individual blood components[171], since the latter contains a greater concentration of anticoagulants and additives with higher chance for coagulopathy and a lesser oxygen-carrying capacity than fresh.[172,173]

Although, hemostatic resuscitation with WB is being used in military settings and in civilian medicine in some Middle East countries and a few hospitals in developed countries, there is an increasing interest by practitioners to return to the use of WB (2-7 days old) in the civilian setting for the treatment of massively hemorrhaging patients and pediatric cases.[174,175] It was also a research priority in the National Heart, Lung, and Blood Institute transfusion medicine state of science symposium summary statement.[176]

The recent in vitro and in vivo studies on cold WB have inspired practitioners to reconsider resumption of WB transfusion for patients with severe hemorrhage. It is of great convenience to be able to use one product to resuscitate a bleeding patient rather than using multiple components.[174] It was believed that cold WB (4°C) could carry activated platelet with

irreversible shape change that harms the recipient, but clinical studies indicated that there is no increase in thrombosis level or adverse events when compared to WB at (20°C).[177,178] Moreover, with respect to donor exposure, resuscitation with WB in trauma patients means that a recipient is exposed to one donor per equivalent unit of blood instead to up to six donors in the case of reconstituted WB from one RBC unit, a unit of whole blood derived platelets pooled from four donors, and plasma unit. A recent study in pediatric patients undergoing cardiac surgery concluded that the fewer the number of donors' exposure to the recipient, the better the outcome for the trauma patient.[179] Transfusion of WB stored at 4°C for 10 to 14 days, for patients with life-threatening bleeding, would significantly lower the logistic burden (both equipment-related and staffing costs) and extend the age of platelet compared to separate component transfusion.[174,180] However, this runs the risk of introducing infectious agents into the patient, which jeopardizes blood safety[181]. Several pathogen inactivation (PI) technologies have been developed for application to plasma and platelet concentrates (PCs),[103] but the riboflavin and ultraviolet (UV) light process for PI (Mirasol PRT System Terumo BCT, Lakewood, Colorado, USA) was the one first utilized to treat WB units[105]. This latter was reported as being an alternative to gamma irradiation to prevent transfusion-associated graft-versus-host disease,[106] and for successfully inactivating white blood cells, viruses, bacteria, and parasites.[105,107,182–184]

Custer *et al*., concluded that PI-treated WB has lower cost-effectiveness of quality-adjusted life-year, compared to current regulations of individual PI-treatment to plasma and PC in Canada [185]. In vitro quality of platelet concentrates from WB treated with PI is less negatively impacted than treatment of the PC component.[186] Moreover, individual treatment of the blood product involves some time expenditure and use of blood centers' equipment.

However, it was concluded that a dose of 80 J/mL$_{RBC}$ of UV light results in an increase in RBC MCV, hemolysis levels, potassium release and microparticle production, in addition to upwards of 44% lowering of the activity of coagulation proteins; for instance, fibrinogen level decreases by as much as 30%.[186,187] Researchers also concluded that altering the RBC additive solution may overcome the negative impact of the treatment. It was still suggested that WB cold storage in conjunction with this method should only be performed for up to 21 days to avoid compromising WB quality.[107,108]

Previous studies assessing the in vitro quality of pathogen-reduced WB demonstrated a reduction in fibrinogen activity.[187,188] RiaSTAP®, (CSL Behring LLC, Kankakee, IL, USA), is a lyophilized purified fibrinogen concentrate made from human plasma administered to patients with fibrinogenemia or afibrinogenemia. It also undergoes intensive microbial inactivation and testing for hepatitis B and C and HIV-1/2, in addition to nucleic acid testing for hepatitis A and C, HIV-1, and parvovirus B19.[189–191] Additionally, it has the potential for more rapid, safer and predictable dosing than cryoprecipitate, and does not require the length of time to prepare than frozen cryoprecipitate does.[189] It can be used along with PI-treated WB to improve hemostasis during damage control resuscitation as it increases clot firmness in patients with fibrinogen deficiencies.[192] Currently, hypofibrinogenemic and afibrinogenemic patients are given purified fibrinogen concentrates RiaSTAP at doses of 70 mg/kg and clot firmness is measured.[193]

In different clinical trials and studies, both ROTEM and TEG have been shown to identify nonresponsive blood components as a cause for impaired clot strength.[144,194] ROTEM was used successfully in the previous chapter to assess the reconstituted WB. Therefore, the investigation

of the role of fibrinogen in modulating the negative impact of PI on WB will use ROTEM technology to assess hemostatic potential.

This study aimed to determine whether ROTEM could be used to test the effect of PI-treated WB in a trauma model. It was initially sought to establish whether ROTEM could detect the effect on WB of riboflavin/UV light (Mirasol) on the hemostatic potential. To model actual transfusions conducted in severe trauma cases, the same model that was used in the previous chapter will be used here with some modification. In this study, the hemostatic function of dilutions of PI-treated WB was investigated with ROTEM using untreated fresh blood, hemodiluted to various hematocrits. Finally, the impact of RiaSTAP supplementation of PI-treated WB, was investigated in this model.

4.2 Materials and methods

WB unit collection and preparation

This study was approved by the research ethics board of Canadian Blood Services (CBS) and healthy volunteers gave informed consent. Whole blood was collected at the CBS netCAD facility (Vancouver, BC, Canada) and all units were held overnight on cooling plates for a minimum of 18 hours. Additionally, plasma units were produced and stored at 4°C for up to 5 days. Hemodiluted blood derived from the WB of healthy donors, collected directly before running the experiment in citrated Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), was prepared by decreasing the hematocrit concentration to 20%, a level chosen to reflect the realistic clinical situation for severe hemorrhage, by dilution with 0.9% saline solution, pH 5.5 (Baxter Corp., Mississauga, Ontario, Canada).

Pathogen reduction of WB and plasma

WB and plasma units were illuminated according to the manufacturer's instructions. PI was achieved with riboflavin and UV (Mirasol system, TerumoBCT, Lakewood, CO) light in which 35 mL of riboflavin solution (500 μmol/L) was added to the WB or plasma before PI treatment. A pooled and split design was used which was ABO-matched, and units were employed in which one product was treated and the other WB was loaded with 35 mL saline and retained as a paired control. The weight of the WB units and the hematocrit HCT (HAEMATOKRIT 210, Hettich Zentrifugen, Tuttlingen, Germany) were utilized to calculate the UV illumination time. Following the treatment, the contents of the illumination bag were drained into the attached storage bag and tested within 6 hours, or they were retained at 4°C for 48 hours to avoid bacterial growth.

WB sampling and preparation for hemostatic functionality

WB and plasma were sampled aseptically in biosafety cabinets and the platelet, RBC count, and hematocrit were obtained with a hematology analyzer (Advia 120, Siemens, Mississauga, ON, Canada). Twenty-four and independent experiments were conducted. PPP was prepared from WB and plasma unit to determine the coagulation profile, as described in the previous chapter.

The preparation of the transfusion model following illumination

To model the effect of WB treatment on transfusion efficacy, we used the dilution model described in the previous chapter. On Day 1, after the illumination process was completed, the reconstitution was combined with ABO-matched hemodiluted blood samples as follows: (a) hemodiluted WB with untreated WB unit, and (b) hemodiluted WB with PI-treated WB units at

different ratios: 30% blood replacement (70% hemodiluted whole blood + 30% WB) “HCT ≈ 27.5%”, 50% blood replacement (50% hemodiluted whole blood + 50% WB) “HCT ≈ 33.5%”, and 70% blood replacement (30% hemodiluted whole blood + 70% WB) ‘’HCT ≈ 37.5%”. Eight independent experiments were conducted on the entire series.

Enriching the fibrinogen level using the fibrinogen concentrate RiaSTAP:

Using aseptic technique, aliquots of fibrinogen were prepared from RiaSTAP (CSL Behring GmbH, USA). To enhance clot firmness, RiaSTAP at a final concentration of 1 µg/µL was added into both the control- and the PI-treated WB at, with or without reconstituting them with hemodiluted blood. The MCF parameter indicates overall clot strength and is a reflection of fibrinogen efficacy, other parameters were not considered because they were variable. The response of PI-treated WB to RiaSTAP was determined, and reported as the delta between WB with or without reconstitution with hemodiluted blood. Eight independent experiments were performed.

The hemostatic profile generation by ROTEM

ROTEM (Tem International GmbH, Munich, Germany) was used to determine the hemostatic profile of the PI-treated WB, and the blood samples reconstituted with hemodiluted blood and those enriched with RiaSTAP. Each sample was incubated in a water bath at 37 °C to mimic the physiological temperature of human blood, and the mechanical and electronic calibration of each ROTEM channel was checked before each study according to the manufacturer's recommendations.

Statistical analysis

First, the normality of the distribution of the data was tested using GraphPad 6 Prism software (GraphPad Software, Inc., 2016, La Jolla, CA, USA). When not normally distributed, a transformation was applied using Minitab 16 software (Minitab Inc., 2013, State College, PA, USA). A statistical analysis was performed using a one-way ANOVA to determine the differences between PI-treated and control WB and the delta of the MCF response to RiaSTAP, and the two-way ANOVA to compare WB, hemodiluted blood, and its replacement with different ratios of WB. In cases where the transformation was not possible, nonparametric analyses were carried out using the Kruskal-Wallis test on different WB samples before and after their dilution with hemodiluted blood. Data were reported at the mean and one standard deviation (±SD). The Bonferroni correction was used to adjust the p-value to account for multiple comparison.

4.3 Results

The hemostatic profile of PI-treated WB versus control WB

The illumination of the WB samples resulted in a significantly different hemostatic profile with respect to clotting time (CT) and clot forming time (CFT) as compared with the untreated samples, (n=24; Figure 4-1). CT and CFT increased significantly in the PI-treated WB as compared to the control WB, at 369.0±49.6 vs. 423.5±35.8 ($p < 0.01$), and 112.5 ±26.3 vs. 175.3±16.4 ($p < 0.001$), respectively with using kaolin. There was a significant decrease in the rate of the fibrin–platelet interaction observed, as expressed by the alpha value in the pathogen-

reduced WB (alpha of PI-treated units 58.3±1.2 vs. control WB 67.8±2.3). The MCF was significantly reduced in the pathogen-reduced WB 55.6 ± 3.1 mm as compared to the control WB (48.8 ± 2.9 mm) ($p \leq 0.01$).

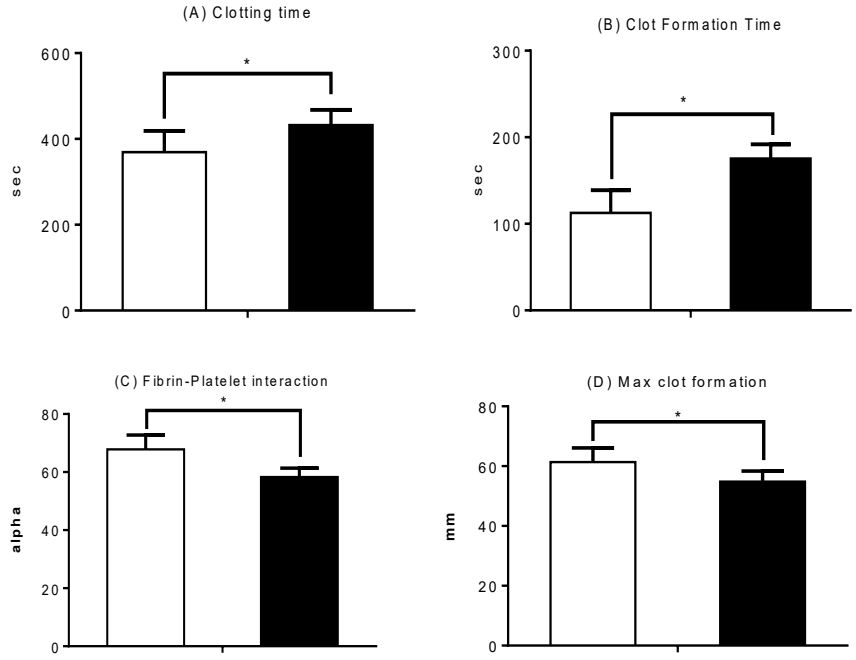


Figure 4-1: Hemostatic profile of pathogen-reduced WB versus control WB.

Results are displayed as the mean ±SD of 24 replicates. The black bar represents the PI-treated BCPC, and the white bar represents the control WB during the storage time. Statistical analysis by one-way analysis of variance, and (*) represents a significant difference between the two study arms ($p < 0.001$).

The coagulation profile of pathogen-reduced plasma:

It was crucial to determine the impact of the treatment on the quality of PPP obtained from pathogen-reduced WB and plasma. The PPP obtained from pathogen-reduced WB showed a significant two-fold increase in the CFT compared to that of the PPP from the control WB, at $p < 0.05$. The rate of clot building decreased, albeit insignificantly, since the SD was high in both groups. The MCF had slightly but insignificantly decreased. Treating plasma units resulted

in a nearly five-fold increase in the CFT compared to that of the control plasma unit, at $p < 0.001$. The alpha and MCF values decreased significantly, at $p < 0.05$, Table 4-1.

Table 4-1: The coagulation profile of plasma following illumination.

N=6	PPP from control WB unit	PPP from pathogen reduced-WB unit	PPP from plasma unit	PPP from pathogen reduced plasma unit
CFT (sec)	564.2±249.2	1320±290	315.0 ± 96.9	1492.6±173.1
alpha (rate)	61.5±9.8	49.8±8	60.6±10.0	44.0±6.2
MCF (mm)	23.2±2.2	20.2±1.2	25.0±2.6	21.3±1.5

Modeling the use of PI-treated WB in the treatment of trauma

The control samples were collected from the WB of healthy donors and hemodiluted with 0.9% normal saline to a hematocrit of 20%. The replacement of hemodiluted blood in the test mix with PI-treated or non-treated WB was conducted at different ratios to create 30%, 50%, and 70% blood replacement. The PI-treated or non-treated WB were tested separately but without the use of kaolin (Fig 3). ROTEM traces of the PI-treated WB were negatively impacted as compared to the control WB as demonstrated by the reduction in the fibrin-platelet interaction rate and the MCF, and delays in the CFT, $p < 0.01$. The CFT was 115.3 ± 17.7 secs. and 163 ± 17.3 secs., and the rate of the fibrin-platelet interaction was 68.3 ± 4.6 vs. 59.1 ± 2.0, while the MCF was 61.6 ± 3.0 vs. 55.5 ± 2.4 mm. (Figure 4-2A, B, and C).

To model the worst potential trauma scenario, PI-treated or non-treated WB was used in subsequent experiments. Replacing the hemodiluted blood with the PI-treated WB at 50% or 70% resulted in an increasing alpha and MCF and a shortened CFT. The overall effect of PI-treatment disappeared when 50% blood replacement or higher when comparing with hemodiluted blood ($p \geq 0.05$; Fig 3). The ROTEM profile of 30% blood replacement with

control WB but not PI-treated WB showed superior procoagulant activity when compared to the hemodiluted blood.

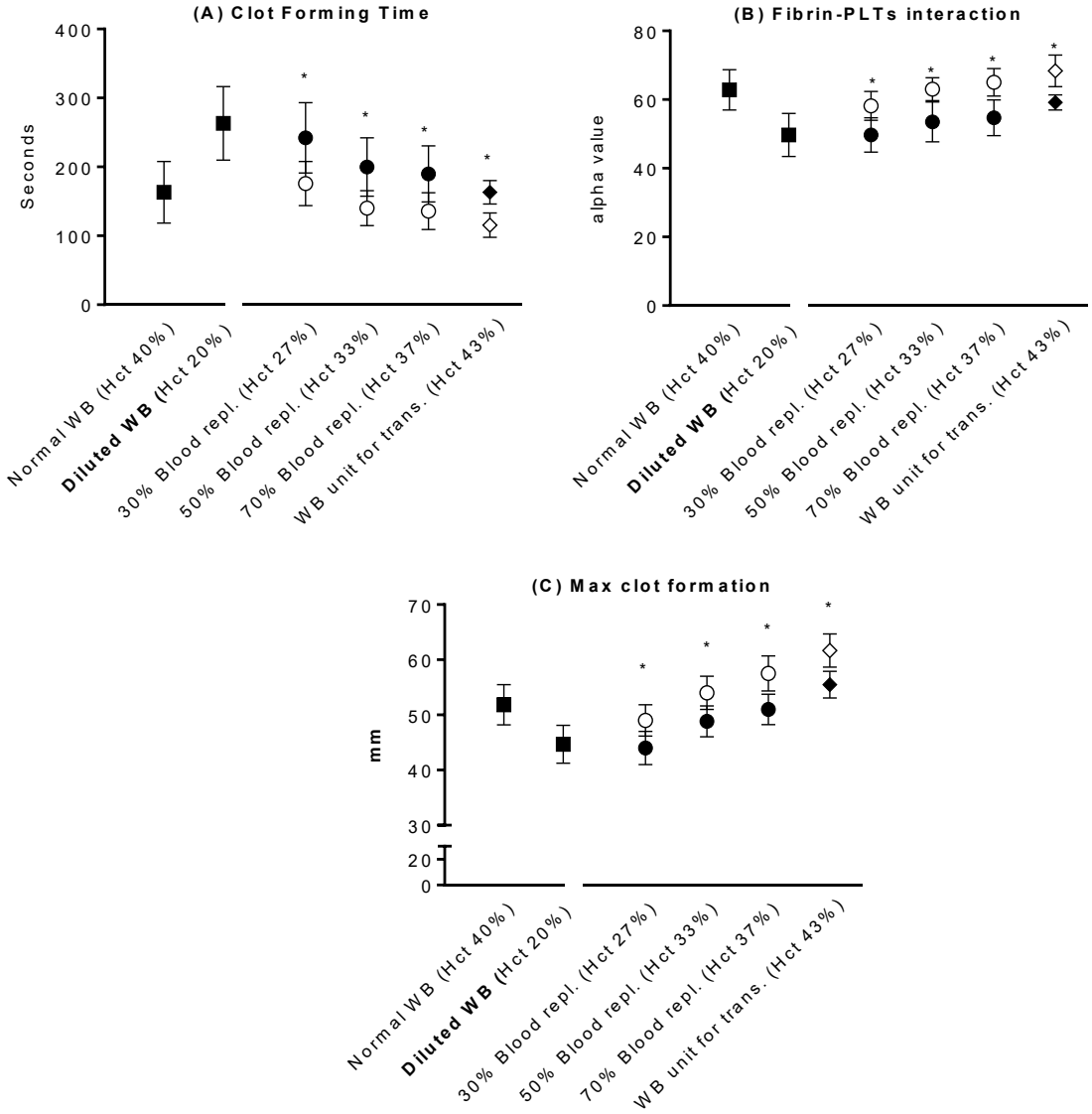


Figure 4-2: In vitro simulation of hemostatic functionality in vivo: WB.

Clot forming time (a), rate of fibrin-platelet interaction (b), and clot maximum amplitude (c) in hemodiluted blood reconstituted with treated or control WB. After the pathogen inactivation process was completed. The symbol (■) refers to normal WB before or after the hemodilution with an approximate hematocrit level of 40 or 20% respectively. The symbols (●) and (○) refer to the *in vitro* replacement of the hemodiluted blood with the trauma transfusion package from treated or non-treated plasma and platelets respectively. The replacement was at three different concentrations: 30% blood replacement (70% hemodiluted whole blood + 30% WB) "HCT ≈ 27.5%", 50% blood replacement (50% hemodiluted whole blood + 50% WB) "HCT ≈ 33.5%", 70% blood replacement (30% hemodiluted whole blood + 70% WB) ''HCT ≈ 37.5%". The hemostatic functionality of the WB unit alone is indicated by (◊), for the control WB, and (♦), for the PI-treated WB. * Significant difference between the two study arms ($p < 0.01$). Results are displayed as means of 8 replicates ±SD.

Supplementation of PI-treated WB with RiaSTAP following illumination

Following the dose response curve of the clot firmness for PI-treated WB enriched with RiaSTAP at a final concentration of 1 µg/µL was used to compensate for the decrease in the fibrinogen level post-treatment (Figure 4-3).

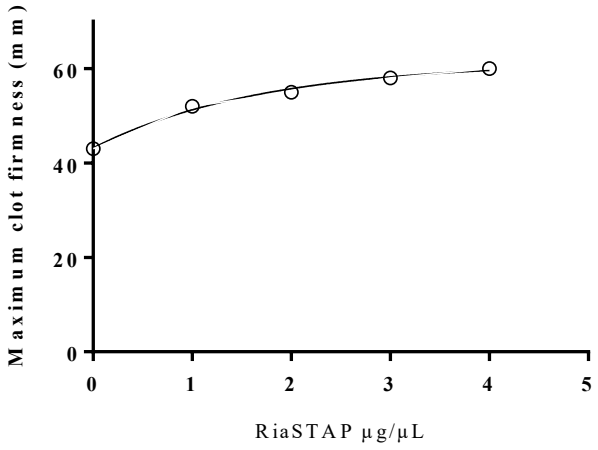


Figure 4-3: The dose response curve to RiaSTAP.

The addition of RiaSTAP to a final concentration of 1 µg/µL resulted in significant improvement of clot strength, reported as delta MCF between the supplemented and control group ($p < 0.01$). The delta of the clot strength was 6.8 ± 0.5 mm between the PI-treated WB and the control and decreased 1.4 ± 0.5 mm to after inducing the RiaSTAP ($p < 0.01$). The overall response to RiaSTAP supplementation resulted in decreasing the delta of the MCF between the PI-treated on non-treated WB. There was no significant difference between the ratios of blood replacement with PI-treated WB and enriched with RiaSTAP (NS) (Figure 4-4) as the delta between WB with or without reconstitution with hemodiluted blood.

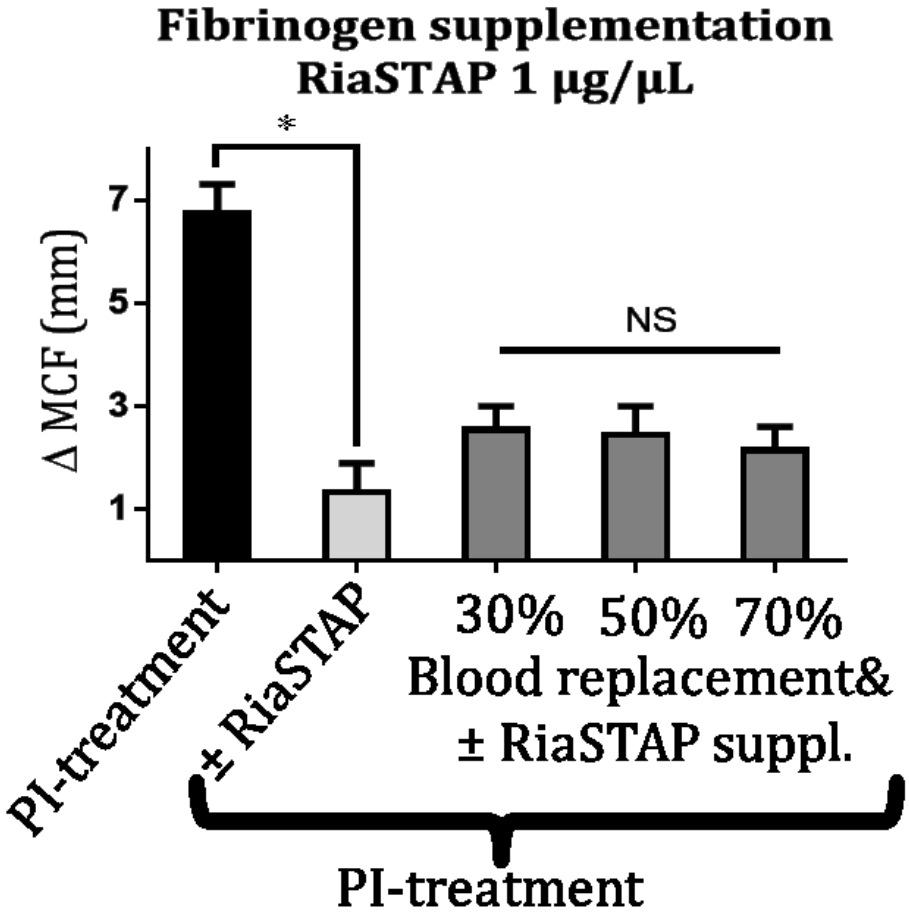


Figure 4-4: Supplementation PI-treated WB with RiaSTAP following treatment.

WB responsiveness to RiaSTAP, (*) indicates a significant difference in the response to the addition of RiaSTAP of the treated WB when compared to the respective control ($p < 0.01$). Results are displayed as the mean ±SD of 8 replicates. The addition of RiaSTAP to a final concentration of 1 µg/µL resulted in a significant clot strength, reported as MCF ($p < 0.01$). The delta of clot strength was steady between different ratio of blood replacement when the WB is PI-treated and enriched with RiaSTAP (NS).

4.4 Discussion

This study investigated whether PI impacts the hemostatic profile of WB in an *in vitro* model of transfusion in trauma patients using ROTEM. These data support the previous observation that PI has a negative impact on the hemostatic characteristics of WB, but optimization can be

performed to decrease this effect, such as altering the storage of the WB following treatment, the amount of blood volume replaced, or whether or not the blood clotting factors have been compensated.[187] These investigations have focused on the Mirasol pathogen inactivation treatment as applied to WB. Mirasol treatment lowered the hemostatic profile of the PI-treated WB samples, but the products demonstrated possible efficacy for transfusions as suggested by *in vitro* and *in vivo* assessments.[188,195]

The reduction observed in the CT, CFT, and alpha value of the pathogen-reduced WB and plasma units might have resulted from a reduction in the residual coagulation factor activity, and particularly in fibrinogen and factor FVIII which are major contributors to the coagulation pathway and are reported to be affected by Mirasol treatment.[186,187,196] Platelets and αIIbβ3 impacted by Mirasol treatment possess impaired functionality, and overall reduction in thrombus formation. The anaerobic rate, α-degranulation, and phosphatidylserine/-ethanolamine exposure increase significantly following treatment.[197] These changes affect the platelets ability to detect fibrinogen activity at the expected level,[151,198] and result in changes in their biochemical mechanisms, and a decrease in the thrombus formation rate as shown by the alpha value and MCF.

However, the results reported here demonstrated that treatment of WB could be superior to reconstitution of WB from PI-treated plasma and PC that was reported in the previous chapter. Using PI-treated and leukoreduced WB to treat trauma patients could enhance the procoagulability and accelerate hemostasis for several reasons. It is obvious that coagulation factors better maintained their functionality when comparing PPP obtained from PI-treated WB and plasma, table 4.1. Therefore, coagulation factors in PI-treated WB but not in PI-treated

plasma have a higher potential to oppose coagulopathy and reduce bleeding needs. Moreover, the moderate microvesiculation of platelet and RBC, in addition to an increased resistance to shrinkage caused by energy depletion emphasizes that the comprehensive treatment could be better than individual component treatment.[199]

Nonetheless, no study has reportedly been conducted to date involving the impact of PI-treated WB samples in patients with severe hemorrhage; it is thus important to determine the impact of the PI on the quality of the WB used for massive hemorrhage. In this study, there was an attempt to mimic as closely as possible the *in vivo* state by replacing hemodiluted blood with PI-treated vs control WB prepared for massive transfusion scenario. A hemodiluted blood with a hematocrit level of 20% was prepared, as reported earlier. This is because it has diluted coagulation factors and that results in a decrease in clot firmness and clotting time.[160,162]

The evaluation of these simulated transfusion configurations by ROTEM demonstrated that the parameters measured in the clot profile were influenced in this model by the use of PI-treated WB. The ratio of blood replacement affected the CFT, alpha value and MCF of the hemostatic test.

Additionally, Mirasol treatment significantly reduced the efficacy in the groups with 30% blood replacement and higher. This large delta value (MCF: 7.8 mm, $p < 0.01$) present between the hemodiluted blood reconstituted with PI-treated and non-treated WB could be related to the decrease in activity of the major coagulation factors in the treated WB.

Simulating trauma transfusion scenarios, these study results suggest that hemostasis would be more greatly compromised by PI-treated WB than they were in the previous study when a reconstituted WB containing PI-treated plasma and BCPC but not RBC was used [chapter 3].

Therefore, RiaSTAP could be the ideal supplement for WB after PI with Mirasol, as it is currently available to treat patients with dysfunctional fibrinogen or reduced fibrinogen levels.[200–202] The normal plasma fibrinogen level is in the range of 2. 0–4.5 μg/μL,[203] while the critical plasma fibrinogen level below which hemorrhages can occur is approximately 1.0 μg/μL.[204] Our lab has already observed a 29% reduction in the plasma fibrinogen (2.62 ± 0.20 to 1.85 ± 0.14μg/μL) following illumination.[186] However, RiaSTAP was added at a final concentration of 1 μg/μL to the Mirasol treated WB and compared with the control WB without the addition of RiaSTAP. The delta value decreased significantly between the WB enriched with RiaSTAP pre- and post-treatment with Mirasol, which enforced the hypothesis that RiaSTAP could be used to correct fibrinogenemia post-treatment with Mirasol. Surprisingly, the delta of MCF, reflecting the supplementation with RiaSTAP in the PI-treated WB and WB without treatment or RiaSTAP, was steady at all dilution ratios with hemodiluted blood and despite increased blood replacement ratio.

Several different pathogen inactivation techniques are currently on the market and can be applied to PC and plasma. Only the Mirasol technology has been applied so far to WB to mitigate pathogens and avoid the challenge of using treated blood components in every massively bleeding patient. Although *in vivo* radiolabel and recovery studies on PI-treated WB in animals in different settings have demonstrated variable changes in *in vitro* quality, none of these changes have translated into significant alterations in post-transfusion WB variables.[186,195,205] A

clinical trial is currently underway with human RBCs following WB treatment (AIMS Study; NCT02118428).[206]

As with all models, this study has its limitations. Fibrinogen levels for hemodiluted blood or PI-treated WB were not measured. Whether these ROTEM signatures are predictive for the clinical use of pathogen-reduced WB and WB spiked with RiaSTAP, remains to be determined with clinical trials. The study reported here suggests that if WB is superior to a balanced transfusion strategy (reconstituted WB: RBCs, plasma, and PCs in a 1:1:1 ratio) for trauma patients,[172 207] the effects of Mirasol treatment on clot firmness might be modified by coagulation factor supplementation post-treatment.

Importantly, this study was performed without leukoreduction of the WB and while Mirasol will inactivate leukocytes and prevent their proliferation, it is unknown whether the inactivated WBC could affect the quality of the ROTEM hemostatic test. Even with these limitations, this study suggests a potential solution to the apparent reduction in hemostatic capability of WB caused by treatment with Mirasol; the use of fibrinogen supplementation appears to largely correct the Mirasol defect.

Chapter 5: Conclusion

Platelet transfusion is based on recipient need; it is essential that patients suffering from hemorrhage receive platelet concentrates along with blood products as a part of their damage control resuscitation protocol. The potential for an ineffective clinical response to platelet transfusion remains a significant concern. Currently, no *in vitro* tests have been developed for routine use to demonstrate the quality of transfusion products prior to transfusions. Previous studies have also failed to reveal any correlation between corrected count increment, as measured by 1-hour CCI, or the WHO bleeding score, with PC age, and no optimal tool has yet been discovered to reflect PC quality.[57–59]

In the best interests of the patient, stored platelets should be transfused with clear pre-transfusion functionality testing. Key points to consider are: Will the patients receive the greatest possible benefit of their blood transfusions? What is the best way to measure blood product quality? It is of the utmost importance to determine whether clinical efficacy can be predicted by *in vitro* quality tests performed prior to transfusions.

In the interest of proposing means for resolving these essential challenges, this study has assessed whether thromboelastography (TEG) and/or rotational thromboelastometry (ROTEM), both being techniques currently employed in operating suites to assess the hemostatic states of patients susceptible to bleeding, might be applied to measure the quality of PCs. Unlike other *in vitro* platelet tests, TEG and ROTEM offer a promising opportunity to assess platelet function in a full hemostatic reaction, despite the system's lack of endothelial cells.

In its proposition for this novel study, this thesis research has sought to address these questions by differentiating between TEG responses to WB, PRP and PPP in fresh blood samples from healthy individuals, to determine the most desirable sample reconstitution method to obtain meaningful TEG measurements from PCs. In addition, the findings from the preliminary studies for this thesis have demonstrated that clot formation time, rate of platelet fibrin interaction, and maximum clot firmness are favorable parameters to reflect the usual, storage-related changes in PCs. Also, a baseline for platelet contribution, using cytochalasin D, was established to differentiate between hyper-, moderate-, and minimally-responsive platelets.

Once the assay optimizations between TEG and ROTEM were achieved and presented interchangeable results, our research then moved on to detect poor quality PCs, and revealed their correlation with other *in vitro* tests (pH, platelet activation using CD62P, and platelet apoptosis using annexin V). The project also examined the contribution of PMVs to clot formation in PCs. Once our project categorized the traditional storage conditions of PCs by TEG/ROTEM, it progressed to determine the effect of pathogen inactivation treatment (PI) using riboflavin/UV light (Mirasol) on the hemostatic potential of BCPC, plasma, and WB.

5.1 The significance of the thesis

The work described in the thesis addresses these challenges, by confronting the need for innovative approaches to platelet concentrate function assessments. As TEG and ROTEM were designed for use with whole blood samples, the results in **Chapter 2** describe their adaptation to evaluate the procoagulant activity of stored platelets. The study differentiates between TEG responses to WB, PRP and PPP (Appendix1), and provides a number of parameters for TEG and

ROTEM read-outs which provide the most meaningful reflection of platelet quality; this work allowed us to confirm that the results from the two techniques are interchangeable, Figure 5-1 and Table 5.1.

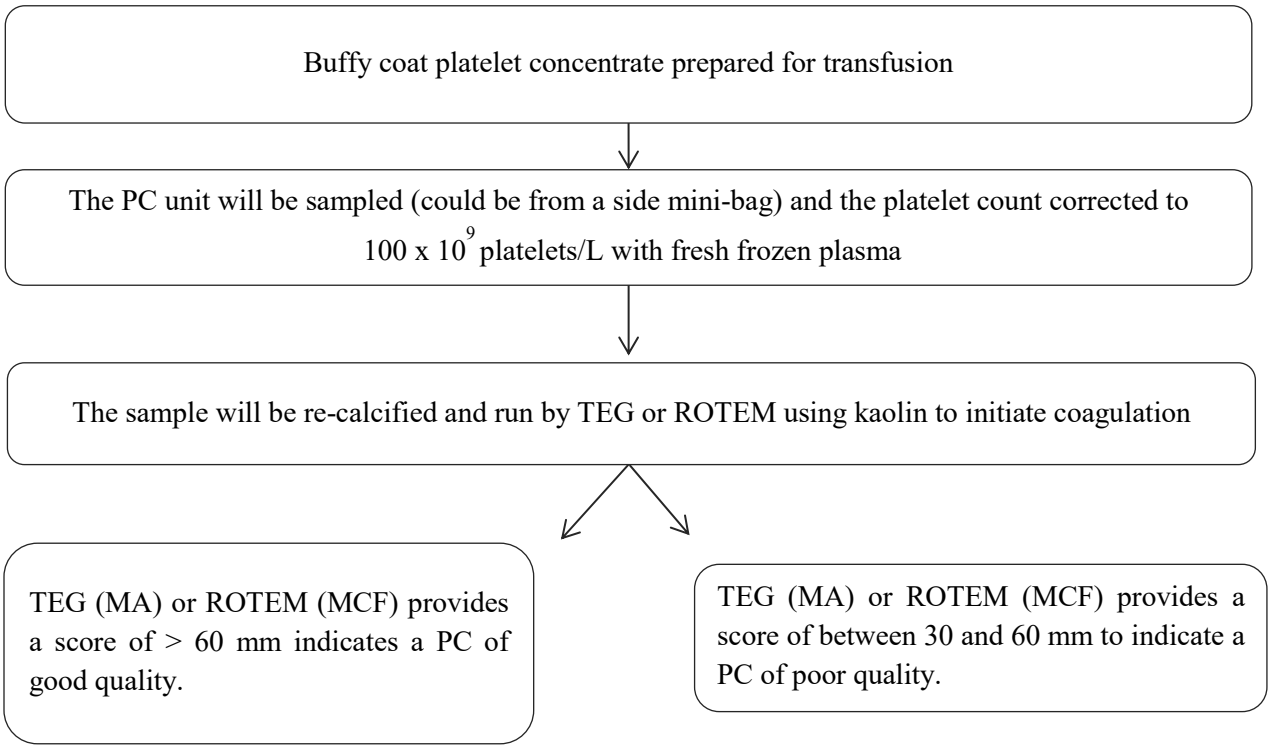


Figure 5- 1: TEG/ROTEM algorithm to guide the functional quality of platelet in PCs prior to transfusion.

Table 5.1. Summarizing TEG and ROTEM measurements using kaolin of buffy coat PCs as a function of storage time at platelet concentration 100 x 10^9/L on Days 2, 5, and 8.

	Day 2		Day 5		Day 8	
	TEG	ROTEM	TEG	ROTEM	TEG	ROTEM
R-time/CT (min.)	6.8 ± 0.9	7.93 ± 0.5	7.5 ± 0.8	8.3 ± 0.3†	7.7 ± 1.2	8.6 ± 0.4
K-time/CFT (min.)	0.9 ± 0.2	1.05 ± 0.2	0.9 ± 0.08	1.08 ± 0.1†	0.9 ± 0.13	1.2 ± 0.1
Alpha	76.5 ± 1.6	77.6 ± 2.5	77.2 ± 2	77 ± 2.1	75.1 ± 3.4	76.2 ± 1.9
MA/ MCF	67.9 ± 1.5	66.4 ± 0.9	66.7 ± 1.7	66.2 ± 1.1	64.4 ± 5.3	65.6 ± 1.5
Kaolin was used as an initiator of the coagulation intrinsic pathway prior to recalification with CaCl2. Results are reported as means of eight independent replicates ±SD.						

The clot strength of the PC represented by MA or MCF provides a score of between 30 and 60 mm to indicate a PC of poor quality, and a score of less than 30 mm to indicate a non-responsive PC when platelet count is 100 x 10^9/L. In the presence of cytochalasin D, an inhibitor of actin polymerisation, TEG shows potential in assessing platelets (55%) and fibrinogen (45%) in regards to the potential for clot formation in PC, unlike these components' contribution in WB.

The system is important in its potential to detect poorly stored PC in cases of incorrect routine PC production or storage. TEG displays a high correlation with other *in vitro* tests such as CD62P and annexin V cell surface expression. The findings indicate that high PMVs contribute toward the hemostatic analysis of PCs by revealing accelerations in the rate of clot formation and clot strength; this builds on current knowledge that units with high PMV might be beneficial for patients with severe bleeding.

The findings reported in **Chapter 3** provide an *in vivo* simulation of an *in vitro* situation of multiple pathogen-reduced components in transfusion packages employed for MTP. Previous studies demonstrate that PI treatment has a negative impact on all *in vitro* parameters. Moreover, this treatment method accelerates platelet storage lesions. Hess *et al.* reported a mathematical analysis used to predict the PI effects on components using published reports[140].

As part of this thesis work, a further step was taken to test their model in the laboratory using ROTEM. Different packages of blood components (RBC, plasma, and BCPC) were created with varied degrees of hematocrit; their hemostatic functionality was then studied in relation to trauma treatment. Only PI-treated plasma, BCPC or controls were employed, but the RBC unit was not PI-treated.

The findings of these studies demonstrate that the BCPC illumination has an impact on platelet functionality, albeit to a lesser degree than that of other *in vitro* tests, which suggest a greater amount of deterioration of the platelet. These studies were built upon previously published investigations in which the blood combination ratio was crucial for the best clot formation. Creating hemodiluted blood with a hematocrit 20% was crucial for establishing a baseline for the hemostatic level used in assessing the products; this determination might be applicable to future research. Moreover, this assessment might further serve to guide the decision to perform transfusions with pathogen inactivated components for various levels of hemorrhage, but this conclusion should nonetheless be interpreted with caution and consideration should be given regarding alternative means to promote hemostasis. For example, should the patient undergo massive transfusions, ROTEM suggests that greater than 50% blood replacement using PI-treated platelets and plasma would threaten hemostasis.

Experimental results simulating transfusions of PI-treated WB to patient with low hematocrit, as described in **Chapter 4**, suggest that clot signatures were affected in this model by the use of Mirasol-treated WB. The ratio of hemodiluted blood to transfusion packages affects the CFT, alpha value and MCF of the clot signature. A level of 30% blood replacement or higher with Mirasol-treated WB reduces the hemostasis efficacy in patients with severe bleeding. This study suggests the need for compensation with coagulation factors like fibrinogen (RiaSTAP) to enhance coagulation. Significant reductions in the delta value of MA in PI-treated WB, as compared to the control, and supplementations RiaSTAP as compared to the control, warrant further investigations.

The effectiveness of RiaSTAP would likely increase PI usage in WB and undoubtedly cause improved compliance among patients. This is because the finding in this study shows that fibrinogen supplementation following the PI-treatment could correct the potential reduction in hemostasis caused by PI-treatment. RiaSTAP is considered the most efficient fibrinogen replacement product. This is because it provides the optimum and consistent fibrinogen concentration in low volume. It is readily available in the emergency setting and easy to use. Therefore, future research focused on applying our findings clinically is expected to benefit patients undergoing severe bleeding and PI-treated WB transfusion, to achieve the adequate hemostasis. Also, these findings will provide direction for future studies aimed to measure compensations with other reduced coagulation factor by the impact of the PI-treatment, such as Factor VIII.

5.2 The implications of adapting TEG and ROTEM in blood centers

In many aspects, TEG and ROTEM are revealed as being significantly more effective than other *in vitro* tests of platelet function due to their potential to reflect the comprehensive hemostatic profile. Although none of the currently used *in vitro* quality tests have advanced to the level of characterizing PC quality prior to blood transfusions, the successful attempts conducted by both Bontekoe *et al.* and ourselves suggest that potential exists for applying a hemostatic analysis for blood products pre-transfusions to patients. The Bontekoe *et al.* study showed that TEG could be used to assess fresh or stored apheresis PC; they further suggested that the technology could be adapted to differentiate between good and bad storage conditions[121]. In this thesis, further steps were taken to substantiate this claim.

One of the most important properties of TEG and ROTEM is the development in its purpose of usage. It was initially designed to measure global hemostatic function. It was subsequently employed clinically for use with a patient undergoing liver transplant surgery, and then for a patient with coagulopathy. The use of TEG and ROTEM are increasing in many surgery suites for cardiac patients undergoing surgeries and patients with certain bleeding disorders to detect requirements for different blood products.

As there is always a percentage of unsuccessful platelet transfusions resulting due to low or absent procoagulant function, ROTEM and TEG have been identified as suitable methods for validating effective blood products and ensure that patients will receive the most suitable one.

The procoagulant effect of platelet microvesicles (PMVs) in the unit has also been demonstrated for the first time in the setting of platelet transfusion product assessment by a hemostatic analysis. Previously, there has been some uncertainty concerning that high PMVs present in

animal and human thrombi [208,209], but our study has confirmed the PMVs impact on clot formation, described in Chapter 2. These observations demonstrate the potential of producing a PMV-rich PC and then validating it via the TEG/ROTEM.

From previous studies conducted *in vitro* into pathogen reduction in blood products and the improvements obtained in minimizing their impact, it is anticipated that TEG/ROTEM will have an expanded capacity with the ability to assess the hemostatic impact on any changes in the treatment procedure or the additive solution. For instance, TEG/ROTEM might offer a screening platform for the development of new platelet and plasma products or product treatments. They could be employed to assess such new plasma products as lyophilized plasma in simulation models very close to the actual trauma transfusion scenario.

Our findings suggest that clotting and clot forming time from TEG/ROTEM are likewise useful in determining the significant impact of pathogen reduction on coagulation factors. This builds upon existing knowledge that pathogen reduction can decrease coagulation factor activity, and that it has demonstrated that removing cellular material from plasma facilitates the ability of TEG/ROTEM to reflect coagulation factor activity, allowing the development of an optimized pathogen reduction technique. This study has furthered our understanding of the potential benefits of a comprehensive treatment of whole blood rather than of individual treatments. For instance, the findings show that the Mirasol treatment of individual products (plasma or platelets) increases clotting time two- to four-fold when testing the PPP.

5.3 Future directions

One major aspect of this work evaluated the potential functionality of low or highly responsive platelet concentrates (**Chapter 2**). The findings indicate that maximum clot formation and the rate of platelet-fibrin interactions may be parameters that reflect functionality. Due to the high standard of operations within the Canadian Blood Services, we were unable to detect units from routine production that possessed low quality; we were thus required to artificially simulate units exhibiting low responsiveness to platelet agonists. Consequently, it is unclear whether gas permeability, and the shaking or non-shaking of blood units, are the only factors that might reduce clot formation rate and strength. Further criteria must exist related to donor platelet responsiveness, or the level of hemostasis in the recipient. Future studies are required to investigate and correlate findings *in vivo* and *in vitro*.

To determine whether PMV-rich PC or WB has a better coagulability than normal for specific patients, experiments might be designed and tested using similar protocols as those described in **Chapter 2**. That would be an efficient means of validating the potential for recipients to benefit from PMV.

Platelet concentrate is an essential element for prophylactic and therapeutic transfusions. Platelet transfusions have been shown to be useful for the treatment of trauma patients with severe bleeding. Therefore, the potential to assess PC units more rapidly in order to minimize bleeding and maximize blood hemostasis in patients as soon as possible calls for further work to accelerate the testing time. In this study, the test processing time was of one hour's duration, but it might in various ways be decreased to 30 minutes through the use of multiple agonists to decrease clotting time.

It would be much more desirable for patients to receive PC units previously assessed for their functionality than random units of unknown functionality. This might reduce mortality and accelerate healing. TEG and ROTEM, have achieved adequate success in reflecting PC functionality; as such, they hold promise as an optimized technique, not only for PCs but also for plasma and reconstituted WB assessments in blood centers.

References

1. Hogshire LC, Patel MS, Rivera E, Carson JL. Evidence review: periprocedural use of blood products. J Hosp Med. 2013 Nov;8(11):647–52.
2. Shah A, Stanworth SJ, McKechnie S. Evidence and triggers for the transfusion of blood and blood products. Anaesthesia. 2015 Jan;70 Suppl 1:10–9, e3-5.
3. Sturk A, Burt L, Hakvoort T, Cate J, Crawford N. The effect of storage on platelet morphology. Transfusion. 1982;22(2):115–20.
4. Slichter SJ, Harker LA. Thrombocytopenia: mechanisms and management of defects in platelet production. Clin Haematol. 1978 Oct;7(3):523–39.
5. Hanson SR, Slichter SJ. Platelet kinetics in patients with bone marrow hypoplasia: evidence for a fixed platelet requirement. Blood. 1985 Nov;66(5):1105–9.
6. Pandey S, Vyas GN. Adverse effects of plasma transfusion. Transfusion. 2012 May;52 Suppl 1(1537–2995):65S–79S.
7. Murphy MF, Brown M CP. Guidelines for the use of platelet transfusions. Br J Haematol. 2003 Jul;122(1):10–23.
8. New H V, Stanworth SJ, Engelfriet CP, Reesink HW, McQuilten ZK, Savoia HF, et al. Neonatal transfusions. Vox Sang. 2009;96(1):62–85.
9. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, et al. Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. Crit Care Med. 2013 Feb;41(2):580–637.
10. Slichter SJ. Evidence-based platelet transfusion guidelines. Hematol Am Soc Hematol Educ Progr. 2007;172–8.

11. Working Party:, Association of Anaesthetists of Great Britain & Ireland, Obstetric Anaesthetists' Association, Regional Anaesthesia UK. Regional anaesthesia and patients with abnormalities of coagulation. Anaesthesia. 2013 Sep;68(9):966–72.

12. Lieberman L, Bercovitz RS, Sholapur NS, Heddle NM, Stanworth SJ, Arnold DM. Platelet transfusions for critically ill patients with thrombocytopenia. Blood. 2014 Feb;123(8):1146–51.

13. Chen Y, Corey SJ, Kim O, Alber MS. Systems biology of platelet-vessel wall interactions. Adv Exp Med Biol. 2014;844:85–98.

14. Thon JN, Italiano JE. Platelets: production, morphology and ultrastructure. Handb Exp Pharmacol. 2012;(210):3–22.

15. Jones CI. Platelet function and ageing. Mamm Genome. 2016 Aug;27(7–8):358–66.

16. Ghoshal K, Bhattacharyya M. Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. ScientificWorldJournal. 2014:781857.

17. Heemskerk JWM, Kuijpers MJE, Munnix ICA, Siljander PRM. Platelet collagen receptors and coagulation. a characteristic platelet response as possible target for antithrombotic treatment. Trends Cardiovasc Med. 2016 Nov;15(3):86–92.

18. Manon-Jensen T, Kjeld NG, Karsdal MA. Collagen-mediated hemostasis. J Thromb Haemos. 2016 Mar;14(3):438–48.

19. Stegner D, Nieswandt B. Platelet receptor signaling in thrombus formation. J Mol Med. 2011 Feb 7;89(2):109–21.

20. Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thromb Haemost. 2009 Aug;102(2):248–57.

21. Hoffman M, Monroe DM. A cell-based model of hemostasis. Thromb Haemost. 2001

Jun;85(6):958–65.

22. Smith SA. The cell-based model of coagulation. J Vet Emerg Crit Care. 2009 Feb 1;19(1):3–10.

23. Yun S, Sim E, Goh R, Park J, Han J. Platelet activation: the mechanisms and potential biomarkers. Biomed Res Int. 2016;2:90.

24. Plow EF, Freaney DE, Plescia J, Miles LA. The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. J Cell Biol. 1986 Dec;103(6 Pt 1):2411–20.

25. Kazmi RS, Boyce S, Lwaleed BA. Homeostasis of hemostasis: the role of endothelium. Semin Thromb Hemost. 2015 Sep;41(6):549–55.

26. Koseoglu S, Flaumenhaft R. Advances in platelet granule biology. Curr Opin Hematol. 2013 Sep;20(5):464–71.

27. Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. Nat Struct Mol Biol. 2009 Sep;16(9):961–6.

28. Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. Blood. 2014 May 1;123(18):2759–67.

29. Thomas MR, Storey RF. The role of platelets in inflammation. Thromb Haemost. 2015 Aug 31;114(3):449–58.

30. Cox D, Kerrigan SW, Watson SP. Platelets and the innate immune system: mechanisms of bacterial-induced platelet activation. J Thromb Haemost. 2011 Jun;9(6):1097–107.

31. Del Conde I, Crúz MA, Zhang H, López JA, Afshar-Kharghan V. Platelet activation leads to activation and propagation of the complement system. J Exp Med. 2005 Mar 21;201(6):871–9.

32. Klinger MHF, Jelkmann W. Role of blood platelets in infection and inflammation. J Interferon Cytokine Res. 2002 Sep;22(9):913–22.

33. Simon TL. The collection of platelets by apheresis procedures. Transfus Med Rev. 1994 Apr;8(2):132–45.

34. Devine D V, Serrano K. The platelet storage lesion. Clin Lab Med. 2010 Jun;30(2):475–87.

35. de Wildt-Eggen J, Nauta S, Schrijver JG, van Marwijk Kooy M, Bins M, van Prooijen HC. Reactions and platelet increments after transfusion of platelet concentrates in plasma or an additive solution: a prospective, randomized study. Transfusion. 2000 Apr;40(4):398–403.

36. J L. Standards for blood banks and transfusion services. 29th ed. Bethesda, MD: AABB; 2014.

37. European Committee on Blood Transfusion: Guide to the preparation, use and quality assurance of blood components. 18th ed. Strasbourg: EDQM; 2015.

38. Benjamin RJ, McDonald CP. The international experience of bacterial screen testing of platelet components with an automated microbial detection system: a need for consensus testing and reporting guidelines. Transfus Med Rev. 2014 Apr;28(2):61–71.

39. Jacobs MR, Smith D, Heaton WA, Zantek ND, Good CE, PGD Study Group. Detection of bacterial contamination in prestorage culture-negative apheresis platelets on day of issue with the Pan Genera Detection test. Transfusion. 2011 Dec;51(12):2573–82.

40. Aster RH. Effect of acidification in enhancing viability of platelet concentrates current status. Vox Sang. 1969 Jul;17(1):23–7.

41. Mourad N. A simple method for obtaining platelet concentrates free of aggregates.

Transfusion. 1968;8(1):48.

42. Devine D, Bradley A, Maurer E, Levin E, Chahal S, Serrano K, et al. Effects of prestorage white cell reduction on platelet aggregate formation and the activation state of platelets and plasma enzyme systems. Transfusion. 1999 Jul;39(7):724–34.

43. The 2009 National Blood Collection and Utilization Survey Report. Washington (DC): US Department of Health and Human Services; 2011.

44. The 2011 National Blood Collection and Utilization Survey Report. Washington (DC): US Department of Health and Human Services; 2013.

45. Stroncek DF, Rebulla P. Platelet transfusions. Lancet. 2007 Jan;370(9585):427–38.

46. Gando S. Disseminated intravascular coagulation in trauma patients. Semin Thromb Hemost. 2001 Dec;27(6):585–92.

47. Murphy S, Gardner FH. Platelet preservation. N Engl J Med. 1969 May 15;280(20):1094–8.

48. Murphy S. Radiolabeling of PLTs to assess viability: a proposal for a standard. Transfusion. 2004 Jan;44(1):131–3.

49. AuBuchon JP, Herschel L, Roger J. Further evaluation of a new standard of efficacy for stored platelets. PG - 1143-50. Transfusion. 2005;45(7):1143–50.

50. AuBuchon JP, Herschel L, Roger J, Murphy S. Preliminary validation of a new standard of efficacy for stored platelets. Transfusion. 2004 Jan;44(1):36–41.

51. Murphy MD. What's so bad about old platelets? Transfusion. 2002 Jul;42(7):809–11.

52. Murphy S, Gardner FH. Effect of storage temperature on maintenance of platelet viability: Deleterious effect of refrigerated storage. N Engl J Med. 1969 May;280(20):1094–8.

53. Heuft H-G, Goudeva L, Krauter J, Peest D, Buchholz S, Tiede A. Effects of platelet

concentrate storage time reduction in patients after blood stem cell transplantation. Vox Sang. 2013 Jul;105(1):18–27.

54. Slichter SJ, Bolgiano D, Jones MK, Christoffel T, Corson J, Rose L, et al. Viability and function of 8-day-stored apheresis platelets. Transfusion. 2006 Oct;46(10):1763–9.

55. Shanwell A, Diedrich B, Falker C, Jansson B, Sandgren P, Sundkvist L, et al. Paired in vitro and in vivo comparison of apheresis platelet concentrates stored in platelet additive solution for 1 versus 7 days. Transfusion. 2006 Jun;46(6):973–9.

56. Slichter SJ, Bolgiano D, Corson J, Jones MK, Christoffel T, Pellham E. Extended storage of autologous apheresis platelets in plasma. Vox Sang. 2013 May;104(4):324–30.

57. Sigle JP, Medinger M, Stern M, Infanti L, Heim D, Halter J, et al. Prospective change control analysis of transfer of platelet concentrate production from a specialized stem cell transplantation unit to a blood transfusion center. J Clin Apher. 2012;27(4):178–82.

58. Goodrich RP, Li J, Pieters H, Crookes R, Roodt J, Heyns A du P. Correlation of in vitro platelet quality measurements with in vivo platelet viability in human subjects. Vox Sang. 2006 May;90(4):279–85.

59. MacLennan S, Harding K, Llewelyn C, Choo L, Bakrania L, Massey E, et al. A randomized noninferiority crossover trial of corrected count increments and bleeding in thrombocytopenic hematology patients receiving 2- to 5- versus 6- or 7-day-stored platelets. Transfusion. 2015 Aug;55(8):1856–65; quiz 1855.

60. Labrie A, Marshall A, Bedi H, Maurer-Spurej E. Characterization of platelet concentrates using dynamic light scattering. Transfus Med Hemotherapy. 2013;40(2):93–100.

61. Mittal K, Kaur R. Platelet storage lesion: An update. Asian J Transfus Sci. 2015;9(1):1–3.

62. Forum I. Evaluation of stored platelets. Vox Sang. 2004;86(3):203–23.

63. Slichter SJ, Bolgiano D, Corson J, Jones MK, Christoffel T, Bailey SL, et al. Extended storage of buffy coat platelet concentrates in plasma or a platelet additive solution. Transfusion. 2014;54(9):2283–91.

64. Mintz PD, Anderson G, Avery N, Clark P, Bonner RF. Assessment of the correlation of platelet morphology with in vivo recovery and survival. Transfusion. 2005 Aug;45(2 Suppl):72S–80S.

65. Curvers J, De Wildt-Eggen J, Heeremans J, Scharenberg J, De Korte D, Van Der Meer PF. Flow cytometric measurement of CD62P (P-selectin) expression on platelets: a multicenter optimization and standardization effort. Transfusion. 2008;48(7):1439–46.

66. Levin E, Serrano K, Devine D. Standardization of CD62P measurement: results of an international comparative study. Vox Sang. 2013 Jul;105(1):38–46.

67. Jain A, Marwaha N, Sharma RR, Kaur J, Thakur M, Dhawan HK. Serial changes in morphology and biochemical markers in platelet preparations with storage. Asian J Transfus Sci. 2015;9(1):41–7.

68. Kriebardis A, Antonelou M, Stamoulis K, Papassideri I. Cell-derived microparticles in stored blood products: innocent-bystanders or effective mediators of post-transfusion reactions? Blood Transfus. 2012 May;10(Suppl 2):s25–38.

69. Aatonen MT, Öhman T, Nyman TA, Laitinen S, Grönholm M, Siljander PR-M. Isolation and characterization of platelet-derived extracellular vesicles. J Extracell Vesicles. 2014 Aug 6;3:103–20.

70. Aatonen M, Grönholm M, Siljander PR-M. Platelet-derived microvesicles: multitalented participants in intercellular communication. Semin Thromb Hemost. 2012 Feb;38(1):102–13.

71. Sinauridze EI, Kireev DA, Popenko NY, Pichugin A V, Panteleev MA, Krymskaya O V, et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost. 2007 Mar;97(3):425–34.

72. Vandromme MJ, McGwin G, Marques MB, Kerby JD, Rue LW, Weinberg JA. Transfusion and pneumonia in the trauma intensive care unit: an examination of the temporal relationship. J Trauma. 2009 Jul;67(1):97–101.

73. Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, et al. Duration of red-cell storage and complications after cardiac surgery. N Engl J Med. 2008 Mar 20;358(12):1229–39.

74. Piccin A, Murphy WG, Smith OP. Circulating microparticles: pathophysiology and clinical implications. Blood Rev. 2007 May;21(3):157–71.

75. Xiao HY, Matsubayashi H, Bonderman DP, Bonderman PW, Reid T, Miraglia CC, et al. Generation of annexin V-positive platelets and shedding of microparticles with stimulus-dependent procoagulant activity during storage of platelets at 4 degrees C. Transfusion. 2000 Apr;40(4):420–7.

76. Chang CP, Zhao J, Wiedmer T, Sims PJ. Contribution of platelet microparticle formation and granule secretion to the transmembrane migration of phosphatidylserine. J Biol Chem. 1993 Apr 5;268(10):7171–8.

77. Kraemer L, Raczat T, Weiss DR, Strobel J, Eckstein R, Ringwald J. Correlation of the hypotonic shock response and extent of shape change with the new ThromboLUX ™. Vox Sang. 2015 Aug;109(2):194–6.

78. DiMinno G, Silver MJ, Murphy S. Stored human platelets retain full aggregation potential in response to pairs of aggregating agents. Blood. 1982 Mar;59(3):563–8.

79. Murphy S. Utility of in vitro tests in predicting the in vivo viability of stored PLTs. Transfusion. 2004 Apr;44(4):618–9; author reply 619.

80. Semple JW, Italiano JE, Freedman J. Platelets and the immune continuum. Nat Rev Immunol. 2011 Apr;11(4):264–74.

81. Boudreau LH, Duchez A-C, Cloutier N, Soulet D, Martin N, Bollinger J, et al. Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. Blood. 2014 Oct;124(14):2173–83.

82. Kelly AM, Garner SF, Herbert N, RGN SEN, Godec TR, Kahan BC, et al. The effect of variation in donor platelet function on transfusion outcome: a semi-randomised, double blind, controlled trial (PROmPT). Blood. 2014;124(21):595.

83. Li J, Kuter DJ. The end is just the beginning: megakaryocyte apoptosis and platelet release. Int J Hematol. 2001 Dec;74(4):365–74.

84. Johnson L, Schubert P, Tan S, Devine D V, Marks DC. Extended storage and glucose exhaustion are associated with apoptotic changes in platelets stored in additive solution. Transfusion. 2016 Feb;56(2):360–8.

85. Johansson PI, Stissing T, Bochsen L, Ostrowski SR. Thrombelastography and tromboelastometry in assessing coagulopathy in trauma. Scand J Trauma Resusc Emerg Med. 2009;17(1):45.

86. Kang Y. Thromboelastography in liver transplantation. Semin Thromb Hemost. 1995;21:34–44.

87. Bolliger D, Seeberger MD, Tanaka KA. Principles and practice of thromboelastography in clinical coagulation management and transfusion practice. Transfus Med Rev. 2012;26(1):1–13.

88. Manufacturer's package insert. Boston (Mass), Haemonetics Corp.; 2010.

89. Lang T, Bauters A, Braun SL, Pötzsch B, von Pape K-W, Kolde H-J, et al. Multi-centre investigation on reference ranges for ROTEM thromboelastometry. Blood Coagul Fibrinolysis. 2005 Jun;16(4):301–10.

90. Perry D, Fitzmaurice D, Kitchen S, Mackie I, Mallett S. Point-of-care testing in haemostasis. Br J Haematol. 2010 Sep;150(5):501–14.

91. Mulcahy, Andrew A, Kapinos K, Briscombe B, Uscher-Pines L. Toward a sustainable blood supply in the United States: an analysis of the current system and alternatives for the future. Santa Monica, CA: RAND Corporation; 2016.

92. Spiess BD, Tuman KJ, McCarthy RJ, DeLaria GA, Schillo R, Ivankovich AD. Thromboelastography as an indicator of post-cardiopulmonary bypass coagulopathies. J Clin Monit. 1987 Jan;3(1):25–30.

93. Ganter MT, Hofer CK. Coagulation monitoring: current techniques and clinical use of viscoelastic point-of-care coagulation devices. Anesth Analg. 2008 May;106(5):1366–75.

94. Williams B, Mcneil J, Crabbe A, Tanaka KA. Practical Use of Thromboelastometry in the Management of Perioperative Coagulopathy and Bleeding. Transfus Med Rev. 2016;1–15.

95. Kirchner C, Dirkmann D, Treckmann JW, Paul A, Hartmann M, Saner FH, et al. Coagulation management with factor concentrates in liver transplantation: a single-center experience. Transfusion. 2014 Oct;54(10 Pt 2):2760–8.

96. Girdauskas E, Kempfert J, Kuntze T, Borger MA, Enders J, Fassl J, et al. Thromboelastometrically guided transfusion protocol during aortic surgery with circulatory arrest: a prospective, randomized trial. J Thorac Cardiovasc Surg. 2010 Nov;140(5):1117–24.e2.

97. Fassl J, Matt P, Eckstein F, Filipovic M, Gregor M, Zenklusen U, et al. Transfusion of allogeneic blood products in proximal aortic surgery with hypothermic circulatory arrest: effect of thromboelastometry-guided transfusion management. J Cardiothorac Vasc Anesth. 2013 Dec;27(6):1181–8.

98. Sankarankutty A, Nascimento B, Teodoro da Luz L, Rizoli S. TEG® and ROTEM® in trauma: similar test but different results? World J Emerg Surg. 2012 Aug 22;7 Suppl 1:S3.

99. da Luz LT, Nascimento B, Rizoli S. Thrombelastography (TEG®): practical considerations on its clinical use in trauma resuscitation . Scand J Trauma Resusc Emerg Med. 2013;21(1):29.

100. Salooja N, Perry DJ. Thrombelastography. Blood Coagul Fibrinolysis. 2001 Jul;12(5):327–37.

101. Görlinger K, Saner FH. Prophylactic plasma and platelet transfusion in the critically Ill patient: just useless and expensive or even harmful? BMC Anesthesiol. 2015;15(1):86.

102. Mathai J. Problem of bacterial contamination in platelet concentrates. Transfus Apher Sci. 2009 Oct;41(2):139–44.

103. Lozano M, Cid J. Pathogen inactivation: coming of age. Curr Opin Hematol. 2013;20(6):540–5.

104. Cap AP, Pidcoke HF, Keil SD, Staples HM, Anantpadma M, Carrion R, et al. Treatment of blood with a pathogen reduction technology using ultraviolet light and riboflavin inactivates Ebola virus in vitro. Transfusion. 2016;56:S6–15.

105. Goodrich RP, Doane S, Reddy HL. Design and development of a method for the reduction of infectious pathogen load and inactivation of white blood cells in whole blood products. Biologicals. 2010;38(1):20–30.

106. Fast LD, Nevola M, Tavares J, Reddy HL, Goodrich RP, Marschner S. Treatment of whole blood with riboflavin plus ultraviolet light, an alternative to gamma irradiation in the prevention of transfusion-associated graft-versus-host disease? Transfusion. 2013;53(2):373–81.

107. Allain J-P, Owusu-Ofori AK, Assennato SM, Marschner S, Goodrich RP, Owusu-Ofori S. Effect of Plasmodium inactivation in whole blood on the incidence of blood transfusion-transmitted malaria in endemic regions: the African Investigation of the Mirasol System (AIMS) randomised controlled trial. Lancet. 387(10029):1753–61.

108. El Chaar M, Atwal S, Freimanis GL, Dinko B, Sutherland CJ, Allain J-P. Inactivation of Plasmodium falciparum in whole blood by riboflavin plus irradiation. Transfusion. 2013;53(12):3174–83.

109. Enriquez LJ, Shore-Lesserson L. Point-of-care coagulation testing and transfusion algorithms. Br J Anaesth. 2009;103.

110. Jackson GNB, Ashpole KJ, Yentis SM. The TEG vs the ROTEM thromboelastography/thromboelastometry systems. Anaesthesia. 2009;64:212–5.

111. Luddington RJ. Thrombelastography/thromboelastometry. Clin Lab Haematol. 2005;27(2):81–90.

112. Scarpelini S, Rhind SG, Nascimento B, Tien H, Shek PN, Peng HT, et al. Normal range values for thromboelastography in healthy adult volunteers. Braz J Med Biol Res. 2009;42(12):1210–7.

113. Levin E, Culibrk B, Gyongyossy-Issa MI, Weiss S, Scammell K, LeFresne W, et al. Implementation of buffy coat platelet component production: comparison to platelet-rich plasma platelet production. Transfusion. 2008;48(11):2331–7.

114. Levin E, Jenkins C, Culibrk B, Gyöngyössy-Issa MIC, Serrano K. Development of a quality monitoring program for platelet components: a report of the first four years' experience at Canadian Blood Services. Transfusion 2012;52810-818.

115. Arbaeen AF, Serrano K, Levin E, Devine D V. Platelet concentrate functionality assessed by thromboelastography or rotational thromboelastometry. Transfusion. 2016;16(10):13760.

116. Lang T, Toller W, Gütl M, Mahla E, Metzler H, Rehak P, et al. Different effects of abciximab and cytochalasin D on clot strength in thrombelastography. J Thromb Haemost. 2004;2(1):147–53.

117. Schubert P, Culibrk B, Coupland D, Levin E, Devine D. Impact of sample volume and handling time during analysis on the in vitro quality measurements of platelet concentrates held in syringes. Int J Lab Hematol. 2011 Dec;33(6):579–85.

118. van der Meer PF, Dumont LJ, Lozano M, Bondar N, Wong J, Ismay S, et al. Aggregates in platelet concentrates. Vox Sang. 2015;108(1):96–100.

119. Katori N, Tanaka KA, Szlam F, Levy JH. The effects of platelet count on clot retraction and tissue plasminogen activator-induced fibrinolysis on thrombelastography. Anesth Analg. 2005;100(6):1781–5.

120. Solomon C, Cadamuro J, Ziegler B, Schochl H, Varvenne M, Sorensen B, et al. A comparison of fibrinogen measurement methods with fibrin clot elasticity assessed by thromboelastometry, before and after administration of fibrinogen concentrate in cardiac surgery patients. Transfusion. 2011;51(8):1695–706.

121. Bontekoe IJ, van der Meer PF, de Korte D. Determination of thromboelastographic responsiveness in stored single-donor platelet concentrates. Transfusion.

2014;54(6):1610–8.

122. Venema LF, Post WJ, Hendriks HG, Huet RC, de Wolf JT, de Vries AJ. An assessment of clinical interchangeability of TEG and RoTEM thromboelastographic variables in cardiac surgical patients. Anesth Analg. 2010;111(2):339–44.

123. Kilkson H, Holme S, Murphy S. Platelet metabolism during storage of platelet concentrates at 22 degrees C. Blood. 1984;64(2):406–14.

124. Rendu F, Brohard-Bohn B. The platelet release reaction: granules' constituents, secretion and functions. Platelets. 2001;12(5):261–73.

125. Nesbitt WS, Giuliano S, Kulkarni S, Dopheide SM, Harper IS, Jackson SP. Intercellular calcium communication regulates platelet aggregation and thrombus growth. J Cell Biol. 2003 Mar 31;160(7):1151–61.

126. Tisherman SA, Schmicker RH, Brasel KJ, Bulger EM, Kerby JD, Minei JP, et al. Detailed description of all deaths in both the shock and traumatic brain injury hypertonic saline trials of the Resuscitation Outcomes Consortium. Ann Surg. 2015;261(3):586–90.

127. Rhee P, Joseph B, Pandit V, Aziz H, Vercruysse G, Kulvatunyou N, et al. Increasing trauma deaths in the United States. Ann Surg. 2014;260(1):13–21.

128. Holcomb JB. Optimal use of blood products in severely injured trauma patients. Hematol Am Soc Hematol Educ Progr. 2010;2010:465–9.

129. Duchesne JC, Barbeau JM, Islam TM, Wahl G, Greiffenstein P, McSwain N. Damage control resuscitation: from emergency department to the operating room. Am Surg. 2011;77(2):201–6.

130. Holcomb JB, Tilley BC, Baraniuk S, Fox EE, Wade CE, Podbielski JM, et al. Transfusion of plasma, platelets, and red blood cells in a 1:1:1 vs a 1:1:2 ratio and mortality in patients

with severe trauma: the PROPPR randomized clinical trial. JAMA. 2015;313(5):471–82.

131. Holcomb JB, del Junco DJ, Fox EE, Wade CE, Cohen MJ, Schreiber MA, et al. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. JAMA Surg. 2013 Feb;148(2):127–36.

132. Pohlman TH, Walsh M, Aversa J, Hutchison EM, Olsen KP, Lawrence Reed R. Damage control resuscitation. Blood Rev. 2015;29(4):251–62.

133. Prowse C. Component pathogen inactivation: a critical review. Vox Sang. 2013;104(3):183–99.

134. Devine D V, Schubert P. Pathogen inactivation technologies: the advent of pathogen-reduced blood components to reduce blood safety risk. Hematol Oncol Clin North Am. 2016 Jun;30(3):609–17.

135. Schlenke P. Pathogen inactivation technologies for cellular blood components: an update. Transfus Med hemotherapy. 2014 Jul;41(4):309–25.

136. Reddy HL, Doane SK, Keil SD, Marschner S, Goodrich RP. Development of a riboflavin and ultraviolet light-based device to treat whole blood. Transfusion. 2013;53(1):12047.

137. Schubert P, Coupland D, Culibrk B, Goodrich R, Devine D. Riboflavin and ultraviolet light treatment of platelets triggers p38MAPK signaling: inhibition significantly improves in vitro platelet quality after pathogen reduction treatment. Transfusion. 2013;53(12):3164–73.

138. Bihm DJ, Ettinger A, Buytaert-Hoefen KA, Hendrix BK, Maldonado-Codina G, Rock G, et al. Characterization of plasma protein activity in riboflavin and UV light-treated fresh frozen plasma during 2 years of storage at -30 degrees C. Vox Sang. 2010;98(2):108–15.

139. Ostrowski SR, Bochsen L, Windelãv NA, Salado-Jimena JA, Reynaerts I, Goodrich RP. Hemostatic function of buffy coat platelets in additive solution treated with pathogen reduction technology. Transfusion. 2011;51(2):344–56.

140. Hess JR, Pagano MB, Barbeau JD, Johannson PI. Will pathogen reduction of blood components harm more people than it helps in developed countries? Transfusion. 2016;56(5):1236–41.

141. Validation Task Force of the International Society of Blood Transfusion Working Party on Information Technology. ISBT Guidelines for validation of automated systems in blood establishments. Vol. 98 Suppl 1, Vox sanguinis. 2010 Feb.

142. Maurer-Spurej E, Chipperfield K. Past and future approaches to assess the quality of platelets for transfusion. Transfus Med Rev. 2007;21(4):295–306.

143. Holme S. In vitro assays used in the evaluation of the quality of stored platelets: correlation with in vivo assays. Transfus Apher Sci. 2008;39(2):161–5.

144. Johansson PI, Sorensen AM, Larsen CF, Windelov NA, Stensballe J, Perner A, et al. Low hemorrhage-related mortality in trauma patients in a Level I trauma center employing transfusion packages and early thromboelastography-directed hemostatic resuscitation with plasma and platelets. Transfusion. 2013;53(12):3088–99.

145. Shore-Lesserson L, Manspeizer HE, DePerio M, Francis S, Vela-Cantos F, Ergin MA. Thromboelastography-guided transfusion algorithm reduces transfusions in complex cardiac surgery. Anesth Analg. 1999;88(2):312–9.

146. Anna Ågren Malin Kardell, Anders Östlund, and Agneta Taune Wikman GE. In vitro combinations of red blood cell, plasma and platelet components evaluated by thromboelastography. Blood Transfusion, 2014 Oct; 12(4) 491–496.

147. Snyder E, Raife T, Lin L, Cimino G, Metzel P, Rheinschmidt M, et al. Recovery and life span of 111indium-radiolabeled platelets treated with pathogen inactivation with amotosalen HCl (S-59) and ultraviolet A light. Transfusion. 2004;44(12):1732–40.

148. AuBuchon JP, Herschel L, Roger J, Taylor H, Whitley P, Li J, et al. Efficacy of apheresis platelets treated with riboflavin and ultraviolet light for pathogen reduction. Transfusion. 2005 Aug;45(8):1335–41.

149. Hornsey VS, Drummond O, Morrison A, McMillan L, MacGregor IR, Prowse C V. Pathogen reduction of fresh plasma using riboflavin and ultraviolet light: effects on plasma coagulation proteins. Transfusion. 2009 Oct;49(10):2167–72.

150. Larrea L, Calabuig M, Roldán V, Rivera J, Tsai H-M, Vicente V, et al. The influence of riboflavin photochemistry on plasma coagulation factors. Transfus Apher Sci. 2009 Dec;41(3):199–204.

151. Harr JN Ghasabyan A, Chin TL, Sauaia A, Banerjee A, Silliman CC. MEE. Functional fibrinogen assay indicates that fibrinogen is critical in correcting abnormal clot strength following trauma. Shock. 2013;39(1):45–9.

152. Smith J, Rock G. Protein quality in Mirasol pathogen reduction technology-treated, apheresis-derived fresh-frozen plasma. Transfusion. 2010;50(4):926–31.

153. Jambor C, Reul V, Schnider TW, Degiacomi P, Metzner H, Korte WC. In vitro inhibition of factor XIII retards clot formation, reduces clot firmness, and increases fibrinolytic effects in whole blood. Anesth Analg. 2009;109(4):1023–8.

154. Theusinger OM, Baulig W, Asmis LM, Seifert B, Spahn DR. In vitro factor XIII supplementation increases clot firmness in Rotation Thromboelastometry (ROTEM). Thromb Haemost. 2010;104(2):385–91.

155. Coene J, Devreese K, Sabot B, Feys HB, Vandekerckhove P, Compernolle V. Paired analysis of plasma proteins and coagulant capacity after treatment with three methods of pathogen reduction. Transfusion. 2014;54(5):1321–31.

156. Benjamin RJ, McLaughlin LS. Plasma components: properties, differences, and uses. Transfusion. 2012;52:9S–19S.

157. van Rhenen D, Gulliksson H, Cazenave J-P, Pamphilon D, Ljungman P, Klüter H, et al. Transfusion of pooled buffy coat platelet components prepared with photochemical pathogen inactivation treatment: the euroSPRITE trial. Blood. 2003 Mar;101(6):2426–33.

158. Mirasol Clinical Evaluation Study Group. A randomized controlled clinical trial evaluating the performance and safety of platelets treated with MIRASOL pathogen reduction technology. Transfusion. 2010 Nov;50(11):2362–75.

159. Snyder E, McCullough J, Slichter SJ, Strauss RG, Lopez-Plaza I, Lin J-S, et al. Clinical safety of platelets photochemically treated with amotosalen HCl and ultraviolet A light for pathogen inactivation: the SPRINT trial. Transfusion. 2005 Dec;45(12):1864–75.

160. Schochl H, Frietsch T, Pavelka M, Jambor C. Hyperfibrinolysis after major trauma: differential diagnosis of lysis patterns and prognostic value of thrombelastometry. J Trauma. 2009;67(1):125–31.

161. Shakur H, Roberts I, Bautista R, Caballero J, Coats T, Dewan Y, et al. Effects of tranexamic acid on death, vascular occlusive events, and blood transfusion in trauma patients with significant haemorrhage (CRASH-2): a randomised, placebo-controlled trial. Lancet. 2010;376(9734):23–32.

162. Kashuk JL, Moore EE, Sawyer M, Wohlauer M, Pezold M, Barnett C, et al. Primary fibrinolysis is integral in the pathogenesis of the acute coagulopathy of trauma. Ann Surg.

2010;252(3):434–42.

163. Koch CG, Li L, Duncan AI, Mihaljevic T, Cosgrove DM, Loop FD, et al. Morbidity and mortality risk associated with red blood cell and blood-component transfusion in isolated coronary artery bypass grafting. Crit Care Med. 2006;34(6):1608–16.

164. Norton R, Kobusingye O. Injuries. N Engl J Med. 2013;368(18):1723–30.

165. Johansson PI, Ostrowski SR, Secher NH. Management of major blood loss: An update. Acta Anaesthesiol Scand. 2010;54(9):1039–49.

166. Savage SA, Zarzaur BL, Croce MA, Fabian TC. Redefining massive transfusion when every second counts. J Trauma Acute Care Surg. 2013;74(2):396–400.

167. Rahbar E, Fox E, del Junco D, Harvin J, Holcomb J, Wade C, et al. Early resuscitation intensity as a surrogate for bleeding severity and early mortality in the PROMMTT study. J Trauma Acute Care Surg. 2013;75:15–20.

168. American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Practice guidelines for perioperative blood transfusion and adjuvant therapies: an updated report by the American Society of Anesthesiologists Task Force on Perioperative Blood Transfusion and Adjuvant Therapies. Vol. 105, Anesthesiology. 2006 Jul.

169. Lundsgaard-Hansen P. Treatment of acute blood lLoss. Vox Sang. 1992;63(4):241–6.

170. Johansson PI, Hansen MB, Sorensen H. Transfusion practice in massively bleeding patients: time for a change? Vox Sang. 2005;89(2):92–6.

171. Nepstad I, Reikvam H, Strandenes G, Hess JR, Apelseth TO, Hervig TA. Comparison of in vitro responses to fresh whole blood and reconstituted whole blood after collagen stimulation. Blood Transfus. 2014 Jan 19;12(1):50–5.

172. Spinella PC, Perkins JG, Grathwohl KW, Beekley AC, Holcomb JB. Warm fresh whole blood is independently associated with improved survival for patients with combat-related traumatic injuries. J Trauma. 2009 Apr;66:S69–76.

173. Bjerkvig CK, Strandenes G, Eliassen HS, Spinella PC, Fosse TK, Cap AP, et al. "Blood failure" time to view blood as an organ: how oxygen debt contributes to blood failure and its implications for remote damage control resuscitation. Transfusion. 2016;56:S182–9.

174. Spinella PC, Pidcoke HF, Strandenes G, Hervig T, Fisher A, Jenkins D, et al. Whole blood for hemostatic resuscitation of major bleeding. Transfusion. 2016;56 Suppl 2:S190-202.

175. Snyder EL, Whitley P, Kingsbury T, Miripol J, Tormey CA. In vitro and in vivo evaluation of a whole blood platelet-sparing leukoreduction filtration system. Transfusion. 2010 Oct;50(10):2145–51.

176. Spitalnik S, Triulzi D, Devine D, Dzik W, Eder A, Gernsheimer T, et al. 2015 Proceedings of the National Heart, Lung, and Blood Institute's State of the Science in Transfusion Medicine Symposium. Transfusion. 2015 Sep;55(9):2282–90.

177. Becker GA, Tuccelli M, Kunicki T, Chalos MK, Aster RH. Studies of platelet concentrates stored at 22 C and 4 C. Transfusion. 1973 Mar;13(2):61–8.

178. Strandenes G, De Pasquale M, Cap AP, Hervig TA, Kristoffersen EK, Hickey M, et al. Emergency whole-blood use in the field: a simplified protocol for collection and transfusion. Shock. 2014 May;41:76–83.

179. Jobes DR, Sesok-Pizzini D, Friedman D. Reduced transfusion requirement with use of fresh whole blood in pediatric cardiac surgical procedures. Ann Thorac Surg. 2015 May;99(5):1706–11.

180. Reddoch KM, Montgomery RK, Rodriguez AC, Meledeo MA, Pidcoke HF,

Ramasubramanian AK, et al. Refrigerated platelets are superior compared to standard-of-care and respond to physiologic control mechanisms under microfluidic flow conditions. Blood. 2014 Dec;124(21):2895.

181. Glynn SA, Busch MP, Dodd RY, Katz LM, Stramer SL, Klein HG, et al. Emerging infectious agents and the nation's blood supply: responding to potential threats in the 21st century. Transfusion. 2013 Feb;53(2):438–54.

182. Keil SD, Hovenga N, Gilmour D, Marschner S, Goodrich R. Treatment of platelet products with riboflavin and UV light: Effectiveness against high titer bacterial contamination. J Vis Exp. 2015 Aug;(102):e52820.

183. Tonnetti L, Thorp AM, Reddy HL, Keil SD, Goodrich RP, Leiby DA. Evaluating pathogen reduction of Trypanosoma cruzi with riboflavin and ultraviolet light for whole blood. Transfusion. 2012 Feb;52(2):409–16.

184. El Chaar M, Atwal S, Freimanis GL, Dinko B, Sutherland CJ, Allain J-P. Inactivation of Plasmodium falciparum in whole blood by riboflavin plus irradiation. Transfusion. 2013 Dec;53(12):3174–83.

185. Custer B, Agapova M, Martinez RH. The cost-effectiveness of pathogen reduction technology as assessed using a multiple risk reduction model. Transfusion. 2010;50(11):2461–73.

186. Schubert P, Culibrk B, Karwal S, Serrano K, Levin E, Bu D, et al. Whole blood treated with riboflavin and ultraviolet light: Quality assessment of all blood components produced by the buffy coat method. Transfusion. 2015;55(4):815–23.

187. Pidcoke HF, McFaul SJ, Ramasubramanian AK, Parida BK, Mora AG, Fedyk CG, et al. Primary hemostatic capacity of whole blood: A comprehensive analysis of pathogen

reduction and refrigeration effects over time. Transfusion. 2013;53(1):137–49.

188. Cancelas JA, Rugg N, Fletcher D, Pratt PG, Worsham DN, Dunn SK, et al. In vivo viability of stored red blood cells derived from riboflavin plus ultraviolet light-treated whole blood. Transfusion. 2011 Jul;51(7):1460–8.

189. Elliott BM, Aledort LM. Restoring hemostasis: fibrinogen concentrate versus cryoprecipitate. Expert Rev Hematol. 2013;6(3):277–86.

190. Gröner A. Reply. Pereira A. Cryoprecipitate versus commercial fibrinogen concentrate in patients who occasionally require a therapeutic supply of fibrinogen: risk comparison in the case of an emerging transfusion-transmitted infection. Haematologica. 2008 Feb;93(2):20–5.

191. Screening Donated Blood for Transfusion-Transmissible Infections: Recommendations. World Health Organization. Geneva; 2009.

192. Ziegler B, Schimke C, Marchet P, Stogermuller B, Schochl H, Solomon C. Severe pediatric blunt trauma--successful ROTEM-guided hemostatic therapy with fibrinogen concentrate and no administration of fresh frozen plasma or platelets. Clin Appl Thromb Hemost. 2013;19(4):453–9.

193. Franzblau EB, Punzalan RC, Friedman KD, Roy A, Bilen O, Flood VH. Use of purified fibrinogen concentrate for dysfibrinogenemia and importance of laboratory fibrinogen activity measurement. Pediatr Blood Cancer. 2013 Mar;60(3):500–2.

194. Thiruvenkatarajan V, Pruett A, Adhikary S. Coagulation testing in the perioperative period. Indian J Anaesth. 2014 Sep;58(5):565–72.

195. Okoye OT, Reddy H, Wong MD, Doane S, Resnick S, Karamanos E, et al. Large animal evaluation of riboflavin and ultraviolet light-treated whole blood transfusion in a diffuse,

nonsurgical bleeding porcine model. Transfusion. 2015 Mar;55(3):532–43.

196. Theusinger OM, Goslings D, Studt J-D, Brand-Staufer B, Seifert B, Spahn DR, et al. Quarantine versus pathogen-reduced plasma-coagulation factor content and rotational thromboelastometry coagulation. Transfusion. 2016 Nov;(1537–2995).

197. Van Aelst B, Feys HB, Devloo R, Vanhoorelbeke K, Vandekerckhove P, Compernolle V. Riboflavin and amotosalen photochemical treatments of platelet concentrates reduce thrombus formation kinetics in vitro. Vox Sang. 2015 May;108(4):328–39.

198. Ostrowski SR, Bochsen L, Windelov NA, Salado-Jimena JA, Reynaerts I, Goodrich RP, et al. Hemostatic function of buffy coat platelets in additive solution treated with pathogen reduction technology. Transfusion. 2011;51(2):344–56.

199. Qadri SM, Chen D, Schubert P, Perruzza DL, Bhakta V, Devine D V, et al. Pathogen inactivation by riboflavin and ultraviolet light illumination accelerates the red blood cell storage lesion and promotes eryptosis. Transfusion. 2016 Dec;(1537–2995 (Electronic)).

200. Karri J V, Cardenas JC, Johansson PI, Matijevic N, Cotton BA, Wade CE, et al. In vitro efficacy of RiaSTAP after rapid reconstitution. J Surg Res. 2014 Aug;190(2):655–61.

201. Prüller F, Münch A, Preininger A, Raggam RB, Grinschgl Y, Krumnikl J, et al. Comparison of functional fibrinogen (FF/CFF) and FIBTEM in surgical patients - a retrospective study. Clin Chem Lab Med. 2016 Mar;54(3):453–8.

202. Armand R, Hess JR. Treating coagulopathy in trauma patients. Transfus Med Rev. 2017 Jan;17(3):223–31.

203. Gaffney PJ, Wong MY. Collaborative study of a proposed international standard for plasma fibrinogen measurement. Thromb Haemost. 1992 Oct 5;68(4):428–32.

204. Rossaint R, Bouillon B, Cerny V, Coats TJ, Duranteau J, Fernández-Mondéjar E, et al.

Management of bleeding following major trauma: an updated European guideline. Crit Care. 2010;14(2):R52.

205. Goodrich RP, Murthy KK, Doane SK, Fitzpatrick CN, Morrow LS, Arndt PA, et al. Evaluation of potential immune response and in vivo survival of riboflavin-ultraviolet light-treated red blood cells in baboons. Transfusion. 2009 Jan;49(1):64–74.

206. Owusu-Ofori S, Kusi J, Owusu-Ofori A, Freimanis G, Olver C, Martinez CR, et al. treatment of whole blood with riboflavin and UV light. Shock. 2015 Aug;44:33–8.

207. Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney P, Mehta S, et al. Damage control resuscitation: directly addressing the early coagulopathy of trauma. J Trauma. 2007 Feb;62(2):307–10.

208. 3rd OAP, Mackman N. Microparticles in hemostasis and thrombosis. PG - 1284-97 LID - 10.1161/CIRCRESAHA.110.233056 [doi].

209. Geddings JE FAU - Mackman N, Mackman N. New players in haemostasis and thrombosis. PG - 570-4 LID - 10.1160/TH13-10-0812 [doi]. ;

Appendix

Appendix A: A comparison between blood components within 6 hours of collection

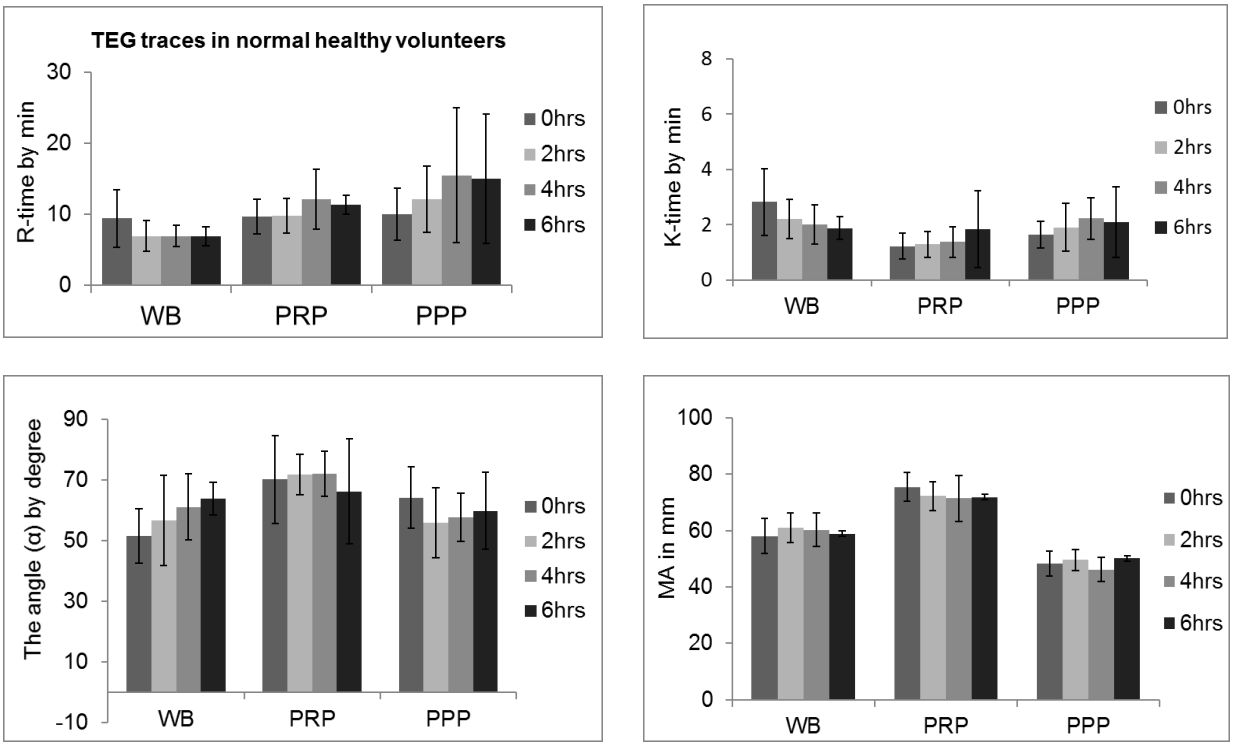
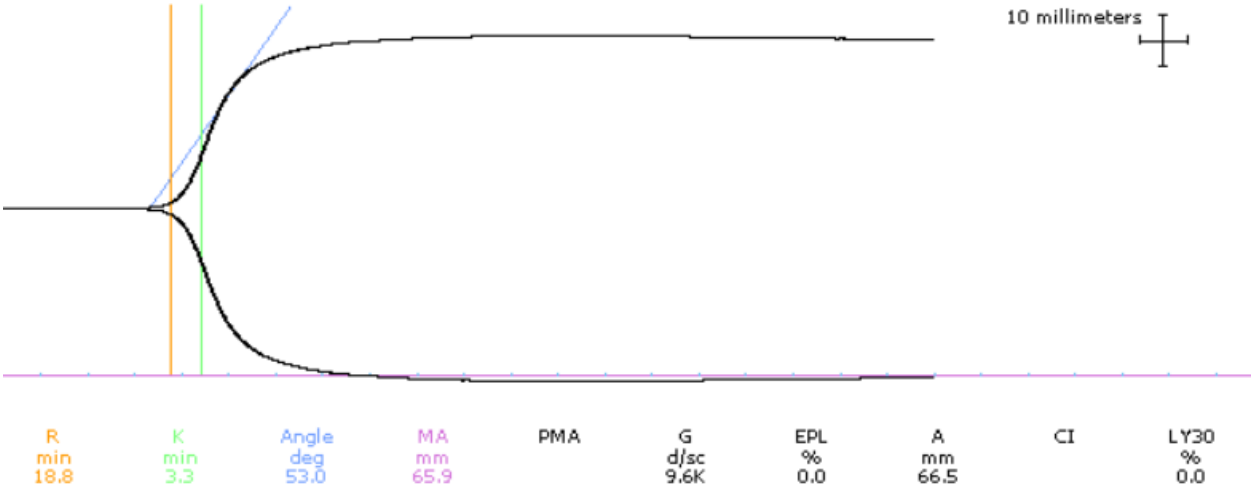


Figure 5: TEG traces of blood components of normal healthy volunteers from 0 to 6 hrs after blood collection.

There is significant difference in MA between WB, PRP, and PPP ($p < 0.005$), n= 8. There was no significant change in MA of the different blood components across all time points

Appendix B: TEG signatures of PCs with good and poor in vitro quality

(A)



(B)

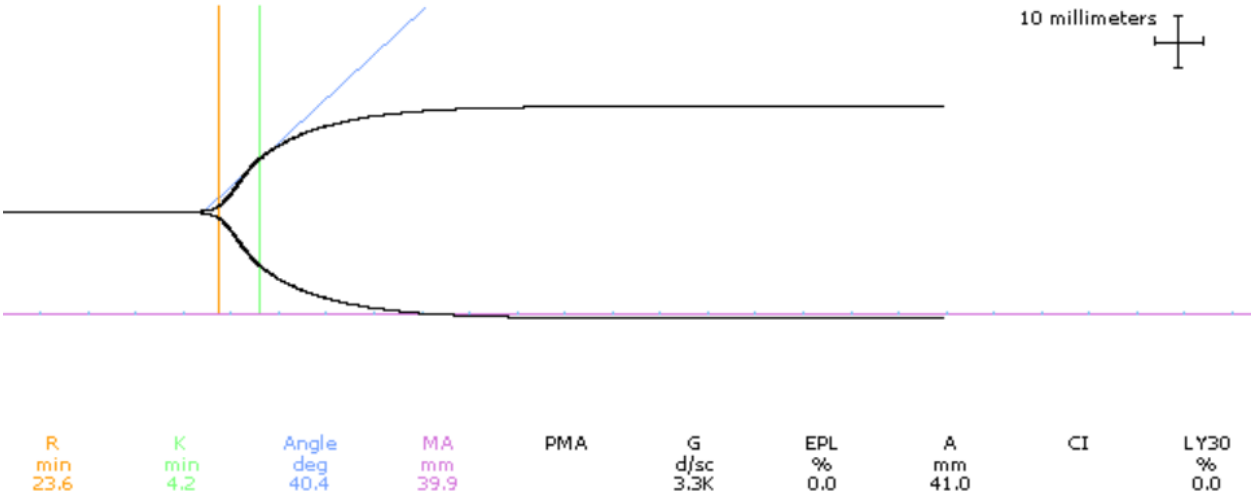


Figure B-1: Representative TEG signatures of (A) PCs with good in vitro quality and (B) PCs with poor in vitro quality stored in no shaking condition and air impermeable bags at 30°C (nS&nA at 30°C), day 5.