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Free oscillation rheometry in the assessment of platelet quality

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ABSTRACT

Platelets play an important role in the haemostatic process in order to seal damaged blood vessels. The platelets form a platelet plug at the damaged area and prevent blood loss. Once the damage to the vessel wall has been covered, the platelets retract the coagulum, to allow the blood to flow freely in the vessel. Free oscillation rheometry (FOR) can be used for analysis of coagulation as measured by clotting time and changes in clot elasticity (G'). Clot G' provides information about the fibrin network in the coagulum and the platelets' ability to retract the coagulum. FOR analysis is performed using the ReoRox[®] 4 instrument. The blood sample is added to a cylindrical sample cup, which is set into free oscillation. The frequency and damping of the oscillation is recorded over time as the blood coagulates. The change in G' is calculated from the frequency and damping measured. Patients with malignant haematological diseases are often thrombocytopenic and require platelet transfusions to prevent or stop bleeding. To ensure good haemostatic function in the recipient it is important that the quality of the platelets used for transfusion is well preserved. The aim of this thesis was to determine the quality of platelet concentrates (PCs), during storage, using various *in vitro* methods, including FOR, and to investigate how various preparation processes affect the quality. We also investigated whether FOR can be used to evaluate the haemostatic status in subjects at risk for thrombosis or bleeding as well as how the haemostatic status was affected by a platelet transfusion.

We show that FOR can provide information about the coagulation properties in subjects at risk of thrombosis (pregnant women) or bleeding (thrombocytopenic patients). We also show that the coagulation as measured by FOR is influenced by red blood cells and the fibrinogen concentration. However, the presence of functional platelets accounted for 90% of the G' . Furthermore we present data that FOR can provide information on the haemostatic effect of platelet transfusions and on the function of the transfused platelets.

PCs produced by two different cell separators showed similar quality during storage for 7 days as assessed by FOR analysis. Leukocytes in the PCs can cause transfusion-associated graft-versus-host disease which can be prevented by gamma irradiation of the PCs. Gamma irradiation did not affect the quality of PCs during 7 days of storage as analysed by FOR. The clotting time was unchanged during the storage period. The capacity of platelets to retract the coagulum was reduced from days 1 to 5 of storage as seen by a prolonged time to reach maximum G' and the reduced mean change in G' per minute. However, if sufficient time is allowed for the platelets to regain their function, the clot will be fully retracted (as seen by a well maintained maximum G'). The FOR parameters were similar for 5- and 7-day old PCs, which, combined with other *in vitro* tests (e.g. hypotonic shock response, changes in pH, swirling, lactate and glucose), support the prolongation of the platelet storage period to 7 days. Intercept[™] treatment of PCs can be performed to inhibit replication of contaminating bacteria in PCs. Intercept[™] treatment of PCs did not diminish the clot-promoting capacity of the platelets as assessed by FOR clotting time.

In conclusion, FOR is a promising method for assessing hyper- and hypocoagulability. It can provide information on the haemostatic effect of platelet transfusions and the function of the transfused platelets. FOR was also shown to be useful for analysing PC quality during different preparation and storage conditions.

LIST OF THE PAPERS

This thesis is based on the work presented in the following papers:

- I. Nahreen Tynngård, Tomas L. Lindahl, Sofia Ramström, Gösta Berlin. Effects of different blood components on clot retraction analysed by measuring elasticity with a free oscillating rheometer. *Platelets* 2006;**17**:545-554.
- II. Nahreen Tynngård, Tomas L. Lindahl, Sofia Ramström, Tuulia Räf, Olof Rugarn, Gösta Berlin. Free oscillation rheometry detects changes in clot properties in pregnancy and thrombocytopaenia. *Accepted for publication in Platelets* 2008.
- III. Nahreen Tynngård, Tomas L. Lindahl, Marie Trinks, Monika Studer, Gösta Berlin. The quality of platelet concentrates produced by COBE Spectra and Trima Accel cell separators during storage for 7 days as assessed by in vitro methods. *Transfusion* 2008; **48**:715-722.
- IV. Nahreen Tynngård, Monika Studer, Tomas L. Lindahl, Marie Trinks, Gösta Berlin. The effect of gamma irradiation on the quality of apheresis platelets during storage for 7 days. *Accepted for publication in Transfusion* 2008.
- V. Nahreen Tynngård, Britt Marie Johansson, Tomas L. Lindahl, Gösta Berlin, Mona Hansson. Effects of intercept pathogen inactivation on platelet function as analysed by free oscillation rheometry. *Transfusion and Apheresis Science* 2008;**38**:85-88.

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ABBREVIATIONS

ACD	Acid-citrate-dextrose
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP	Apheresis
ATP	Adenosine triphosphate
AU	Arbitrary units
BC	Buffy-coat
CAD	Compound adsorption device
cAMP	Cyclic adenosine monophosphate
CCI	Corrected count increment
CO ₂	Carbon dioxide
CPD	Citrate-phosphate-dextrose
CRP	Collagen-related peptide
CT	Clot time
C.V.	Coefficient of variation
D	Damping
FITC	Fluorescein isothiocyanate
FNHTR	Febrile nonhaemolytic transfusion reactions
FOR	Free oscillation rheometry
Fq	Frequency
FS	Forward scatter
G'	Elasticity
G'max	Maximum elasticity
G' max-slope	Maximum change in elasticity per minute
GP	Glycoprotein
GPO	Glycine-proline-hydroxyproline
G-protein	Guanyl-nucleotide-binding protein
HLA	Human leukocyte antigen
HSR	Hypotonic shock response
HTR	Haemolytic transfusion reactions
LDH	Lactate dehydrogenase
MA	Maximum amplitude
MCF	Maximum clot firmness
MFI	Mean fluorescence intensity
PAR	Protease-activated receptor
PAS	Platelet additive solution
PC	Platelet concentrate
PCT	Photochemical treatment
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein
PKC	Protein kinase C
PLC	Phospholipase C
PMTs	Photomultiplier tubes
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PVC	Polyvinyl chloride
R	Reaction time

sP-selectin	Soluble P-selectin
SS	Side scatter
TAFI	Thrombin activatable fibrinolysis inhibitor
TA-GVHD	Transfusion-associated graft-versus-host disease
TCA	Tricarboxylic acid cycle
TF	Tissue factor
TRALI	Transfusion-related acute lung injury
TRAP	Thrombin receptor activating peptide
TXA ₂	Thromboxane A ₂
vWF	von Willebrand factor

INTRODUCTION

“The life of the flesh is in the blood”

Leviticus 17:11

Blood transfusion is the process of transferring blood or blood-based products from one person into the circulatory system of another. Blood transfusions are given to patients suffering massive blood loss due to trauma and to replace blood loss that occurs during surgery. Blood transfusions may also be used to treat a severe anaemia or thrombocytopenia caused by a blood disease. The present thesis will focus on one of the blood components used for transfusion, namely the platelets and their biology, involvement in haemostasis, their storage and transfusion.

History of blood transfusion

Long ago blood was recommended for the treatment of various ailments such as lunacy, fits, melancholia, palsy and bad disposition. A myth claims that Pope Innocent VIII on his deathbed in 1492 received blood from 3 boys as a last attempt at revival [1]. The first documented successful transfusion took place in 1665 when blood from a dog was transfused to another dog. The first fully documented blood transfusion to humans occurred already in 1667 when a French philosopher-mathematician and physician, Jean-Baptiste Denis, transfused a boy with blood from a sheep. Later in 1667 Denis treated a madman at the request of the madman's wife with blood from a “gentle calf” to treat his lunacy. The transfusion was successful, and the madman was transfused once more. This time the man developed transfusion reactions. Two months later the madman was transfused again, but he died. The madman's wife accused Denis of having killed her husband, but the subsequent trial proved that she had poisoned her husband with arsenic and Denis was exonerated [1]. This incident led to the prohibition by the French Parliament of further transfusions to humans. The Royal Society in England and the magistrates in Rome followed suit. James Blundell, a British obstetrician, performed the first recorded transfusion from human to human in 1818. In the following years, Blundell performed 10 transfusions, of which 5 were successful. In the hope that it would be less troublesome than blood, milk was introduced in 1873-1880 for intravenous therapy. In 1900, Karl Landsteiner discovered the human blood groups A, B and O, and in 1907 it was suggested that cross-matching blood between donor and recipient might improve the safety of transfusions. The early transfusions were made directly from donor to recipient. In the early 20th century, however, it was discovered that by adding anticoagulant and refrigeration it was possible to store the blood for a few days prior to transfusion. This opened the way for blood banks. In 1943 and 1957, respectively, acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose (CPD) were introduced as blood preservatives and made it possible to extend the storage period [1]. In the 1930-1940's, it was discovered that blood could be separated into plasma and red blood cells by centrifugation and the components could be stored separately with improved quality. In modern transfusion medicine, blood is usually separated into its components - red blood cells, platelets and plasma - which are stored and transfused separately. Increased knowledge about these products has resulted in better storage conditions.

Platelets

Any disruption of the vessel wall needs to be repaired. The repair system is called haemostasis, which gives rise to a blood clot that seals the damage. It is important that the haemostatic system is functional; a dysfunctional system can lead to either bleeding or thrombosis. The haemostatic process can be divided into two parts: primary and secondary haemostasis. Primary haemostasis involves the platelets that adhere and aggregate at the site of the wound, as reviewed by Nieswandt & Watson [2] and Offermanns [3]. Secondary haemostasis involves the coagulation cascade, leading to a fibrin network that stabilises the platelet plug, as reviewed by Dahlbäck [4] and Monroe & Hoffman [5]. A schematic drawing of the process is shown in Figure 1.

It was Bizzozero who, in 1881-1882, first discovered the platelets and their involvement in haemostasis [6]. Platelets are fragments released from the megakaryocyte. They circulate at a concentration of $150\text{-}350 \times 10^9$ cells/L and, due to the laminar blood flow, they circulate close to the endothelium of the vessel wall and thus can rapidly attach to the vascular damage. Platelets are small a-nucleated blood cells with an approximate size of $3.6 \times 0.7 \mu\text{m}$ [7] and have a cytoskeleton containing actin which is involved in clot retraction [8]. α granules, dense granules and lysosomes are packed into the cytoplasm. The α granules store matrix adhesive proteins and glycoprotein (GP) receptors. Dense granules contain adenosine diphosphate (ADP), Ca^{2+} and nucleotides [7]. The platelet lifespan in a healthy individual is 8-10 days. Platelets are removed from the circulation due to senescence, which accounts for the majority of the loss in a healthy individual, and due to a fixed number of platelets per day required for maintenance of the vessel integrity. The survival of platelets in a thrombocytopenic patient is reduced because the majority of the platelets are required for the haemostatic maintenance process [9].

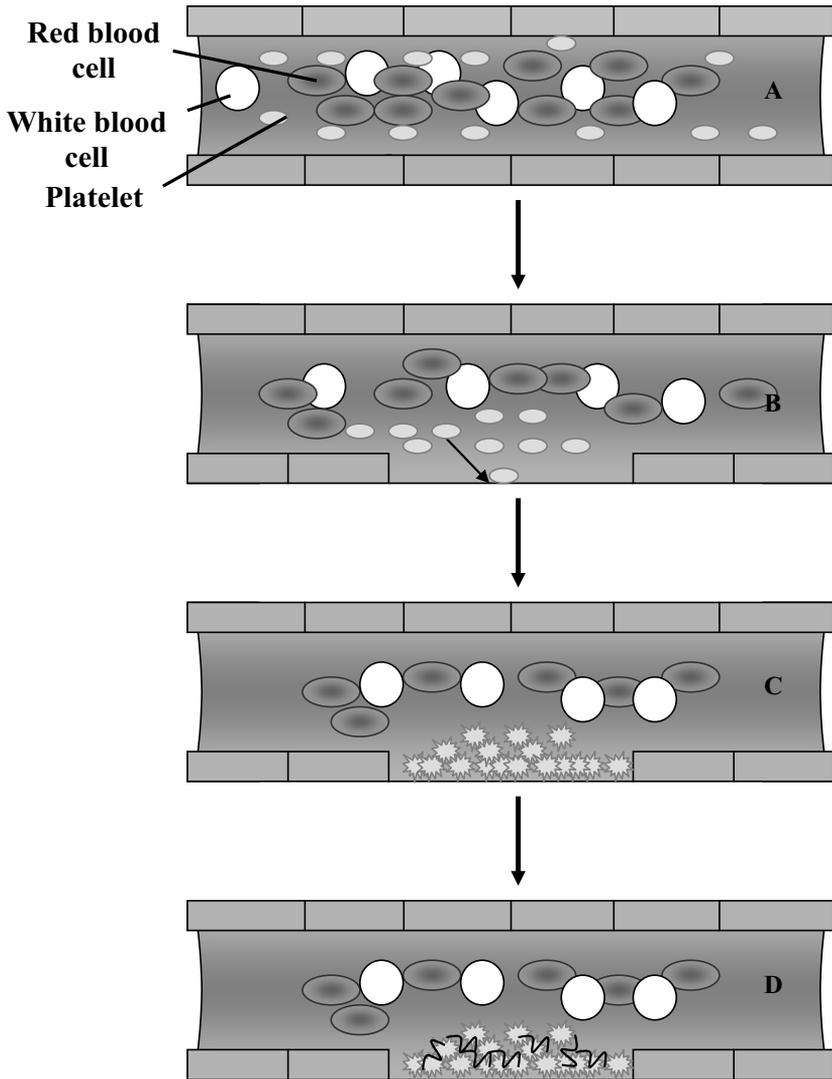


Figure 1: The formation of a platelet plug at site of vascular injury.

The laminar flow of the blood cells in an intact vessel with the platelets circulating close to the endothelium of the vessel wall (A). The platelets recognise vessel wall damage and adhere to the damaged site (B). Platelets undergo shape change, secretion and aggregate, forming a platelet plug (C). The platelet plug is stabilised by fibrin formed in the coagulation cascade (D).

Platelets contain a number of receptors involved in haemostasis (Figure 2). Platelet receptors can be classified according to type and signalling mechanism. Here I will focus on a few receptors of importance.

Integrins

Integrins consist of non-covalently associated heterodimers of α and β subunits including GPIa/IIa ($\alpha_2\beta_1$) and GPIIb/IIIa ($\alpha_{IIb}\beta_3$) [3]. These receptors need to undergo a conformational change to be able to bind a ligand. GPIa/IIa functions as an adhesion receptor for collagen. Activation of GPVI or other receptors results in the activation of GPIa/IIa during which GPIa/IIa undergoes a conformational change to a high affinity state [10]. GPIIb/IIIa is a receptor for fibrinogen, von Willebrand factor (vWF) and fibronectin [11]. It is absent or deficient in patients with Glanzmann's thrombasthenia [12]. GPIIb/IIIa is involved in platelet-platelet interactions as well as adhesion to the subendothelium via vWF under high shear [13,14]. GPIIb/IIIa is also important for clot retraction, and patients with Glanzmann's thrombasthenia cannot reduce clot volume [15,16]. GPVI interacting with collagen and GPIb binding to vWF induce a conformational change in the GPIIb/IIIa complex, enabling it able to bind its ligands [17-19]. Binding of ligand to GPIIb/IIIa enables spreading and full aggregation [3,20].

GPIb

GPIb, which is present as a complex with GPIX and GPV (the GPIb-IX-V complex), is a receptor for vWF, thrombin and P-selectin [19,21,22]. It is involved in the initial adhesion of platelets to vessel wall damage in vessels with high shear [19]. GPIb functions as a cofactor that localises thrombin to the platelet surface supporting the cleavage of the protease-activated receptor (PAR), PAR-1 [22]. Thrombin stimulation of platelets results in the internalisation of GPIb-IX-V complex concomitant with the exposure of the GPIIb/IIIa complex [23].

GPVI

GPVI belongs to the immunoglobulin superfamily [24]. Interaction of GPVI with collagen on the platelet surface causes clustering of GPVI. This leads to an increase in intracellular Ca^{2+} through a signalling cascade [25,26]. GPVI binds to the glycine-proline-hydroxyproline (GPO) sequence in collagen [26]. Collagen-related peptide (CRP) is a synthetic peptide (the cross-linked repetitive sequence of GPO) that can activate platelets through the GPVI receptor [27] and induces a much greater degree of GPVI activation than collagen [25].

P-selectin

P-selectin is localised to the α granule membranes in resting platelets [28]. When the platelets are activated and the granule content released, the P-selectin translocates to the platelet cell surface [29] where it initiates adhesion to leukocytes. P-selectin can also be released in soluble form during activation, soluble P-selectin (sP-selectin). It may also be involved in aggregation by stabilising platelet aggregates [30].

G-protein coupled receptors

Guanyl-nucleotide-binding protein (G-protein) coupled receptors are membrane-bound receptors with seven transmembrane spanning regions. They include the P2Y₁ and P2Y₁₂ ADP receptors [31] the PAR-1 and PAR-4 thrombin receptors [32,33] and the thromboxane A₂ (TXA₂) receptor [3]. PAR-1, PAR-4 and the TXA₂ receptors are coupled to G_{12/13} and G_q [7,34]. P2Y₁ is coupled to G_q [31,35] and P2Y₁₂ is coupled to G_i [31,36]. Both G_{12/13} and G_q signalling results in shape change, aggregation, secretion of ADP and formation/secretion of TXA₂. The ADP and TXA₂ can then bind to their respective G-protein coupled receptors

[3,7]. G_i signalling result in decreased adenylyl-cyclase activity and thereby reduced levels of the platelet inhibitor cyclic adenosine monophosphate (cAMP) [37].

Activation of $P2Y_1$ by ADP thus leads to an intracellular increase in Ca^{2+} , aggregation and shape change [35,38] whereas activation of $P2Y_{12}$ by ADP results in lower levels of cAMP [31,36].

Activation of the PAR receptors begins when thrombin cleaves the receptor near the N-terminus to create a new N-terminus that serves as a tethered ligand, which binds to the receptor and thereby mediate receptor signalling [32,33,39,40]. Thrombin cleavage of PAR-1 releases a new N-terminus beginning with the sequence SFLR [32,39]. Synthetic peptides mimicking the new N-terminus (beginning with the sequence SFLR i.e. the thrombin receptor activating peptide, TRAP) were found to be effective agonists and bypassed the requirement for receptor proteolysis [39]. PAR-4 can be activated by the peptide GYPGQV, which mimics the thrombin-cleaved N-terminus of PAR-4 [33]. It has previously been thought that PAR-1 mediates platelet responses at low thrombin concentration and is responsible for a rapid response, whereas PAR-4 activation has been thought to result in a more sustained activation and only at high thrombin concentrations [41-43]. However, more recent publications suggest that this might not be correct [44,45].

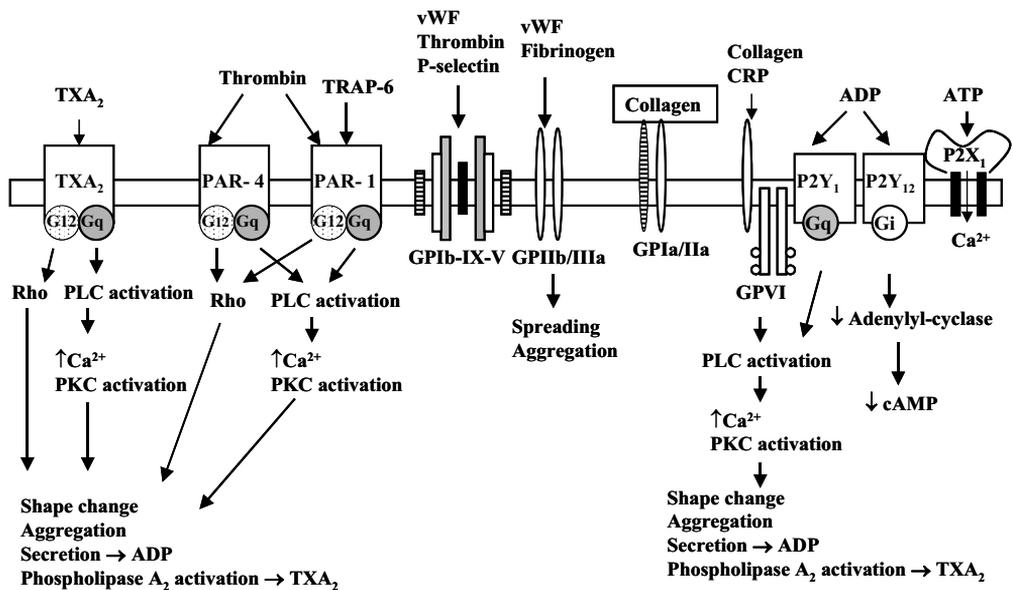


Figure 2. Platelet membrane receptors.

Platelet receptors with their ligands and signalling responses following ligand binding. Adenosine diphosphate (ADP); adenosine triphosphate (ATP); collagen-related peptide (CRP); cyclic adenosine monophosphate (cAMP); glycoprotein (GP); protease-activated receptor (PAR); phospholipase C (PLC); protein kinase C (PKC); thrombin receptor activating peptide (TRAP); thromboxane A₂ (TXA₂); von Willebrand factor (vWF).

Haemostasis

Primary haemostasis

When a vessel's endothelial cell layer is damaged, platelets adhere to fibrillar collagen exposed in the damaged endothelium. At high flow, as in arteries or capillaries, the platelets must first bind via GPIb to wWF immobilised on the damaged endothelium. This causes the platelets to roll along the matrix [19,46,47]. The GPVI receptors can then interact with collagen. At low shear rate (such as venous flow), GPIb binding is not required, and GPVI can interact directly with collagen [2].

GPVI interacting with collagen and the signals generated by GPIb lead to activation of GPIIb/IIIa [18,19] and GPIa/IIa [10]. Firm adhesion and spreading is then mediated by the binding of GPIIb/IIIa to vWF, fibrinogen and fibronectin in the matrix [11,13,19,20] and by GPIa/IIa binding collagen [18]. Adhesion and platelet activation leads to the production and secretion of ADP and TXA₂ [3]. The intracellular Ca²⁺ level increases, resulting in a negatively charged surface through exposure of phosphatidylserine (PS) [48]. PS, which is normally localised to the cytosolic side of the membrane, translocates to the outer side of the membrane [49,50]. The activated platelets undergo shape change, during which the cytoskeleton of the platelets is reorganised. New actin filaments are formed, and the filopodia are extended. Dense and α granules are centralised through the action of actomyosin contractile forces. The centralisation is necessary for efficient secretion. Platelet shape change facilitates platelet-platelet interactions [3].

The released ADP and TXA₂ promote further platelet activation and lead to the recruitment of more platelets to the injured site. The result is platelet aggregation [3], which is mediated through the activated GPIIb/IIIa receptor. Activated GPIIb/IIIa on a platelet can bind its ligands fibrinogen or wWF, which in turn bind to an activated GPIIb/IIIa on an adjacent platelet, thus creating a cross-linked matrix. Cross-linking via fibrinogen/wWF leads to the formation of an aggregate, a platelet plug [51].

Platelet adhesion at the site of injury forms a temporary plug in the damaged vessel and localises the procoagulant events to the site of injury. Aggregation of the platelets prevents additional blood loss from the injured vessel.

Secondary haemostasis

Secondary haemostasis involves the coagulation cascade and leads to fibrin formation (Figure 3).

In the **initiation phase**, FVIIa associates with tissue factor (TF) on a TF-bearing cell. The FVIIa/TF complex activates FX and FIX to FXa and FIXa, respectively [5]. FXa then associates with FVa and prothrombin to form prothrombinase complexes on TF-bearing cells and prothrombin is cleaved to thrombin [52]. This generates a small amount of thrombin. In the **amplification phase**, the thrombin formed during the initiation phase activates platelets that are adhered at the site of injury [52] and activates FV, FVIII and FXI on the platelet surface [5,53-55]. The amplification phase is followed by the **propagation phase**. In this phase FIXa formed during the initiation phase forms the tenase complex with FX and FVIIIa on the negatively charged platelet which activates FX to FXa [56]. FXIa on activated platelets can also activate more FIX to FIXa [5]. FXa forms a prothrombinase complex with FVa and prothrombin on the platelet surface leading to a burst in thrombin generation [5,57,58].

Thrombin converts fibrinogen into fibrin, which is cross-linked with the action of FXIIIa. The cross-linked fibrin stabilises the platelet plug [5]. Activation of FX, FIX and the conversion of prothrombin to thrombin require Ca^{2+} [4,58,59].

Secondary haemostasis is regulated through the actions of activated protein C, which inactivates FVIIIa [56,60] and FVa [61]. Protein C is activated by thrombin bound to the membrane protein thrombomodulin on the surface of intact endothelial cells [4]. Thus the procoagulant factor thrombin has an anticoagulant activity and thus limits the growth of the clot. Another regulatory pathway is by tissue factor pathway inhibitor which forms a complex with TF/FVIIa and FXa [62,63] and inhibits thrombin generation. Antithrombin regulates secondary haemostasis by inactivating of FXIa, FXa, FIXa and thrombin by complex formation [64]. The activity of antithrombin is facilitated by heparin-like molecules present on the surface on endothelial cells [4].

Clot retraction

Once the clot formation is complete, clot retraction occurs during which the platelets pull the fibrin network strands, leading to a reduction in clot volume. This process concentrates the clot to the injured area and facilitates clot lysis as well as recanalisation [65,66]. The clot retraction involves the GPIIb/IIIa receptors on the platelets and actin/myosin complexes in the platelets. The active conformation of the GPIIb/IIIa receptors with bound ligands binds myosin that will interact with underlying actin filaments. Once tethered to actin, myosin applies the contractile force on the actin filaments [67]. As the clusters of GPIIb/IIIa with its bound fibrin are pulled into the open canalicular system of the platelets [8], the clot is pulled in on itself, which reduces its overall size.

Fibrinolysis

When the damaged vessel is repaired, the fibrin network is degraded by the fibrinolytic system. Plasminogen binds to fibrin in the clot. The tissue plasminogen activator activates plasminogen to plasmin on the fibrin surface [68,69], which in turn degrades the fibrin into fibrin degradation products and D-dimer. Fibrinolysis is regulated by inactivation of plasmin by α_2 -antiplasmin [68] or inactivation of tissue plasminogen activator by plasminogen activator inhibitor type 1 [70,71]. Fibrinolysis is also regulated through the actions of thrombin activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis. For TAFI to inhibit fibrinolysis, TAFI needs to be activated by thrombin [5].

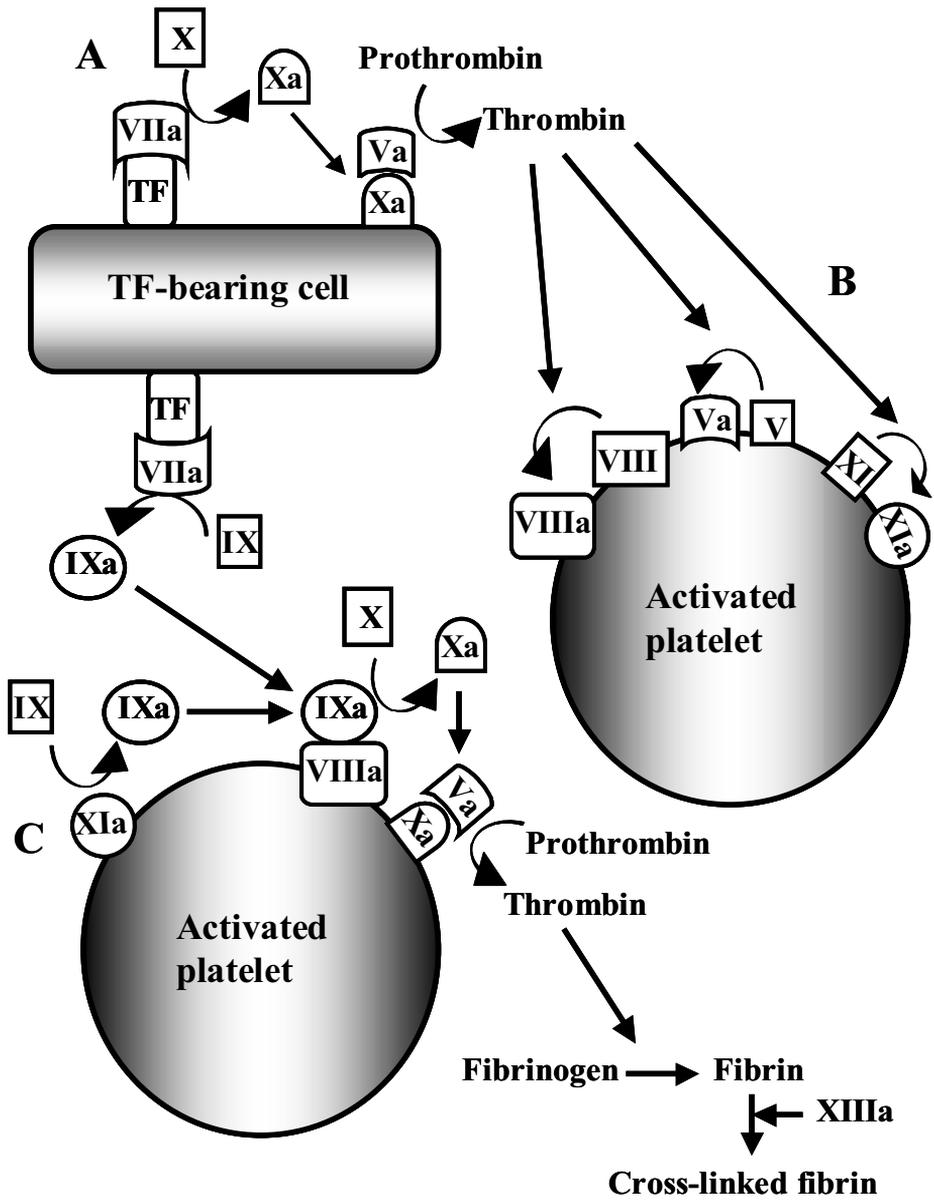


Figure 3. The coagulation cascade.

The coagulation cascade with the initiation phase (A), amplification phase (B) and the propagation phase (C) leading to cross-linked fibrin supporting the platelet plug.

Platelet transfusions

The successful treatment of malignant haematological diseases is dependent on transfusion of blood components as these patients have a lack of blood cells caused by their illness or chemotherapy treatment. Platelets are transfused to patients who are severely thrombocytopenic or to patients with platelet dysfunction to prevent bleeding or induce primary haemostasis. Platelets must be viable to be effective in haemostasis.

Prophylactic platelet transfusions are widely adopted as standard practice. A platelet count of $20 \times 10^9 /L$ was previously used as the target level for prophylactic transfusions [72]. This was based on a study from 1962 which reported a quantitative relationship between the platelet count and the frequency of haemorrhage in patients with acute leukaemia, showing that with a platelet count of $\geq 20 \times 10^9 /L$, there was less risk of gross haemorrhage [73]. It was reported that prophylactic platelet transfusions to patients with acute leukaemia when the platelet count was $< 20 \times 10^9 /L$ resulted in a lower proportion of patients with bleeding events and fewer bleeding events compared with patients who only received therapeutic transfusions [74]. More recent studies have shown that lowering the prophylactic platelet threshold to $10 \times 10^9 /L$ results in decreased platelet utilisation without increasing major bleedings [75-77].

Preparation and storage of platelet concentrates

Many factors influence the quality of the platelets during storage. These include the preparation method of the platelets [78], the plastic material of the storage bag [79] and the ability of bags to exchange gas across its surface [80]. Other important factors that effect the quality are the storage temperature [78,81,82], the type of anticoagulant used and the platelet concentration in the bag [78]. Agitation during storage has also been found to be important [78].

The platelet concentrate (PC) can be prepared by the apheresis method (AP) or from whole blood using the buffy-coat (BC) or platelet-rich plasma (PRP) method. The use of AP PCs has increased to approximately 75-80% in the U.S.A and 50% in Europe. In the U.S.A, platelets derived from whole blood are produced by the PRP method, whereas the BC method is used in Europe [83].

In the AP procedure, the blood from the donor is processed with a cell separator with an in-line centrifuge for platelet collection. The platelets are transferred to a collection bag, whereas the red cells and most of the plasma are returned to the donor. Various cell separators are available for platelet collection and have different collection principles, as reviewed by Burgstaler [84]. It has previously been reported that the cell separators induce various degrees of platelet activation due to their different collection principles [85,86].

The PRP and BC methods require pooling of platelets from several donors (usually 4-6) to obtain a platelet dose equivalent to that which can be obtained from a single donor by AP collection [83,87,88]. Since only one donor is required to produce a transfusion unit by AP technique the risk for immunisation is reduced as the recipient in this case is exposed to less antigen stimulation [89].

Using the PRP method to prepare PCs, whole blood units are centrifuged by soft spin to prepare PRP followed by a high-speed centrifugation to obtain a platelet pellet. Most of the

plasma is removed, and the platelets are stored in a reduced volume of remaining plasma. Alternatively, the platelets are re-suspended in a synthetic medium [90,91].

With the BC processing method whole blood is centrifuged at high-speed to prepare a BC. The BCs are pooled in a pooling container (often with a storage medium) and centrifuged to a platelet-rich supernatant that is transferred to the storage container [91].

Platelets have usually been stored for up to 5 days at room temperature with constant agitation, which is necessary for the maintenance of platelet viability [79,92,93]. As early as 1960 Baldini et al. reported that platelets stored at 4°C had decreased viability compared with fresh platelets [81]. In 1969 it was shown that the optimum storage temperature is 22°C [82], which was confirmed in other studies [78,94,95]. Recent studies have shown that cooling irreversibly reorganises the GPIb-IX-V receptors into clusters on the platelet surface. The clustered receptors are recognised by the $\alpha_M\beta_2$ receptor on macrophages which ingest the platelets [96].

Changes in pH in the PCs has been shown to greatly effect platelet viability [78-80]. To maintain pH during *in vitro* storage of platelets at 22°C, containers with sufficient permeability for oxygen and carbon dioxide (CO₂) are required [80,97]. Early storage bags made of polyvinyl chloride (PVC) plasticised with di(2-ethylhexyl) phthalate had poor oxygen permeability and therefore only allowed storage up to 3 days. Second-generation storage bags made of polyolefin [98] and PVC plasticised with tri(2-ethylhexyl) trimellitate [99] had increased gas permeability and allowed the storage period to be extended to 5 days. New storage containers made of PVC plasticised with butyryl-tri-n-hexyl-citrate have been developed. They have been shown to have good gaseous permeability and result in good platelet *in vivo* viability following transfusion [100,101].

Platelet metabolism

The main energy source for platelets is the hydrolysis of adenosine triphosphate (ATP). Platelets must generate ATP continuously to meet their energy needs [102]. Platelets can generate ATP through two different metabolic pathways, as shown in Figure 4. One pathway requires oxygen, the tricarboxylic acid cycle (TCA) whereas the other, glycolysis, does not require oxygen. It has been suggested that the TCA cycle accounts for 85% of the ATP [97,103,104]. In the TCA cycle the substrate is free fatty acids in plasma [105] or acetate (if added to a synthetic medium). The end product is CO₂ [104]. Glucose metabolism may generate 15% of the ATP [104]. During glycolysis, glucose is converted to lactate and a free hydrogen ion [104]. The hydrogen ion can be buffered by the bicarbonate in the plasma and converted to CO₂ and water [90,104]. The buffer capacity of the medium particularly bicarbonate will therefore also effect the pH level [106-108]. However, plasma only contains enough bicarbonate to buffer a lactate increase to about 28 mmol/L. Above that level, the pH will fall rapidly, which might result in a loss in platelet viability [90]. The decrease in pH is not entirely attributed to lactate formation. The amount of CO₂ also contributes considerably to the pH because CO₂ is a volatile acid [109]. It is therefore of great importance with gas permeable storage bags to keep the glycolysis to a minimum and allow the CO₂ that has formed to leave the PC through the walls of the container.

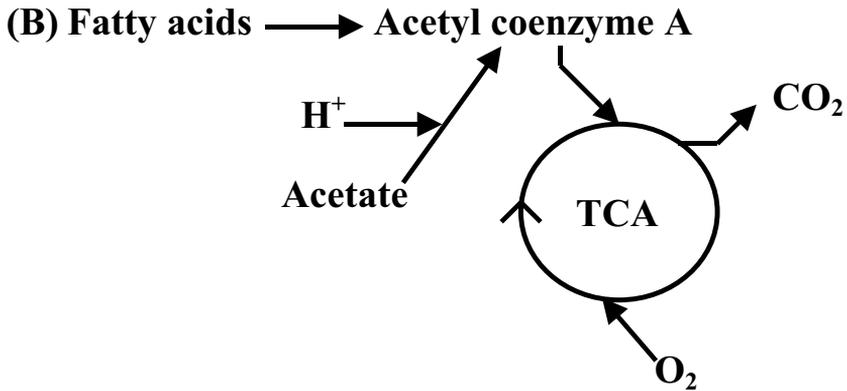
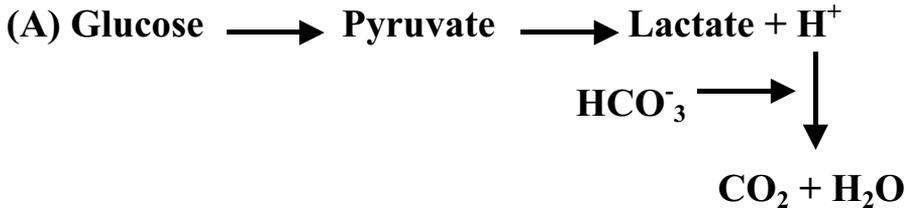


Figure 4. Platelet metabolism.

The glycolysis (A) and the tricarboxylic acid cycle, TCA (B).

By using a synthetic medium, it is possible to include components in the storage environment that are good for platelet quality. It might also reduce the adverse transfusion reactions as most of these are caused by compounds present in plasma [110]. Guppy et al. proposed that acetate should be included in the storage medium as an oxidisable fuel because 70-100% of the ATP might be produced aerobically [103]. The presence of acetate in the platelet additive solution (PAS) was shown to reduce lactate production [107,111]. Based on these findings, the additive solution PAS-II (T-Sol™) containing acetate was designed [112]. It is possible to store platelets for 7 days in a medium containing 70% PAS-II and 30% plasma [112]. PAS-III (InterSol™), an additive solution that is used for pathogen inactivation procedures, has a similar composition to PAS-II, with the addition of phosphate [113]. The composition of different additive solutions is shown in Table I.

Table I: Composition of different platelet storage solutions

	Plasma-Lyte-A ^[114]	PAS-I ^[112]	PAS-II ^[112]	PAS-III ^[115]	PAS-IIIM ^[115]	Composol ^[116]
NaCl	90	70	115.5	77	69	90
KCl	5	10			5	5
MgCl ₂	3				1.5	1.5
Na ₃ citrate		30	10	10	10	11
Na acetate	27		30	30	30	27
Na phosphate		5		26	26	
Na gluconate	23					23
Mannitol		30				

NOTE. All values are expressed as mmol/L.

Platelet additive solution (PAS).

PAS-II is also called T-Sol™, PAS-III is also called InterSol™ and PAS-IIIM is also called SSP+.

Transfusion reactions

Febrile nonhaemolytic

Transfusion of platelets can result in transfusion reactions in the recipient. The most common of these reactions are the febrile nonhaemolytic transfusion reactions (FNHTR). FNHTR can be caused either by human leukocyte antigens (HLA) present on the surface of remaining leukocytes in the PC reacting with the antibodies of the recipient, or by cytokines released from the leukocytes in the PC. The storage period of the PCs affect the likelihood of such a reaction as increased storage results in increased cytokine release. Removal of leukocytes reduces the risk. FNHTR is characterised by fever, chills and discomfort [117-119].

Haemolytic

Haemolytic transfusion reactions (HTR) can be caused by transfusion of ABO incompatible PCs. Antibodies in the plasma of the PC directed against an antigen on red blood cells of the recipient can cause haemolysis [120]. The risk is reduced if PAS is used as the storage medium. Clinical manifestations include fever, chills, pain, renal failure and disseminated intravascular coagulation [120].

Alloimmunisation

Platelets express HLA-A, HLA-B and human platelet antigens [119]. Leukocytes in the PCs or transfusion of platelets which differ in human platelet antigens to those of the recipient can cause alloimmunisation, i.e. platelet-reactive alloantibodies are formed in the thrombocytopaenic recipient [119,121]. Antibodies in the recipient directed against donor platelets may cause refractoriness to platelet transfusions [122]. Platelets collected by AP technique from a single donor expose recipients to a limited number of HLA specificities [123] and could thus reduce the risk of alloimmunisation. HLA alloimmunisation is reduced by removing contaminating leukocytes from the PC.

Transfusion-related acute lung injury

Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related deaths [119]. The main cause is believed to be antibodies in the PC reacting with the leukocytes of the recipient leading to leukocyte aggregation and severe pulmonary reaction. Clinical manifestations include fever, chills, dyspnoea and hypoxia [118,119,124].

Transfusion-associated graft-versus-host disease (TA-GVHD)

Leukocytes in the PCs can also cause transfusion-associated graft-versus-host disease (TA-GVHD). TA-GVHD is mediated by the engraftment of allogenic T-lymphocytes [125]. The risk of developing TA-GVHD depends on the viability and number of contaminating lymphocytes, the susceptibility of the recipient's immune system to their engraftment and the degree of HLA disparity between donor and recipient. The risks of TA-GVHD is highest in recipients with immunodeficiency [126,127]. TA-GVHD is associated with a more than 90% mortality rate [125], and there is no effective therapy.

Leukocyte filtration is not effective enough to produce the level of lymphocyte removal required to prevent TA-GVHD [126]. PCs are gamma irradiated prior to transfusion to immunosuppressed patients to inhibit the proliferation of remaining lymphocytes and thus reduces the risk of TA-GVHD [125]. Luban et al. showed that the frequency of proliferating T-cells in the PCs decreased with irradiation dose. No growth was detected when a dose of 25 Gy was used [127].

Conflicting results have been reported with respect to whether gamma irradiation is harmful to the *in vitro* and *in vivo* properties of platelets [128-131]. An irradiation dose of 25 Gy has been recommended, and irradiation can be performed at any time during the storage period [126].

Transfusion-transmitted bacteraemia/sepsis

PCs have been shown to be associated with greater risk of transfusion-transmitted bacteraemia than red blood cells. Bacterial infections transmitted by blood transfusion are associated with the rapid onset of sepsis and account for >10% of transfusion-associated fatalities [132]. Bacterial contamination is a problem because PCs are stored at room temperature. The introduction of a small number of contaminating bacteria during component preparation can result in high numbers during storage at this temperature [133].

Several methods have been developed to screen for bacteria-contaminating PCs during the last decade. These include analysis of glucose levels, oxygen tension, detection by solid-phase laser cytometry, dielectrophoresis and automated bacterial culture [119].

The photochemical treatment (PCT) process with amotosalen HCl (formerly named S-59) called the Intercept™ Blood System for Platelets has been developed by the CERUS Corporation for pathogen inactivation. The PCT process inactivates contaminating bacteria, viruses, and protozoa [113,133-135]. It also enables storage for longer time than five days [113]. The Intercept™ system inactivates leukocytes and thereby eliminates the need for gamma irradiation to prevent TA-GVHD [136,137]. When added to the blood component, the amotosalen crosses the membrane or cell wall of the pathogen. Amotosalen reversibly intercalates into helical regions of DNA and RNA. Upon illumination with UVA light (320-400 nm), amotosalen reacts with pyrimidine bases to form both covalent monoadducts and cross-links with nucleic acid, preventing replication [138,139]. Platelets, on the other hand, are a-nucleated cells and do not need nucleic acid transcription and translation for haemostatic function. PAS-III is used in the pathogen inactivation procedure [113]. A transfusion unit is suspended in 35% plasma and 65% PAS-III. The mixture, containing 150 µmol/L amotosalen HCl, is exposed to 3 J/cm² long-wavelength ultraviolet light for 3 min to inactivate pathogens. The levels of residual amotosalen and free photoproducts are then reduced by compound adsorption device (CAD) treatment for 4-16 hours [139]. The time of CAD treatment has been shown to influence the platelets [140]. Recently reported studies show that transfusion of Intercept™ treated PCs reduces *in vivo* recovery and survival [141], reduces corrected count increments (CCI) and shortens transfusion intervals compared with platelets in plasma or PAS-II [135,142-145]. Despite these drawbacks, Intercept™ platelets seem to be able to provide adequate haemostasis [143-145].

Quality testing of platelet concentrates

Platelet quality during storage can be evaluated by determining the recovery and survival of the transfused platelets in thrombocytopaenic patients [146]. Calculation of CCI corrects for the patient's blood volume and the number of platelets in the PC when evaluating the post-transfusion platelet count increment. A 1-hour post transfusion CCI of 10-20000 is considered a good response while a CCI of less than 7500 is a poor response and indicates transfusion failure [147]. An alternative approach is to measure the recovery and survival of fresh or stored radiolabelled platelets (^{51}Cr) in healthy volunteers [146]. However, as these *in vivo* studies are expensive and complex to perform, several studies use *in vitro* tests to assess *in vivo* viability, as discussed below.

Bleeding time

Ivy bleeding time measurement can be performed if the patient receiving a platelet transfusion is relatively stable. The bleeding time has been shown to be inversely proportional to the platelet concentration between platelet concentrations of 100–10 x 10⁹ /L [148]. It compares the shortening of the bleeding time with the increase in platelet concentration achieved. The bleeding time is affected by a number of factors unrelated to the platelet function. It is also complex to perform, resulting in operator variability. Because the thrombocytopaenic patients are often leukopaenic, there is risk of infection caused by the skin incision [90,149].

Blood gases

pH, pCO₂ and pO₂ can be measured in the PCs to evaluate the PC quality. The pH has been shown to greatly affect the quality of the PCs. pH falls during storage of PCs at 22°C [79,109] which is due to glycolysis [80]. pH values of 6.4-7.4 do not correlate to *in vivo* viability [150]. A pH drop below 6.0 is associated with loss of viability [78,79] as is pH values greater than 7.4-7.6 [80]. pO₂, and pCO₂ reflect the permeability of the container. Increased pCO₂ leads to a pH drop, which could be prevented by CO₂ egress from the storage container.

Metabolic parameters

Glucose and lactate concentrations in the PCs are commonly used as quality parameters. A decrease in glucose and an increase in lactate concentration during storage have been reported in several studies [151-154]. Depletion of glucose and increase in lactate concentration to more than 28 mmol/L can result in low ATP levels and a rapid pH reduction which might result in a loss of platelet viability [90,91,155]. Measurement of cytoplasmic leakage of lactate dehydrogenase (LDH) can also be used as a quality parameter and reflects platelet membrane damage. LDH has been shown to correlate to platelet survival ($r = -0.64$; [156]).

Swirling

Discoid platelets exposed to a light source reflect light and thereby producing the “swirling” phenomenon. Swirling is routinely used to evaluate the quality of PCs. Swirling is determined by examining a PC against a light source while gently rotating the container or gently squeezing the PC [157]. The presence of swirling is informative because it is highly effective in predicting a pH value in an adequate range. The presence of swirling was associated in 94% of cases with a pH value in the range of 6.7-7.5 [157]. This pH range is associated with adequate *in vivo* survival [79,80].

Hypotonic shock response (HSR)

Adding a hypotonic solution to platelets results in initial swelling followed by a gradual decline as the platelets resume their baseline size. This can be measured spectrophotometrically at 610 nm [158]. Osmotic swelling of a cell increases cytosolic calcium and the release of ATP from the cell. This initiates several signalling processes in the cell. These responses lead to eventual K^+ and Cl^- ion and water egress from the cell and correction in cell size [159]. Kim & Baldini found that response to hypotonic stress was positively correlated with the platelet viability index, which is a combined measure of post-transfusion platelet recovery and survival [160]. In contrast, Valeri et al. found that it correlated with post-storage platelet recovery *in vivo* but not with survival [158].

Platelet activation

Platelets become activated during the preparation and storage of PCs. Platelet activation can be measured by the release of P-selectin [28] (soluble or surface bound), the active conformation of GPIIb/IIIa [161], and GPIb expression. Surface expression of GPIIb/IIIa, P-selectin and GPIb can be measured by flow cytometry. Increased P-selectin expression during storage has been reported by several authors [85,162-165], whereas GPIb has been shown to decrease during storage [164,166]. It is, however, unclear whether the level of *in vitro* platelet activation in stored PCs correlates with *in vivo* survival and haemostatic function of platelets after transfusion [167]. Rinder et al. demonstrated that platelets characterised by increased *in vitro* activity are rapidly cleared from the circulation *in vivo* [168]. P-selectin and GPIb were shown to be involved in regulating post-transfusion platelet clearance by mediating adhesive interactions of platelets with counter receptors on macrophages and endothelial cells [169,170]. Significant negative correlations between P-selectin exposure and post-transfusion platelet recovery and survival have been reported [156,168,171]. However, in a recent study no such correlation could be detected [172].

Aggregation

PRP is used for measurement of aggregation. PRP is stirred in a cuvette at 37°C between a light source and a detector. Aggregation is initiated using an agonist such as collagen, thrombin or ADP. When platelets aggregate, the light transmission increases and is recorded as the rate of aggregation and maximum response. By comparing responses to different agonists platelet defects can be identified [173]. Aggregation measurements on platelets from PCs do not appear to be able to predict platelet recovery as platelets stored at 4°C have better aggregability than those stored at 22°C [174] despite low recovery and survival.

AIMS OF THE THESIS

The overall aim of this thesis was to assess whether coagulation analysis by free oscillation rheometry (FOR) can be used in the field of platelet transfusion therapy to evaluate of the haemostatic status of thrombocytopaenic patients and to assess the function of platelets in PCs.

The thesis had four primary aims:

1. Evaluate how various blood components influence the FOR analysis.
2. Determine whether FOR can be used in clinical practice to assess coagulation properties in hyper- and hypocoagulable states.
3. Determine if FOR can be used to study the effects of a platelet transfusion to patients with thrombocytopaenia.
4. Evaluate the quality of PCs using *in vitro* methods, including FOR, and determine how different PC preparation processes affect the platelet quality.

METHODOLOGY

Free oscillation rheometry (FOR)

FOR using the ReoRox[®] 4 instrument (Medirox AB, Nyköping, Sweden) makes it possible to monitor the coagulation process and to obtain information about clotting time and coagulum elastic properties in whole blood and plasma samples [175,176]. Measurements with the ReoRox[®] 4 can be performed at 37°C.

The ReoRox[®] 4 is a small, robust and portable instrument that can be used for bedside analysis. It can be used for measurements in fresh non-anticoagulated whole blood samples, re-calcified citrated whole blood samples and PRP and plasma samples. ReoRox[®] 4 has four measuring channels allowing four samples to be analysed simultaneously. The instrument is equipped with a cylindrical sample cup, which is set into free oscillation along its longitudinal axis every 2.5 seconds, with a frequency of about 10 Hz. The frequency (Fq) and damping (D) of the oscillation are recorded as a function of time [176].

When the blood is in fluid form, the D is low and the Fq high. As the blood begins to coagulate, the viscosity increases. The result is an increased D because a larger portion of blood participates in the oscillation and the frequency is reduced [175]. When the thrombin concentration in the sample has grown sufficiently large to begin the formation of fibrin strands, the clotting time is reached. The clotting time is determined as the time when the sum of change in D and Fq reach a preset value, C:

$$\sqrt{(\Delta Fq^2 + \Delta D^2)} \geq C \text{ [177]}$$

In the present thesis a “high sensitivity state detector” was used where C=0.01. This algorithm results in a clotting time which is almost identical to the time obtained with the manual reference method, i.e. visual inspection of tilting tubes in a water bath [177]. The clotting time is evaluated using the ReoRox[®] 4 Viewer v.2.11k (Medirox AB).

One important function of the platelets after a vessel wall injury is to retract the coagulum when the injury has been temporarily covered to allow the blood to flow freely in the blood vessel. The elasticity (G') measured by FOR gives information about the fibrin network in the coagulum and the ability of the platelets to retract the coagulum.

The change in G' over time is measured using a reaction chamber, which consists of a sample cup with a 6 mm cylinder (bob) attached to a shaft in the centre of the cup. The sample cup and the bob are made of plastic. However, for measurement of whole blood, gold-coated parts are advantageous to avoid detachment of the clot due to clot retraction [176]. The change in G' can be calculated in SI units from the Fq and D data using the formula $G' = 1.444 (\omega^2 - k)$, where k is chosen so that the minimum of the G' curve is set to 10 Pa. ω is calculated using the formula $\omega = 2\pi * f$, where $f = f_0 + f_s$, f_0 being the frequency of the empty cup. Maximum elasticity (G'max), the maximum change in elasticity per minute (G' max-slope) and the time between clotting time and G'max (time to G'max) were evaluated using a program designed in-house. The mean change in elasticity per minute (i.e. G'max/time to G'max) was calculated. A FOR elasticity curve is shown in Figure 5.

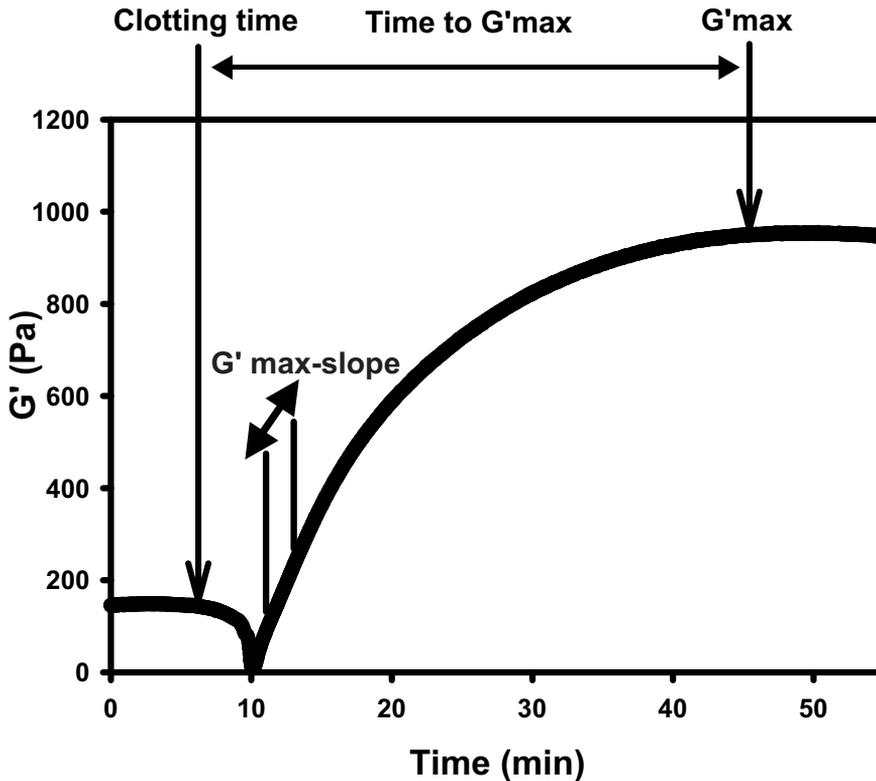


Figure 5. A FOR elasticity curve.

The change in elasticity (G') over time of coagulating blood measured with free oscillation rheometry (FOR) using ReoRox[®] 4. The clotting time, maximum elasticity (G' max), maximum change in elasticity per minute (G' max-slope) and time to G' max are indicated in the figure.

Comparison of FOR and thromboelastography

Thromboelastography, a method similar to FOR, has been widely used to evaluate coagulation properties, as reviewed by Luddington [178]. We performed a comparative study of the clotting time and the measuring range for clot elasticity between ReoRox[®] 4 and two commercially available thromboelastographs, TEG[®] 5000 (Haemoscope Corporation, Niles, IL, U.S.A) and ROTEM[®] (Pentapharm GmbH, Munich, Germany). The TEG[®] 5000 consists of an oscillating sample cup. A pin is suspended in the blood sample by a torsion wire and is monitored for motion [179,180]. The TEG[®] analysis gives a curve from which the reaction time (R), the α angle (which reflects the kinetics of clot formation) and the maximum amplitude (MA; the maximum strength/stiffness of the clot) can be obtained [180]. The ROTEM[®], in contrast, has a fixed sample cup with a pin suspended in the blood sample. The pin oscillates and the movement is registered [181]. The ROTEM[®] analysis gives rise to a curve from which the clot time (CT), α angle, and the maximum clot firmness (MCF) can be determined [178].

We compared the ability of the three instruments to measure differences of coagulum properties at various platelet concentrations. Platelets from one PC, obtained by the AP technique, were diluted in autologous plasma to a concentration of 400, 300, 200, 150, 100, 50, 10 and 0 $\times 10^9$ platelets/L. Samples of each dilution were re-calcified with CaCl_2 (final concentration 0.02 mol/L), activated with thromboplastin (final concentration 0.05 arbitrary units (AU)/mL) and added to each instrument in appropriate volumes. All measurements were performed at 37°C. The clotting time and G'max measured with ReoRox[®] 4 were compared to the corresponding parameters R and MA for TEG[®] and CT and MCF for ROTEM[®].

The clotting time was similar for all concentrations with a mean of 3.48 ± 0.19 min for ReoRox[®] 4, 3.11 ± 0.53 min for TEG[®], and 3.61 ± 0.10 min for ROTEM[®]. The ReoRox[®] 4 was shown to measure a wider range of elasticity (Figure 6).

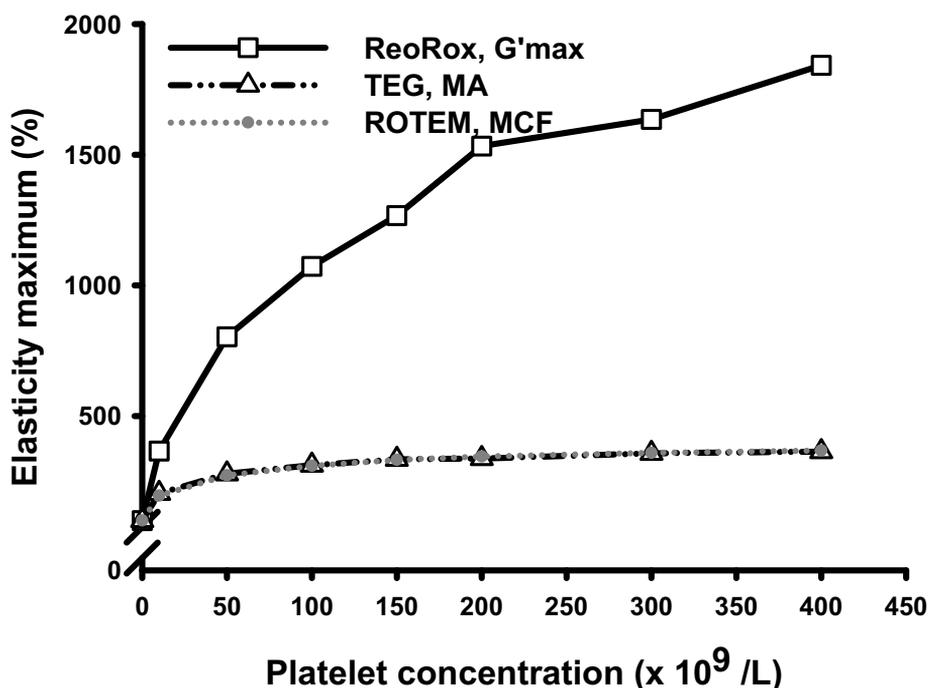


Figure 6. Maximum clot elasticity measured by ReoRox[®] 4, TEG[®] and ROTEM[®].
 The maximum clot elasticity (G'max, MA and MCF) measured in plasma with platelet concentrations of 0-400 $\times 10^9$ /L using the ReoRox[®] 4, TEG[®] 5000 and ROTEM[®] instruments. The elasticity for each platelet concentration is given as a percentage of the value for plasma alone for each instrument.

Flow cytometry

Flow cytometry is used for analysis of specific characteristics of single cells. Characteristics that can be studied are cell size, cytoplasmic complexity, DNA or RNA and membrane-located proteins such as receptors. The analysis is based on the differences in the light scattering properties of the cells and on fluorescent signals. The cells are arranged in a stream in front of a laser beam. Physiological and chemical properties are analysed on single cell level. The sample, which must be in a single cell suspension, is carried by a sheat fluid into the flow chamber. The pressure of the sheat fluid on the cell sample align the cells in a single file in the middle of the stream, so called hydrodynamic focusing. Inside the flow chamber the cells pass through a laser beam, usually an argon laser with 488 nm wavelength, causing the light to scatter. The light scattered in the extension of the incoming laser light is referred to as forward scatter (FS) [182]. The FS detector converts the FS light to a voltage pulse that is related to the cell size. The side scatter (SS) light can also be detected and reflects the cytoplasmic complexity/granularity of the cell.

Fluorochromes, bound to or incorporated into the cells, that have been excited by the laser will emit light of different colours, which can also be detected. The different light colours are separated by filters and detected by sensors called photomultiplier tubes (PMTs). The most common fluorochromes that can be excited at 488 nm are fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin-chlorophyll protein (PerCP). The voltage pulses from the FS, SS detectors and the PMTs are amplified and converted to a digital signal, which is exported to a computer for analysis [182].

Flow cytometry is used to characterise phenotypic alterations of platelets that are related to cellular activation and haemostatic function [183]. The activation state and reactivity of the circulating platelets can be determined [184]. Antibodies directed against activation-dependent epitopes are used to evaluate activation. Activation markers of interest that can be studied in such a way include P-selectin (CD62P), by the use of anti P-selectin, and the active conformation of GPIIb/IIIa, by the use of PAC-1 or indirectly by the use of anti-fibrinogen [183-185]. Another activation marker that can be studied is the exposure of PS using annexin V. *In vitro* platelet reactivity towards various agonists such as ADP, collagen, TRAP, thrombin [184] and CRP can be analysed using flow cytometry.

The platelets in a sample can be detected based on their FS and SS properties as shown in Figure 7. As an alternative, the platelets can be detected by the use of an antibody directed against the platelet-specific receptor GPIb (CD42b). Once the platelets have been identified they can be analysed with respect of the property of interest (i.e. the activation epitope). The fluorescent signal can be expressed as the percentage of positive cells (relative to an irrelevant (isotype) control antibody) or as the mean fluorescence intensity (MFI) of the entire platelet population as shown in Figure 7 [184].

Monoclonal antibodies more reliably saturate all specific epitopes and result in less non-specific binding than polyclonal antibodies. Thus they are preferable in whole blood flow cytometry. Antibodies directly fluorescence-conjugated are preferable because they eliminate the need to add secondary antibodies, which could result in higher background fluorescence and decreased sensitivity [184].

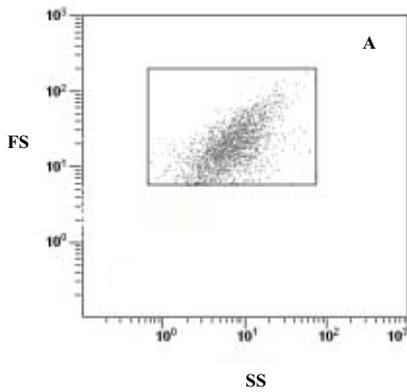
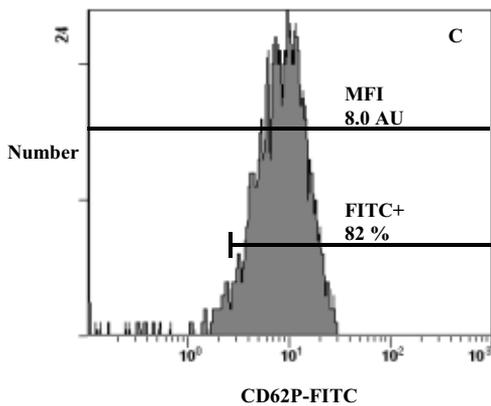
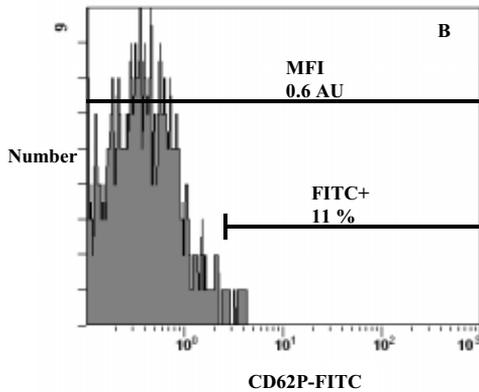


Figure 7. Flow cytometry plots.

The detection of the platelet population based on their forward scatter (FS) vs. side scatter (SS) properties (A). The spontaneous binding of fluorescein isothiocyanate (FITC) conjugated CD62P to platelets (B). The binding of CD62P-FITC to platelets following stimulation with thrombin receptor activating peptide (TRAP), TRAP-6 (C). The binding can be expressed as percentage of positive platelets (platelets within the FITC+ region) or mean fluorescence intensity (MFI in arbitrary units, AU) of the entire platelet population.



ANALYTICAL PROCEDURES

FOR analysis

Paper I

Non-anticoagulated blood, citrated blood, PRP or plasma samples were activated with CRP (final concentration 0.0016 mg/mL), an activator of the platelet receptor GPVI, or thromboplastin (final concentrations between 0.00-0.18 AU/mL as indicated in Paper I). Coagulation was analysed by FOR with the ReoRox[®] 4 using gold-coated reaction chambers. Citrated blood was also analysed in the presence of GPIIb/IIIa blocker, abciximab (final concentration 0.02 mg/mL). Non-anticoagulated blood from a patient with Glanzmann's thrombasthenia was analysed by FOR. Citrated blood without and with extra fibrinogen supplemented (+2 and +4 g/L extra) was analysed by FOR. The citrated blood, plasma and PRP samples were re-calcified prior to activation with a final CaCl₂ concentration of 0.01-0.02 mol/L to obtain a free CaCl₂ concentration of 1.5-1.8 mmol/L. All non-anticoagulated samples were analysed immediately after blood collection. The clotting time, G'max and G' max-slope were evaluated.

Paper II

Non-anticoagulated blood was activated with thromboplastin (final concentration 0.055 AU/mL). The coagulation was monitored over time by FOR using gold-coated reaction chambers. FOR analysis was performed immediately after blood collection. The clotting time and G'max were evaluated.

Papers III and IV

The PC sample was diluted to a concentration of 100×10^9 platelets/L in plasma from an AB D+ donor. The diluted PC sample was re-calcified with CaCl₂ (final concentration 0.02 mol/L to obtain a free CaCl₂ concentration of 1.5-1.8 mmol/L) and activated with TRAP-6 (amino acid sequence SFLLRN, final concentration 0.09 mmol/L). Clotting time and G' were analysed by FOR using gold-coated reaction chambers. The clotting time, G'max, time to G'max and mean change in G' per minute were evaluated.

Paper V

The platelet concentration in the PC samples was adjusted to 100×10^9 /L by dilution in plasma from an AB D+ donor. The diluted PC samples were re-calcified with CaCl₂ (final concentration 0.02 mol/L to obtain a free CaCl₂ concentration of 1.5-1.8 mmol/L) and activated with TRAP-6 (final concentration 0.086 mmol/L). The clotting time was analysed in a polyamide sample cup with ReoRox[®] 4.

Flow cytometry

Paper I

Dose-response analysis of the GPIIb/IIIa inhibitor abciximab on platelet activation was performed by flow cytometry. Platelet activation was measured in blood as platelet-bound fibrinogen on activation by TRAP-6. Blood was incubated for 5 min in the presence of abciximab at various concentrations between 0 and 0.02 mg/mL. The blood was then incubated with anti-GPIIb-PE and anti-fibrinogen-FITC for 10 minutes followed by 10 min activation with TRAP-6 (final concentration 0.3 mg/mL). Flow cytometry was performed with an Ortho Cytoron Absolute Flow Cytometer using the ImmunoCount II software (v 2.00; Ortho, Raritan, NY, U.S.A) and 1000 events were collected based on their FS and PE expression. The percentage of platelets binding fibrinogen, normalised to the value for an irrelevant control antibody with corresponding fluorescence intensity, was determined.

Papers III and IV

Platelet surface markers were measured by flow cytometry. The sample from the PC was diluted in autologous plasma to a platelet concentration of 300×10^9 platelets/L. The diluted PC sample was then incubated with anti-CD62P-FITC, anti-insulin-FITC, anti-CD42b-FITC or anti-IgG2a-FITC for 20 minutes. The diluted PC sample was also incubated with anti-CD62P-FITC for 10 minutes followed by 10 minutes activation with TRAP-6 (final concentration 0.06 mmol/L). Flow cytometry analysis was performed with the Beckman Coulter Epics XL-MCL instrument (Beckman Coulter Inc., Fullerton, CA, U.S.A) with a computer software program (Expo 32 ADC, Beckman Coulter Inc.). The fluorescence intensity was monitored daily with fluorescent beads (Flow set, Beckman Coulter Inc.). Five thousand events were collected based on their forward and side scatter properties. The percentage of platelets expressing P-selectin (CD62P) or GPIb (CD42b), normalised to the value for an irrelevant control antibody (anti-insulin and IgG2a, respectively), as well as the MFI values were determined.

HSR

Papers III and IV

HSR, which reflects the ability of the platelets to regain normal size after exposure to a hypotonic solution, was measured according to Valeri et al. [158]. The platelet concentration was adjusted to 300×10^9 platelets/L by dilution with autologous plasma. HSR was measured with a dual-beam spectrophotometer (UV-2101 PC, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 610 nm. The beams of the spectrophotometer were zeroed simultaneously with 0.6 mL of autologous plasma mixed with 0.3 mL of NaCl in the reference and test positions. After that, 0.6 mL of diluted PC mixed with 0.3 mL of NaCl was placed in the test position and light transmission was recorded for 11 minutes. Finally, 0.6 mL of diluted PC mixed with 0.3 mL of water was placed in the test position and transmission was once again recorded for 11 minutes. The results were expressed as the percentage of recovery at 10 minutes after exposure to the hypotonic solution.

Statistical analysis

Mean \pm SD and Student's T-test were used for statistical analyses. Paired or unpaired two-tailed Student's T-test analysis was used for comparisons between groups. Three-way analysis of variance (ANOVA), coefficient of variation (C.V.), correlation coefficient and the two-tailed significance of the correlation were also used for statistical analysis. The median, percentiles and outliers are presented in the box plots.

RESULTS AND DISCUSSION

Paper I

The purpose of **Paper I** was to determine the influence of various blood components on the FOR analysis. The effect of the oscillation-induced stress on clot G' was assessed. We studied how the coagulation process and change in G' over time were influenced by citrate anticoagulation, various platelet concentrations ($0-200 \times 10^9/L$), fibrinogen concentration and different haematocrit ($0-40\%$). The importance of the GPIIb/IIIa receptor on platelet function was investigated by comparing the FOR parameters of blood in the presence and absence of a GPIIb/IIIa receptor inhibitor, abciximab, and by analysing blood from a patient with Glanzmann's thrombasthenia.

The stress induced by the oscillation reduced the elasticity by 8%, which is less than the 50% reported for TEG[®] [186]. Non-anticoagulated blood samples ($n=5$) and re-calcified citrated blood samples ($n=5$) had similar clotting times and G' max-slope values, whereas G' max was higher in non-anticoagulated samples ($p<0.05$). The clotting time decreased with increasing platelet concentration when CRP was used as the activator ($n=5$) but not with thromboplastin ($n=4-8$). G' max and G' max-slope values increased with increasing platelet concentrations for both activators. FOR analysis of blood samples without or with extra fibrinogen supplemented ($n=10$) showed that an increasing concentration of fibrinogen resulted in decreased clotting time and increased G' . Analysis of samples with haematocrits of 0%, 20%, 30% and 40% ($n=5$) showed that the G' decreased with increasing concentrations of red blood cells. The difference was significant ($p<0.05$) below 30% compared with 40%. Treatment of blood samples with the GPIIb/IIIa receptor inhibitor, abciximab, resulted in prolonged clotting time ($p<0.01$) and a reduction in G' . G' max was decreased from 1137 ± 225 Pa to 135 ± 52 Pa with abciximab, demonstrating that the platelets contribute by 90% to the maximum elasticity. This is higher than reported for TEG[®], where the platelets only contributed 50-55% of the maximum elasticity [187,188]. The reason for this discrepancy is the narrow measuring range for TEG[®]. Blood from a patient with Glanzmann's thrombasthenia also had low G' , further indicating the importance of functional GPIIb/IIIa receptors. The concentration of fibrinogen and red blood cells as well as the concentration and function of the platelets all affect the elasticity. The presence of functional platelets was the major determinant of clot elasticity

Paper II

Paper II sought to test whether FOR can be used to measure the clot properties of blood from subjects at risk of thrombosis or bleeding and to study the effect of platelet transfusions. Pregnant women represented subjects at risk of thrombosis as hypercoagulability is known to occur during pregnancy. Leukaemia/lymphoma patients with severe thrombocytopenia represented the subjects at risk of bleeding.

Coagulation was measured by FOR in blood obtained from pregnant women in different pregnancy trimesters (n=58) and compared with coagulation in blood from non-pregnant women (n=30). Coagulation was also analysed in blood from patients with severe thrombocytopenia before and after a platelet transfusion (n=20) and compared with that of healthy blood donors (n=60).

There was no difference in platelet concentration between the pregnant women and the female blood donors. The clotting time was shorter and the G' higher in pregnant women than in non-pregnant women. G'max increased gradually during pregnancy and was higher ($p<0.01$) in all pregnancy trimesters compared with that of non-pregnant women. Shorter clotting time and high G' suggest increased coagulation. Others, using TEG[®], have also reported hypercoagulability in pregnant women [189,190]. By the third trimester, G'max had increased by 75%, which is higher than the 14-19% increase in elasticity reported by others using TEG[®] [189-191].

The platelet concentration and G'max were lower in the patients prior to transfusion compared with the blood donors ($p<0.01$). There was an increase in both platelet concentration and G'max after the platelet transfusion, but the patients still had lower platelet concentration and G'max values after the transfusion compared with blood donors ($p<0.01$). There were patients with similar platelet concentration but different G'max. There was no difference in clotting time between the patients and the blood donors. Three of the patients had reported bleeding prior to transfusion, all of whom had a G'max below the mean value for the patients.

There was a different increase in G' following platelet transfusion in patients with similar platelet count increment after the transfusion, suggesting a difference in the function of the transfused platelets. The G' thus provides information on the function of transfused platelets in contrast to the post-transfusion platelet count that only gives information on the concentration of circulating platelets. The results of this study suggest that the G' provides information on the haemostatic status of thrombocytopenic patients, not only on the platelet concentration but also on functional properties of the platelets.

Paper III

The use of single-donor PCs has increased worldwide in the recent years [192]. Single-donor PCs are collected using the AP technique. Different cell separators are available for collecting single-donor platelets. Until recently, PCs have been stored for a maximum of five days due to the risk of bacterial contamination. The introduction of bacterial detection systems has made it possible to extend the storage period from 5 to 7 days providing the platelet function is well maintained. Prolonging the storage period would be advantageous as it would limit wastage due to outdated and thereby reduce the costs.

In **Paper III**, platelet function was assessed by *in vitro* analyses including FOR of PCs during prolonged storage for 7 days to compare the quality of PCs produced by the COBE[®] Spectra[™] and Trima[®] Accel[™] cell separators. The COBE[®] Spectra[™] has a dual-stage channel for the separation of platelets from other blood cells. In the first stage the red blood cells and white blood cells are removed. The platelets then flow into the second stage where they are concentrated [84]. The Trima[®] Accel[™] has a single-stage channel where the cells separate at maximum radius and over full channel circumference [193]. Ten PCs collected with each separator were included in the study.

Swirling and the percentage of GPIb-expressing platelets were well maintained for 7 days for both Spectra[™] and Trima[®] produced PCs. HSR was well maintained during the storage period and with no difference between Spectra[™] and Trima[®] PCs. HSR has previously been shown to be well maintained for 7 days in Spectra[™] and Trima[®] PCs [171,194]. Dumont & VandenBroeke in contrast reported a decrease of 15% in HSR during 7 days of storage in PCs collected by the Spectra[™] [151]. pH was well maintained during storage and was within an acceptable range (6.4-7.4). Glucose decreased and lactate increased during storage ($p < 0.05$) as expected, with significant differences between day 5 and 7 but were always at acceptable levels (i.e. glucose was always available and lactate was never above 28 mmol/L). Glucose and lactate were similar in both types of PCs during the storage period. The platelets were gradually activated, as measured by P-selectin expression (both surface bound and soluble), during the storage period, with a significant increase between day 5 and 7 ($p < 0.05$). Previous studies have also reported increased P-selectin expression on platelets collected by the Spectra[™] and Trima[®] cell separators [151,171,195,196]. No difference in P-selectin could be detected between PCs produced by the two cell separators in our study. Others have reported different P-selectin expression on platelets when comparing various separators [197-199]. Platelets exhibited reduced response to the activator TRAP-6 during storage as measured by P-selectin expression and the response was similar in both types of PCs.

There was no difference in the FOR parameters for Spectra[™] and Trima[®] PCs. The clotting time was well maintained during the storage period. G'max was slightly increased from day 1 to 5 ($p < 0.05$). Gutensohn et al. also reported higher elasticity in stored PCs than in fresh PCs as assessed by thromboelastography [200]. However, the longer the storage time, the longer the time to obtain maximum clot retraction (prolonged time to G'max, reduced mean change in G' per minute). The prolonged time to reach G'max could be attributed to a reduction in the ability of the platelets to respond to activation. No difference was detected in the FOR parameters between 5- and 7-day old PCs.

Several of the *in vitro* parameters showed that platelets stored for 7 days have a good quality (e.g. HSR, pH, swirling and acceptable levels of glucose and lactate) despite a gradually

increased activation state and a gradually decreased ability to be activated. The FOR analysis also showed that platelets stored for 5 or 7 days have similar clotting properties. AuBuchon et al. [152] and Dumont et al. [171] found a reduction in recovery and survival after autologous transfusion of platelets stored for 7 days compared with platelets stored for 5 days. Dumont et al., however, concluded that the magnitude of the changes would not result in the diminution of transfusion efficacy in a clinical situation. These findings support our results suggesting that the storage period can be prolonged from 5 to 7 days.

Paper IV

A rare, but often lethal, transfusion complication is TA-GVHD. TA-GVHD is caused by remaining T-lymphocytes in the blood product. However, the development of TA-GVHD can be prevented by gamma irradiation of the blood product.

In **Paper IV** *in vitro* methods including FOR analysis, were used to investigate the quality, of PCs treated with 25 Gy gamma irradiation during storage for 7 days and compared with non-irradiated control PCs. Platelets were collected using the AP technique from 20 donors, and the platelets from each donation were divided into two standard units (PCs). One PC was irradiated; the other, non-irradiated, PC was used as a control.

Swirling, HSR and the percentage of platelets expressing GPIb were well maintained for 7 days of storage in both types of PCs. Irradiated and non-irradiated PCs had similar HSR, as has been shown previously [156]. pH was well maintained during the storage period and at an acceptable range (6.4-7.4). No difference could be found in pH between irradiated and control PCs, which is in accordance with some previous reports [201,202]. There are also reports that have shown a difference in pH between irradiated and control PCs [156,203]. Glucose decreased and lactate increased during the storage period ($p < 0.05$) to a similar extent in both types of PCs. Others have reported similar levels of glucose and lactate in irradiated and non-irradiated PCs [156,201]. Surface-bound activation marker P-selectin as well as soluble P-selectin increased from day 1 to 7 ($p < 0.05$). Previous studies have also reported increased P-selectin expression in irradiated and non-irradiated AP PCs [153,171,201,204]. The percentage of P-selectin expressing cells was higher in the irradiated PCs on day 1 of storage but no other difference was detected between irradiated and non-irradiated PCs with respect to P-selectin. P-selectin expression induced by TRAP-6 was reduced during storage ($p < 0.05$) in both irradiated and control PCs, with no difference between the two types of PCs. Others have also found a reduced response to TRAP during storage of AP PCs [153,196,205]. There was no difference between irradiated and non-irradiated PCs for any of the FOR parameters. The clotting time and G'max of 7-day old PCs were well maintained, and G'max was increased in 5- and 7-day old PCs ($p < 0.05$). It took longer time to obtain G'max (prolonged time to G'max, reduced mean change in G' per minute) in 5- and 7-day old PCs compared with 1-day old PCs. This result suggests that the capacity of the platelets to retract the coagulum is reduced during storage, but if sufficient time is allowed for the platelets, the clot will be fully retracted.

The results presented in this study show that gamma irradiation of platelets does not affect PC quality as assessed by *in vitro* tests including FOR.

Paper V

Bacterial contamination of PCs can cause transfusion-transmitted infections. The introduction of a few bacteria at component collection can grow to high numbers during room temperature storage. A pathogen inactivation procedure has been developed to prevent transfusion-transmitted infections [113]. This procedure, called the Intercept™ Blood System for Platelets, uses amotosalen HCl and UVA light to inactivate contaminating viruses and bacteria [113]. As this treatment minimises the risk of bacterial transmission, PCs treated with Intercept™ might be used for an extended period of storage provided platelet function is well preserved.

We investigated the quality of fresh Intercept™ treated AP PCs (n=27) as measured by FOR clotting time and compared it with fresh platelets in the additive solution, T-Sol™ (n=47) and PCs in plasma (n=46). All PCs were analysed when they were available for transfusion (<26 hours after collection). Platelets in T-Sol™ and plasma had similar clotting time. The Intercept™ treated platelets responded well to a platelet activator, TRAP-6, as measured by clotting time. The clotting time was slightly shorter for Intercept™ treated platelets than platelets in T-Sol™ and plasma. We attribute this to a slight pre-activation of the Intercept™ treated platelets, as was also shown by higher sP-selectin values for Intercept™ PCs.

It has been reported that fresh Intercept™ treated platelets respond to TRAP activation to a similar extent as non-treated platelets when assayed by P-selectin expression using flow cytometry [153]. The study reported that the response of Intercept™ treated platelets to TRAP was lower than for non-treated platelets from day 5 of storage. We did not address the issue of storage time in our study.

The correlation of our FOR results with *in vivo* viability and function of Intercept™ treated platelets needs to be further elucidated. It has been reported that Intercept™ treated PCs have reduced *in vivo* platelet viability compared with platelets in plasma, T-Sol™ and InterSol™ [135,141,143-145] but the platelets seem to be able to provide adequate haemostasis [143-145].

GENERAL DISCUSSION

An imbalance in the haemostatic process can lead to thrombotic or bleeding events. Patients with thrombocytopenia are at risk of bleeding events and require platelet transfusion to prevent or stop bleeding. The purpose of a platelet transfusion is to supply platelets with good haemostatic properties. A recent review stated that an ideal platelet transfusion concentrate should contain platelets with a low activation state and a high activation capacity, and lack of markers that could result in platelet destruction and removal following transfusion [206]. The decision to transfuse platelets and the success of a transfusion is usually based on the number of circulating platelets in the patient and the count increment following transfusion. Changes in coagulation can be evaluated by analysing clotting factors, D-dimers, prothrombin time and activated partial thromboplastin time in plasma. Unfortunately, these methods do not measure the combined effect of plasma and cellular components on coagulation. Other available methods such as aggregometry and platelet adhesion assay (PFA-100[®]) are not suitable for thrombocytopenic patients as they require a normal number of platelets. Improved methods are required that include contributions from all blood components to identify patients at risk of thrombosis or bleeding and that can provide information on the function of the transfused platelets.

The quality of PCs can be evaluated by measuring platelet viability following transfusion. However such measurements are expensive and complicated to perform, and *in vitro* methods are often used to evaluate PC quality as surrogate tests to *in vivo* viability. The value of currently used tests in predicting *in vivo* viability is not clear and few of them directly measure the platelets haemostatic function. In the 1970's HSR was shown to correlate to *in vivo* viability [158,160]. However, in a recent study Slichter et al. was unable to detect such a correlation [172]. pH in the range of 6.4-7.4 has been shown not to correlate with *in vivo* viability [150], but pH values below 6.0 or above 7.4 have been shown to result in low viability [78-80]. The relevance of increased platelet activation on *in vivo* viability is not clear. Some studies have shown a negative correlation between P-selectin expression and *in vivo* viability [85,156,168,171] but some report only a low correlation [85,168]. Furthermore, a recent study found no correlation between P-selectin expression and *in vivo* viability [172]. A new technique, the cone and plate analyzer (Impact-R), has recently been tested for evaluation of PC quality [196]. As the cone and plate method requires the presence of red blood cells, the PC sample must be suspended with red cells, which makes the method complicated for analysis of PC quality. Therefore, new improved methods that are easy to use are needed to evaluate the haemostatic quality of platelets in PCs during storage and might be used to predict the survival and the post-transfusion haemostatic efficacy of the platelets.

FOR analysis using the ReoRox[®] 4 instrument can be used to monitor the coagulation process. ReoRox[®] 4 is a small portable instrument that can be used for bedside analysis and is easy to use. FOR analysis of whole blood offers assessment of clot properties based on contribution of all blood components, as is the case *in vivo*. This makes FOR an interesting option when assessing subjects at risk of hyper- or hypocoagulation (i.e. thrombosis or bleeding). FOR can also be used to analyse PRP samples and provides information on the ability of platelets to support the formation of a clot and to induce clot retraction. Hence, it can also be used in analysis of PC quality.

In this thesis we assessed the use of FOR to measure the coagulation properties in subjects at risk of thrombosis (i.e. pregnant women) or bleeding (i.e. thrombocytopenic patients).

Moreover, we explored the use of FOR to determine the function of platelets in PCs during storage and, in combination with other *in vitro* tests, to determine the effects of different preparation and storage conditions.

FOR was shown to be promising for assessment of clot properties in subjects with hypercoagulability and hypocoagulability. As the elastic properties of whole blood are influenced by the concentration of the platelets, red blood cells and fibrinogen, it might be helpful to measure the concentration of these factors to interpret the elasticity curve.

The finding that 90% of the maximum elasticity is dependent on functional platelets suggests that FOR can be used to measure platelet function. Platelets were shown to contribute only by 50-55% to the maximum elasticity measured by thromboelastography, a technique similar to FOR [187,188]. This result and our data on the wider elasticity measuring range of FOR suggest that FOR is to be preferred to thromboelastography for analysing platelet function.

FOR was shown to provide information on the haemostatic function of transfused platelets. This makes FOR advantageous to the current method of measuring the post-transfusion platelet count increment that only provide information on the increase in the concentration of circulating platelets following transfusion. The use of FOR in clinical practice to assess clot properties in subjects at risk of hyper- or hypocoagulability needs to be further evaluated.

Until recently, the storage period for PCs has been limited to 5 days mainly because of the risk of bacterial growth. The introduction of bacterial detection systems and bacteria inactivation procedures has made it possible to extend the storage period from 5 to 7 days provided the platelet function is well maintained. An extension of the storage period would result in less wastage of PCs caused by outdating and lead to increased PC supply. When FOR was used to analyse the platelet quality of PCs during prolonged storage for 7 days, we found that the clotting time was well maintained throughout storage. Maximum elasticity (G'max) was slightly increased in 5- and 7-day old PCs, and a recent review suggested that such an increase in clot elasticity is an indication of increased GPIIb/IIIa signalling [206]. Concomitant with the increased G'max, the time to G'max was prolonged and the mean change in G' per minute decreased, showing that it takes longer for the platelets to attain full clot retraction. The relevance of these changes on *in vivo* viability and function needs to be further studied.

CONCLUSIONS

- Free oscillation rheometry (FOR) can be used to analyse clotting time and clot elasticity in non-anticoagulated whole blood, re-calcified citrated whole blood, re-calcified citrated plasma and re-calcified citrated platelet-rich plasma.
- An increased concentration of fibrinogen and reduced concentration of red blood cells influence the elasticity as measured by FOR. Functional GPIIb/IIIa receptors are important for clotting time and clot elasticity.
- Clot properties, as measured by FOR, vary between subjects at risk of thrombosis or bleeding. FOR can detect the progressive increase in hypercoagulability occurring during the course of pregnancy. FOR can also provide information about the haemostatic status of thrombocytopenic patients and on the haemostatic effect of platelet transfusions.
- Single-donor apheresis platelet concentrates (PCs) stored for seven days have good quality (i.e. have well maintained hypotonic shock response, swirling, pH, acceptable levels of glucose and lactate) despite increased activation. The FOR results further support the prolongation of the storage time from five to seven days.
- No difference in platelet quality as analysed by FOR and other *in vitro* tests was observed between Spectra™ and Trima® produced PCs despite differences in collection principles.
- Platelets gamma irradiated with 25 Gy on the day of collection have similar quality to non-irradiated platelets during storage for seven days as measured by FOR and other *in vitro* tests.
- Intercept™ treated platelets have good clot-promoting capacity as measured by FOR.

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