A STUDY ON THE EFFECTS OF SEROTONIN REUPTAKE INHIBITORS ON PLATELET FUNCTION IN WHOLE BLOOD AND PLATELET CONCENTRATES

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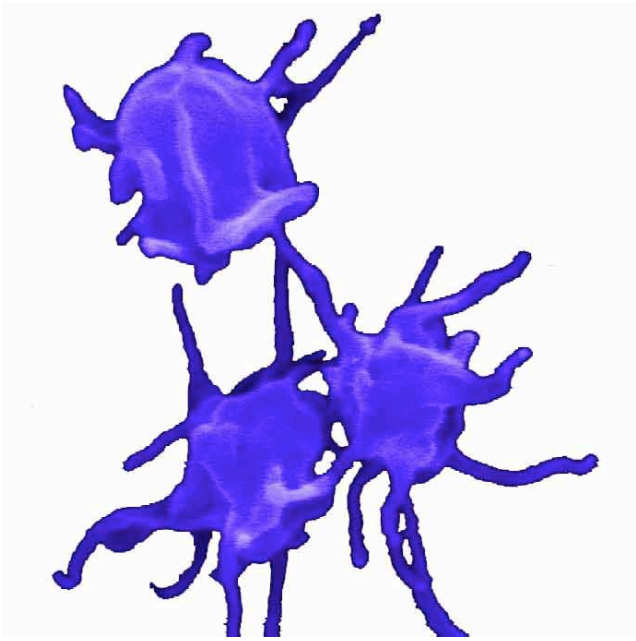

A thesis submitted for the degree of
Master of Pharmacy

Centre of Pharmacy, The Gade Institute, Institute of Medicine
University of Bergen, Norway
May 2010

Acknowledgments

First of all I would like to thank my supervisors Tor Audun Hervig and Steinar Hustad. Your knowledge, guidance and encouragement are highly appreciated.

I would also like to thank Linda Skordal and Solfrid Bjørsvik for giving me extensive training in laboratory methods, and for always taking time to answer my questions. A special thanks goes out to Judith Eriksen for being patient and for never giving up when nothing went our way during the serotonin analysis. I must also acknowledge the staff at the Blood bank that was involved in my project.

I would like to express my gratitude to Torun Apelseth for giving me statistical advice and Ina Nepstad for giving my valuable advice during the writing process.

I would also like to thank my parents, my brothers and my friends who support and encourage me in everything I do.

My special thanks go out to Terje for always believing in me.

At last I would like to thank Marianne for being the best friend one can ever get.

I could never have done this without any of you.

Bergen, May 2010

Anne-Grete

Bergen, May 2010

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Abstract

Background: Several studies report that patients who use serotonin reuptake inhibitors (SRIs) to treat depression may have increased risk of bleeding, particularly from the gastrointestinal tract. This may be related to low concentrations of serotonin in platelets. Several blood banks defer SRI users from platelet donation. Search in Medline have not revealed any study clarifying the effect of SRIs on platelets stored for transfusion.

Methods: We conducted a prospective blinded randomized control study in blood donors using SRI (n=8) and in donors without medication (n=10). The test group consisted of 4 men and 4 women, and the median age was 40 years (range: 29-54 years). The control group consisted of 3 women and 7 men, and the median age was 39 years (range: 21-54 years). Platelet function was compared in whole blood and in platelet concentrates stored for up to five days. In addition, quantitative analyses of serotonin, tryptophan and tryptophan metabolites were undertaken.

Results: Light transmission aggregometry, trombelastography and flow cytometric analysis of glycoprotein expression did not detected altered platelet function in blood donors using SRI. Routine tests for platelet confirmed high quality of platelet concentrate obtained from donors using SRIs. Blood donors using SRI have markedly lower platelet serotonin compared to blood donors without medication.

Conclusion: The results from our pilot study indicate that patients using SRIs do not have altered platelet function and can be accepted as platelet donors. Our results are limited to platelets stored for 5 days and for low doses of SRI. There is a need for larger studies to finally clarify the influence of SRI on platelets stored transfusion.

Abbreviations

ADP	Adenosin diphosphate
BBB	Blood brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
CYP	Cytochrom P450
DDD	Defined daily doses
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
DTS	Dense tubular system
EDTA	Ethylenediaminetetraacetic acid
FSC	Forward scatter
GIT	Gastrointestinal tract
GP	Glycoprotein
HPLC	High performance liquid chromatography
LTA	Light transmission aggregometry
LC-ESI-MS/MS	Liquid chromatography- electronspray ionization- tandem mass spectrometry
MAO	Monoamin oxidase
MP	Microparticle
NAD	Nicotinamide adenin dinucleotide
NADP	Nicotinamide adenin dinucleotide phosphate
NET	Norepinephrine transporter
NSAID	Non-steroidal anti-inflammatory drug
OCS	Open canalicular system
PAS	Platelet additive solution
pCO_2	Partial pressure CO_2
PC	Platelet concentrate
PLT	Platelet count
pO_2	Partial pressure O_2
PRP	Platelet- rich plasma
PPP	Platelet- poor plasma
rpm	Rotation per minute
SDC	Single donor concentrate
SERT	Serotonin transporter
SNRI	Serotonin norepinephrine reuptake inhibitor
SRI	Serotonin reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SSC	Side scatter
TCA	Tricyclic antidepressant
TEG	Thrombelastography
TF	Tissue factor
TRAP	Thrombin receptor activator peptide
TXA_2	Thromboxane A_2
vWF	von Willebrand factor
5-HT	5-hydroxytryptamine (serotonin)
5-HIAA	5-hydroxyindoleacetic acid

1 Introduction

1.1 Background

The Word Health Organization predicts that depression will become the leading contributor to disease burden in the western world within 2030 (1). The main biochemical theory of depression is the monoamine hypothesis, which claims that depression is caused by functionally deficient monoamergic transmission in the central nervous system (CNS) (2). The restoration of serotonin and norepinephrine deficiencies to normal levels as a therapeutic strategy is based on this hypothesis (3). In moderate and severe depression, pharmacological intervention is one of the main forms of effective treatment and the selective serotonin reuptake inhibitors (SSRIs) have dominated the antidepressant marked since the introduction in the 1990 (4). More recently, the serotonin norepinephrie reuptake inhibitors (SNRIs) have been introduced as an alternative to the SSRIs. Serotonin reuptake inhibitor (SRI) is used as generic name for SSRI and SNRI.

It is presumed that the mechanism of action of the SRIs is presynaptic blockade of the neuronal reuptake of norepinephrine and/or serotonin in the central nervous system, resulting in an increase in transmission by monoamine neurons (5, 6). In the last few years, numerous case reports and population-based cohort studies suggested that antidepressant with a relevant blockade action on the serotonin reuptake increase the risk of bleeding episodes, particularly from the gastrointestinal tract (GIT) (7-11). Studies indicate that patients using SSRI have increased transfusion requirements during orthopedic surgery have also been published (12, 13). In addition to serving as a neurotransmitter in the CNS, serotonin is involved in a variety of biological processes, including haemostasis. In humans, platelets are unable to synthesize serotonin, but almost all circulating serotonin is transported into platelet dense granules (14). Upon activation, platelets release serotonin, which acts as a vasoconstrictor and an amplifier of the aggregation response (15, 16). Currently, it is well established that platelets and neurons act similarly with respect to serotonin uptake, release and metabolism, and human platelets have been used as models for the study of several aspects regarding the function of CNS serotonergic neurons (17). Because serotonin promotes platelet aggregation, the underlying mechanism of impaired haemostasis

in patients using SRIs is suggested to be due to the depletion of serotonin in platelet as a result of the reuptake inhibition (18-20).

The purpose of platelet transfusion is to provide platelets with satisfying haemostatic properties to patients suffering from thrombocytopenia or impaired platelet function. Blood donors are recruited among voluntary, healthy individuals. Accordingly, the main principle is that blood donors should be drug-free. However, as the numbers of blood donors is below the need to ensure the blood supply, compromises must be made. Currently, there is word-wide agreement that blood donors needing substitution for hypothyreosis and woman using anticonseption therapy are eligible for donation. When it comes to drugs against depression, there is different practice between countries. In Norway, individuals using antidepressant drugs may be donors, which is not the case in Denmark (21). Search in Medline database, has not revealed any study clarifying the effect of SRIs on platelets stored for transfusion. We therefore conducted a prospective blinded randomized control study, aiming to compare platelet function in platelet concentrate (PC) during storage obtained from blood donors using SRIs as only medication and blood donors without medication. Additional *in vitro* platelet aggregation studies at baseline (day of donation) and quantitative analyses of serotonin and the serotonin precursor tryptophan were also performed.

1.2 Platelets

Platelets are small, irregularly shaped fragments derived from the cytoplasm of the megakaryocyte. After passage from the bone marrow into the bloodstream, the platelets circulate for less than 10 days before ending their short life in the spleen. The platelets normally range from 2 to 4 μm in diameters, and they only contain mitochondrial DNA since they lack a nucleus (22). The platelet concentration in healthy individuals varies between 250,000 and 400,00 platelets per μl of blood and the major biological role of the platelets is the maintenance of the haemostasis by sealing rifts in the vascular tree (22).

1.2.1 Morphology

Organelles

Platelets contain organelles that are essential for normal platelet function. The mitochondria are responsible for oxidative metabolism, which supplies energy. Peroxisomes are small organelles presents in platelets, assumed to play an important role in the lipid metabolism. Platelets also possess three types of granules. α- granules are the most abundant granules in the platelets and contain factors, which are released upon activation to facilitate vessel repair, platelet aggregation and coagulation of blood (23). The dense bodies are another type of granules, which contain factors facilitating platelet aggregation and adhesion as well as factors able to induce vasoconstriction (24). The third types of granules are the lysosomes, which contain hydrolytic enzymes.

Cytoskeleton

The platelet cytoskeleton consists of three main components. The microtubuli coil and the membrane skeleton are situated immediately below the plasma membrane, while a network of long- interconnecting actin filaments fill the cytoplasm (25). The dynamic behaviour of the cytoskeleton is responsible for the rapid change in shape upon activation of platelets. The cytoskeleton also regulates activities of membrane proteins, and binds signal molecules, directing them to specific cellular location (25).

Tubular systems

The surface-connected open canalicular system (OCS) is a unique feature of the platelet anatomy. It consists of a series of conduits that begin as indentations of the plasma membrane and permeate the interior of the platelet. The OCS provides a way for the platelets to communicate with the outside by allowing entry of external elements and by providing a "road system" for the release of the granule contents in addition to function as a storage compartments for glycoproteins (26). The platelets also possess another tubular system called the dense tubular system (DTS). The dense tubular system is a closed channel network, originating from the endoplasmatic reticulum of the megacaryocyte. The membrane system

functions to release calcium, regulating platelet activation (27). It is probably also a major site of prostaglandin and tromboxane synthesis (28).

The plasma membrane

The plasma membrane consists of a bilayer of phospholipids in which cholesterol, glycolipids and glycoproteins are embedded. In activated platelets the distribution of phospholipids is asymmetric, giving rise to a negatively charged membrane inside (29). The negatively charged phospholipids serve as a catalytic surface, which is able to accelerate several steps in the coagulation sequence (29). Numerous glycoproteins are present on the platelet surface, which facilitate interactions with subendothelial structure, blood cells or other platelets.

Platelet surface molecules

Many molecules cover the platelet surface. The molecules that are most important for platelet function and the molecules that we used to measure platelet activation in this study, are addressed here. For didactic reasons they are separated into two groups; receptors and activation markers.

Platelet receptors

When the corresponding ligand binds to a receptor, a well defined response is performed. The receptors relevant for this study are summarized in Table 1.1.

Table 1.1 Agonists, ligands and receptors important for platelet function

Function	Receptors	Ligand/Agonist
Adhesion	GPIb/V/IX	vWF
	GPIa/IIa	Collagen
Activation and amplification	PAR-1	Thrombin
	$P2Y_1$, $P2Y_{12}$	ADP
	$5\text{-}HT_{2A}$	Serotonin
	TPαβ, TPβ	TXA_2
	α_{2A}	Epinephrine
Aggregation	Activated GPIIb/IIIa	Fibrinogen

TXA_2= tromboxane A_2

Following vascular injuries, the endothelium release von Willebrand factor (vWF), which deposits in the area of damage. The multiple binding sites of vWF, enable binding to platelet receptor GPIb/IX/V and the major matrix protein collagen, resulting in platelets capture at site of injury. ADP released from the dense bodies mediates mobilization of Ca^{2+}, shape change, transient aggregation and platelet secretion predominantly via two important ADP receptors on the platelet surface (30). Serotonin binds to the G-protein-coupled 5-HT_{2A} and amplifies together with ADP the platelet response. TXA_2 mediates platelet aggregation and shape change via two distinct platelets receptors (31), while epinephrine synergize with others agonist like ADP. Thrombin is one of the most potent platelet agonists and activates the G-protein-coupled receptor PAR-1 (32). The platelet receptor GPIIb/IIIa, also termed CD61, plays a key role in platelet aggregation. GPIIb/IIIA is present at high density on the platelet surface, but in a low-affinity ligand-binding state on resting platelets. Activation by agonists including ADP, serotonin, thrombin and collagen causes conformation change and promotes the binding to fibrinogen (33).

Platelet activation markers

GPIbα, also termed CD42b, is a subunit of the GPIb complex, which constitutes the binding site for vWF (34). Decreased expression of CD42b is shown to be a reliable marker for platelet activation (35). P-selectin, also termed CD62P, is a glycoprotein present in the membrane of α granules in resting platelets. Upon platelet activation, the protein is secreted and expressed on the platelet surface (36) to mediate the attachment of neurophiles and monocytes to platelets and endothelial cells (37). Increased expression of P-selectin is widely used as a marker of platelet activation (38, 39).

1.2.2 Platelet Function

Hemostasis is a complex physiological process, which prevents extensive bleeding after vessel wall injury and ensuring viable coagulation by keeping the blood in an uncoaculated state. The process of stopping a bleeding by sealing rifts in the vascular tree requires the combined activity of platelets, plasma factors and blood vessels. For didactic reasons, haemostasis is usually divided into primary and secondary haemostasis.

Primary hemostasis

The first response after injury of small blood vessels is the process of arteriolar vasoconstriction to reduce local blood flow. Vasoconstriction not only reduces extensive blood loss, but it also promotes the adherence of platelets to exposed subendothelial material. For this to occur, platelets must undergo activation by physiology platelet agonists. These activators are present at the site of vascular injury, giving a local response. Based on their ability to induce platelet activation, agonist can be classified as either weak (e.g., epinephrine, serotonin, ADP) or strong (e.g. collagen and high-dose thrombin). Platelet activation results in several phenomena:

1. Adhesion

Platelet adhesion is the initial step in the formation of a platelet plug. vWF-dependent adhesion occurs predominantly at medium and high shear rates conditions as found in small arteries and arterioles, probably because high shear rates cause conformational change in vWF and/or platelets GPIb (40) . Under static or low shear conditions, platelets may also adhere directly to collagen through the platelet collagen receptors GPIa/IIa and GPVI (41).

2. Shape change

Upon activation by agonists and exposure to collagen, disk- shaped platelets turn into spheres with extensive formation of pseudopodia originating from the plasma membrane. The response originates from a change in the assembly of the cytoskeleton, and is fast and energy requiring (42). The presence of the swirling phenomenon correlates with the platelets discoid shape, and it disappears when platelets become spherical (43).

3. Secretion

Interactions between platelets and vascular cells are modulated by granular components released from activated platelets. ADP is considered an essential physiological agonist as it contributes significantly to the full aggregation response induced by other platelet activation markers like serotonin (44). Adhesive glycoproteins, coagulation factors, plasma protein e.g. released from the α-granules, facilitate vessel repair, platelets aggregation and coagulation of blood.

4. Aggregation

The platelet aggregation is characterized by the formation a hemostatic plug. Activated GPIIb/IIIa receptors on adherent platelets results in the recruitment of additional layers of platelet via cross-linking activated platelets through fibrinogen bridges.

Secondary haemostasis

The hemostatic plug is friable and must be stabilized by fibrin to avoid being washed away when the vasoconstriction reverses. The classical model of coagulation describes a sequentially conversion of pro-enzyme to enzyme, along either an intrinsic or extrinsic pathway. Activation of any of these two pathways leads to the formation of thrombin and subsequently fibrin. The absence of platelets and the separation into two pathways has made the coagulation cascade theory inadequate for the explanation of hemostasis *in vivo*. The cell-based model of hemostasis presented by Hoffmann et al. has been accepted as a more accurate explanation of *in vivo* hemostasis (45). In this model, coagulation occurs not as a cascade but in three overlapping stages: Initiation, amplification and propagation.

1. Initiation

Initiation of coagulation is triggered by tissue factor (TF), which is an integral membrane protein expressed by cells generally found outside the vasculature. Once an injury occurs in the vessel wall, TF will be exposed. Coagulation factor VII is activated and binds to TF. The TF-FVIIa complex is capable of activating both FIX and FX, and FXa can further activate FV. A FXa/Va complex on the surface of the TF-bearing cells converts small amounts of prothrombin to thrombin, which diffuse away from the surface of TF-bearing cells and act on platelets close to the site of injury. Initiation is summarised in Figure 1.1 B.

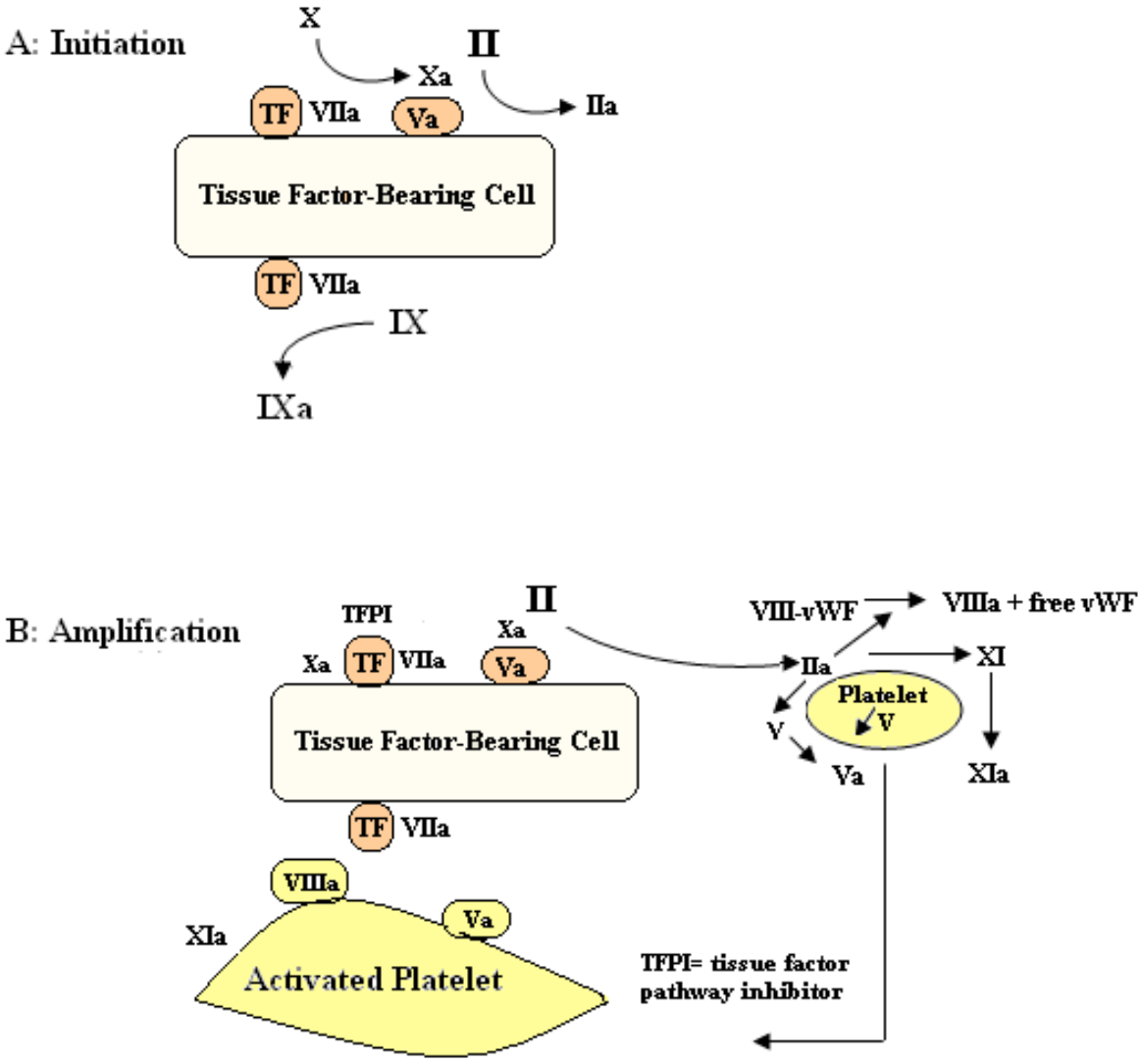
2. Amplification

At this stage, the process moves from the TF-bearing cell to the platelet surface. The small amount of thrombin generated on the TF-bearing cells amplifies the initial procoagulant signal by activating platelets that have leaked from the vasculature at the site of injury. In addition, thrombin activates FXI and FV on

platelet surface, and cleaves of FVIII from vWF. Amplification is summarised in Figure 1.1 B.

3. Propagation

During the propagation phase, FIXa binds to FVIIa on the platelet surface, which also causes further generating of FXa. The FXa/FVa complex generates a burst of thrombin, which removes fibrinpeptides from fibrinogen to form fibrin monomers. The fibrin monomers polymerize spontaneous for form an insoluble gel, which is stabilized by FIIIa. The fibrin aggregate and the platelets act together to stop further bleeding. The propagation is summarised in Figure 1.1 C.



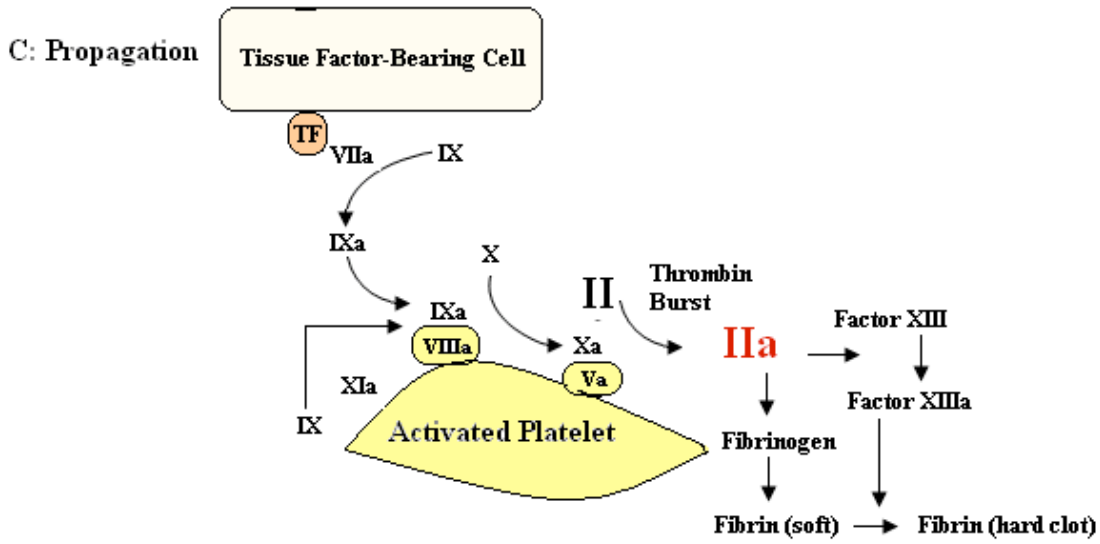


Figure 1.1. The cell-based model of fibrin formation composes three overlapping phases. A) Initiation Phase: TF- bearing cell activate a FX/V complex and the overall outcome is the generation of small amounts of thrombin, which diffuse away from the surface of TF-bearing cells to platelets. B) Amplification phase: The small amount of thrombin generated during initiation activates platelets and FV, FXI and FVIII. C) Propagation phase: Formation of a FX/V and FIX/VIII complex on activated platelet surface leads to a burst of thrombin generation. The figure is adapted by Hoffman, 2003 (46).

1.2.3 Platelet-derived microparticles

Microparticles (MP) are circulating membrane vesicles derived from platelets (47). They contain cytoplasmic components and possess membrane receptors inherited from their parental cell. Platelet microparticles are released from activated platelets and defined as vesicles less than 1 μm in diameter (48). MPs support procoagulant activity and contribute to formation of the hemostatic plug (49).

1.3 Tryptophan

Tryptophan is one of the eight essential amino acids, and both excessive intake and deficiency of tryptophan are detrimental to health. As in the case for all other amino acid, only the L isomer is used in protein synthesis (50). It is believed that tryptophan can play a rate-limiting role in protein synthesis, possibly because tryptophan is the amino acid with the lowest overall concentration in the body (51).

In addition to the principal role as a constituent of protein synthesis, tryptophan is involved in different metabolic pathways. Approximately 90 % of the tryptophan

catabolism results in the formation of kynureinine, which is a key component in the synthesis of a range of biologically active compounds (51). Its most important metabolites include kynurenic acid, which is a glutamate receptor antagonist, and quinolinic acid, which is a glutamate receptor agonist (52). Kynurenine may ultimately be converted to the coenzymes nicotamide adenine dinucleotide (NAD) and NAD phosphate (NADP), which are essential for electron transfer reactions in living cell (51). A large fraction of the body's NAD and NADP may be formed from tryptophan, while remainder is obtained from the diet (53). Persons who consume a diet that is low in tryptophan may develop deficiency of these coenzymes, a condition that is known as pellagra and may be associated with high morbidity and mortality. Less then 5 % of dietary tryptophan is used for serotonin synthesis (54), despite the fact that serotonin has wide-ranging effects throughout the body. Melatonin, which is known for its role in regulating diurnal rhythms in synthesised from serotonin in the pineal gland (51).

Thus, the kynurenine and serotonin pathways are interconnected and their integrity depends upon the availability of adequate amounts of tryptophan. This may be a problem in patients with carcinoids, which are neuroendocrine tumors usually present in the gut. A large tumor may produce substantial amounts of serotonin, and this may deplete body stores of tryptophan and cause pellagra like symptoms because of low levels of NAD and NADP (55).

1.4 Serotonin

In the late 1940s, Rapport et al discovered a unknown substance in the serum after blood had clotted (56). The substance was identified as a vasoconstrictor substance and the chemical name 5-hydroxytryptamine (5-HT) was given.

1.4.1 Biosynthesis, storage and metabolism

Serotonin is synthesised from the dietary amino acid tryptophan and the pathway involves two enzymatic steps. First, L-tryptophan is hydroxylated to 5-hydroxy-tryptophan (5-HTP) by the action of *tryptophan hydroxylase.* In the second step 5-HTP is decarboxylated to form 5-HT by L-amino *acid decarboxylase.* While both enzymes are necessary for the conversion of tryptophan to serotonin, the first step is considered the rate limited step (57).

About 95 % of all human serotonin is located outside CNS and the enterochromaffin cells in the GIT are almost entirely responsible for the synthesis of all peripheral serotonin (58). The gut is also a major storage site for serotonin. Unlike the enterochromafinn cells, platelets lack the enzymes to synthesize serotonin. Instead, serotonin released into the circulation from the enterochromafinn cells is rapidly taken up by platelets and stored in platelets dense granules, leaving a very small fraction freely circulating (14). Only a small fraction of the total body serotonin is synthesised in the CNS. Because the blood-brain-barrier (BBB) is impermeable to serotonin, the amount of serotonin produced centrally is dependent on the amount of tryptophan available to cross BBB by the action of a non-specific carrier protein (59, 60). *Monoamine oxidase (MAO*) and *aldehyd dehydrogenase* is responsible for degradation of serotonin respectively through oxidative deamination and oxidation to form 5-hydroxyindoleacetic acid (5-HIAA). Circulating serotonin is mainly metabolized in the liver, except a small amount, which is degraded in the endothelium of lung capillaries. 5-HIAA in the brain is transported to the peripheral where it is excreted in the urine along with peripheral 5-HIAA (57). Figure 1.2 summarizes the life cycle of serotonin.

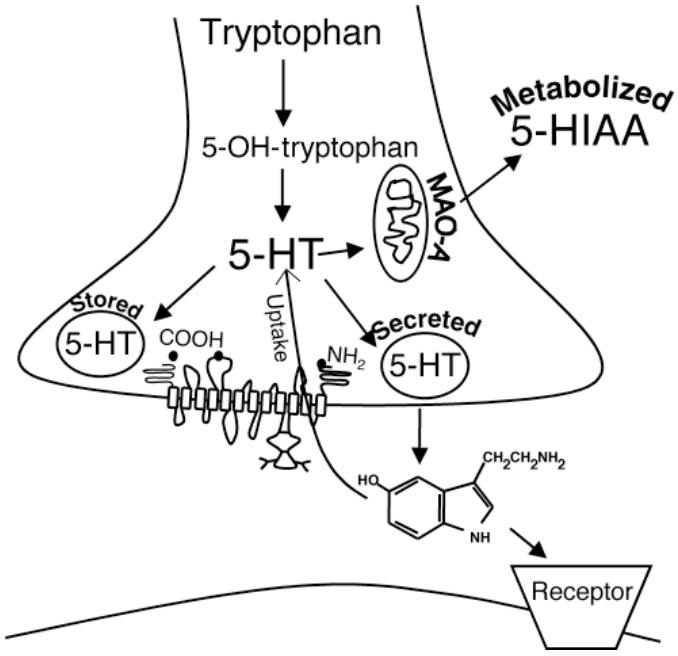


Figure 1.2: The synthesis, release, reuptake and metabolism of serotonin in a serotonergic neuronal synapse. Adapted from Ni et al., 2003 (61)

1.4.2 Serotonin receptors

The diverse effect of serotonin is mediated via 5-HT receptors, localized both in the central and peripheral nervous system, as well as in a number of nonneural tissues including the gut, blood and the cardiovascular system. Currently, there are 15 known serotonin receptor subtypes, divided into seven families (5-HT_{1-7}) based on their structure, pharmacology and transduction signal (62). With the exception of 5-HT_3, which is a ligand-gated ion channel, 5-HT receptors belong to the family of G-protein- coupled receptors (62). The earlier discovered 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 have been subjects to extensive research, and are consequently best understood of all the serotonin receptors.

1.4.3 Serotonin transport

Serotonin transporter (SERT) is a plasma membrane protein that selectively transports serotonin back into the cell cytoplasm for metabolism or vesicular repackaging. SERT possesses twelve transmembrane domains, and represents the first step in the action of terminating serotonin at its extracelluar cognate receptors. The serotonin transporter belongs to a family of Na^+/Cl^- dependent transporter that include transporter for norepinephrine, dompamin, glycine and GABA (61). Serotonin together with Na+ and Cl- are transported in the same direction, whereas K+ ions are transported out of the cell. SERT is the drug target for the SRIs, but also other psychostimulant drugs like cocaine and amphetamine (63).

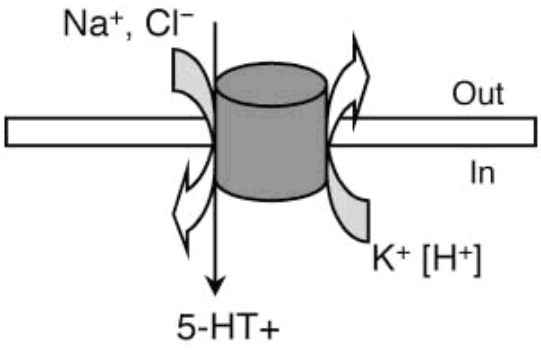


Figure NN: The serotonin transporter (SERT) is a sodium chloride-dependent neurotransmitter symporter. SERT removes serotonin from extracelluar space by high affinitet reuptake into cells. The figure is adapted from: NI et al. 2006 (61)

1.4.4 Serotonin physiology

Currently, we now that serotonin plays an important role in a variety of biological processes, and the profusion of 5-HT receptors reflects the complex mechanism of action of serotonin.

Serotonin and the CNS

In the CNS, serotonin is almost exclusively synthesized in neurons originating in the raphe nuclei, a cluster of nuclei found in the brain stem (64). In human brain, these neurons possess the largest and most complex efferent system we know, and it is assumed that virtually all brain regions express multiple serotonin receptors. Serotonin is believed to play an important role in almost all brain regulated activities ranging from temperature regulation, appetite, sleep cycles and emesis among others (65). The most clinical aspect has been the role of serotonin in human behaviour disorders. Currently, serotonin is believed to be involved in psychiatric disorders like depression, anxiety, obsessive-compulsive disorders and eating disorders (66, 67).

Serotonin and the gastrointestinal tract

A variety of different neurotransmitters have shown to interfere with gastrointestinal motor and sensory functions, including serotonin. Low pH, acetylcholine, sympathetic nerve stimulation and elevated intraluminal pressure, all contribute to the release of serotonin from the enterochromaffin cells in the gastrointestinal tract (68). Serotonin regulates digestion by initiating peristaltic and secretory reflexes (69). Currently, there is increasing evidence that serotonin contribute to the pathophysiology of gastrointestinal disorders such as inflammatory bowel disease and irritable bowel syndrome (70, 71).

Serotonin and the cardiovascular system

The effects of serotonin on the cardiovascular system are numerous and complex. Serotonin mediates its central neurotransmitter effect on the cardiovascular system by activating sympathetic and parasympathetic pathways (72, 73). Serotonin receptors are also represented on vascular smooth muscle cells and endothelial cells, causing either vasocontriction or vasodilation dependent on the receptor expressed (74).

Serotonin and platelets

Platelets lack the enzymes to synthesize serotonin, but have an extensive capacity to actively take up serotonin into dense granule from plasma (14). Upon activation, the release of serotonin from platelets is believed to cause multiple responses even though serotonin is classified as a weak platelet agonist. The receptor that mediates serotonin's effect on platelet function has been pharmacologically classified as the $5HT_2$-receptor (75). Serotonin is not assumed to cause platelet aggregation itself, but it enhances aggregation induced by ADP (16). In addition, serotonin causes vasoconstriction of blood vessels that have suffered endothelial damage by the interaction with $5HT_2$-receptor in the vessel wall (15). Serotonin can also promote the release of nitrix oxide from endothelial cells, which brings out a relaxing response on the vessel walls (57).

However, recent studies have expanded the classical paradigm, in which serotonin mediates its action by the interaction with membrane bound receptors. It has been postulated that intracellular serotonin can facilitate platelet alpha granule release by activating G-protein-dependent signalling pathways (76). In addition, the revealing that serotonin is covalently cross-linked to a variety of adhesion proteins and clotting factors on the platelet cell surface (77) have given rise to many new questions concerning the role of serotonin in hemostasis, as well in other organ systems.

Endogenous serotonin content has also been linked to platelet function during storage. Earlier studies from our group has reported that reduced intra-platelet serotonin content is related to impaired platelet function (78).

1.5 Antidepressant drugs

Depression is a common psychiatric condition worldwide, characterised with a persistent and substantial functional impairment. A variety of biochemical theories have arisen to explain the mechanism of depression, and a corresponding variety of different drug have been used in treatment. Functional deficiencies of serotonin and norepinephrine in synaptic regions have become the leading theory regarding the patophysiology of depressive symptoms (3), and drugs that

selectively inhibit the reuptake of the central monoamines have shown to be effective in treating symptoms associated with depressive illnesses (79). Defined daily doses (DDD) is the assumed average maintenance dose per day for a drug used for its main indication in adults (80). The consumption of some of the frequently prescribed class of antidepressant in Norway in 2009 is given in Table 1.2. The numbers are from the Norwegian prescription database (81).

Table 1.2: Antidepressant drug consumption in Norway in 2009

Antidepressant class	DDDs/1000 inhabitants/year
TCA	3.46
SSRI	34.56
SNRI	6.67

TCA= tricyclic antidepressant

1.5.1 Selective serotonin reuptake inhibitor

SSRI were developed as a result of the need for less CNS-toxic and cardiotoxic drugs than TCA. Despite the improvement in side effect profiles, the SSRI are generally not considered more clinically efficacious at treating depression than any previous antidepressant drugs (82). They are, however, considered as first-line drug of choice for treating moderate to severe depression due to the fact that SSRI are better tolerated and are safer in overdose than other classes of antidepressants.

As the name implies, SSRIs interfere with the neurotransmitter serotonin in the CNS. By blocking the reuptake of serotonin into the presynaptic cell by inhibiting SERT, the concentration of serotonin in the synaptic cleft is increased. This means that activation of the serotonin receptors can take place to a much greater extent. The antidepressant effect is related to secondary adaptive changes in the CNS and altered expression of membrane receptors and emerges after 2-4 weeks (5). As well as being used by patient with depression, SSRI have also shown to be effective in treating patient with anxiety disorders, panic attack and obsessive-compulsive disorder (83-85). In Norway, six different SSRIs are available, and they are considering to be equally concerning antidepressiv effect and side effect profile (86, 87). They differ, however, in their pharmacokinetic properties as seen in Table 1.2.

Table 1.2: Pharmacokinetic parameters of SSRIs

Drug	Daily dose (mg)	D_V (kg/L)	$t_{1/2}$	Active metabolite	CYP inhibition
Fluoxetine	20-80	20-45	4-6 days	Yes	2D6
Fluvoxamine	50-300	25	15-22 hours	Yes	1A2, 2C19
Paroxetine	20-50	3-12	24 hours	No	2D6
Sertraline	50-200	20	22-36 hours	Yes	2D6
Citalopram	10-60	12-14	24-48 hours	Yes	Minimal
Escitalopram	10-20	12-26	27-32 hours	Yes	Minimal

D_V = distribution volume, $t_{1/2}$ = half life

Fluoxetine differ most from the other SSRIs due to its long half-life and due to its highly biologically active metabolite, norfluoxetine (88). Because of this, fluoxetine appears to have a slower onset of action than other SSRIs. However, the long-half life may be beneficial for patients who have experienced severe withdrawal symptoms in the past. Fluoxetine also differ from the other SSRIs because of its high distribution volume (89, 90).

All SSRIs are highly lipohilic and undergoes extensive hepatic metabolism, which involves the human CYP system. The effect of SSRI on specific CYP isoforms has been one of the major distinguishing factors among this class of antidepressants. Fluoxetine and paroxetine are potent inhibitors of CYP2D6, which can lead to increased plasma concentrations of coadministered drugs metabolised by this enzymes like certain TCA and antipsychotics (89). Because of long half-life of fluoxetine and norfluoxetine, potential CYP inhibiton and drug-drug interactions may occur even weeks after discontinuation of fluoxetine. Fluvoxamine markedly inhibit CYP1A2 and CYP2C19, and have been reported to impair the elimination of certain TCA and various neuroleptics with clinical implications (91). Citalopram and escitalopram have weak inhibitory effect on various CYP isoforms *in vitro* and appear to have more favourable drug-interactions profile *in vivo* compared to the others SSRI (91).

1.5.2 Serotonin- norepinephrine reuptake inhibitors

In the recent years, there has been an increased interest in antidepressants with broader mechanism of action due to failure of SSRIs to treat subgroups of

depressed patients. The antidepressant activity of SNRIs is based on the inhibition of SERT and the norepinephrine transporter (NET). It has been suggested that this dual-action antidepressant group may increase clinical utility in severe depression compared to the SSRIs (92). Two different SNRIs are available on the Norwegian marked, venlafaxine and duloxetine. As seen in table 1.3 they possess different pharmacokinetic (88, 91, 93, 94).

Table 1.3 Pharmacokinetic parameters of SNRIs

Drug	Daily dose (mg)	d_V (kg/L)	$t_{1/2}$ (hours)	Active metabolite	CYP inhibiton
Venlafaxine	75-375	3.8- 11.2	~ 5	Yes	Minimal
Duloxetine	60-120	11-50	8-17	No	CYP 2D6

D_V = distribution volume, $t_{1/2}$ = half life

Venlafaxine

Venlafaxine was the first marketed antidepressant in the SNRI class. It is a potent selective serotonin reuptake inhibitor at lower doses as venlafaxine has a 30-fold higher affinity to SERT than NET (95). By increasing the daily dose to >150 mg, venlafaxine exert dual antidepressant effect because its noradrenic effect becomes more apparent (95). Earlier reviews of the safety of venlafaxine has, suggested that there is an increased risk of cardiac events associated with venlafaxine compared to SSRIs (96, 97). A newly published nested case-control study have, however, questioned this association (98).

Duloxetine

Duloxetine is a relatively new antidepressant in the SNRI class. It has high affinity for both SERT and NET in contrast to venlafaxine (99). As well as being effective in the treatment of emotional/physcological symptoms associated with depression, duloxetine has shown to be effective in treating pain in general and painful physical symptoms in depression (100). It is suggested that this additional effect gives rise to better response rates and remission from depressive symptoms compared to SSRI. In Norway, duloxetine is also licensed for treating diabetic naturopathic pain and stress urinary incontinence in woman.

1.6 Transfusion Medicine

Modern transfusion practice is based on the use of blood components produced from whole blood donations or apheresis procedures. The use of blood components rather than whole blood has the advantageous of more effective utilization of blood donations, optimal preservation of blood components *in vitro* and more effective treatment by specific replacement of deficiency. National regulations and international guidelines ensure high quality and safety of all procedures related to all fields of transfusion medicine (101, 102). The bloods banks must ensure that individuals fulfil certain criteria before being accepted as blood donors, and that they give their informed written consent before donation. Testing for HIV, hepatitis B and C must be performed at every donation, and the national blood banks must have a system for reporting the outcome of every transfusion.

1.6.1 Preparation of platelet concentrate

Platelet collection follows one of two alternatives, preparation of whole blood or platelet apheresis. Whole blood-derived platelet concentrate can be prepared from either buffy-coat or from platelet-rich plasma. It is required 4- 6 donations to obtain a standard dose of platelets (102). Platelet apheresis techniques enable the blood bank to collect up to 12 times the platelet dose that can be recovered from one whole blood donation, giving the advantage of exposing the patient to fewer donors. This reduces the risk for immunisation as the recipient is exposed to less antigen stimulation (103). The platelets are stored either in plasma or in a combination of plasma and additive solution. Many drugs like aspirin and non-steroidal anti-inflammatory drug (NSAIDs) are known to impair platelet functions and it is therefore crucial to ensure that the blood donors have not ingested such drugs during the last five days before donation.

1.6.2 Platelet additive solution

Platelet additive solution (PAS) is used as a substitute for plasma in order to make plasma available for other needs and reduce the risk of possible circulatory overload following transfusion of larger volume. 70-80 % of the plasma is replaced with PAS, giving the additional benefits of reduced allergic and febrile transfusion reactions (104) and decreased transfusion of unwanted antibodies since these side effects are related to plasma content (105). In addition, the use of

synthetic medium makes it possible to include components that support good platelet function. Several platelets additive solutions with different composition are currently available. Most additive solutions use acetate as metabolic fuel for the platelets, which also has the benefit of generating buffer capacity. Alternatively, glucose can be used. Adding another buffering substance like phosphate to maintain a neutral pH is possible. Citrate is added as an anticoagulant. The latest generation of additive solutions often contains magnesium and potassium, which have shown to limit platelet activation and therefore reducing the platelet storage lesion (106).

4.5.3 Storage of platelets

Platelets are stored with continuous agitation and the temperature should be +20°C to + 24°C (102). Because of bacterial contamination and storage-related deterioration, the platelet storage time is limited to 5 days. If platelet bacterial testing is performed, the storage period can be extended to 7 days (102). Platelets are particularly sensitive to storage conditions, and numerous morphologic, biochemical and functional derangements demonstrate that platelets tend to become activated during storage. These include loss of disc shape (43), increased surface glycoprotein expression (107) and increases release of platelet α-granules (108). All of these findings have proven to demonstrate platelet activation (43, 109). The phenomenon concerning changes in platelet structure and function arising during storage is generally referred to as the platelet storage lesion. Conventional assays, like record of the swirling phenomenon, hypertonic shock response, pH, pO_2 and pCO_2 and mean platelet volume are used as quality markers to ensure the platelets integrity during storage. It is difficult to prove strong correlation between *in vitro* test results and *in vivo* platelet function. There is, however, good evidence suggesting that that many platelet storage abnormalities observed *in vitro* reverse during transfusion (109).

2 Subjects and materials

2.1 Study participants

This prospective blinded randomized control study was approved by the local Research Ethics Committee (University of Bergen, Norway). The subjects were recruited from the Blood Bank, Haukeland University Hospital. Blood donors who provided information on SRI and had adequate periphereal venous access and blood platelet concentration sufficient for undergoing platelet apheresis were asked to participate in the test group. Individuals fulfilling the same criteria, but not using any medications, were used as controls. Only subjects who gave written consent were included in the study. A total of 18 individuals were included in the study, whereas 8 blood donors using SRI for at least one year were included in the test group. All donors met usual requirements for an acceptable donation, were healthy and had not taken any drugs influencing platelet function for at least 5 days prior to donation. The characteristics of the donors included in the study are given in Table 2.1 and 2.2.

Table 2.1 Characteristics of the individuals in the test group

Number	Sex (M=Male) (F=Female)	Age (yrs)	Drug	Dose
1	F	54	Citalopram (Cipramil)	10mg x 1
2	F	35	Paroksetin (Seroxat)	10mg x 1
3	M	36	Escitalopram (Cipralex)	10mg x 1
4	F	50	Escitalopram (Cipralex)	20mg x 1
5	M	40	Venlafaxine (Efexor)	75mg x 1
6	M	40	Venlafaxine(Efexor)	75mg x 3
7	M	45	Venlafaxine (Efexor)	75mg x 2
8	F	29	Escitalopram (Cipralex)	10mg x 1

Table 2.2: Characteristics of the individuals included in the control group.

Number	Sex (M=Male) (F=Female)	Age (yrs)	Drug
1	M	32	None
2	M	54	None
3	F	44	None
4	M	29	None
5	M	43	None
6	M	42	None
7	F	28	None
8	F	35	None
9	M	21	None
10	M	50	None

The median age was 40 years (range: 29-54 years) in the test group and 39 years in the control group (range: 21-54 years). While there was an equal distribution of men and woman in the test group, three woman and seven men were included in the control group.

2.2 Blood sample collection

Single donor concentrates (SDC) were collected by single-needle apheresis employing the elutriation principle to provide leukocyte-reduced platelets (Fenwal Amicus Cell Separator, Baxter Healthcare Corp., Deerfield, IL, USA). During the apheresis procedure, whole blood is removed from the donor and anticoagulated with a citrate solution. Following centrifugation, the constitutes of the blood are separated and the platelets are collected. The remaining blood components are returned to the donor.

Prior to the apheresis procedure whole blood from the donors was collected in a satellite bag and immediately transferred to three citrate tubes (4.5 ml 0.129M/3.8% Sodium Citrate, BD Vacutainer, BD bioscience, Plymouth, UK) and one EDTA tube (3 ml K_3EDTA, Greiner Vacuette, Greiner Bio-One International AG, Kremsmuenster, Austria). Testing on day was completed within 2 hours of collection

2.3 Sample preparation

2.3.1 Citrated whole blood

Citrated whole blood was obtained as described under blood sample collection in section 2.2.

2.3.2 Platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

PRP was obtained by centrifugation (Heraeus labofuge A, Heraeus Sepatech, Germany) of 3.8% citrated whole blood for 15 min at 110 G. The PRP was transferred to a plastic tube, and the sample was then centrifuged for 10 min at 1740 x g to prepare PPP.

2.3.3 EDTA plasma

The EDTA vacutainer tube was immediately put on ice, before being centrifuged (Mikro 200R, Hettich Zentrifugen, Hettich, Kirchlengern, Germany) at 2000 x g and 4°C for 10 min. The plasma was then obtained and stored at -80°C until use.

2.3.4 Platelet concentrate

SDCs were stored in 37 % plasma and 63 % PAS (Intersol, Fenwal, Lake Zurich, IL, USA) in plastic bags under routine conditions at 22°C on a horizontal agitator (Helmer, Noblesville, IN, USA). A maximum of 4.5 ml platelet concentrate was removed from each SDC before performing a particular analysis.

3 Methods

Tests of platelet function attempt to measure a defined event in the platelet activation process in an individual patient. A range of methods is available, making it possible to identify platelet dysfunction, monitor anti-platelet therapy and assessing the quality of stored platelets. In this study, we used following methods:

3.1 Light transmission platelet aggregometry

3.1.1 Principles of the method

Light transmission platelet aggregometry (LTA) measures the change in light transmission as a result of *in vitro* platelet-to-platelet aggregation following addition of an agonist. Classical Born aggregometry measures light transmission through PRP relative to autologous PPP. A beam of infrared light is directed through the two samples and detected by a silicon photodiode. PPP is considered to give 100% light transmission, because all light passes through the sample. No light is able to pass PRP, and PRP is therefore defined to allow 0% light transmission. Addition of an agonist to the PRP leads to increasing transmittance of light as larger and larger aggregates form. The optical aggregation output is proportional to the continuously measured difference in transmission between PPP and PRP. A range of agonists with different concentration can be used in LTA, making it possible to obtain additional information if the platelets are exposed simultaneously to more that one activator or a combination of activators and inhibitors.

The aggregation profile can be divided into two phases:

1) Primary aggregation, which is characteristic with a reversible aggregation response.

2) Secondary aggregation, which possesses an irreversible aggregation response.

To evaluate the aggregation curve, Max aggregation and aggregation velocity expressed as change over time (%/min) are calculated. Max aggregation = aggregation amplitude, represents the change in light transmission from the addition of an agonist (baseline) to the highest point on the aggregation curve. This parameter can easily be obtained by reading directly of the chart at the end of the aggregation profile. Change in aggregation velocity over time is determined by drawing a tangent to the steepest part of the curve. The slope is calculated, and expressed as percentage

aggregation per minute (%/min). Figure 3.1 shows how calculations of slope and max aggregation were done.

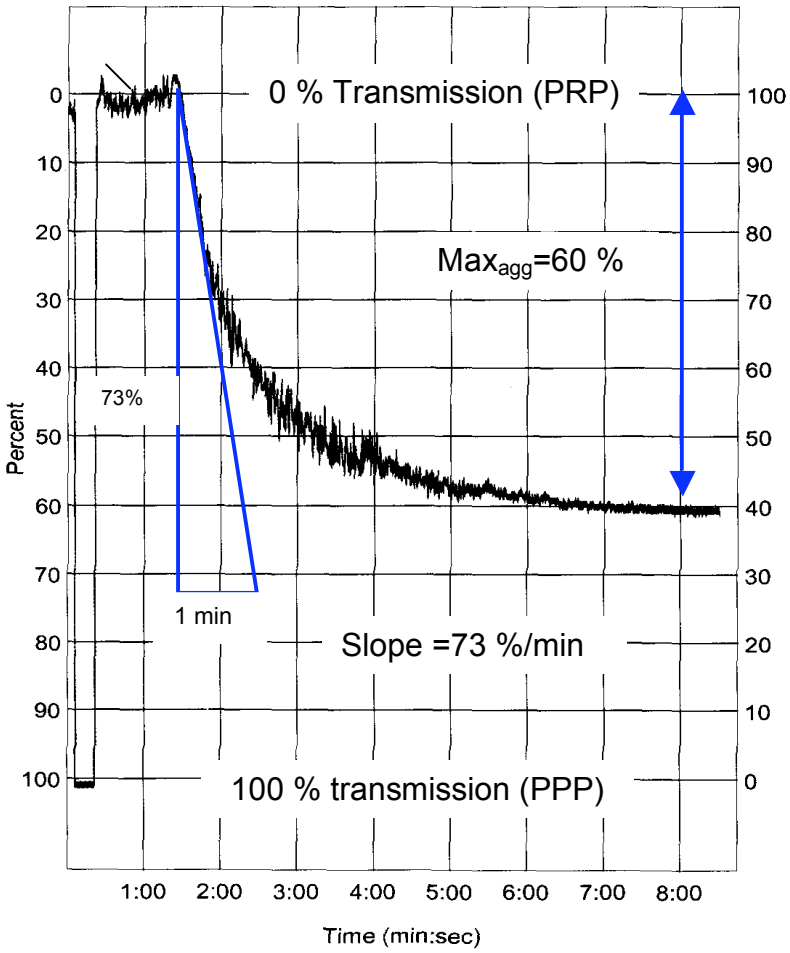


Figure 3.1 Calculation of max aggregation and slope. Max aggregation is obtained by reading directly of the chart at the highest point one the aggregation curve. The slope is calculated by constructing a triangle with a one minute base. The height is calculated and the value is represented as aggregation/minute (slope).

3.1.2 Practical test procedure

The LTA analysis was performed on day of donation on a Whole Blood Lumi-Aggregometer (Chrono-log Corporation, Model 560-Ca, Havertown, PA, USA). Two cuvettes, one containing PRP (sample) and one containing PPP (reference), are placed in chambers, pre-varmed to 37°. 100 % baseline is established using PPP, and 0 % baseline is achieved by the use of PRP. The addition of 50 µl ADP (Sigma-Aldrich, St.Louise, MO, USA) to 450 µl PRP induces platelet aggregation. The PRP is stirred at 1000 rpm and allowed to run for a minimum of 5 minutes. Final concentration of agonists obtained in sample includes 5 µM ADP and 2 µM ADP.

3.2 Thrombelastography

3.2.1 Principles of the method

Thrombelastography (TEG) measures viscoelastic changes during coagulation of blood *in vitro*. In classical TEG analysis, whole blood is added to a cup and incubated at 37°C. If not immediate processing using native blood is possible, the use of recalcified citrated blood allows for prolonged sample stability. When the cup is placed in the instrument, it oscillates back and forth six times per minute with an angle of 4°45. The addition of caolin to the cup activates the coagulation cascade, leading to the formation of thrombin and subsequently the formation of fibrin and a clot. A pin attached to a stationary wire is immersed into the sample and monitors the strength and stability of the clot formation. The dynamic changes occurring as the clot formation progresses is measured, and converted into a curve. Different phases of the clotting process give rise to different parameters in the thromboelastogram. A standard TEG tracing obtained from citrated whole blood is shown in figure 3.2.

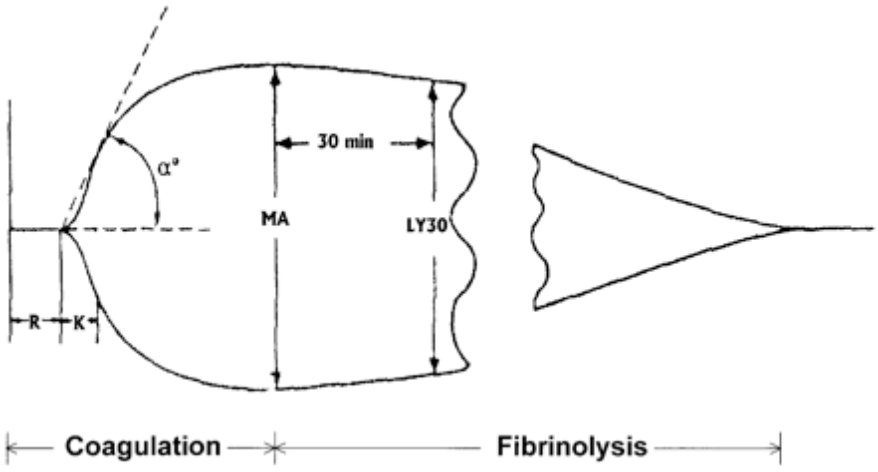


Figure 3.2: A TEG tracing gives information about the coagulation factors (R), fibrin (angle), platelet function (MA) and fibrinolyse (LY30). The figure is adopted from Rai et al., 2003(110)

The parameters obtained from the TEG tracing indicates following:
R-time represents the time from initiation of the test to the first formation of fibrin.
k-time is the time taken from the start of the clot formation until the amplitude of the tromboelastogram reaches 20 mm and indicates clot kinetic.
α-angle is the angel between the baseline and the tangent to the curve and represents the kinetic of fibrin formation and cross-linking.

Maximal amplitude (MA) indicates the strength of the clot, and is a direct measurement of the highest point on the TEG curve.
LY30 represents the cloth strength 30 minutes after MA is reached and provides information about the fibrinolytic activity.

TEG is a rather simple and rapid procedure to perform, and its application is increasing. Some current clinical applications include monitoring haemostasis in trauma patients, assessing coagulation problems during liver and cardiac surgery, and monitoring treatment response in patient with bleeding disorder (111).

3.2.2 Practical test procedure

The TEG analyses were performed on a TEG® 5000, version 4.2 (Haemoscope Corporation, Niles, IL, USA).
Day 0: 1 ml citrated whole blood was added to a caolin vial (Haemoscope Corporation, Niles, IL, USA) and gently mixed by inversion. 20 μL $CaCl_2$ (B.Brown, Melsungen, Germany) and 340 μl of the caolin-treated citrate blood in the vial were then added to a neutral cup (Haemoscope Corporation, Niles, IL, USA) and the test was run.
Day 1 and 5: 330 μl platelet concentrate together with 30 μl $CaCl_2$ (B.Brown, Melsunge, Germany) was added to a neutral cup (Haemoscope Corporation, Niles, IL, USA) and the test was run.

3.3 Platelet count

3.3.1 Principles of the method

Estimation of platelet concentration is a frequently used quality assessment to confirm that platelet units meet requirements for an acceptable donation. The impedance method is the most accurate method of platelet count (PLT) estimation in PC and is based on the measurement of changes in electrical resistance caused by cells in a buffered electrolyte solution. The non-conducting blood cells are forced through an aperture and thereby alter electrical charge between two electrodes. Pulses that are proportional to the volume of an individual cell are generated, and the count is determined from the total number of pulses produced from a measured volume of blood.

3.3.2 Practical test procedure

Platelet concentrations were determined by the impedance method (CELL-DYN Sapphire, Abbott Diagnostic, IL, USA) on day 1 and 5 of platelet storage and in PRP on day of donation. The PRP was diluted 1:10 with PBS prior to the analysis.

3.4 Analysis of pH, pO_2 and CO_2

3.4.1 Principles of the method

Ion selective electrodes are used to measure pCO_2 and pH in clinical laboratories. The principle of the operation is measurement of the potential of a specific ion in solution against a stable reference electrode of constant potential. The method is based on the technique of potentiometry, which is the measurement of electrical potential without a current flowing. pO_2 are measured based on the process of amperometry which is the measurement of a current flowing through at a constant potential.

3.4.2 Practical test procedure

Examination of pH and blood gas was performed on storage days 1 and 5 (ABL 725, Radiometer Copenhagen, Denmark; Modular, Hitachi High-Technologies corp., Tokyo, Japan).

3.5 Recording of swirling

3.5.1 Principles of the method

Visual inspection of the swirling phenomenon is a simple and frequently used test for assessing the quality of stored platelet concentrates before transfusion. The swirling effect correlates with the *in vitro* transformation of platelets from disk to spiny spheres. When platelets are held up to a light source and gently agitated, discoid platelets will scatter incident light at all angels due to change in orientation relative to the incident light. Platelets, which have undergone transformation to spheres, have immediately very little ability to change orientation. The swirling phenomenon is consequently not observed in transformed platelets. Degree of swirling is usually scored from 0 (no swirling) to 3 (maximal swirling).

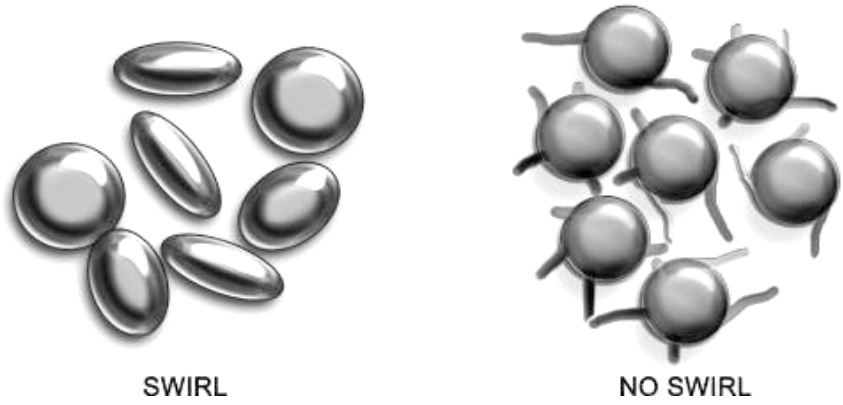


Figure 3.3: A, when platelets are held up to a light source and gently agitated, swirling platelet scatter incident light at all angles because platelets change orientation relative to the incident light. B, The swirling phenomenon is not observed in discoid platelets because of their decreased ability to change orientation and scatter light. Adapted by Maurer-Spurej & Chipperfield, 2007 (112).

3.5.2 Practical test procedure

Visual inspection of the swirling phenomena was performed on SDC on day 1 and 5 of platelet storage.

3.6 Flow cytometry

3.6.1 Principles of the method

Flow cytometry is a technique for measuring and analyzing single particles, usually cells, as they pass down a fluid stream through a beam of light. The important feature of flow cytometric analysis is that measurements are obtained from each separate particle within the suspension, rather than an average value of the whole population. Flow cytometric analysis uses the principles of light scattering and fluorescence to measure multiple cellular parameters. The use of flow cytometry for cell sorting is also possible. The application of the flow cytometric technology is numerous in both research and in medicine. The major clinical application is diagnosis of hematologic malignancy.

A flow cytometer is made of 3 components: the fluid system, the optics and the electronics.

The fluid system:

A fluid system is responsible for carrying the particles in the sample to the laser intercept. For accurate data collection, it is important that the particles and the cells

pass through the laser beam one at a time. Most flow cytometries accomplish this by injecting the sample in the centre of a sheath fluid and subsequently using the principle of hydrodynamic focusing. Due to a pressurized laminar flow, the sheath fluid causes the sample to remain in a central core as it flows within the flow cell one by one along their long axis.

The optic part:
Optics are central to flow cytometry for the detection of scatter and fluorescent light signals. The optical system in a flow cytometer consists of excitation optics and collection optics. The excitation optics is made up of the laser and lenses that are responsible for focusing the laser beam. The fluid system flows perpendicularly to the beam, and the point at which stream and light beam intersect is called the interrogation point. As a cell passes through the interrogation point it will scatter light at all angels. The extent to which this occurs is dependent on the physical properties on the particles in the sample. Forward scatter (FSC) or low angel scatter is the amount of light scattered in the forward direction. The magnitude of the forward scatter is roughly proportional to the size of the particle. Side scatter (SSC) is light scatter at larger angels and is caused by granularity and complexity inside the cell. Forward and side scatter are used for preliminary identification of cells.

The addition of labelling cell surfaces or antigens in the cells cytoplasm by antibodies conjugated with fluorescents like PE and FITC, allows for the investigation of cell structure and function. Absorption of light from the laser beam as the cell passes through the interrogation point causes an electron in the fluorochrom molecule to be raised to a higher energy level. The excited electron is unstable, and drops to its original ground state and emits the excess energy as a photon of light. This transition of energy is termed fluorescence, and can be detected in a flow cytometer along with scattered light.

The collection optics are responsible for collecting light as it emerges after intersection with the stream. A combination of optical filters and mirrors direct scattered and fluorescent light to appropriate detectors. Emitted SSC and fluorescence signals are directed to the photomultiplier tubes located 90 degrees to the laser beam. FSC signals are diverted to photodiodes along the axis of the laser beam.

The electronics

Particles are analysed individually but interpreted collectively. Light signals generated as particles flow through the laser beam are converted to electronic signals by the photodetectors. The electronic signals are proportional to the optical signals striking the detectors. Numerous options for displaying and analyzing collected data are available in today's software program. Histograms and dot plots are most frequently used for representing flow cytometric data. Single parameters such as FSC, SSC or a fluorochrom signal can be displayed as a single-parameter histogram, where the horizontal axis represents the intensity of the detected signal and the vertical axis represents the number of events (cells) counted. Dot plots provide a two-parameter display of data. In a dot plot each cell represents one dot, positioned on the horizontal axis and vertical axis according to the intensities detected for that cell.
Figure 3.4 summarizes the most important components in a flow cytometer.

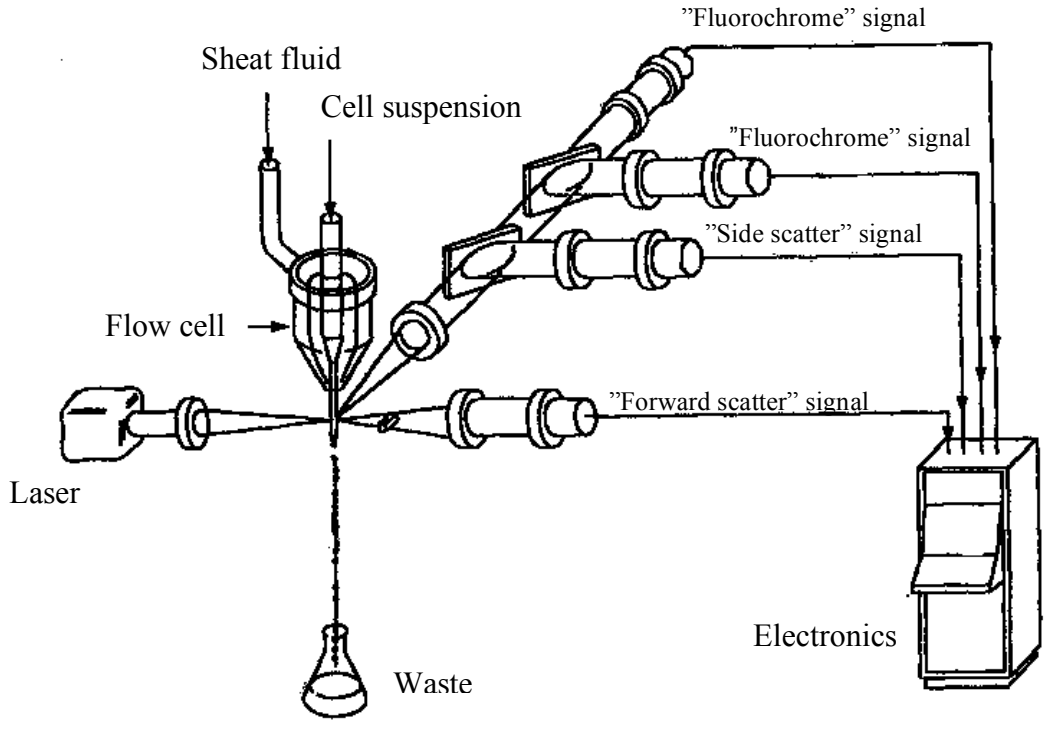


Figure 3.4: Components of a generalized "four parameter" flow cytometer. The figure is adapted from Givani, 2001 (113) and modified.

3.6.2 Practical test procedure

Flow cytometric platelet activation analysis was performed on platelets stored under routine blood bank condition on storage day 1 and 5. The platelet population was identified by light-scatter characteristics and FITC-conjugated CD61- spesific monoclonal antibody (DAKO, Glostrup, Denmark). Light scatter and fluroresence data from 10,000 CD61-positive events were collected with al detectors in logarithmic mode. The platelets were exposed to agonists, including thrombin receptor activator peptide (TRAP) (Sigma-Aldrich, St.Louise, USA) and two different concentrations of ADP (Sigma-Aldrich, St.Louise, MO, USA) before stained with PE-conjugated CD62P- spesific monoclonal antibody (DAKO, Glostrup, Denmark) and PE-conjugated CD42b specific monoclonal antibody (BD Pharmingen, San Diego, CA, USA). The final agonist concentrations in the samples include 100 μM TRAP, 10 μM ADP and 1 μM ADP. For each activation point, a negative matched IgG control conjugated with PE was carried out in parallel. Median fluorescence intensity of positive cell population was estimated for each antigen. The fluroresence resulting from the binding of matched IgG control was set to 1%. In addition to expression of platelet surface glycoproteins, the extent of platelet microparticles generation was examined. A nonfluorescent Size Calibration kit (Molecular Probes, Eugene, ORE, USA) containing suspended polystyrene mircrospheres with different diameter was used to determine sizes. MPs were defined as elements with size less than 1.0 μm.

The flow cytometric analysis was performed on a FACS Calibur (BD Biosciences, San Jose, CA, USA) equipped with CELLQuest® software (BD Biosciences, San Jose, CA, USA).

3.7 Chromatography and mass spectrometry

We used high performance liquid chromatography (HPLC) combined with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to determine tryptophan and tryptophan metabolites and HPLC together with a fluorescent detector to determine serotonin.

HPLC is an improved form of column chromatography used to separate, identify, purify and quantify chemical compounds. Chromatography uses the principle of

differences in surface interaction between analyte and eluent molecules to separate compounds within one sample. In HPLC, the analytic instrument composes two main phases. The stationary phase (absorbend) is the solid support situated within the column. The mobile phase (eluent) in HPLC refers to the solvent, which is continually added to the column. When injecting a sample into the instrument it will migrate through the column according to its affinity to the mobile phase and stationary phase. Compounds having stronger interaction with the mobile phase than the stationary phase will elute more quickly than compounds having stronger affinity to the mobile phase. In HPLC, applied pressure ensures that the analyte and the mobile phase are forced through the densely packed column. Quantitative analysis of biomolecules by the use of HPLC is achieved by preparation of standard curves.

3.7.1 LC-ESI-MS/MS

3.7.1.1 Principal of the method

The effluent from the HPLC-column is introduced into the mass spectrometer and enters the ionization chamber through a capillary to which a 3-5 kV charge has been applied. Charged droplets emerge from the tip of the capillary and the charge-to-volume ratio increases as the droplets evaporate. Positive charges are repelled and free proton-adducts of the molecules emerge. The adducts are selected in an electromagnetic field in the first quadropole according to their mass-to-charge ratios (m/z). They then enter the second quadropole, where they are collided with gas molecules, usually nitrogen, and fragmented to form product ions. Usually the most abundant product ion is selected in the third quadropole according to its mass/charge ratio and allowed to reach the detector. Because MS/MS is a selective method, several ions may be measured even if they are not chromatographically separated. This is achieved by monitoring several molecular transitions intermittently, so called multiple reaction monitoring (MRM). The amount of analyte in a sample does not correlate directly with the ion-current intensity of its mass spectrometric signal. To calculate analyte concentrations, an internal standard is contained in the sample. The internal standard should be chemically similar to the compound that is to be measured, ideally an isotopic analogue. Peak area ratios are calculated between the analyte and the internal standard and used to calculate concentrations by using a standard curve, which is obtained by analysing several samples containing know amounts of the compound to be determined.

3.7.1.2 Practical test procedure

LC-ESI-MS/MS was used for the quantitative analysis of tryptophan and tryptophan metabolites in EDTA plasma. The plasma was first deproteinized by adding an equal volume of thrichoracetic acid (Merck Whitehouse Station, NJ, USA) in water (60 g/l) containing the isotope labelled internal standards (100 nmol/l of $^2H_5$-KA and $^2H_4$-AA, 200 nmol/l of $^2H_4$-kyneruine, and 2 μmol/l of $^2H_5$- tryptophan). A robotic workstation (MicrolabAT Plus, Reno, NV, USA) carried out the precipitation step, before the solution was left on ice for 60 min and finally centrifuged at 5796 x g at 4ºC for 15 minutes. The supernatant (60 μl) was added to a new vial before being placed in a cooled (8ºC) autosampler. The analysis was performed on an API 4000 triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) with electronspray ionization. The instrument was equipped with Analyst v.1.4.1 software (Applied Biosystems/MDS SCIEX, Foster City, CA, USA).

3.7.2 HPLC combined with fluorescence detection

3.7.2.1 Principle of the method

Fluorescence detectors excite the analytes in the eluate with a laser and measure fluorescence. Detection techniques based on fluorescence are very sensitive independent of the sample concentration.

3.7.2.2 Practical test procedure

Preparation of platelet extracts:

250 μl 24% percloric acid solution (Merck, Whithouse Station, NJ, USA) containing 40 mmol dithiothreitol (DTT) (Sigma-Aldrich, St.Louise, MO, USA) per litre was mixed with 750 μl sample (PRP or SDC). After centrifugation at 8000 x g for 4 min (Biofuge A, Heraeus Sepatech, Germany) 600 μl of the supernatant was added to a new tube, and neutralized with 150 μl 3 M K_2HPO_4 (Sigma-Aldrich, St.Louise, MO, USA) before stored at -80°C until use.

On the day of HPLC analysis, the precipitated potassium perchlorate was removed by a new centrifugation (Mikro 200R, Hettich Zentrifugen, Hettich, Kirchlengern, Germany) and samples of the supernate (20 μl) were injected directly into the liquid chromatograph.

Preparation of standard:

A standard stock solution of 1 mmol/l serotonin (Sigma-Aldrich, St.Louise, MO, USA) was prepared in water containing 10 mmol/l DTT (Sigma-Aldrich, St.Louise, MO, USA). Neutralized perchloric acid (Merck, Whithouse Station, NJ, USA) with the same composition as obtained in the standard extraction procedure of platelets, was then used to dilute the stock solution to obtain standard samples with concentration of serotonin within the range of 0.6 µM to 6.0 µM. Two different standard curves were then obtained as shown in figure 3.5 and figure 3.6.

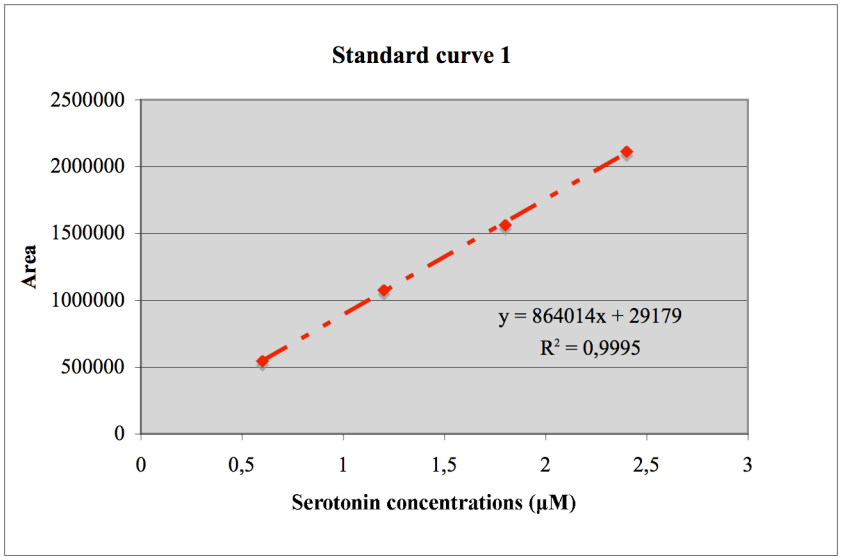


Figure 3.5: Standard curve obtained from samples with serotonin concentrations in the range of 0.6 µM to 2.4 µM.

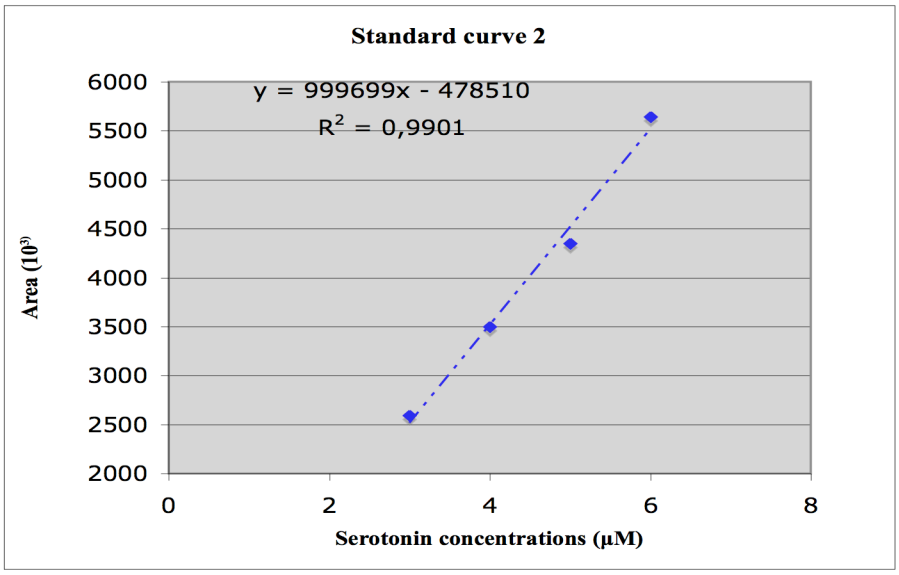


Figure 3.6: Standard curve obtained from samples with serotonin concentrations in the range of 3.06 µM to 6.0 µM

Liquid chromatographic analyses of serotonin:

The liquid chromatograph consisted of a Spectra System P4000 pump (SP Thermo Separation products, San Jose, CA, USA), a Gilson 232 XL autosampler (Gilson, Villiers-le-Bel, France) and a LaChrom L-7480 fluorescence detector (Merck, Whitehouse Station, NJ, USA) For the chromatographic separation of serotonin a cation-exchange column (Zorbax SCX, 4.6x150 mm, Agilent, Santa Clara, CA, USA) with sodium acetate (pH 5.2, 150 mmol/l) (Merck, Whithouse Station, NJ, USA) containing 10 ml of 1-propanol (Merck, Whithouse Station, NJ, USA) per litre as the mobile phase was used. The instrument was equipped with PC1000 System Software (SP Thermo Separation products, San Jose, CA, USA).

3.8 Statistical analysis

Descriptive statistics and statistical hypothesis testing were performed by the use of SPSS16 for Mac (SPSS, Chicago, IL, USA). Because most data obtained showed a non-symmetrical distribution, only non-parametric tests were performed. The Mann Whitney U test was used to compare the observations from the test and the control group. A p-value less than 0.05 was considered significant.

4 Results

4.1 Platelet aggregation

Data from the platelet aggregation testing by the LTA method are given in Table 4.1.

Table 4.1. The results from the platelet aggregation assay

Parameter	Agonist	Test group *(n=8)*	Control group *(n=10)*	*P*
Max aggregation (%)	20 μM ADP	35.5 (23-65)	28 (14-73)	0.247
	50 μM ADP	53.5 (34-72)	59 (27-80)	0.350
Velocity (%/min)	20 μM ADP	54 (45-71)	47 (35-82)	0.230
	50 μM ADP	60.5 (49-100)	66 (34-440)	0.460

The results from the calculated parameters max aggregation and the velocity are given as the median (range).

As expected, the platelet aggregation response for both the test- and the control group was much greater when adding 50 μM ADP compared to adding 20 μM ADP. The lowest concentration of ADP induced reversible aggregation for all individuals, except one, which showed irreversible aggregation. The highest concentration of ADP produced irreversible aggregation for all individuals, and gave and much higher calculated values for max aggregation and velocity. The p-values (Table 4.1) indicate no statistical significant differences concerning max aggregation response and velocity (%/min) response using ADP as agonist for blood donors using SRI compared to blood donor using no medication. Figure 4.1 show examples of the aggregations curves for the two concentrations of ADP for one individual included in the study.

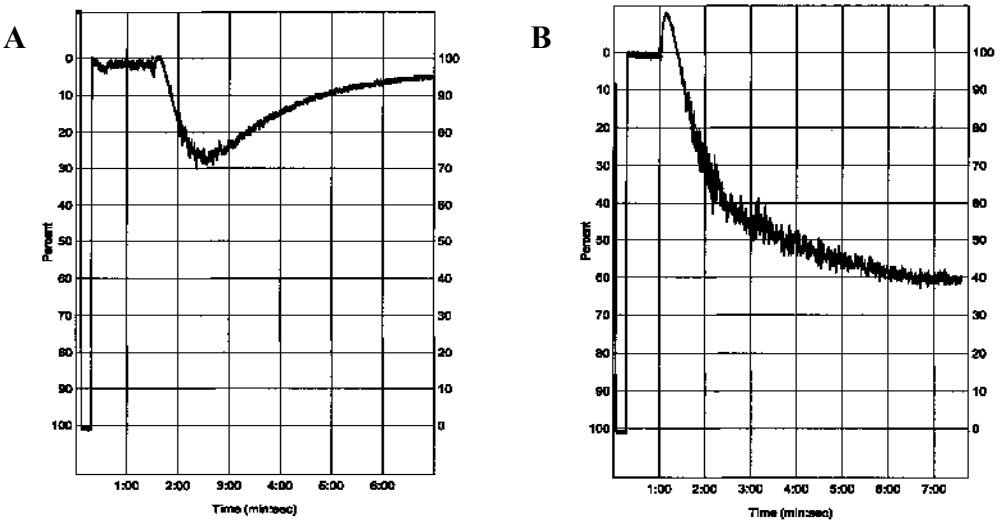


Figure 4.1: The use of 20 μM ADP as agonist gave a reversible aggregation response (A), whereas 50 μM ADP induced an irreversible aggregation response (B).

4.2 Thrombelastography

Data from the thrombelastography analysis are given in Table 4.2.

Table 4.2: The results from the TEG analysis

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
R (min)	0	10.7 (8.2-12.0)	9.25 (7.5-14.2)	0.722
	1	13.3 (9.7-20.8)	14.05 (8.1-20.9)	0.756
	5	17.2 (12.4-28.70)	17.75 (11.8-21.5)	0.964
k (min)	0	2.9 (1.8-3.6)	2.4 (1.8-3.5)	0.167
	1	1.8 (1.1-3.9)	1.9 (1.0-4.8)	0.656
	5	2.1 (1.2-4.2)	2.2 (1.2-5.0)	0.721
Angle (°)	0	51.9 (48.3-63.3)	56.9 (48.0-66.7)	0.142
	1	64.50 (50.5-74.6)	64.2 (40.1-75.3)	0.657
	5	63.3 (42.4-74.1)	60.45 (44.9-74.8)	0.286
MA (mm)	0	59.0 (53.6-67.2)	62.85 (53.7-67.9)	0.131
	1	65.3 (63.2-77.8)	66.8 (55.3-74.2)	1.000
	5	70.7 (63.3-74.8)	69.6 (58.2-74.30)	0.563

The results from the TEG analysis are given as the median (range) of the parameters R, k, angle and MA for the test and the control group.

There were no significant difference between the test group and the control group regarding the parameters obtained from the thromboelastography analysis. This observation was done for analysis in both PRP and SDC and on both day 1 and day 5 of platelet storage. Figure 4.2 and 4.3 show examples of TEG curves obtained from one individual included in the study.

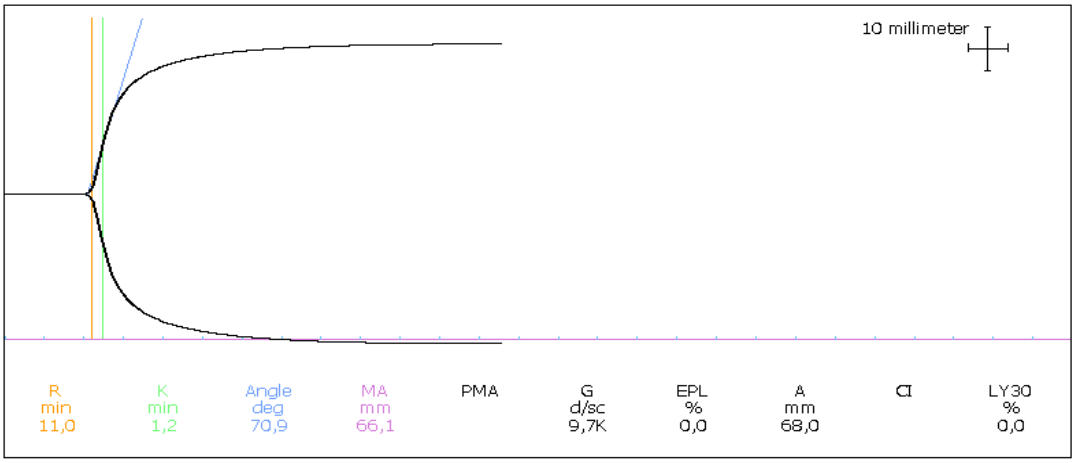


Figure 4.2: TEG tracing obtained from a SDC on storage day 1.

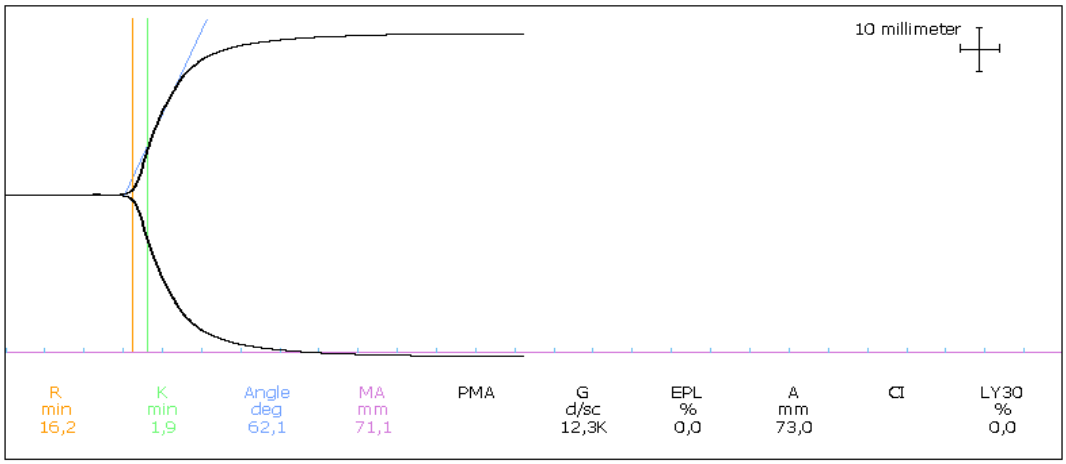


Figure 4.3: TEG tracing obtained from the same SDC as in figure 4.2, but now on storage day five.

4.3 Platelet content

Table 4.3 presents the results from the calculation of the platelet content in SDC based on PLT and measurement of the weight of the SDC. The parameter is expressed as total numbers of platelets in a SDC. During day 1 to day 5 of platelet storage, the platelet content was decreased in all SDC.

Table 4.3: The results from the calculation of the platelet content in SDC

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
Platelet content (=10^9)	1	366 (316-480)	418 (244-537)	0.328
	5	296 (249-409)	343 (211-465)	0.285

The results from the platelet count in SDC for the test group and the control group are given as the median (range).

The calculated p-values (Table 4.3) indicate that there are no statistical significant differences concerning the total numbers for platelet in SDC for the test group and the control group on both storage days. Except one case, all SDC met the requirement regarding a minimum content of $240x10^9$ platelets.

4.4 pH, pO2, pCO2

Analysis of blood gas

Data from the analysis of blood gas in SDC during storage are given in Table 4.5.

Table 4.5: The results from the analysis of blood gas

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
pO_2 (kPa)	1	15.23 (12.50-19.57)	16.03 (11.50-21.32)	0.722
	5	17.47 (15.95-19.95)	17.85 (12.60-19.51)	0.965
pCO_2 (kPa)	1	3.68 (3.31-4.99)	3.78 (2.61-4.33)	0.505
	5	3.15 (2.53-3.45)	3.09 (2.38-4.08)	0.213

The results from the analysis of pO2 and pCO2 in SDC during storage are given as the median (range).

pO_2 increased from day 1 to day 5 of storage, whereas pCO_2 decreased from day 1 to day 5 for all SDCs. The p-values (Table 4.5) indicate that no statistical significant differences were detected regarding the analysis of blood gas on both storage days for blood donors using SRI and blood donors using no medication.

Analysis of pH

In Table 4.6 the results from the pH analysis in SDC during storage are presented.

Table 4.6: The results from the pH analysis

Parameter	Day	Test-group *(n=8)*	Control-group *(n=10)*	*P*
pH	**1**	7.11(7.00-7.21)	7.17(7.10-7.23)	0.076
	5	7.02(6.90-7.13)	7.09(7.03-7.14)	0.068

The results from the analysis of pH on storage days 1 and five are given as the median (range).

pH decreased from day 1 to day 5 of storage for all SDCs. No significant difference in median values was observed between the test and the control group on both storage days.

4.5 Swirling

No difference was obtained between the test group and the control group regarding the recording of the swirling phenomenon, since all SDCs were given the score 3 on storage days 1 and 5.

4.6 Platelet glycoprotein expression and microparticle formation

Spontaneous and agonist-induced glycoprotein expression

The results from the spontaneous glycoprotein expression are presented in Table 4.7.

Table 4.7: Spotaneous glycoprotein expression

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
Spontaneous CD62P expression	1	50.3 (21.9-56.7)	33.1 (15.0-38.5)	0.041
	5	35.1 (21.5-50.5)	27.5 (14.2-46.1)	0.266
Spontaneous CD42b expression	1	294.6 (222.7-381.8)	355.5 (257.1-382.0)	0.247
	5	277.7 (201.0-388.9)	310.6 (246.9-349.1)	0.230

The results from the spontaneous expression of CD62P and CD42b are given as the median (range).

A p-value of 0.041 indicates a statistical significant expression of spontaneous CD62P on day 1 of platelet storage. Surprisingly, the observation shows that it is the test group that have greater spontaneous CD62P expression relative to the control group. Spontaneous CD62P expression on day 5 and spontaneous CD42b expression on day 1 and 5 show no significant difference for the blood donors using SRI and blood donors without medication.

Platelet activation capacity expressed as upregulation of CD62P and downregulation of CD42b in response to agonists were evaluated by comparing calculated median activation ratios (agonist-induced glycoprotein expression/spontaneous glycoprotein expression) for the test and control group. The results from the study of agonist-induced CD62P expression are given in Table 4.8.

Table 4.8: Flow cytometric analysis of CD62P

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
Median activation ratio 1 μM ADP	1	0.97 (0.91-1.04)	0.94 (0.86-1.03)	0.286
	5	0.97 (0.92-1.06)	0.96 (0.84-1.07)	0.594
Median activation ratio 10 μM ADP	1	1.00 (0.94-1.30)	0.96 (0.90-1.20)	0.374
	5	1.02 (0.95-1.37)	0.98 (0.81-1.41)	0.286
Median activation ratio TRAP	1	2.30 (1.76-6.04)	4.18 (2.89-6.61)	0.760
	5	3.35 (1.95-7.17)	4.06 (2.15-7.57)	0.408

Flow cytometric analysis of CD62P expression in response to agonists was evaluated by the calculation of median activation rations. The results are given as the median (range).

The p-values (Table 4.8) indicate that there were no statistical significant differences in calculated median activation ratios for CD62P in the test and control group. This observation was done on both day 1 and day 5 of platelet storage. Figure 4.4 presents dot plots obtained from samples from the same SDC, incubated with anti-CD61 (FL1) and anti-CD62P (FL2), but with different agonists.

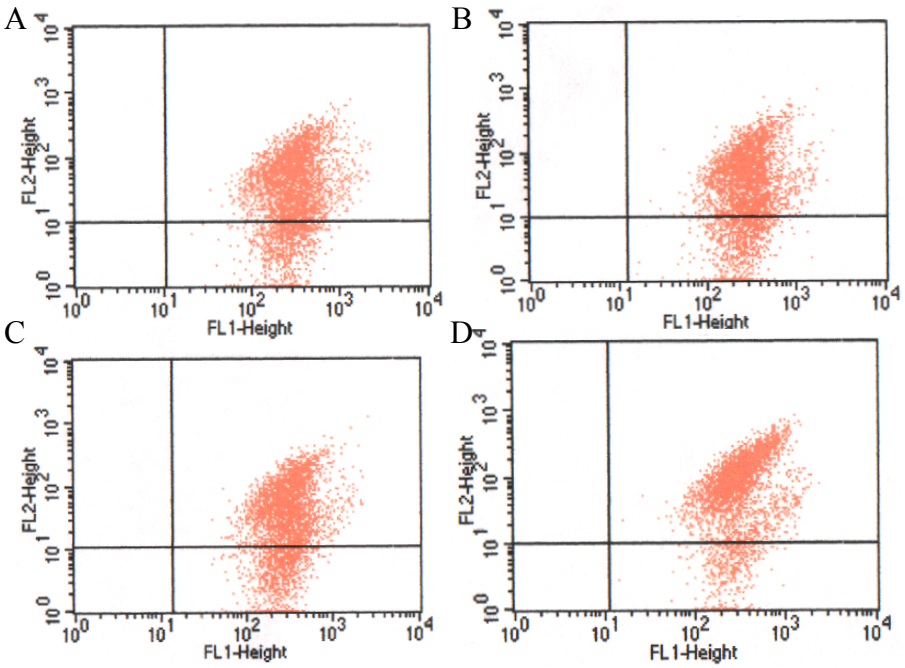


Figure 4.4: Dot plots representing CD61 (FL1) and CD62P (FL2) expression at: A) Baseline, B) induced by 1 µM ADP C) induced by 10 µM ADP and D) induced by 100 µM TRAP (C) for the same individual.

As seen in Table 4.8, the platelet activation response to ADP gave a median activation close to 1 for both groups and on both storage days, whereas TRAP had greater ability to induce changes in CD62P expression relative to the baseline. This trend can also be observed in Figure 4.4. Here, CD62P expression at baseline (A), induced by 1 µM ADP (B) and induced by 10 µm ADP (C) show almost identical dot plots, reflecting the fact that the quantitative percentages of cells expressing CD62P is similar. In contrast, the platelets stimulated with 100 µm TRAP (D) show greater expression of CD62P, as more dots are situated in the upper right quadrant.

The results from the study of agonist-induced CD42b expression are given in Table 4.9.

Table 4.9: Flow cytometric analysis of CD42b expression

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
Median activation ratio 1 μM ADP	1	0.99 (0.91-1.19)	0.98 (0.86-1.37)	0.929
	5	0.96 (0.86-1.11)	1.03 (0.99-1.35)	0.006
Median activation ratio 10 μM ADP	1	0.93 (0.63-1.05)	0.88 (0.62-1.29)	0.374
	5	0.92 (0.74-0.98)	0.96 (0.81-1.24)	0.183
Median activation ratio TRAP	1	0.52 (0.26-0.69)	0.45 (0.24-0.63)	0.155
	5	0.49 (0.29-0.62)	0.47 (0.38-0.70)	0.790

Flow cytometric analysis of CD42b expression was evaluated by the calculation of median activation rations. The data are given as the median (range).

A p-value of 0.006 (Table 4.9) reveals a significant difference between the test and control group, when it comes to the median activation ratio for 1 μm ADP on day 1. The CD42b expression is highest in the control group.

The expression of CD42b on day one for 1 μm ADP and for the other activation steps, show no difference between the test and control group. Figure 4.5 show dot plots obtained from samples from the same SDC incubated with anti- CD61 (FL1) and CD42b (FL2). Each dot plot represents one activation step.

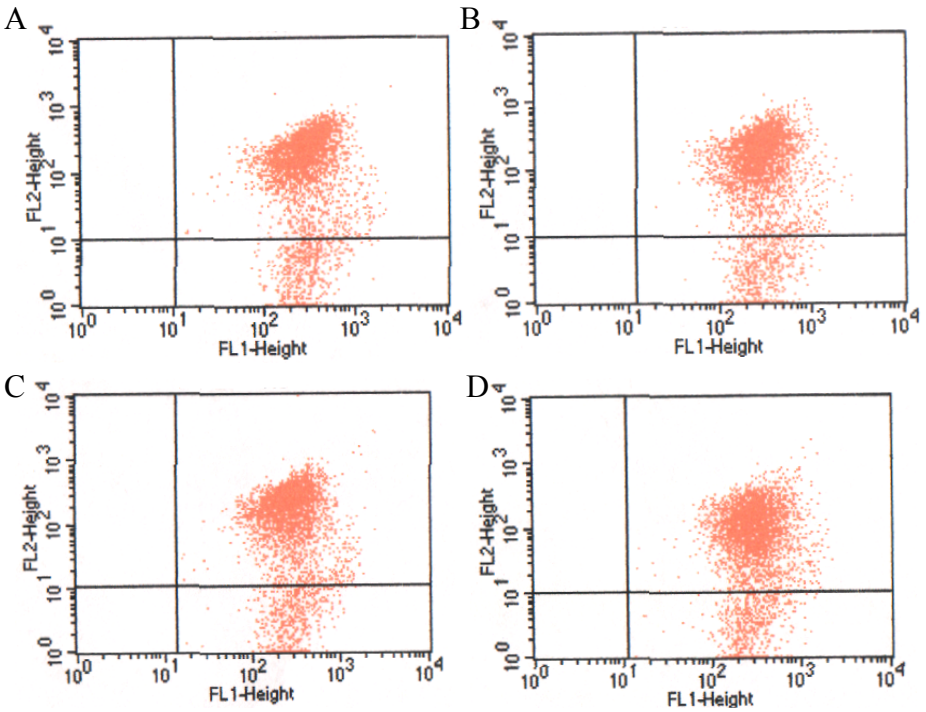


Figure 4.5: Dot plots representing CD61 (FL1) and CD42b (FL2) expression at: Baseline (A), induced by 5 μM ADP (B), induced by 50 μM ADP and induced by 500 μM TRAP (C)

As seen in Table 4.10, the analysis of agonist induced CD42b expression also gave median platelet activation ratios close to 1 in response to ADP, whereas TRAP had

greater ability to induce a decrease in CD42b expression. This observation is, however, more difficult to detect when studying the dot plots in figure 4.5 compared to the dot plots in figure 4.4. Here, the dot plots are almost equal for the different activations steps, reflecting that the quantitative percentages of cells expressing CD42b is much similar. However, it is the magnitude of the fluorescence intensity, which makes it possible to observe a difference in CD42b expression for platelets stimulated ADP and TRAP. This difference in fluorescence intensity must be due to the fact that expression of CD42b per platelet is greater for samples stimulated with ADP compared to TRAP.

Agonist induced microparticles formation

Figure 4.6 show examples of dot plots obtained from flow cytometric analysis of two different SDC activated with TRAP on the same storage day.

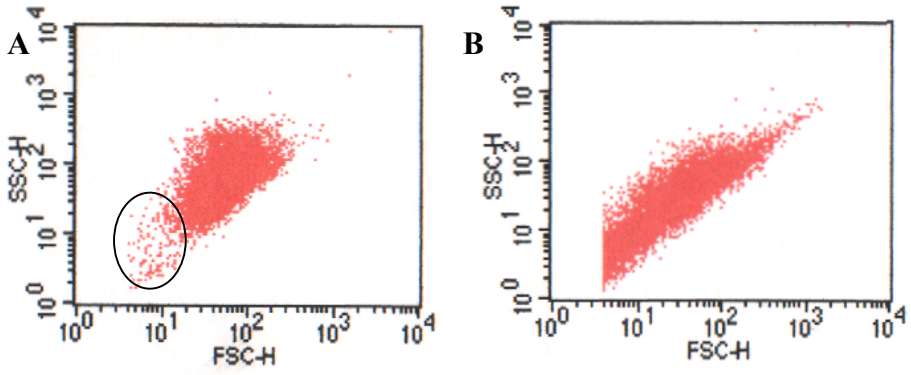


Figure 4.6: Dot plots from two different SDC activated with the same agonist on the same platelet storage day. In A, a circle is drawn around the microparticle population, which is easily detected. In B, the platelet population is shifted to the left in the dot plot, and the microparticles cannot be observed

Technical problems are the reason for the observed phenomenon in Figure 4.6. In A, the microparticle population is easily observed in the dot plot and a circle is drawn around the population. However, in B, the platelet population is shifted to the left in the dot plot, making it impossible to obtain information about microparticle formation. The trend regarding shifted population was independent of storage days, agonist used, and on the test and control group. Due to this technical challenge, it is problematic to compare platelet microparticle formation for the test and control group.

4.7 Tryptophan, tryptophan metabolites and serotonin

Tryptophan and tryptophan metabolites

The results from the quantitative analysis of tryptophan and some important tryptophan metabolites are given in Table 4.11.

Table 4.10: The results from the analysis of tryptophan and tryptophan metabolites

Parameter	Test group *(n=9)*	Control group *(n=10)*	*P*
Tryptophan (μM)	86.8 (66.85-102.18)	78.45 (68.52-95.15)	0.424
Kynurenine (nM)	1.62 (1.25-2.01)	1.73 (1.08-2.01)	0.824
Kynurenic acid (nM)	40.1 (29.85-82.73)	40.92 (16.64-69.66)	0.328
Xanthurenic acid (nM)	20.7 (2.60-31.88)	16.21 (7.49-34.95)	0.424
Anthralinic acid (nM)	6.67 (4.82-9.47)	6.54 (3.78-10.18)	1.000

Data from the quantitative analysis of tryptophan and tryptophan metabolites are given as the median (range) for the test and control group.

The p-values (Table 4.11) show no statistical significant differences between blood donors using SRI and blood donors using no medication when it comes to tryptophan content and the content of some of the most important metabolites of tryptophan.

Serotonin

Quantitative analysis of serotonin by the use of HPLC was achieved by constructing standard curves with serotonin concentrations within the range of normal serotonin content in human blood. The serotonin content was analysed on day of donation in PRP and in SDC on storage days 1 and 5.

Two analyses from each sample material were carried out in parallel, and the mean value was calculated. The results presented in Table 4.11 and 4.12 are based on this calculation.

Table 4.11. Serotonin content in PRP on donation day

Parameter	Test group *(n=8)*	Control group *(n=10)*	*P*
serotonin content (nmol/10^9 platelet)	<0.6 (<0.6)	1.97 (0.85-2.96)	0.0004

Serotonin content measured in PRP on day of donation is give as the median (range). All the individuals in the test groups had serotonin content <0.6 nmol/10^9 platelet.

Table 4.12: Serotonin content in SDC on storage days 1 and 5.

Parameter	Day	Test group *(n=8)*	Control group *(n=10)*	*P*
serotonin content (nmol/10^9 platelet)	1	<0.6 (<0.6)	1.70 (0.92-2.83)	0.0004
	2	<0.6 (<0.6)	1.89 (1.44-2.59)	0.0004

Serotonin content measured in SDC on storage days 1 and 5 are given as the median (range). All the individuals in the test groups had serotonin content <0.6 nmol/10^9 platelet.

The HPLC instrument we used in this study showed not to compose satisfying stability, and we meet a variety of technical challenges during analysis. This resulted in great variations between the two parallels in many data sets. We chose, however, to present the results as the mean value for the two parallels, and we included all values in statistical hypothesis testing because we observed an obvious difference in the two groups during analysis.

The median values and the ranges from Table 4.11 and 4.12 indicate that the test group composes markedly lower serotonin content that the control group. This is also confirmed by the significant p-values. However, the great variation we experienced in many parallel data sets have to be taken into consideration when evaluating the p-value. Despite this, the results indicate strongly that blood donors using SRI have clearly lower serotonin content in platelets compared to blood donors without medication.

5 Discussion

Our prospective blinded randomized control study aimed to investigate platelet function in whole blood and in apheresis PCs between blood donors using SRI *(n=8)* and blood donors without medication *(n=10).* Qualitative analyses of tryptophan, tryptophan metabolites and serotonin were also undertaken. Light transmission aggregometry, trombelastography and flow cytometric analysis of glycoprotein expression did not detected altered platelet function in blood donors using SRI. The test group showed markedly lower platelet sertonin compared to the control group, while tryptophan and tryptophan metabolites shared no difference between the groups. Routine tests of stored platelets indicate high quality of PC obtained from blood donors using SRIs.
The results from each different test performed in the study will be discussed separately, before an overall view of the study will be taken into consideration.

5.1 Platelet aggregation

Our results obtained from the platelet aggregation response using ADP as agonist, indicate that blood donors using SRI show no abnormalities in aggregation profiles compared to blood donors using no medication. We chose to include ADP as the agonist, because it has been postulated that serotonin dose-dependently enhances platelet activation induced by ADP (16).

There are few published studies that have investigated the effect of SRIs on *in vitro* platelet aggregation. Moreover, the findings from these studies are conflicting. McCloskey et al. (19) compared aggregation responses in plasma between patients using SSRI and patient using a non-SSRI antidepressant (bupropion) and found no difference between the two groups when ADP at final concentration of 5.5 μM was used as a agonist. This finding is in line with our results. However, significant abnormalities in the SSRI patients were detected when 4.5 μg/ml collagen and 0.87 mM arachidonic acid, which is a precursor for TXA_2, were used as agonists. Serebruany et al. (114) studied the platelet aggregation response to 5 μM ADP and 1μg/ml collagen in patients undergoing elective corononary artery stenting. They found that patients using SSRI showed significant depressed aggregation response

compared to patients not using a SSRI. We could not reproduce the finding that the SSRI patients show abnormalities in ADP-induced aggregation.

Most published studies that have investigated the influence of antidepressants on *in vitro* platelet aggregation, have used plasma preincubated with the drugs. Galan et al. (115) demonstrated that incubation with clinical relevant concentrations of escitalopram caused statistical reduction in aggregation induced by 0.5 μM and 2 μM ADP, although no difference were observed for 2.5 μg/ml collagen and 1.4 mM arachidonic acid. In contrast, Sarma et al. (116) reported that venlafaxine concentration at least 1000-fold greater than those used clinically were needed to demonstrate platelet aggregation abnormalities in response to 5.5 μM ADP, 2.2 μg/ml and 0.9 mM arachidonic acid. Serebruany et al. (117) showed that only high therapeutic doses of sertraline caused inhibition of aggregation induced by 5 μM and 10 μM ADP.

LTA has for more than 50 years been in use in hemostasis laboratories and is by many clinicians and researchers considered to be the "gold standard" for the evaluation of patients with defects in platelet function. However, the assay is prone to pre-analytical variables like blood collection, transportation and type of anticoagulant (118). In addition, many investigators standardize the platelet count in PRP before analysis (e.g. at 250×10^9 L^{-1}). In our study, we did not standardize the platelet concentrate because studies have revealed that the processes needed to obtain a uniform platelet concentrate will always lead to some platelet activation and because aggregation response has not shown to change much between platelets counts of 150, 000 per μL and 450, 000 per μL (118).

The effect of SRI on platelet aggregation is a matter of considerable controversy. Because of the lack of standardization of LTA, it may also be problematic to compare results from different studies. There is a need for further studies to clearly identify the effect of SRIs on platelet aggregation.

5.2 Thrombelastography

Our TEG assay results indicate that blood donor using SRI show no abnormalities in the TEG tracing compared to blood donors without medication. Only one published study has investigated the effect of SRI on platelets by the use of assays that measures viscoelastic changes during coagulation. Galan et al. (115) applied ROTEM thrombelastometry to analyse whole blood exposed to serotonin without and with previous incubation with therapeutic doses of citalopram for 2 minutes. The method is considered to be an alternative to TEG, but in the ROTEM, the cup is held stationary while an oscillatory force in applied to the pin (119). As the blood clots, the decrease in the pin's actually oscillation is measured. In standard conditions using citrated blood, Galan and co-workers observed no significant difference between samples exposed to only serotonin and samples exposed to both serotonin and citalopram.

The use of conventional TEG has shown limitations in the assessment of platelet function in patients who use antiplatelet therapy like aspirin and clopidogrel (120). While aspirin inhibits the formation of TXA_2, clopidogrel inhibits the $P2Y_{12}$ receptors on the platelet surface. Normally, activation of the platelet TXA_2 and ADP receptors cause activation of the GPIIb/IIIa receptor, which plays a key role in platelet aggregation. Standard TEG MA is a direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin cloth (121). Standard TEG is insensitive to platelet inhibitors because of the use of citrated blood, which allows for thrombin generation when $CaCl_2$ is added during the testing process. The powerful platelet activator thrombin bypasses the less-potent platelet activators like ADP and TXA_2, making assessment of their contribution to platelet activation impossible. Because serotonin is believed to be an amplifier of platelet aggregation through other activators like ADP, it is possible that the effects of SRI therapy is not detectable in standard TEG for the same reasons as aspirin and clopidogrel.
It would be interesting to study the effect of SRI in the TEG Platelet Mapping assay, which is a modified form of TEG. The particular advantage of the TEG Platelet Mapping is that it measures the platelet contribution to the clot strength in addition to platelet function. This assay has also shown to be suitable for monitoring antiplatelet therapy (122).

5.3 Platelet content

We did not detect a significant difference concerning the platelet content between SDCs obtained from blood donors using SRI and blood donors without medication. A decreased concentration of platelets was observed from day 1 to day 5 during storage. This was expected due to the platelet storage lesion. However, all SDCs used in this study met the requirement of a minimum content of $240x10^9$ platelets (101), except one, which had a content of $211\ x10^9$ platelets on storage day 5. This was not relevant for our study, as none of the SDCs were transfused.

5.4 pH, pCO_2 and pO_2

Metabolic parameters such as pH, pO_2 and pCO_2 are considered as reliable quality markers of platelet concentrate during storage (123). We did not detect a significant difference between SDCs obtained from blood donors using SRI compared to the control group when it comes to pH. As expected, we detected a fall in pH from day one to day five of storage for all SDCs obtained in the study. Platelets are metabolic active, and a fall in pH correlates strongly with a rise in lactate concentration. The subsequent formation of CO_2 during glycolysis also contributes to a fall in pH (124). In our study we found the lowest pH value to be 6.90 and the highest pH value to be 7.23. Previous studies have shown that pH < 6.0 or pH > 7.6 results in low platelet viability (125, 126). Thus, all PC used in our study were in line with quality criteria based on pH measurement.

We observed no significant differences in blood gas during storage between the test and control group. To maintain an adequate pH in platelet during storage at 22°C, it is important that the storage containers have sufficient permeability for O_2 and CO_2 (126). Data from our study shows that all SDCs were able to maintain pO_2 and pCO_2 within acceptable ranges, reflected by the fact that pH did not change to values associated with low *in vivo* viability.

5.5 Swirling

In our study swirling with the score 3 was observed in all SDCs on both day one and day five of storage. The presence of the swirling phenomenon has shown to correlate

with a pH value in the range of 6.7-7.5 (126), which is confirmed from the results from the measurement of pH as referred to in section 4.5.

5.6 Platelet glycoprotein expression and microparticle formation

Platelet glycoprotein expression

The results from the flow cytometric analyses indicate that blood donors using SRIs do not have altered platelet glycoprotein that indicates depressed platelet function. Platelet glycoprotein expression in patients receiving SRI therapy has previously been investigated, but in a limited number of studies. Moreover, no studies have evaluated the effect of SRI on platelets during storage. It is difficult to compare our findings with previous studies for many reasons. First of all, previous studies have mostly aimed to evaluate glycoprotein expression before and after SRI therapy, and not in comparison with a control group. The results from these studies suggest that depressed patients shown increased platelet activation, and that SRI treatment is associated with a beneficial protection against cardiovascular mortality, as SRI can cause normalization of platelet activation (117, 127, 128). Moreover, some of the studies have investigated platelet activation reflected by enhanced content of the soluble form of P-selectin in plasma. P-selectin can be cleaved from the platelet surface by the action of proteases. The prediction that soluble P-selectin reflects platelet activation may be questioned as platelets can shed or reinternalise this protein after activation, and due to the possibility of recycling during later activation (129, 130). We therefore considered the membrane form of P-selectin as the most reliable parameter. In summary, the published studies on platelet glycoprotein expression in patients undergoing SRI therapy show conflicting results. There is a need for further studies to clearly identify this effect of SRIs.

Interestingly, we found significant decreased ADP-induced CD42b expression on day 5 ($p=0.006$) and borderline significant increased spontaneous expression of CD62P on day 1 ($p=0.041$) in the test group. These findings may be without biological importance since none of the other parameters concerning glycoprotein expression were significant. However, there are studies that have revealed that patients with depressive disorders are at increased risk for developing ischemic heart disease, and many studies have reported increased platelet activation in these patients (127, 128).

Also, three of the individuals in the test groups used venlafaxine, which have suggested to promote adverse cardiovascular events by increasing platelet activity in susceptible patients (131).

Several published studies have demonstrated changes in glycoprotein expression on platelet surface as a part of the process known as the platelet storage lesion. In our study we found a relatively high spontaneous expression of CD62P and a low expression of CD42b for both the test and the control groups. In addition, the platelet activation response to ADP gave a median activation ratio close to 1 for both groups and both antibodies, indicating that flow cytometric analyses does not show major significant differences in platelet activation in response to ADP. These findings suggest that preparation and storage of platelet concentrate causes activation of platelets, and that ADP is a too weak agonist to induce further activation. It would have been interesting to study the glycoprotein expression using whole blood flow cytometry to see to what extent the platelets are activated during the processing and storage of platelet concentrates.

Agonist induced microparticle formation

In this study we were not able compare MP formation in the test and control group due to the technical problems explained in chapter 4.6. The unimodal decrease (or left-shift) in forward angle light scatter we observed in our study is previous reported as a typical data presentation under routine flow cytometric settings (132). It has been suggested that this unimodal decrease represents really degranulated platelets (132).

5.7 Relationship to epidemiological studies

The first epidemiological evidence concerning the association between bleeding risk and antidepressant with a relevant blockade on the serotonin reuptake was published in 1999. Since that, only four additional epidemiological studies have investigated the association between SSRI and bleeding risk from the GIT. A systematic review published by Yuan et al. in 2006 (133) concluded that that there is very weak evidence to support a link between bleeding risk from GIT and SSRIs at a population level. However, the same review states that there is greater evidence to support that SSRIs may precipitate GIT bleeding under certain circumstances. The concurrent use

of SRIs and NSAIDs increase the bleeding risk markedly (8, 134). Similar conditions are reported in elderly patients (9). As the participants in our study were relative young (range: 21-54 years) and were asked specifically if they had ingested drugs know to interfere with platelet function, the possibility that SRI precipitate GIT bleeding in given conditions can not be excluded from our study.

5.8 Tryptophan, tryptophan metabolites and serotonin

In our study we observed no difference regarding tryptophan and some of the important tryptophan metabolites in plasma between the test and control group.
We detected, however, a markedly decreased content of platelet serotonin for blood donors using SRI compared to blood donors without medication. This last finding is in line with many other published studies (14, 20, 135), and is probably related to decreased uptake of serotonin from serum. We aimed to investigate if decreased serotonin reuptake might mobilize tryptophan for the synthesis of serotonin and consequently deplete tryptophan stores and lower concentrations of metabolites in the kynurenine pathway. Our results indicate no such effect.
The fact that we observed markedly decreased platelet serotonin in the test group, confirm that the individuals in the study was adherent to the treatment regimen.

5.9 Limitations of the study

The most obvious limitation in our study is the small sample size. Another limitation of the study is that not all available SRI on the Norwegian marked was included in the study. Interestingly, a nested case-control study published by Meijer et al. in 2004 managed to show a moderate linear relationship between the risk of hospitalization because of bleeding and the affinity of the antidepressant on the SERT (136). In this study, fluoxetine, sertraline, and paroxetine were classified with high degree of inhibition of serotonin reuptake, while citalopram, venlafaxine and fluvoxamine were included in the class with intermediate affinity to SERT. Escitalopram was not included in the study. It must be taken into consideration that our study only included one of the drug (paroxetine) in the class with high degree of inhibition of serotonin transport reuptake.

In our study, all individuals included in the test group had used a SRI for at least one year. None of the individuals used maximum doses of the drugs, except one, who used 20 mg escitalopram. The possible effect of dose and duration of treatment have been investigated by de Abajo et al. in 1999 (137). They found no evidence that these factors influenced the risk of upper gastrointestinal bleeding. However, there are studies that have observed *in vitro* dose-dependent inhibition on human platelets (137).

According to the current guidelines, platelet concentrate may be stored for 7 days if there is control of possible bacterial contamination and for five days without such testing. Our results are limited to a storage period of 5 days.

5.10 Conclusion

The results from our pilot study indicate that individuals using SRIs do not have altered platelet function and can be accepted as platelet donors. Routine tests confirm high platelet quality during storage obtained from blood donors using SRIs. Our findings are limited to platelets stored for 5 days and for low doses of the drugs. There is a need for further studies to clearly identify the effect of SRI on platelet during storage. Our results also show that individuals using SRI have markedly lower platelet serotonin.

6 Bibliography

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