

Linköping University Medical Dissertations No. 1068

Platelet Adhesion to Proteins in Microplates:

Applications in Experimental and Clinical Research

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Linköping 2008

The cover image shows some of the interactions that can be studied with the platelet adhesion assay described in this thesis.

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ISBN 978-91-7393-863-1

ISSN 0345-0082

Printed in Sweden by Linköpings Tryckeri AB, 2008

“If at first, the idea is not absurd, then there is no hope for it”

Albert Einstein

Abstract

Platelets are crucial for prevention of blood loss after vessel injury. Platelet adhesion to disrupted vessel walls is mediated by receptors such as the GPIb-IX-V complex that binds von Willebrand factor and the collagen-binding integrin $\alpha_2\beta_1$. Also cross-linking of platelets, mediated by $\alpha_{IIb}\beta_3$ that binds to fibrinogen, results in platelet aggregation that further contributes to hemostasis. Platelets are also important pathophysiologically because of their role in thrombus formation following atherosclerotic plaque rupture. Pharmacological treatments aimed to prevent such events include use of platelet inhibitors such as acetylsalicylic acid (ASA) and clopidogrel. Despite the presence of several different platelet function assays, no one has so far been considered useful for clinical evaluation of the effect of anti-platelet treatment. The aim of this thesis was to evaluate possible applications in experimental as well as in clinical research for a platelet adhesion assay performed during static conditions. In principle, platelets in plasma are allowed to attach to protein coated microplates. Adhered platelets are then detected by induction of an enzymatic reaction followed by spectrophotometric measurements of the developed product. Our results show that the platelet adhesion assay is able to detect experimentally induced activation as well as inhibition of platelets. The assay also seems useful for investigation of synergistically induced platelet activation, especially when the coated surface consists of albumin. This is exemplified by the combination of lysophosphatidic acid and adrenaline, which induced a synergistically increased platelet adhesion to albumin that was dependent on $\alpha_{IIb}\beta_3$ -receptors and on the secretion of ADP. Furthermore, secretion of ADP as well as TXA_2 seems to contribute to several adhesive reactions investigated with this assay. The dependence on secretion, together with results showing that adhesion to collagen and fibrinogen is dependent on $\alpha_2\beta_1$ - and $\alpha_{IIb}\beta_3$ -receptors respectively, indicate that the adhesive interactions occurring in the assay is in accordance with the general knowledge about platelet function. Regarding clinical applications, we found that platelet adhesion was increased for patients with essential thrombocythemia (ET) compared to controls. This is in line with the *in vivo* function of ET-platelets since a common complication for ET-patients is thrombosis. Furthermore, the assay was able to detect effects of treatment with clopidogrel in patients with unstable angina. To some extent it also measured the effects of ASA-treatment. In conclusion, our results suggest that the assay is suitable for experimental research and that further studies should be performed aimed at developing the assay into a clinically useful device.

List of Papers

The thesis is based on the following separate papers. The roman numerals are used when referring to the papers in the text.

- I. Eriksson, A.C. & Whiss, P.A. (2005) Measurement of adhesion of human platelets in plasma to protein surfaces in microplates. *J Pharmacol Toxicol Methods*, **52** (3), 356-365.
- II. Eriksson, A.C., Whiss, P.A. & Nilsson, U.K. (2006) Adhesion of human platelets to albumin is synergistically increased by lysophosphatidic acid and adrenaline in a donor-dependent fashion. *Blood Coagul Fibrinolysis*, **17** (5), 359-368.
- III. Eriksson, A.C. & Whiss, P.A. Characterization of static adhesion of human platelets in plasma to protein surfaces in microplates. *Manuscript*.
- IV. Eriksson, A.C., Lotfi, K. & Whiss, P.A. Enhanced platelet adhesion after in vitro activation in essential thrombocythemia. *Manuscript*.
- V. Eriksson, A.C., Jonasson, L., Lindahl, T.L., Hedbäck, B. & Whiss, P.A. Static platelet adhesion, flow cytometry and serum TXB₂ levels for monitoring platelet inhibiting treatment with ASA and clopidogrel in coronary artery disease. *Manuscript*.

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Abbreviations

α_2 -AR	α_2 -adrenergic receptor
$[Ca^{2+}]_i$	Intracellular Ca^{2+}
$[cAMP]_i$	Intracellular cyclic adenosine monophosphate
AchE	Acetylcholine esterase
ADAM15	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
ApoE	Apolipoprotein E
ASA	Acetylsalicylic acid
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
COX	Cyclooxygenase
CVD	Cardiovascular disease
ECM	Extracellular matrix
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
ERK2	Extracellular signal-regulated kinase 2
ET	Essential thrombocythemia
FcR	Fc receptor
GP	Glycoprotein
ICAM-1	Intercellular adhesion molecule 1
JAK2	Janus kinase 2
LPA	Lysophosphatidic acid
MI	Myocardial infarction
ox-LDL	Oxidized low-density lipoprotein
PAF	Platelet activating factor
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PFA-100	Platelet function analyzer
PI3-kinase	Phosphatidylinositol 3-kinase
PRP	Platelet rich plasma
PSGL-1	P-selectin glycoprotein ligand 1
RGD	Arginine-Glycine-Aspartate
RT	Room temperature
TEG	Thrombelastography
TF	Tissue factor
TGF- β	Transforming growth factor-beta
TP	Thromboxane receptor
TXA ₂	Thromboxane A ₂
vWf	von Willebrand factor

Introduction

Platelets and hemostasis

Platelets are small anucleate cell fragments derived from bone marrow megakaryocytes (George 2000). They circulate in blood for approximately 10 days and are then sequestered and degraded primarily in the spleen. Platelets are crucial for prevention of blood loss after vessel injury, a process known as hemostasis. They contribute to normal hemostasis in several different ways. First of all, they adhere to the exposed extracellular matrix (ECM) of the wounded vessel and prevent blood loss by acting as a physical barrier. The effectiveness of this physical barrier is increased by the ability of platelets to bind to each other in an interaction called aggregation. It has also been established that platelets are important for effective blood coagulation. Coagulation is induced by vessel injury with consequent release of tissue factor (TF). Basically, TF induces a cascade of events where proteases serially cleave each other, which results in the production of a blood clot composed of fibrin (Walsh 2004). The fibrin clot contributes to the physical blocking of blood loss through the wounded vessel. Platelets affect the process of blood coagulation by acting as an attachment site for coagulation proteases (Walsh 2004). This facilitates the interactions between coagulation proteases and it also protects the coagulation proteases from degradation by protease inhibitors. In addition, there are other pathways by which platelets contribute to hemostasis. Secretion of vasoactive substances from platelets such as thromboxane A₂ (TXA₂) contributes to hemostasis by constricting the wounded vessel (Sellers & Stallone 2008). Furthermore, platelets contribute to wound healing by secretion of substances including platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) (Diegelmann & Evans 2004). Thus, there are no doubts that platelets are central for the maintenance of hemostasis. A special focus will now be directed towards the mechanisms of platelet adhesion.

Platelet adhesion receptors

Platelet adhesion is an initial hemostatic process important for prevention of blood loss after vessel injury. This event is dependent on a complex interplay between the exposed ECM and adhesion receptors on platelets (Table I). An important component of the ECM of blood vessels is collagen. Collagens are a large group of proteins comprising at least 28 members

(Farndale 2006) and 8 of those (type I-VI, XII and XIV) can be found in vessels (Bou-Gharios, *et al* 2004). Platelets have been shown to adhere *in vitro* to collagens I through VIII (Saelman, *et al* 1994). However, the nature of the interaction between platelets and collagen seems to be dependent on the type of collagen. Collagens I through IV appeared more effective in supporting platelet adhesion than collagens V through VIII (Saelman, *et al* 1994). Furthermore, adhesion to type VIII collagen required flow while adhesion to type V collagen only occurred during static conditions (Saelman, *et al* 1994). Also, different procedures exist for extraction of collagen from tissues including use of different solvents such as (1) a neutral salt solution, (2) acetic acid or (3) acetic acid with pepsin (Miller & Rhodes 1982). Use of acetic acid, with or without pepsin, results in degradation of the original triple helical structure into collagen monomers (Farndale 2006). This adds further complexity to studies of collagen-platelet interactions since different preparations of the same type of collagen have different morphologies and bind to platelets in different ways (Savage, *et al* 1999). Nevertheless, it has been found that platelets express at least two different receptors for collagen called $\alpha_2\beta_1$ (Santoro 1986, Santoro, *et al* 1988, Staatz, *et al* 1989) and glycoprotein (GP)VI (Moroi, *et al* 1989, Clemetson, *et al* 1999) respectively. The $\alpha_2\beta_1$ -receptor is generally considered the receptor responsible for the adhesive interactions between platelets and collagen, while GPVI mainly acts to induce activating intracellular signalling in platelets (Nieswandt & Watson 2003, Varga-Szabo, *et al* 2008). The GPVI-receptor belongs to the immunoglobulin superfamily (Clemetson, *et al* 1999) and is associated with Fc receptor (FcR) γ -chain (Tsuji, *et al* 1997). Activation with collagen results in tyrosine stimulation of FcR γ -chain and consequent interactions with intracellular signalling molecules such as phosphatidylinositol 3-kinase (PI3-kinase) (Gibbins, *et al* 1998) and Syk (Gibbins, *et al* 1996, Tsuji, *et al* 1997). Several other potential collagen receptors have also been described but their importance for collagen-induced events is currently unknown (Surin, *et al* 2007). Another important receptor for platelet adhesion is the GPIb-IX-V-complex consisting of the four subunits GPIb α , GPIb β , GPIX and GPV in a quantitative relationship of 2:2:2:1 (Phillips & Agin 1977, Berndt, *et al* 1983, Du, *et al* 1987, Modderman, *et al* 1992, Clemetson & Clemetson 1995). GPIb-IX-V has several ligands including P-selectin, thrombin, Mac-1, factor XII and high molecular weight kininogen (Berndt, *et al* 2001). However, the major physiological role seems to be its interaction with subendothelial von Willebrand factor (vWf) during high shear stress. The $\alpha_2\beta_1$ -receptor described above is a member of a group of adhesive receptors called integrins. In general, integrins are adhesive receptors important in

many different physiological processes (Hynes 2002). They are composed of one α - and one β -subunit. In total 18 α - and 8 β -subunits have been described and shown to combine into 24 different integrins. Beyond $\alpha_2\beta_1$, integrins present on platelets include, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_6\beta_1$ (Kasirer-Friede, *et al* 2007). The integrin $\alpha_6\beta_1$ binds to laminin (Sonnenberg, *et al* 1988), which is an important component of basement membranes that are located basolateral to the endothelium (LeBleu, *et al* 2007). Common for $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ are that they are all classified as RGD-receptors meaning that they recognize the amino acid sequence Arginine-Glycine-Aspartate (RGD) on ligands (Hynes 2002). Ligands having an RGD-sequence include *e.g.* fibrinogen, fibronectin, vitronectin and vWf (Takagi 2004) making it clear that the RGD-receptors are more or less unspecific in their binding properties. However, the preferred ligands for $\alpha_5\beta_1$ and $\alpha_v\beta_3$ are fibronectin and vitronectin respectively (Kasirer-Friede, *et al* 2007). Also, the major physiologic ligand for $\alpha_{IIb}\beta_3$ is fibrinogen (Bennett 2001). This ligand-specificity is determined by residues outside the RGD-binding motif (Takagi 2004).

Table I. Some important receptors involved in platelet adhesion and platelet activation. The intracellular signals induced by the platelet activators are also shown. Note that many (most probably all) of the receptors classified as adhesion receptors also induce platelet activation. Likewise, it can be assumed that the collagen receptor GPVI is involved in both platelet activation and platelet adhesion

Platelet adhesion		Platelet activation		
Receptor	Main ligand	Receptor	Agonist	Intracellular signal
GPIb-IX-V	vWf	GPVI	collagen	Tyrosine kinase
$\alpha_2\beta_1$	collagen	P2Y1, P2Y12	ADP	G_q , G_i
$\alpha_{IIb}\beta_3$	fibrinogen	PAR1, PAR4	thrombin	G_q , $G_{12/13}$
$\alpha_5\beta_1$	fibronectin	TP	TXA ₂	G_q , G_{13}
$\alpha_v\beta_3$	vitronectin	α_2 -AR	adrenaline	G_z
$\alpha_6\beta_1$	laminin	LPA ₁ , LPA ₂ , LPA ₃	LPA	$G_{12/13}$

Abbreviations. ADP: adenosine diphosphate, AR: adrenergic receptor, GP: glycoprotein, LPA: lysophosphatidic acid, PAR: protease activated receptor, TP: thromboxane receptor, TXA₂: thromboxane A₂, vWf: von Willebrand factor.

Platelet adhesion to extracellular matrix

Research aimed at elucidating the mechanisms of platelet adhesion *in vivo* has primarily studied platelet adhesion to collagen. Much effort has been made in trying to understand the relative contributions of GPVI and $\alpha_2\beta_1$ for adhesion. An early model of platelet adhesion (known as the two-site, two-step model) suggested that platelet adhesion to collagen is initiated by $\alpha_2\beta_1$ (Santoro, *et al* 1991). This initial interaction would then allow binding to collagen through a second low-affinity receptor resulting in platelet secretion and activation. At the time, Santoro *et al.* were not able to describe the nature of this second low-affinity receptor. However, current knowledge regarding collagen-induced platelet activation suggests that GPVI is the unknown receptor described by Santoro *et al.* Another conflicting theory was described by Nieswandt *et al.* (2001a). This theory states that after initial interactions between GPIb-IX-V and vWf platelets bind collagen through GPVI. The GPVI-collagen interaction activates platelets, which results in activation of $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. Binding of $\alpha_2\beta_1$ to collagen then contributes to firm adhesion. Consequently the models proposed by Santoro *et al.* and Nieswandt *et al.* differ in the order by which the collagen receptors are involved in adhesion. Furthermore, the study by Nieswandt *et al.* also suggested that $\alpha_2\beta_1$ only exert a minor role in platelet adhesion to collagen. They showed that mice lacking β_1 did not differ from wild-type mice when measuring bleeding time. Also β_1 -null platelets, but neither platelets depleted of GPVI nor platelets lacking the GPVI-associated FcR γ -chain that mediates intracellular signalling from GPVI, adhered to fibrillar collagen during static conditions confirming the important role for GPVI in this process. However, a contributory role from $\alpha_2\beta_1$ was evident since adhesion to monomeric collagen was dependent on both GPVI and $\alpha_2\beta_1$ and because fibrillar collagen activated β_1 -integrin and $\alpha_{IIb}\beta_3$ in a GPVI-dependent manner. Other studies have confirmed the important role of GPVI for platelet-collagen interactions. Vessel injury induced by ferric chloride results in collagen exposure and mice lacking FcR γ -chain are less prone to accumulate platelets at the site of injury compared to wild-type mice (Dubois, *et al* 2006). An additional study, using platelets from GPVI^{-/-}-mice in an *in vitro* flow chamber system, has shown that GPVI is important for the initial activation of platelets during adhesion to collagen (Kato, *et al* 2003). Also, GPVI-deficient mice obtained by use of a GPVI-specific antibody, reported moderately prolonged bleeding times when GPVI was absent (Nieswandt, *et al* 2001b). The important role of GPVI is not restricted to mice since GPVI deficiency or use of GPVI-blocking antibodies inhibits adhesion to collagen during

flow for human platelets (Goto, *et al* 2002). GPVI also seems to be interesting from a pathophysiological perspective. Human atheromatous plaques were found to contain collagen I and III, which both were essential for platelet activation (Penz, *et al* 2005). Furthermore, adhesion of human and mice platelets to atheromatous plaques were dependent on GPVI but not $\alpha_2\beta_1$. The pathophysiological role of GPVI is confirmed by results showing that GPVI-deficient mice are protected from thromboembolism induced by injection of collagen and adrenaline (Nieswandt, *et al* 2001b, Lockyer, *et al* 2006). However, it is unlikely that GPVI is solely responsible for platelet adhesion *in vivo*. In contrast to the study by Nieswandt *et al.* (2001b), *in vivo* bleeding times have been reported to be normal in GPVI-deficient mice (Kato, *et al* 2003, Lockyer, *et al* 2006) and there are several studies in support of an important role for $\alpha_2\beta_1$ in platelet adhesion. Absence of $\alpha_2\beta_1$ in knock-out mice resulted in loss of platelet adhesion to collagen during static as well as flow conditions (Chen, *et al* 2002). Absence of $\alpha_2\beta_1$ also resulted in decreased thrombus formation after carotid artery injury but not after injection of collagen (He, *et al* 2003). This indicates that adhesion induced by an exposed surface is dependent on $\alpha_2\beta_1$. The use of different collagen preparations in experimental settings and its influence on the results have been addressed in experiments using $\alpha_2\beta_1$ -deficient mice. Adhesion to fibrillar collagen for platelets from α_2 -deficient mice was comparable to wild-type mice (Holtkotter, *et al* 2002). However, blocking the GPVI-receptor abolished adhesion of α_2 -deficient platelets but not of wild-type platelets. The same relationship has also been observed for β_1 -deficient platelets (Nieswandt, *et al* 2001a) indicating that GPVI must be inhibited in order for adhesion to fibrillar collagen to be $\alpha_2\beta_1$ -dependent. However, adhesion to monomeric collagen was dependent on both GPVI and $\alpha_2\beta_1$ since neither α_2 - nor β_1 -deficient platelets adhered to collagen and adhesion of wild-type platelets was inhibited when blocking GPVI (Nieswandt, *et al* 2001a, Holtkotter, *et al* 2002). All those studies make it reasonable to assume that both $\alpha_2\beta_1$ and GPVI contribute to platelet adhesion to collagen. Consequently, studies have been performed aimed at elucidating the contributory roles of the GPVI and $\alpha_2\beta_1$ -receptors. In one such study, GPVI-deficiency blocked adhesion to collagen during flow, while lack of $\alpha_2\beta_1$ resulted in platelet adhesion with aggregates that tended to disintegrate (Kuijpers, *et al* 2003). It was also found that $\alpha_2\beta_1$ was important for GPVI-induced platelet activation. From these results a model was proposed in which $\alpha_2\beta_1$, activated by GPVI, stabilizes adhesion to collagen and thereby facilitates further GPVI-signalling. Further studies by the same research group showed that the time elapsing between initial collagen interaction and platelet activation (as measured by

intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) levels) differed between platelets (Auger, *et al* 2005). This refined the model suggesting that platelets can engage in two separate pathways when adhering to collagen. Some platelets first interact through GPVI, which activates $\alpha_2\beta_1$ resulting in firm adhesion. Other platelets initially interact through $\alpha_2\beta_1$ and secondly with GPVI. Thus, this model of platelet adhesion merges the earlier models proposed by Santoro *et al.* (1991) and Nieswandt *et al.* (2001a), suggesting that some platelets behave according to the first described model while others behave according to the second.

The hitherto discussion regarding *in vivo* adhesion has only been concerned with platelet adhesion to collagen. However, another interaction of major importance is the binding of GPIb-IX-V to vWf. This interaction can be induced experimentally by the compound ristocetin (Coller, *et al* 1983). *In vitro* studies have also shown that shear stress is important in order for GPIb-IX-V to bind vWf. The conformation of adsorbed vWf is influenced by shear stress (Siedlecki, *et al* 1996) and the conformation of the functional domain of vWf affects its interaction with GPIb-IX-V (Miyata, *et al* 1996). Functional *in vitro* studies have shown that platelet adhesion to vWf increases with elevated shear stress and that this interaction is transient resulting in continuous movement of platelets (Savage, *et al* 1996, Savage, *et al* 1998). However, integrins were necessary for induction of stable and irreversible adhesion. Furthermore, GPIb-IX-V is reported to be important for *in vivo* arterial thrombus formation in mice (Konstantinides, *et al* 2006).

From the discussion above it is evident that platelet adhesion is a very complex process. However, the main events can be described as follows (Figure 1). At high shear stress, GPIb-IX-V is responsible for initiation of platelet adhesion to ECM (Varga-Szabo, *et al* 2008). vWf bound to collagen in the exposed vascular wall interacts weakly with GPIb-IX-V, which induces a rolling phenomenon. Rolling maintains platelets in contact with ECM and consequently enables platelet interaction with collagen through GPVI. This results in platelet activation, which is further amplified through the action of different soluble platelet agonists. Details of platelet activation will be discussed later in this thesis. One important consequence of platelet activation is the induction of high-affinity conformations of integrins such as $\alpha_2\beta_1$. This finally results in firm adhesion through binding of $\alpha_2\beta_1$ to collagen. The $\alpha_{IIb}\beta_3$ -receptor is involved in platelet adhesion by binding to proteins such as vWf and fibronectin in the

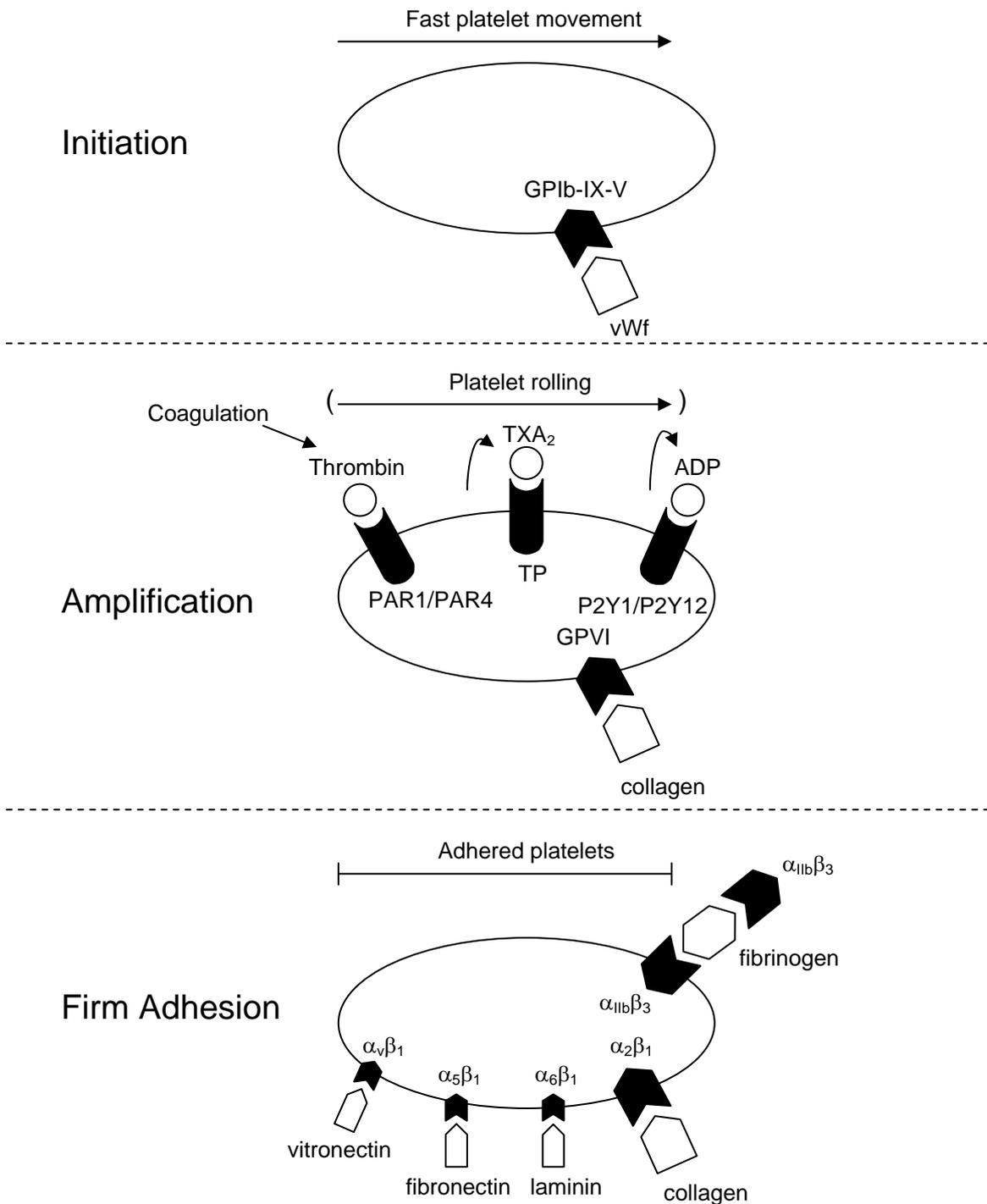


Figure 1. A simplified description of platelet adhesion during dynamic flow conditions. Platelet adhesion can be considered an event occurring in three sequential steps. Adhesion is first initiated by interactions between vWf and GPIb-IX-V, which result in reduced platelet movement. The platelet response is then amplified by platelet activation. Activation is induced by binding of platelets to the exposed surface and by platelet interaction with agonists either secreted from platelets or originating from plasma. Finally, platelets adhere firmly by integrins binding ECM-components. The most important of these interactions is $\alpha_2\beta_1$ that binds to collagen. Also, platelets are cross-linked by binding to plasma fibrinogen, which results in platelet aggregation. In reality, the three steps are not easily distinguished from each other. Platelets are activated during the initiation phase and the amplification- and firm adhesion phases are more or less simultaneous events.

vascular wall. Also, $\alpha_{IIb}\beta_3$ cross-links platelets by interacting with plasma fibrinogen in a process known as platelet aggregation (Bennett 2001). Aggregation creates a platelet plug that finally will seal the vessel wound. It should be evident from the above discussion that collagen and vWf are the major substrates for platelet adhesion. Knowledge about other substrates is relatively scarce but depending on the *in vivo* situation other ECM-components might be important as well. A role for the fibronectin-receptor $\alpha_5\beta_1$ and/or the laminin-binding $\alpha_6\beta_1$ for *in vivo* platelet adhesion in mice has been suggested (Gruner, *et al* 2003). The authors further speculated that effective adhesion to diverse substrates occurring in different areas of the vascular tree or occurring because of different severity of a lesion is ensured by this multitude of adhesive receptors.

Platelet adhesion to endothelial cells

The above discussion was aimed to give a brief introduction to mechanisms responsible for platelet adhesion to ECM. However, some attention must also be addressed towards platelet adhesion to endothelial cells. Physiologically, the endothelium contributes to hemostasis by inhibiting coagulation and platelet activation as well as by stimulating fibrinolysis (van Hinsbergh 2001). The platelet inhibiting effect is achieved by release of platelet inhibitors such as nitric oxide, prostacyclin and prostaglandin E_2 . Furthermore, the endothelial cell surface express platelet repelling proteoglycans such as heparan sulphates as well as ectonucleotidases that degrade the platelet activator adenosine diphosphate (ADP). Consequently, platelet adhesion to the endothelium is only possible when endothelial cells are activated/injured. Unactivated platelets have been shown to roll on activated endothelial cells *in vivo* (Frenette, *et al* 1995). This rolling was dependent on P-selectin expressed on endothelial cells. Ligands for P-selectin present on platelets seem to be GPIb-IX-V (Romo, *et al* 1999) and P-selectin glycoprotein ligand 1 (PSGL-1) (Frenette, *et al* 2000). Interactions between selectins and their ligands normally demand the presence of carbohydrates on the selectin-ligand. In contrary, platelet-endothelium interactions occur in the absence of glycosylations, which probably results in weaker interactions (McEver 2001). However, this might be compensated for by a high density of GPIb-IX-V on platelets and also by the small size of platelets which reduces the force exerted on the bonds.

As for platelet adhesion to ECM, rolling on the endothelial cells is followed by firm platelet adhesion. Several structures have been proposed to be important for this process. It has been shown *in vitro* that $\alpha_{IIb}\beta_3$ on activated platelets interacts with GPIb-IX-V, $\alpha_v\beta_3$ and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells through bridging mechanisms involving fibrinogen, fibronectin and vWf (Bombeli, *et al* 1998). Another study has confirmed the importance of $\alpha_{IIb}\beta_3$ for platelet-endothelium interactions (Tomita, *et al* 2001). Thus, data from different studies have resulted in a model for platelet adhesion including rolling followed by firm adhesion (McEver 2001). Platelet rolling on endothelial cells involves binding of platelet GPIb-IX-V or PSGL-1 to P-selectin on endothelial cells. Firm adhesion requires platelet activation and is accomplished by binding of $\alpha_{IIb}\beta_3$ on platelets to ICAM-1, $\alpha_v\beta_3$ or GPIb-IX-V on endothelial cells via protein bridges.

Recent results suggest that the adhesion process might be more complicated than described above. ADAM15 (a disintegrin and metalloproteinase) is expressed on endothelial cells and interacts with platelet $\alpha_{IIb}\beta_3$ during both static and flow conditions (Langer, *et al* 2005). The amount of adhesion was comparable to platelet adhesion to fibrinogen. Langer *et al.* further showed that binding of ADAM15 to $\alpha_{IIb}\beta_3$ induced platelet secretion of P-selectin and CD40 Ligand, which propose a role for this interaction in inflammation. Also, endothelial PSGL-1 interacts with platelet P-selectin, *i.e.* the two receptors interact irrespective of the cell type they are attached to (da Costa Martins, *et al* 2007).

Platelet activation

Further complexity is added to the adhesive process by taking into account the platelet activating process occurring simultaneously with platelet adhesion. Such stimuli induce the formation of pseudopods on platelets, which result in a larger surface area available for adhesion (George 2000). Furthermore, platelet activating stimuli also increase the affinity of integrins for their respective ligands. Affinity-regulation of integrins can be performed by direct conformational changes of the integrins and possibly also by clustering of receptors (Carman & Springer 2003). Thus, the activating process facilitates the adhesive interactions. Furthermore, it recruits additional platelets by inducing platelet secretion of activating substances (Woulfe 2005). The details of the activating process are complex involving several different mediators that amplify the platelet response (Figure 1). First of all, the interactions

described above involving GPIIb-IIIa, $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ are all known to induce intracellular stimulating events (Gibbins 2004). Also, soluble activators derived from plasma or from activated platelets further contribute to platelet activation. All known physiological soluble platelet activators stimulate platelets through G-protein coupled receptors (Table I) (Woulfe 2005). The G-proteins responsible for platelet activation are derived from the G_q -, G_i -, and $G_{12/13}$ -families. The different G-proteins can generally be seen as inducers of separate intracellular events. In simple terms, G_q increases $[Ca^{2+}]_i$, G_i decreases intracellular production of cyclic adenosine monophosphate ($[cAMP]_i$) and $G_{12/13}$ activates Rho kinase. Increased levels of $[Ca^{2+}]_i$ and decreased levels of $[cAMP]_i$ are well-known general inducers of platelet activation. Rho kinase is specifically important for platelet shape change. One important platelet activator is ADP, which is stored in platelet dense granules and released upon platelet activation (McNicol & Israels 1999). Also, shear stress can induce release of ADP from erythrocytes (Reimers, *et al* 1984). ADP acts in an autocrine/paracrine manner and binds two different platelet receptors called P2Y1 (Jin, *et al* 1998) and P2Y12 (Hollopeter, *et al* 2001). Stimulation of P2Y1 activates G_q -proteins resulting in increased $[Ca^{2+}]_i$ while binding of ADP to P2Y12 activates G_i with the consequence of reduced levels of $[cAMP]_i$ (Oury, *et al* 2006). Another important substance in the regulation of hemostasis is thrombin, which is formed by the actions of the coagulation cascade and contributes to final coagulation by converting fibrinogen to fibrin (Walsh 2004). In addition, thrombin is a strong platelet activator and acts through two different protease activated receptors (PARs) on platelets called PAR1 and PAR4 (Coughlin 2005). Both receptors activate G_q - and $G_{12/13}$ -signalling. Furthermore, activation with thrombin induces a release reaction from platelets resulting in stimulation of G_i -signalling through ADP (Kim, *et al* 2002). TXA_2 is produced by activated platelets and diffuses over the plasma membrane (Puri 1998) in order to act through a single thromboxane receptor (TP) on platelets (Habib, *et al* 1999). This activates both G_{13} and G_q (Knezevic, *et al* 1993, Djellas, *et al* 1999). In analogy with thrombin, G_i -signalling occurring after TP-receptor activation is achieved by autocrine activation by *e.g.* ADP (Paul, *et al* 1999). Another important platelet activator is adrenaline. Adrenaline can be released by platelets after platelet activation (Born & Smith 1970, Paul, *et al* 1999) but is also a stress-hormone that is circulating in blood. It activates platelets through α_2 -adrenergic receptors (α_2 -ARs) (Hoffman, *et al* 1979, Hsu, *et al* 1979, Grant & Scrutton 1980), which stimulates a specific G_i -protein called G_z (Yang, *et al* 2000). Furthermore, lysophosphatidic acid (LPA) is a phospholipid with platelet activating potential (Siess & Tigyi 2004). Even though not as well studied as the above mentioned platelet activators, it has been shown that platelets

contain mRNA coding for three different LPA-receptors called LPA₁, LPA₂ and LPA₃ (Motohashi, *et al* 2000). As evidenced by its ability to activate Rho, LPA seems to activate G_{12/13}-signalling (Retzer & Essler 2000).

From the above discussion, it should be obvious that platelet activation is a complex process involving several different actors. This diversity could be utilized as a back-up system but it is also clear that simultaneous activation results in synergistic platelet activation. A synergistic effect can be defined as an effect of two inducers exceeding the sum of the effects of the inducers used alone (Graff, *et al* 2004). Synergism is often discussed in the context of adrenaline, which has been observed to induce synergistic effects when combined with diverse activators such as thrombin, platelet activating factor (PAF), ADP, LPA, estrogens, histamine, serotonin and thrombopoietin (Roevens, *et al* 1993, Steen, *et al* 1993, Masini, *et al* 1998, Nilsson, *et al* 2002, Haseruck, *et al* 2004, Campus, *et al* 2005, Akarasereenont, *et al* 2006). Several studies report synergistic effects as the result of two compounds activating two different signalling pathways that converge in platelet activation. First of all, many studies suggest that G_i and G_q-signalling seem to be able to cooperate with resultant synergistic effects. One study investigated ADP-induced platelet aggregation by alternately inhibiting the G_i-coupled P2Y₁₂-receptor and the G_q-coupled P2Y₁ (Jin & Kunapuli 1998). Selective stimulation of the G_i-pathway by adrenaline or the G_q-pathway by serotonin was found to reverse the inhibiting effects of inhibitors towards P2Y₁₂ and P2Y₁ respectively. Thus, blocking G_q- or G_i-dependent ADP-signalling could be substituted for by selective activation of the respective pathways. Also, platelet aggregation induced by the thromboxane mimetic U46619 is dependent on direct G_q-stimulation by U46619 and indirect G_i-stimulation by secreted ADP and adrenaline (Paul, *et al* 1999). Finally, phosphorylation of extracellular signal-regulated kinase 2 (ERK2) induced by collagen demanded simultaneous activation of G_q and G_i through TXA₂ and ADP respectively (Roger, *et al* 2004). Further complexity to this field of research is added by findings showing that G_i also can cooperate with G_{12/13} in order to produce synergistic effects (Dorsam, *et al* 2002, Nieswandt, *et al* 2002). Thus, platelet adhesion *in vivo* is certainly an intricate process being affected by several different mediators simultaneously.

Platelets in pathophysiology

Due to the physiological function of platelets to seal wounds after vessel injury it is quite reasonable that hypo-function would result in increased risk of bleeding, while hyper-function would cause thrombosis. Increased bleeding tendency can be the result of either quantitative or qualitative defects. Decreased platelet counts (thrombocytopenia) results in increased bleeding tendency, even though the platelet count must be decreased to levels way below normal before bleeding becomes significant (George 2000). The most important cause of thrombocytopenia is infection, but it can also be drug-induced or be the result of production of autoantibodies (George 2000). Also, particularly high platelet counts occurring for a subgroup of patients with essential thrombocythemia (ET) results in a paradoxical bleeding tendency (van Genderen & Michiels 1994). Regarding qualitative defects, there are a number of rare genetic platelet disorders with increased bleeding risk (Nurden 2005). Such disorders include Glanzmann's thrombastenia and Bernard Soulier's syndrome characterized by the lack of functional $\alpha_{IIb}\beta_3$ and GPIb-IX-V respectively. Also, there are different kinds of Storage pool diseases with defects affecting dense and/or α -granules. In this discussion it is also important to mention bleeding disorders such as von Willebrand's disease and Hemophilia A and B. However, those disorders are not connected to direct defects of platelets but represent 95-97 % of all inherited disorders concerning deficiencies of coagulation proteins (Peyvandi, *et al* 2006).

Thrombosis is a condition occurring in several disease states and being caused by a multitude of factors. First of all, abnormalities of blood, generally known as thrombophilia, result in increased risk of thrombosis. Such conditions can be the result of activating defects of certain coagulation factors or result from deficiencies of physiological anticoagulants (Boekholdt & Kramer 2007). Another cause of thrombosis is venous blood stasis resulting from prolonged immobilization (Line 2001). A common manifestation is deep vein thrombosis of the legs, which can be life-threatening if embolizing to the lungs. Furthermore, the myeloproliferative disorders ET and polycythemia vera are closely connected to thrombosis of arterial, venous or microcirculatory origin (Elliott & Tefferi 2005). However, thrombosis is especially important in cardiovascular diseases (CVDs), which are the leading causes of death globally (Fuster, *et al* 2007). Atherosclerosis is an important contributor to the CVDs. Atherosclerosis is a disorder of the arteries characterized by an inflamed vessel wall with lipid deposits and

infiltration of inflammatory cells and smooth muscle cells (Kher & Marsh 2004). This transformed vessel area is commonly denoted as an atherosclerotic plaque. The ultimate fate of an atherosclerotic artery is plaque rupture, which results in the development of thrombosis. If such thrombosis occurs in coronary vessels the consequence might be the development of a myocardial infarction (MI). The importance of platelets in atherothrombosis has been proved in several ways. Morphological analysis shows infiltration of platelets in thrombi resulting from unstable angina (Kragel, *et al* 1991, Arbustini, *et al* 1995), platelet activation is connected to cardiovascular disease (Fitzgerald, *et al* 1986, Vejar, *et al* 1990, Furman, *et al* 1998) and platelet-inhibiting drugs are useful for the prevention of future events (see below for references). However, the importance of the coagulation system must not be forgotten. Use of anticoagulants in combination with anti-platelet drugs is recommended for patients with acute coronary disease (Pollack & Goldberg 2008). Also, it has been shown *in situ* that platelet deposition to plaques is positively correlated to the plaque content of tissue factor (Toschi, *et al* 1997) and that fibrin is present in thrombi resulting from MI (Kragel, *et al* 1991). Even though this discussion is mainly aimed at describing the role of platelets for the final formation of thrombi, it is interesting to note that platelets might also have a role in early development of atherosclerosis. Mice lacking the anti-atherogenic compound apolipoprotein E (ApoE) are known to develop atherosclerotic disease (Greenow, *et al* 2005). Platelet adhesion to the endothelium has been shown to occur in such atherosclerosis-prone ApoE^{-/-} mice but not in wild-type mice (Massberg, *et al* 2002). Massberg *et al.* (2002) also showed that platelet adhesion preceded development of atherosclerosis and infiltration of leukocytes. Furthermore, inhibition of platelet adhesion through a GPIb-IX-V antibody both reduced leukocyte adhesion and decreased the formation of atherosclerotic lesions. In another study, rabbits fed a cholesterol-rich diet showed platelet adhesion to endothelium on atherosclerosis-prone sites before atherosclerosis could be detected histologically (Theilmeyer, *et al* 2002). In summary, platelets are known to be important for atherothrombosis but might also have a role in early events of atherosclerosis.

Platelet inhibiting treatment

Therapy for patients with increased risk of thrombosis includes the use of anti-coagulants as well as use of anti-platelet drugs. Clinically useful anti-platelet drugs basically acts through four different mechanisms (Figure 2). Acetylsalicylic acid (ASA) was first described as a

platelet inhibitor in the late 1960's (Weiss & Aledort 1967) and it has thereafter been shown to be effective in several clinical conditions. ASA acts by inhibiting the cyclooxygenase (COX)-enzyme thereby reducing the production of TXA₂ (Awtry & Loscalzo 2000). An overview of published clinical studies showed that ASA reduces the risk of MI, stroke and vascular death by 25 % in patients previously diagnosed with unstable angina, MI, stroke or transient ischaemic attack (Antiplatelet Trialists' Collaboration 1994). Other platelet inhibitors are the thienopyridines called ticlopidine and clopidogrel. Both drugs are metabolized by cytochrome P450 in the liver and their respective metabolites act as antagonists of the ADP-binding P2Y₁₂-receptor (Michelson 2008). Because of less side-effects, clopidogrel is more commonly used than ticlopidine. Clopidogrel has been shown to be slightly more effective than ASA in preventing ischaemic stroke, MI and vascular death in patients with previous cardiovascular events (CAPRIE Steering Committee 1996). However, combining ASA and clopidogrel have been shown to be superior compared to ASA alone in patients suffering from unstable angina (Yusuf, *et al* 2001), MI (Chen, *et al* 2005, Sabatine, *et al* 2005) and in patients undergoing percutaneous coronary intervention (PCI) (Mehta, *et al* 2001, Steinhubl, *et al* 2002). A third way of inhibiting platelet function is by antagonizing the $\alpha_{IIb}\beta_3$ -receptor. Substances available for this purpose include abciximab, eptifibatid and tirofiban (Schorr & Weber 2003). A systematic overview has proven clinical benefit of abciximab when used during PCI (Kandzari, *et al* 2004). A final pharmacological agent of importance for prevention of thrombosis is dipyridamole. Dipyridamole increases the concentration of the platelet inhibitor adenosine in plasma and also inhibits cGMP-degrading phosphodiesterases (Schaper 2005). Dipyridamole in combination with ASA has been shown to be more effective than ASA alone in preventing vascular events in patients with previous cerebral ischaemia of arterial origin (Halkes, *et al* 2006).

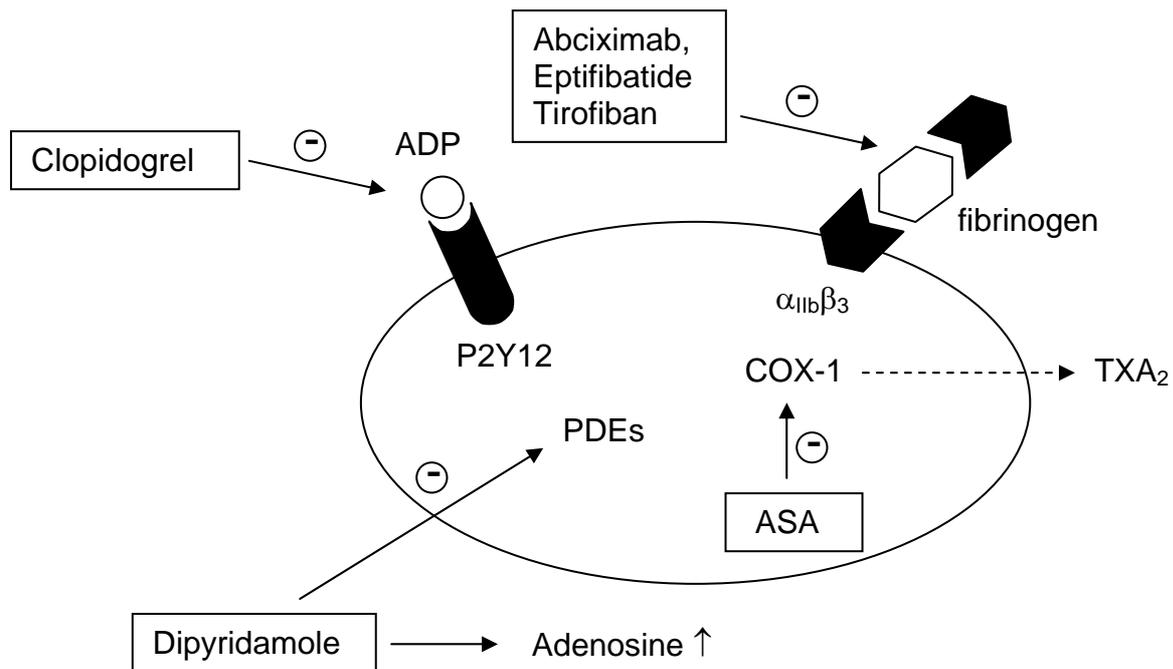


Figure 2. Clinically used platelet inhibitors have distinct pharmacodynamic actions. ASA inhibits COX-1, clopidogrel inhibits ADP-signalling and dipyridamole elevates plasma adenosine and inhibits cGMP phosphodiesterases. The three drugs abciximab, eptifibatide and tirofiban are antagonists of fibrinogen binding.

In general, inter-individual differences in the efficacy of pharmacological treatment are common and can in part be explained by genetic polymorphisms of drug targets or of drug metabolizing enzymes (Evans & Johnson 2001). Of interest for this thesis are inter-individual effects observed for the platelet inhibitors ASA and clopidogrel. In 1993 Helgason *et al.* was the first to suggest that there might be patients that are resistant to the effect of ASA (Helgason, *et al* 1993). Since this report several other investigators have dealt with the issue of ASA-resistance and it has been the topic of many recent reviews. Much confusion in this area exists, primarily because different researchers have defined ASA resistance in different ways. Therefore, efforts have been made in order to find general definitions of this phenomenon. It has been proposed that ASA resistance can be defined either as laboratory resistance or clinical resistance (Mason, *et al* 2005, Sanderson, *et al* 2005, Hankey & Eikelboom 2006). Laboratory resistance is defined as the inability of ASA to inhibit platelets in one or more tests of platelet function, while clinical resistance is the inability of ASA to prevent thrombosis. Still, there are no definite estimates of the prevalence of ASA resistance. The multitude of platelet function assays employed for measuring laboratory resistance have resulted in dispersed results showing that 5 to 60 % of individuals are ASA resistant (Mason, *et al* 2005, Sanderson, *et al* 2005). Also, a drawback with the laboratory resistance definition is that other factors in addition to TXA₂ often contribute to final platelet function.

Consequently, failure to inhibit TXA₂ synthesis is suggested to be the only acceptable definition of ASA resistance since this is a direct measure of the pharmacodynamic action of the drug (Cattaneo 2004). More confusion is added to the concept of ASA-resistance since the mechanism of this effect is not clearly established. However, several possible causes have been proposed including (1) patient non-compliance, (2) prevention of ASA-interaction with COX-1 by concurrent medication with other COX-1 binding drugs, (3) interindividual pharmacokinetics, (4) platelet-independent TXA₂-synthesis by the COX-2 enzyme in cells such as monocytes, macrophages and endothelial cells, (5) platelet activation through TXA₂-independent events and (6) genetic polymorphisms (Mason, *et al* 2005, Hankey & Eikelboom 2006). Of course, there is a close connection between the explanation and the definition of ASA resistance. It is possible to place the mechanisms described above in two separate groups. In the first three explanations, the cause of the resistance is inability of ASA to interact properly with COX-1. This group can be called *Direct ASA-resistance*. In turn, explanations 4 and 5 are concerned with compensatory mechanisms meaning that their ASA resistance occurs despite proper inhibition of COX-1. In analogy with the above nomenclature, this group represents *Indirect ASA-resistance*. The sixth explanation of ASA-resistance concerns genetic polymorphisms in genes coding for proteins such as COX-1 and $\alpha_{IIb}\beta_3$ (Mason, *et al* 2005, Hankey & Eikelboom 2006), which should be interpreted as *Direct* and *Indirect ASA-resistance* respectively. Consequently, the diversity of explanatory models definitely contributes to the difficulties in defining the concept of ASA-resistance. A true definition of ASA resistance might not be possible to formulate until the mechanism of the effect is known.

It is well-established that inter-individual effects occur for clopidogrel as well (Jaremo, *et al* 2002, Gurbel, *et al* 2003, Serebruany, *et al* 2005). As for ASA there is no standardized definition but based on findings showing that the clopidogrel response follows a normal distribution (Serebruany, *et al* 2005) a relevant term to use would be clopidogrel response variability in order to emphasize that the phenomenon can not be described in a dichotomous way (Angiolillo, *et al* 2007). The cause of the response variability is unknown but might involve genetic mechanisms as well as up-regulation of P2Y₁₂-independent signalling pathways (Angiolillo, *et al* 2007).

Platelet function assays

During the years, several assays for the evaluation of platelet function have been developed. One widely used assay that has had a large impact on platelet research is platelet aggregometry, which was introduced in the early 1960's (O'Brien 1962, Born & Cross 1963). This assay measures the changes in light transmittance that occur when platelets aggregate. It can be used both for isolated platelets and for platelets in plasma and a related assay called impedance aggregometry is suitable for analysis in whole blood (McNicol 1996). Nowadays, a multitude of assays exist for investigating platelet function. First of all, analysis of intracellular signal transduction represents narrow and specific ways of determining platelet function. Such assays include measurements of $[Ca^{2+}]_i$ and cyclic nucleotides as well as measurements of the level of tyrosine phosphorylation (McNicol 1996). On a somewhat broader scale are assays that measure the functional consequences of the intracellular signalling. Such assays include measures of platelet secretion, platelet aggregation as described above and platelet adhesion. Platelet secretion can be estimated by direct measurements of released substances from platelets such as TXB₂, platelet factor 4 or beta-thromboglobulin (Wu 1996). Platelet secretion can also be evaluated by means of flow cytometry through measurements of the amount of P-selectin incorporated in the plasma membrane (Michelson, *et al* 2000) or by labelling of platelet dense granules with mepacrine (Wall, *et al* 1995). Flow cytometry can also be used for estimation of other aspects of platelet function such as $\alpha_{IIb}\beta_3$ -activation (Michelson, *et al* 2000). Platelet adhesion assays come in many different types and shapes but can broadly be divided into assays that measure adhesion during static or during flow conditions. Detection of platelet adhesion can be performed by *e.g.* light microscopy (Lyman, *et al* 1971), fluorescence microscopy (Feuerstein & Kush 1986) or radioactivity measurements of ⁵¹Cr-labeled platelets (Cazenave, *et al* 1973, Brass, *et al* 1976). Moreover, platelet adhesion can be estimated by ELISA-measurements of the amounts of P-selectin released after lysing attached platelets with a detergent (Nadar, *et al* 2005). Two adhesion assays that deserves to be mentioned are the Cone and Plate(let) Analyzer and the Platelet Function Analyzer (PFA-100). Both assays utilize whole blood and measure platelet adhesion during flow conditions. The Cone and Plate(let) Analyzer induce flow by the use of a rotating cone (Varon, *et al* 1997). Adhered platelets are stained with May-Grünwald stain and platelet adhesion is evaluated by a computerized image analysis system. In this way information is collected regarding surface coverage, total amount of objects and average size of objects. In the PFA-100, whole blood added to a cartridge flows

through a membrane coated with collagen combined with ADP or adrenaline and the time elapsing before occlusion of the membrane is measured (Kundu, *et al* 1995). Another assay with close resemblance to the PFA-100 is filtragometry. In this assay, blood is drawn continuously from a cubital vein over a nickel filter (Hornstra & ten Hoor 1975). Platelet aggregates are formed in the filter and the grade of occlusion is estimated by measuring the pressure difference over the filter. A last category of platelet assays could be defined as assays measuring hemostasis as a whole. Such assays can be exemplified by thrombelastography (TEG). TEG is an assay system basically consisting of a pin and an oscillating cup (Hobson, *et al* 2006). The pin is connected to a torsion wire and suspended in the cup. Whole blood is added to the cup and during blood coagulation fibrin strands are formed between the cup and the pin. In this way, the viscoelastic properties of the blood are transmitted to the pin. This generates an electrical signal, which forms the basis of the interpretation of the results. This global test of hemostasis is dependent both on platelets and on coagulation proteins. Connected to global tests of hemostasis are animal models of thrombosis. Several genetically modified mouse strains exist, suitable for *in vivo* studies of thrombus formation (Westrick, *et al* 2007). Also, different techniques have been developed for induction of thrombi *in vivo* (Westrick, *et al* 2007). Such techniques include injection of collagen combined with adrenaline as well as induction of vessel injury mechanically or by laser, ferric chloride or photochemically. Consequently, there are several different ways of measuring platelet function and all assays have their specific advantages and disadvantages. In its broadest sense, the assays measuring global hemostasis are definitely closest to the *in vivo* situation. However, details of the hemostatic process can be hard to investigate by such an approach and might need complementary studies with assays measuring more specific aspects of platelet function.

In connection to the issues of drug resistance it would of course be useful if an assay for measurements of platelet function could be used in order to detect increased risk of thrombosis. Also, several attempts using different methodology have been made for evaluating if measures of laboratory ASA resistance can be used to predict clinical events. Non-response to ASA, as measured by optical platelet aggregometry, have been shown to be predictive of thrombotic complications and all-cause mortality (Gum, *et al* 2003). Also, urinary concentrations of 11-dehydro-TXB₂ in patients treated with ASA has been associated with increased risk of cardiovascular events (Eikelboom, *et al* 2002). The PFA-100 has been reported to detect differences between patients receiving ASA-treatment and experiencing

recurrent cerebrovascular events compared to patients with ASA and no recurrent events (Grundmann, *et al* 2003). Finally, whole blood aggregometry was investigated in ASA-treated patients with claudicatio and was shown to detect patients with increased risk for recurrent peripheral thrombosis (Mueller, *et al* 1997). Even though it seems promising, the above mentioned studies have been criticized for different reasons, including small sample sizes and low number of events, suggesting that more studies are needed on this issue (Michelson, *et al* 2005, Sanderson, *et al* 2005). Also, no studies have investigated the effects of changing therapy based on platelet function tests and it is currently not recommended to monitor anti-platelet treatment in patients (Michelson, *et al* 2005, Cattaneo 2007).

Aims

This thesis is focused on platelet adhesion measured by an *in vitro* assay. A primary aim was to describe the assay and to characterize the adhesive events it measures (Papers I + III). We also wanted to investigate the usefulness of the assay for both experimental (Paper II) and for clinical research (Papers IV + V).

Methodological considerations

Static platelet adhesion

The common theme for all papers constituting this thesis is an assay for measuring static platelet adhesion. This assay is based on the principles outlined by Bellavite *et al.* (1994) for measuring adhesion of isolated platelets. The assay, as performed by us, can be described as follows. The assay measures platelet adhesion in 96-well microplates coated with different proteins. Coating is performed by addition of protein solutions to the wells followed by incubation at least overnight and maximally for seven days at 4°C. This range of incubation times was found not to influence platelet adhesion (Paper I). In order to facilitate protein adsorption we used microplates known to bind proteins (Nunc maxisorp, Roskilde, Denmark) and the protein solutions added were also highly concentrated. After washing the microplates twice in 0.9 % NaCl by plate inversion the microplates were ready for use in platelet adhesion experiments. Platelets were prepared by centrifugation of whole blood for 20 min at 205×g (Papers I-II, IV-V) or 220×g (Paper III). This procedure separates blood according to density and results in a lower phase consisting of erythrocytes, a middle phase with leukocytes and an upper phase constituting platelets dissolved in plasma. The upper phase with plasma and platelets are commonly known as platelet-rich-plasma (PRP). Approximately two thirds of the PRP was transferred to a new plastic tube. By leaving one third of the PRP we reduced the probability of transferring other cells than platelets. During the course of this thesis it was found that increasing the volume of blood in tubes when centrifuging seemed to increase the ability of platelets to adhere (unpublished results). Increased *g*-forces during centrifugation is reported to result in PRP with decreased amounts of large platelets and decreased platelet aggregating capacity (Healy & Egan 1984). Consequently, different overall centrifugal forces occurring with different blood volumes could be a source of assay variability. Having discovered this, centrifugation was standardized and always performed with 8 mL whole blood in each tube (Papers III + V). The prepared PRP was then diluted 4 times with 0.9 % NaCl. The reason for using diluted PRP is connected to the below described way of detecting adhesion by spectrophotometric measurements of absorbance. We found that when measuring acid phosphatase activity in different dilutions of PRP, the activity in undiluted plasma deviated from the linearity observed between PRPs diluted 2, 4 and 8 times (unpublished results). These results are in accordance with the ability of non-absorbing solutes to affect the properties of the absorbing compound (Harris 2002). Consequently,

dilution was necessary and was found to be optimal when diluting 4 times (Paper I). Higher dilutions resulted in difficulties in detecting adhered platelets because of too low platelet number. The diluted PRP was then added to the washed and protein coated microplate wells together with test substances whose effects on platelet adhesion were to be investigated. Test substances used varied for the different papers and included activators as well as inhibitors of platelet function. Some inhibitors were incubated in PRP before addition to wells in order to facilitate their effects. The time elapsing between addition of test substance and addition of PRP tended to influence LPA-induced platelet adhesion to albumin (unpublished results). The results indicated that increasing the time between LPA-addition and addition of PRP increased platelet adhesion. It has previously been shown that albumin binds LPA (Tigyi & Miledi 1992) and that albumin inhibits LPA-induced platelet aggregation (Tokumura, *et al* 1987, Haseruck, *et al* 2004). This makes us suggest that LPA binds surface-attached albumin in our assay. This binding might then facilitate LPA-binding to platelets making activation more efficient. An activating effect observed in our assay compared to the inhibiting effects observed for aggregation could be connected to structural differences between surface-attached albumin and albumin in solution. These results made us standardize the time period between addition of test substance and PRP to 20 min (Paper V). After addition of PRP the microplates were left for 1 h at room temperature (RT) to allow platelets to attach to the surface. The two major differences between our assay and the assay described by Bellavite *et al.* (1994) now deserve some attention. First, Bellavite *et al.* (1994) investigated adhesion of washed isolated platelets instead of platelets in plasma. As the name implies, isolated platelets are platelets dissolved in a buffer without presence of other blood cells or plasma constituents. Accordingly, the different environmental conditions for isolated platelets compared to platelets in plasma could most probably result in deviating results between the two assays. This has already been recognized for assays such as platelet aggregometry and platelet flow cytometry for which it is possible to measure platelet activity in different preparations of blood (McNicol 1996). Analysing platelet function in different environments is an effective way of gathering complementary information regarding platelet function. The second major difference from the study by Bellavite *et al.* is that they consequently add the same number of platelets/well while we just add PRP diluted 4 times irrespective of the actual platelet count. Using the same number of platelets is an effective way of standardising an assay. However, one advantage when omitting platelet counting is that the results might be more representative of the actual platelet activity since it includes the variable of platelet count. This could be important when trying to investigate platelet function in individual patients. However, our

results consistently showed that there were no associations between platelet adhesion and platelet count (Papers IV + V). Thus, platelet count does not seem to affect adhesion of platelets in plasma in this assay. After platelets had been incubated in the wells for 1 h the microplates were washed twice in 0.9 % NaCl by plate inversion. A sodium citrate/citric acid buffer (0.1 mol/L, pH 5.4) containing 0.1 % Triton X-100 and 1 mg/ml *p*-nitrophenyl phosphate was added to all wells. Background absorbance was measured at 405 nm followed by incubation for 40 min at RT during continuous gentle agitation. Triton X-100, which is a detergent, will be incorporated in the platelet membranes thereby creating pores. This allows *p*-nitrophenyl phosphate to enter the platelets and/or acid phosphatase to leave the platelets. Consequently, *p*-nitrophenyl phosphate interacts with acid phosphatase producing *p*-nitrophenol. After the 40 min incubation, 2 mol/L NaOH was added to all wells. NaOH exerts a dual function. First of all it raises the pH-value, which stops the reaction by inactivating acid phosphatase. Secondly, the pH-change transforms the produced *p*-nitrophenol to an optically active compound and absorbance was consequently measured at 405 nm. In paper I we show that the absorbance values obtained by measuring *p*-nitrophenol closely correlates with platelet counts measured by an automatic cell counter. Thus, we conclude that this enzymatic way of detecting platelet adhesion gives a good estimate of the amount of adhered platelets. However, since the number of platelets added is not standardised it is not possible to compare adhesion between two individuals simply by evaluating the absorbance values. Thus, a different procedure was employed for this purpose. To a separate microplate, the buffer solution containing Triton X-100 and *p*-nitrophenyl phosphate was added to wells containing either 0.9 % NaCl or PRP diluted 4 times. In parallel, this microplate was then treated in exactly the same way as the microplates with adhered platelets and absorbance was measured at 405 nm. Thus, this separate microplate measured the absorbance values at 0 % and 100 % adhesion and these values were used in order to calculate the percentage of adhered platelets.

Platelet P-selectin surface expression on adhered cells

An important aspect of platelet activation is the ability of platelets to release contents of intracellular granules. P-selectin is a component of α - and dense granules, which gets incorporated in the plasma membrane after granule release (McNicol & Israels 1999). Measurements of the surface expression of P-selectin on platelets have been widely used for evaluation of platelet function by flow cytometry (Michelson, *et al* 2000) and it can also be utilized for estimating the activity of adhered isolated platelets (Whiss & Andersson 2002).

Based on the assay reported by Whiss and Andersson (2002) we analysed P-selectin on adhered platelets in plasma in order to investigate the importance of granule release for platelet adhesion (Paper II). Platelets were allowed to adhere in the same way as described for the static adhesion assay followed by addition of 0.04 % paraformaldehyde (PFA) in order to fix the platelets. After 5 min incubation with PFA, the microplates were washed twice with 0.9 % NaCl. A phosphate buffered saline (PBS)-solution containing 5 % bovine serum albumin (BSA) was added. Addition of BSA was performed in order to block unspecific binding of antibodies added in subsequent steps of the assay. Excess BSA was removed by a single wash in 0.9 % NaCl after 30 min incubation. The expression of P-selectin was then measured by an enzyme linked immunosorbent assay (ELISA). A primary P-selectin antibody was added followed by incubation for 30 min. After washing twice in 0.9 % NaCl containing 0.05 % Tween 20, an alkaline phosphatase-conjugated secondary antibody was added and the incubation and washing procedure was repeated. Finally, *p*-nitrophenyl phosphate dissolved in diethanolamine buffer (pH 9.8) was added. The microplates were incubated for 10 min, which allowed *p*-nitrophenyl phosphate to react with alkaline phosphatase on the secondary antibodies. Absorbance of the developed product was measured at 405 nm.

Visualization of adhered platelets by fluorescence microscopy

Another way of detecting platelet adhesion, not involving enzymatic reactions, is by fluorescence microscopy. In paper III we employed this procedure in order to get complementary visual information regarding platelet adhesion. Platelets were allowed to attach to surfaces as described above for the static platelet adhesion assay. Adhered platelets were fixed with 4 % PFA and permeabilised with 0.1 % Triton X-100. Platelets were then stained for actin by addition of phalloidin followed by visual inspection of adhered platelets by using a Zeiss AxioObserver inverted fluorescence microscope.

Plasma levels of insulin and oxidized LDL

Plasma levels of insulin and oxidized low-density lipoprotein (ox-LDL) was measured by two separate commercial ELISA-kits (Mercodia, Uppsala, Sweden) in order to establish if those parameters correlated with synergistic adhesion to albumin induced by LPA and adrenaline

(Paper II). The principles were the same for both assays and they are therefore described as an entity. Briefly, peroxidase-conjugated antibodies towards insulin and ox-LDL respectively, reacted with sample insulin or ox-LDL bound to insulin- or ox-LDL-antibodies attached to microplate wells. Amount of plasma insulin and ox-LDL was estimated after measuring the absorbance of the product developed when adding 3,3',5,5'-tetramethylbenzidine, which reacted with antibody-bound peroxidase.

Serum TXB₂-analysis

TXB₂ is a metabolite formed after decay of TXA₂ (Hamberg, *et al* 1975). In paper V we measured serum levels of TXB₂ in order to estimate the pharmacodynamic effect of ASA. A commercial enzyme immuno assay (EIA) kit was used according to manufacturers' instructions (Cayman Chemical, Ann Arbor, Michigan, USA). Serum samples were diluted in EIA buffer and added to microplate wells together with TXB₂ Acetylcholine esterase (AChE) tracer and TXB₂ antiserum. The plates were then incubated for 18 hours at RT. During this incubation two processes occur. First, the TXB₂ antiserum attaches to the surface of the microplate wells. Secondly, the TXB₂ AChE tracer and the TXB₂ from serum bind in a competitive way to the attached TXB₂ antiserum. After incubation, the microplates were washed five times with wash buffer followed by the addition of Ellman's reagent containing the substrate of AChE. The microplates were incubated for 1.5 hours to allow interaction between AChE and its substrate, which produce a yellow coloured product. Absorbance was measured at 405 nm and amount of TXB₂ present in serum was calculated with the use of a data analysis tool developed by Cayman Chemical.

Flow cytometry

In paper V, P-selectin expression and binding of fibrinogen to platelets was measured by flow cytometry. FITC-conjugated antibodies directed towards P-selectin and fibrinogen respectively was added to whole blood. This was followed by platelet activation through addition of ADP or the PAR-1 activating peptide SFLLRN. After incubation for 10 min the reactions were stopped and surface binding of the respective antibodies were evaluated by

flow cytometry using the instrument Beckman Coulter Epics XL-MCL (Beckman Coulter Inc., Fullerton, CA) with computer software program (Expo 32 ADC, Beckman Coulter Inc.).

Allele specific PCR

Allele-specific PCR was used in Paper IV in order to investigate occurrence of the Val617Phe-mutation in the gene coding for Janus kinase 2 (JAK2) in patients with ET. This mutation has earlier been described in approximately 50 % of ET-patients (Tefferi & Elliott 2007). The assay was performed essentially as described by Baxter *et al.* (2005). White blood cell DNA was extracted and mixed with a common reverse primer and two forward primers. The two forward primers served to amplify the wild-type and the mutant gene respectively. After 35 PCR-cycles, the amplified products were visualized through agarose gele electrophoresis.

Results and Discussion

Detection of platelet adhesion

In this thesis we utilized platelet acid phosphatase for enzymatic detection of adhesion in microplates. The use of acid phosphatase for detection of cell adhesion was first described in 1992 when an assay for measurements of neutrophil adhesion in microplates was published (Bellavite, *et al* 1992). This assay was further developed for measurements of adhesion of washed platelets (Bellavite, *et al* 1994) and we have shown that this assay can also be used for platelets in plasma (Paper I). Apart from acid phosphatase other enzymes such as lactate dehydrogenase could be utilized in this kind of assay. However, acid phosphatase seems superior to lactate dehydrogenase since lactate dehydrogenase (in contrast to acid phosphatase) is released from platelets, which results in underestimation of the degree of adhesion (Vanickova, *et al* 2006). One aspect that must be taken into consideration when using acid phosphatase is that this enzyme is present in most cell types present in blood (Radzun & Parwaresch 1980). This could complicate an assay performed in plasma because of possible interference from other cells than platelets. However, we found that platelets constituted 97.9 % of the cells in plasma (Paper I) and we found no evidence of other cell types attached to the surface when adhered cells were visualized by fluorescence microscopy (Paper III).

Platelet adhesion vs. platelet aggregation

When investigating attachment of platelets to surfaces it is interesting to know whether platelets adhere as single cells or if they tend to aggregate. Basal- as well as ADP-induced platelet adhesion to collagen was found to be dependent on $\alpha_2\beta_1$ -receptors (Paper III). Also, fluorescence microscopy images showed homogenous attachment of platelets to collagen rather than the production of platelet islands. However, further experiments showed that a small part of adhesion to collagen seemed to be dependent on $\alpha_{IIb}\beta_3$. There are two explanations for this finding. First, cryptic RGD-sequences in collagen might be exposed after degradation (Farndale 2006), making it possible that such motifs are exposed after surface attachment of collagen. It is also possible that a small amount of platelets aggregate after attachment to collagen. This interpretation is in accordance with previous studies measuring

static platelet adhesion to collagen (Wu, *et al* 1996, Patel, *et al* 2003). Consequently, we conclude that adhesion to collagen in our assay is mainly dependent on $\alpha_2\beta_1$ with a minor contribution from $\alpha_{IIb}\beta_3$ -receptors. Also, the fluorescence microscopy images showed homogenous attachment of platelets adhered to albumin and fibrinogen, which indicates that platelet aggregation is of minor importance on these surfaces as well. When comparing studies measuring static platelet adhesion it is striking that 20-30 % adhesion most often seem to be maximal adhesion after 1 h incubation (Thiagarajan & Kelly 1988, Bellavite, *et al* 1994, Wu, *et al* 1996). Except for some occasional donors or patients this was also true for the results constituting this thesis. Thiagarajan and Kelly (1988), found that adhesion to vitronectin consequently were in the range of 25-35 % after 1 h incubation. Incubating non-attached platelets on new vitronectin-surfaces resulted in adhesion of 30-50 % of platelets. The conclusion from these studies was that adhesion occurs until the surface is saturated with platelets and that there are no specific sub-populations of platelets that are more prone to attach. However, it is also important to take the incubation time into consideration. We report in paper I that percentage adhesion is increased after 1 h incubation compared to incubation for 15 or 30 min. Also, platelet adhesion in microplates is increased after 2 h incubation compared to 1 h for isolated platelets (Bellavite, *et al* 1994) as well as for platelets in plasma (unpublished observations). Consequently, the surfaces were not saturated after 1 h since more platelets adhered with longer incubation time. We suggest that these results might be explained in terms of random motion, since this is the way platelets initially make contact with the surface in a static microplate assay. The amount of favourable interactions between platelets and the surface would therefore increase with time making it more likely with high adhesion values after long incubation periods. Consequently, every incubation period might have a specific maximal value of adhesion. Thus, 30 % adhesion might correspond to a “saturated” surface after incubation for 1 h, while another percentage adhesion is interpreted as saturated adhesion at another incubation period. This model is in accordance with our findings throughout this thesis that the amount of added platelets does not correlate with percentage platelet adhesion. If percentage platelet adhesion was only dependent on the available surface area, percentage adhesion and platelet count would be assumed to be inversely correlated. Low platelet count would allow a high percentage of platelet adhesion and vice versa. Furthermore, our results from Paper IV shows that adhesion of ET-platelets was increased compared to adhesion of platelets from healthy donors. Thus, high percentage adhesion was possible for ET-platelets despite the fact that the total number of platelets added to the wells was higher for ET-patients compared to healthy controls. However, it must be

stressed that adding an increasing number of platelets might increase the probability of aggregate formation. This is indicated by an image obtained from a blood donor having platelet count (as measured by acid phosphatase activity) in the same range as ET-patients (Figure 3). In this image platelets are not distinctively separated from each other making it likely that platelets are aggregated. Also, the surface was not saturated since addition of 10 μM ADP increased adhesion from 24 % to 32 %. Thus, it is possible that when increasing the probability of platelet adhesion, either by addition of an increased amount of platelets or by increasing incubation time, platelet aggregation might occur. Consequently, by regulating those two variables it might be possible to study the adhesion process both in the presence and absence of simultaneous platelet aggregation.

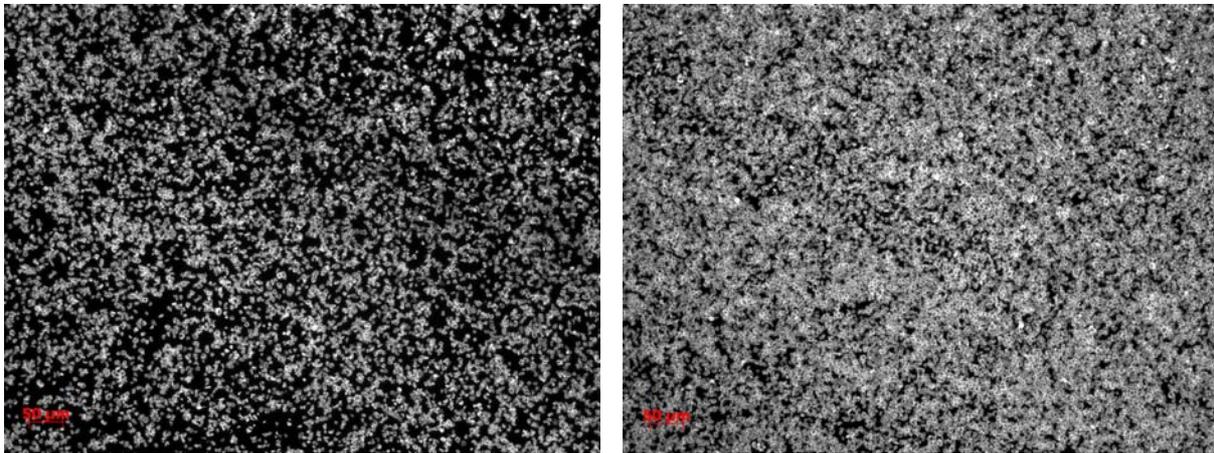


Figure 3. Platelet adhesion to fibrinogen was visualized by staining platelets with phalloidin. The right figure shows platelet adhesion from a blood donor having particularly high platelet count. Visual inspection of the image suggests the presence of platelet aggregates. Percentage adhesion was 24 %. The left figure shows platelet adhesion (27 %) from a donor with normal platelet count.

Static vs. dynamic platelet adhesion

One major methodological concern that demands discussion is the fact that adhesion is performed in a static environment. The absence of a dynamic flow could be a source for questioning the *in vivo* relevance of results from such an assay. We also show that the shear stress dependent receptor GPIb-IX-V is not involved in static platelet adhesion (Paper III). However, we claim that the static adhesion assay is able to generate important information regarding platelet function despite the absence of flow. We have shown that adhesion to collagen and fibrinogen is dependent on the $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ receptors respectively (Paper III). In paper III we also show that the majority of platelet adhesion events studied is dependent on ADP- and/or TXA_2 -secretion. These results apply very well to the general model about how

platelets function (Varga-Szabo, *et al* 2008). Consequently, the fundamental mechanisms of platelet adhesion can easily be detected by this static assay. We therefore claim that the assay generates results that most probably are relevant to the *in vivo* situation.

Studies of platelet function are generally performed by simultaneous use of different platelet function assays. Some platelet function assays can also be performed with platelets in different environments such as isolated platelets in buffer, platelets in plasma or platelets in whole blood. It is generally accepted that different preparations of platelets generate different kinds of information. Investigating the effects of a novel compound on isolated platelets might show that the compound is able to elicit a response in platelets. However, the same compound might be without effect in whole blood because of interference with plasma or other cells. Consequently, in order to get the complete picture of how a compound affects platelets it is necessary to investigate its effect in different environments. In accordance with this we claim that the static adhesion assay might be a complement to adhesion assays performed during flow conditions. Even though we found ristocetin-induced adhesion to be inhibited by a GPIb-IX-V antibody (Paper III), it is obvious that a flow-dependent assay is more suitable for investigating shear stress dependent events. However, static adhesion in the presence of soluble activators might simulate adhesion events occurring directly after platelet rolling. Possibly, static adhesion might turn out to be superior to adhesion during flow in measuring these late adhesion events.

Influence of ions and proteins in platelet adhesion

Dependence on Mg^{2+} for platelet adhesion

We show in paper I that platelet adhesion is affected by Mg^{2+} and that the effect of Mg^{2+} is different depending on which surface is investigated. Basal levels of adhesion to albumin and fibrinogen were decreased by Mg^{2+} , while basal adhesion to collagen was increased from practically no adhesion to approximately 10 %. The effect of Mg^{2+} is also interesting to discuss for adhesion induced by soluble activators. We report in paper II that adrenaline and LPA act synergistically to induce platelet adhesion to albumin in the presence of externally added Mg^{2+} . From these studies we also concluded that LPA is not able to induce platelet adhesion to albumin on its own. However, further research on this subject showed that LPA is able to induce platelet adhesion on its own if addition of external Mg^{2+} is omitted (Figure 4)

(Eriksson, *et al* 2006). Absence of externally added Mg^{2+} also increased the synergistic effect observed for LPA combined with adrenaline. Collectively, our results show that Mg^{2+} has an ambivalent role in platelet adhesion. It inhibits adhesion to surfaces coated with albumin and fibrinogen but increases adhesion to collagen. There are several studies that have shown that Mg^{2+} acts as a platelet inhibitor. Aspects of platelet function that is inhibited by Mg^{2+} include aggregation, adhesion to fibrinogen, P-selectin expression, ATP- and TXA_2 -secretion and thrombin-induced increase in pH_i as well as intracellular activating events such as phosphoinositide breakdown, $[Ca^{2+}]_i$ mobilization and protein kinase C activation (Gawaz, *et al* 1994, Sheu, *et al* 2002, Hsiao, *et al* 2004). Also, Mg^{2+} is able to inhibit platelets by elevating intracellular levels of cAMP (Hardy, *et al* 1995, Sheu, *et al* 2002) but it does not affect cGMP (Sheu, *et al* 2002). From these studies it is clear that Mg^{2+} is able to inhibit several pathways leading to platelet activation. However, the precise mechanism by which Mg^{2+} acts is still unknown. It has long been known that platelet aggregation is dependent on the presence of divalent cations such as Ca^{2+} or Mg^{2+} (Marguerie, *et al* 1980). The study by Marguerie *et al.* (1980) also showed that ADP-induced aggregation and fibrinogen binding was greater in the presence of Ca^{2+} compared to Mg^{2+} . Furthermore, combining Ca^{2+} and Mg^{2+} resulted in fibrinogen binding levels between those occurring for Ca^{2+} and Mg^{2+} added separately. Further research on this subject has shown that Mg^{2+} can competitively replace binding of Ca^{2+} to the α_{IIB} -subunit of $\alpha_{IIB}\beta_3$ (Gulino, *et al* 1992). Thus, it might be possible that Mg^{2+} exerts its platelet inhibiting effect by substituting for Ca^{2+} in the $\alpha_{IIB}\beta_3$ -receptor (Sheu, *et al* 2002). This mechanism is interesting regarding our studies. All our experiments were performed in PRP meaning that both Ca^{2+} and Mg^{2+} were present. As described above, the addition of external Mg^{2+} inhibited platelet adhesion to fibrinogen and albumin (Paper I) and also adhesion to albumin induced by LPA combined with adrenaline (Figure 4) (Eriksson, *et al* 2006). In papers II and III we show that adhesion to fibrinogen and adhesion to albumin induced by soluble activators are dependent on $\alpha_{IIB}\beta_3$. Thus, the inhibiting effect of Mg^{2+} on albumin- and fibrinogen surfaces might be the result of displacement of Ca^{2+} by Mg^{2+} on $\alpha_{IIB}\beta_3$ (Gulino, *et al* 1992). However, bearing the multiple effects of Mg^{2+} in mind it is probable that other mechanisms for inhibition of platelet function operate as well. Opposite to the inhibiting effect of Mg^{2+} on albumin and fibrinogen, Mg^{2+} induced platelet adhesion to collagen (Paper I). Mg^{2+} is known to be important for adhesion to collagen and this effect was first described by Shadle and Barondes (1982). The stimulating effect of Mg^{2+} can be explained by the receptor dependency for adhesion to this surface. Adhesion to collagen in the

present assay was shown to be mediated by the $\alpha_2\beta_1$ -receptor (Paper III), which is a receptor known to mediate Mg^{2+} -dependent adhesion to collagen (Santoro 1986, Santoro, *et al* 1988, Staatz, *et al* 1989). The need for addition of external Mg^{2+} despite the fact that the assay is performed in plasma that by itself contain Mg^{2+} could be explained by findings showing that Ca^{2+} acts as an antagonist of Mg^{2+} -induced adhesion to collagen (Santoro 1986, Staatz, *et al* 1989). Consequently, it is possible that external Mg^{2+} shifts the equilibrium.

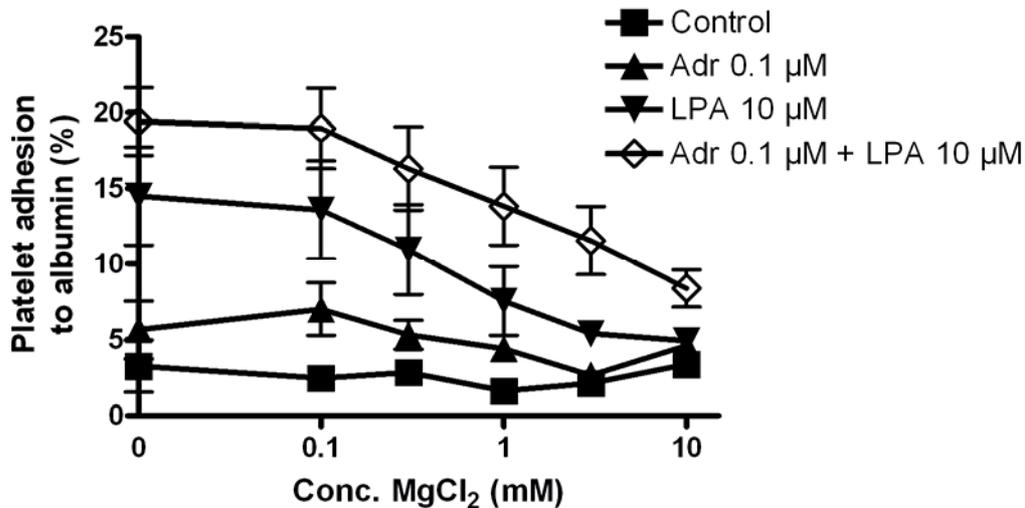


Figure 4. The effect of increasing doses of Mg^{2+} on adhesion to albumin was investigated. Mg^{2+} dose-dependently inhibited both adhesion induced by LPA and adhesion induced by the combination of LPA and adrenaline. Data are presented as mean \pm SEM (n=6).

Platelet adhesion to collagen

Studies of platelet-collagen interactions are complicated for two related reasons. First of all, collagen is not just one molecule but at least 28 different types exist (Farndale 2006). Also, different preparative procedures generate collagens with different properties. Savage *et al.*, (1999) studied collagen type I attached to glass coverslips and found that fibrillar collagen formed a striated pattern compared to pepsin-solubilized collagen that formed spiraled structures made from polymerization of collagen monomers (Savage, *et al* 1999). They also found that platelet adhesion during flow conditions was dependent on $\alpha_2\beta_1$ for the polymerized pepsin-solubilized collagen but not for the fibrillar collagen. Similarly, differences between collagen preparations also occur when investigating platelet adhesion during static conditions. In a previous study, platelet adhesion to monomeric as well as fibrillar collagen type I was achieved by $\alpha_2\beta_1$ and GPIV, but GPVI was only important for

adhesion to fibrillar collagen (Nakamura, *et al* 1998). The monomeric nature of the collagen was conserved in that study by keeping the collagen in an acidic environment. We coated our microplates with monomeric collagen type I with additional small amounts of fibrillar collagen type III in papers I, II and IV. Although not investigated, we propose that the monomeric collagen formed polymeric structures resembling those reported by Savage *et al.* (1999), since we diluted the collagen in 0.9 % NaCl with no intention to keep the pH acidic. Our results from Paper III showing that adhesion to collagen was dependent on $\alpha_2\beta_1$ is in accordance with the above described receptor dependency for adhesion to collagen polymerized from monomers. The fibrillar collagen most probably had a minor role since adhesion did not occur in the absence of Mg^{2+} (Paper I). Adhesion to collagen in the absence of Mg^{2+} has been observed for fibrillar but not for monomeric collagen (Nakamura, *et al* 1998).

In Paper I we noticed that platelet adhesion to collagen in the presence of Mg^{2+} is highly variable between individuals. We suggest that this might be caused by variations in the expression of $\alpha_2\beta_1$, which has been shown earlier to influence platelet adhesion to collagen (Kunicki, *et al* 1993).

Platelet adhesion to fibrinogen

Fibrinogen is important for normal platelet function in hemostasis because it mediates platelet-platelet adhesion (aggregation) (Bennett 2001). It is known that in order to bind fibrinogen in solution platelets need to be activated (Marguerie, *et al* 1979). However, unstimulated platelets are able to bind surface-bound fibrinogen (Coller 1980), which we also describe in papers I-V. A model explaining these interactions were proposed by Coller (1980) based on the assumption that there is equilibrium between activated and unactivated fibrinogen receptors. If this is true, the high local fibrinogen concentration occurring on a surface would increase the probability of platelet-fibrinogen interactions. Further studies revealed that this model could not account for all adhesion of unactivated platelets occurring on fibrinogen surfaces (Savage & Ruggeri 1991). The sequences recognized by $\alpha_{IIb}\beta_3$ on stimulated vs. unstimulated platelets were different indicating different adhesion mechanisms depending on platelet activity. Also, it is not surprising if the nature of platelet interactions with fibrinogen differs depending on whether fibrinogen is attached to a surface or free in solution. This is because surface characteristics of the adsorbing material are known to

influence conformation of different attached proteins including fibrinogen (Bergkvist, *et al* 2003, Kim & Somorjai 2003, Baugh & Vogel 2004). Furthermore, experiments performed on transfected Chinese hamster ovary (CHO)-cells show that increasing affinity and inducing clustering of $\alpha_{IIb}\beta_3$ both increase adhesion to fibrinogen, although affinity regulation seems to be most important (Hato, *et al* 1998). This is in accordance with our studies showing increased adhesion to fibrinogen in the presence of soluble activators (Papers I, III-V). The binding of platelets to surface-attached fibrinogen might be of physiological importance since fibrinogen has been found in vessel walls (Smith & Staples 1980) and is suggested to be able to attach to exposed ECM (Ruggeri 2002).

Platelet adhesion to albumin

In all papers we describe attachment of platelets to an albumin-coated surface in the presence of soluble activators. This might at first seem somewhat peculiar since albumin is generally considered a negative control-surface where platelets cannot adhere. However, this phenomenon has been observed earlier when studying platelet adhesion during flow conditions (Feuerstein & Kush 1986). That study also found platelet adhesion to albumin to be especially efficient in areas previously exposed to platelets. This made the authors speculate about release of substances from platelets that might replace or cover the original albumin, which then would facilitate adhesion of the new platelets entering the area. In our studies of static platelet adhesion we found that adhesion to albumin only occurred after platelets had been preactivated with a soluble activator. This closely resembles the results from the study performed during flow conditions. It might be suggested that in the study by Feuerstein and Kush platelets were activated by the flow conditions, while we achieved the same activating effect by adding a soluble activator. We also find it likely that platelet adhesion to an albumin-coated surface in our assay is in fact mediated by adhesion to secreted fibrinogen. This conclusion is based on our results showing that platelet adhesion induced by the combination of adrenaline and LPA (Paper II) and by ADP (Paper III) is blocked by antagonists of the $\alpha_{IIb}\beta_3$ -receptor. This was compelling when we investigated platelet adhesion to albumin and fibrinogen in parallel. By using several different antibodies and antagonists of the $\alpha_{IIb}\beta_3$ -receptor we found their inhibiting properties to be remarkably similar on both surfaces indicating similar adhesive mechanisms (Paper III). Also, it has earlier been shown that albumin is able to attach to a fibrinogen-surface (Gogstad, *et al* 1982) as well as to surfaces coated with RGD (Olivieri & Tweden 1999). This makes the interaction between

albumin and fibrinogen in our assay highly likely. However, it must also be stated that albumin contains arginines (R), glycines (G) and aspartates (D) scattered over the primary sequence (Meloun, *et al* 1975). We speculate that conformational changes induced by surface adsorption might bring those amino acids into close apposition resulting in true RGD-sequences with consequent $\alpha_{IIb}\beta_3$ -dependent binding to albumin. Irrespective of the exact mechanism of interaction between platelets and albumin, it is important to discuss the physiological importance of this interaction. We suggest that this interaction might occur *in vivo* and that it might be especially important in atherosclerosis. These conclusions are based on studies showing that albumin is present in normal arteries (Smith & Staples 1980) and that the amount of albumin is indicated to be increased in atherosclerotic plaques (Stastny & Fosslien 1992).

Platelet adhesion in experimental research

Our initial studies showed that basal platelet adhesion can be increased and decreased by different known platelet activators and inhibitors respectively (Paper I). Adhesion to albumin, collagen and fibrinogen was significantly increased by ADP (1 μ M), adrenaline (1 μ M) and ristocetin (1 mg/mL). Distinct inhibiting effects on platelet adhesion were observed for prostacyclin and forskolin. IC₅₀-values for inhibition of platelet adhesion to collagen and fibrinogen respectively were 0.1 and 1.9 μ M for forskolin and 1.2 and 0.8 μ M for prostacyclin. This property of the assay was essential and was exploited in all the other papers constituting this thesis. Because the assay is performed in 96-well microplates it is easy to gain a lot of results by only a few experiments. In other words, applications for the adhesion assay might be found through screening approaches investigating the effects of known or potential platelet activators/inhibitors. By such a procedure, we found the assay to be potentially useful for studies of synergistically increased platelet adhesion induced by 10 μ M LPA combined with 0.1 μ M adrenaline. A synergistic effect was seen for platelet adhesion to albumin but not to collagen, fibrinogen or a plasma surface (Paper II). Although LPA and adrenaline acted in synergy also on a fibronectin surface, this effect was relatively weak compared to the albumin surface. Studies of synergistic effects are important since this is the most probable way by which platelet activation occurs *in vivo*. Consequently, further screening has been performed in order to find other synergistic effects that could possibly be

studied with the adhesion assay. By these means, additional synergistic interactions were detected as exemplified by synergistically increased adhesion to albumin induced by adrenaline combined with ristocetin. Such synergistic effects were consequently included as *in vitro* activators in our study on patients with unstable angina (Paper V). Common for several of the synergistic interactions investigated in Paper V is that ristocetin constitutes one of the activators while the other activator couples to a G-protein coupled receptor. Beyond those interactions we have preliminary results indicating that ristocetin also acts synergistically when combined with PAF-16 or the PAR1-activating peptide SFLLRN (Figure 5). This suggests that the platelet adhesion assay might be a suitable model system for investigating interactions between G-protein coupled signalling and tyrosine kinase coupled receptors (represented by ristocetin). Another common theme for the synergistic effects observed in this assay is that they only seem to occur on surfaces with low basal adhesion. As described above, such surfaces include albumin and fibronectin (Paper II) as well as collagen when omitting addition of Mg^{2+} (unpublished observations). This probably reflects that an interaction between two soluble activators is more easily detected when the surrounding environment is relatively scarce of other platelet activating factors. Thus, we have been able to detect several synergistic interactions, but more detailed studies on the mechanisms of synergistic platelet adhesion in this thesis have only been performed for the combination of LPA and adrenaline (Paper II). These compounds are interesting because of their potential links to atherothrombosis. Siess *et al.* (1999) have shown that LPA is the most important platelet-activating lipid in mildly oxidized LDL and that it is present in atherosclerotic plaques. This prompted the authors to suggest that LPA might be important for atherothrombosis. Also, adrenaline is connected to atherothrombosis since plasma levels of adrenaline are elevated in patients with MI (Stubbs, *et al* 1999). LPA and adrenaline have been shown to synergistically increase platelet aggregation (Nilsson, *et al* 2002, Haseruck, *et al* 2004), which confirms that the adhesion assay measures an aspect of platelet function that can be detected by more traditional platelet function assays. In accordance with platelet aggregometry (Nilsson, *et al* 2002) we also concluded that the synergistic effect was very donor-dependent. To describe the donor-dependency, we defined synergy as adhesion exceeding the sum of the adhesion values obtained for either activator used alone, *i.e.* adhesion exceeding the additive effect. By this procedure we found 46 of 53 donors (87 %) to respond synergistically to the combination of 10 μ M LPA and 0.1 μ M adrenaline on adhesion to albumin. It is also important to note that among the donors

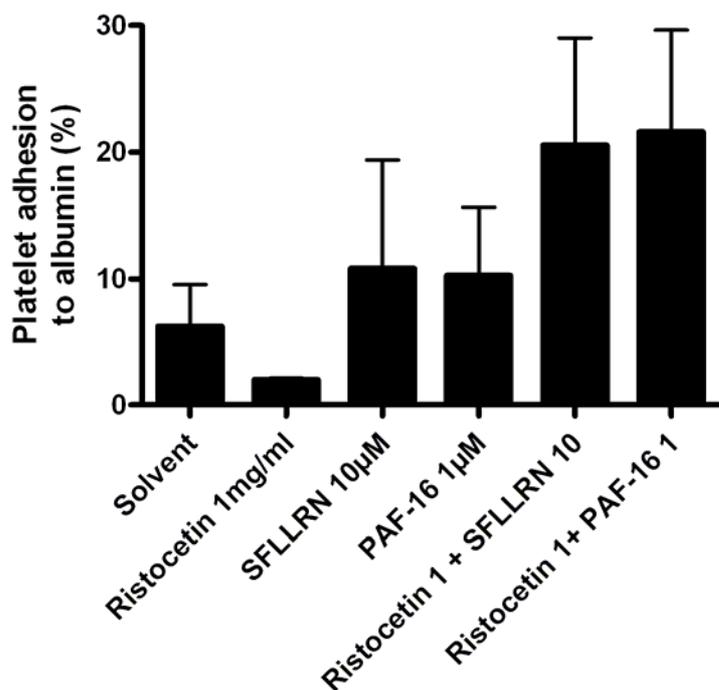


Figure 5. Platelet adhesion to albumin was investigated without added Mg^{2+} in the presence of ristocetin combined with either SFLLRN or PAF-16. Both combinations tended to increase adhesion synergistically. Results are presented as mean + SEM (n=2).

responding synergistically, values of synergistic effects varied from hardly detectable synergy to approximately 15 % increased adhesion as compared to the additive effect. The donor-dependency is by itself very interesting since such properties might turn out to be novel risk factors for disease. Because of this we tried to find possible causes of the inter-individual effects. Our approach was to mainly focus on circumstances that potentially could act directly to influence the LPA and adrenaline signalling pathways. Concerning LPA, we measured plasma levels of ox-LDL since ox-LDL desensitizes platelets to LPA stimulation (Siess, *et al* 1999). However, ox-LDL was not found to correlate with platelet activation induced by LPA and adrenaline in our assay. Adrenaline might be a better candidate for being responsible for the inter-individual effects since the responses to adrenaline is known to be highly variable (Kambayashi, *et al* 1996, Pyo, *et al* 2003). We assessed this possibility in two different ways. First, we measured levels of insulin in plasma since insulin has been reported to be able to decrease levels of platelet α_2 -ARs *in vitro* (Kahn & Sinha 1992). Also, our results showed that adrenaline acted through α_2 -ARs since the synergistic effect was totally abolished by the addition of the α_2 -AR antagonist yohimbine. However, we found no correlation between the synergistic effect and insulin-levels, but interpretations of these results must be done with

caution. First of all, levels of plasma insulin are not a direct measure of the amount of α_2 -ARs. Secondly, it is uncertain whether the α_2 -AR density actually influences adrenergic signalling. Different studies have reported that the effect of adrenaline is positively (Kambayashi, *et al* 1996) or negatively (Varani, *et al* 1999) correlated as well as uncorrelated (Nakahashi, *et al* 2001) to the amount of α_2 -ARs. Also, platelets are able to receive inhibiting signals through β_2 -adrenergic receptors (β_2 -ARs) (Kerry & Scrutton 1983). Speculatively, variation in β_2 -AR expression might contribute to the donor-dependency of the synergistic effect between LPA and adrenaline. Because of these uncertain connections between receptor density and adrenergic signalling we decided to investigate the relationship between synergistic effects and the activating capacity of 1 μ M adrenaline, which induce platelet adhesion on its own. Platelets responding synergistically to adrenaline and LPA appeared to be more easily activated with adrenaline as single activator. However, it must be stated that the connection was only a non-significant trend ($p = 0.07$, $r^2 = 0.22$, $n = 16$). Another study has suggested that variation between donors in platelet response to LPA is caused by different abilities of different donors to secrete ADP after LPA stimulation (Haseruck, *et al* 2004). This model might explain the inter-individual effects in our assay since the synergistic effect was dependent on ADP secreted from dense granules. Furthermore, secretion from alpha granules occurred as indicated by P-selectin expression on adhered cells. Thus, it is definitely possible that ADP-secretion could explain the donor-dependency observed by us. If this is the case, an important future task would be to elucidate why individuals differ in their secretion response.

Platelet adhesion in clinical research

The platelet adhesion assay has been used in two different clinical settings in order to study platelet adhesion in patients with ET (Paper IV) and unstable angina (Paper V). These groups of patients were suitable for our studies since both groups are prone to develop thrombosis. Two questions were addressed in the two studies respectively. In paper IV we investigated the ability of the platelet adhesion assay to detect disturbed platelet function in ET-patients compared to controls. Paper V dealt with the issue of monitoring platelet-inhibiting treatment. Concurrently, the two studies complemented each other by being concerned with two important issues for managing patients with thrombosis risk.

Platelet adhesion for ET-patients

In paper IV we investigated platelet adhesion in 30 ET-patients, diagnosed according to the WHO classification of tumors, and 14 healthy controls. Platelets from patients with ET were generally found to be more adhesive than platelets from healthy controls. This is in line with the increased thrombotic risk observed for this patient group but in direct conflict with other studies evaluating *in vitro* platelet activity in patients with ET. Decreased responses of ET-platelets to activating stimuli *in vitro* have been observed for aggregation of platelets in PRP (Ginsburg 1975, Yamamoto, *et al* 1984, Avram, *et al* 2001, Cesar, *et al* 2005), whole blood flow cytometry (Jensen, *et al* 2000) and for platelet activity as measured by PFA-100 (Cesar, *et al* 2005). Opposite to this, assays that measure the *ex vivo* platelet activity often show that ET-platelets are highly reactive (Wu 1978, Rocca, *et al* 1995, Jensen, *et al* 2000, Jensen, *et al* 2001). The decreased *in vitro* platelet activity observed by others has been explained by the concept of platelet exhaustion. Increased platelet activity *in vivo* results in degranulated platelets that are not able to respond *in vitro*. We have two reasons to suggest that this model is in line with our measures of increased *in vitro* platelet activity. First of all, we found that the prevalence of ET-patients having increased adhesion compared to controls was dependent on the platelet stimuli. Approximately 50 % of ET-patients had platelet adhesion above the mean + two standard deviations of controls when investigating adhesion to collagen or fibrinogen in the presence of ADP or ristocetin. However, when using comparatively weaker stimuli represented by adhesion to albumin in the presence of ADP or the combination of adrenaline and LPA, only 25 % of patients responded with adhesion above two standard deviations of the control mean. Thus, a strong stimulus resulted in a greater fraction of highly activated platelets for ET-patients. Secondly, the relatively long one hour incubation of platelets in microplates allows extensive platelet stimulation. We therefore suggest that the conditions employed in this assay were favourable for platelet function. Such conditions might be necessary in order to detect increased activity of ET-platelets *in vitro*. It is possible that the favourable environment stimulates secretion from *in vivo* exhausted platelets and that this secretion contributes to the increased platelet adhesion for ET-platelets compared to controls. This conclusion is strengthened by our results showing that ADP-secretion is important in the adhesion assay (Paper III). We also made some efforts to investigate the influence of the Val617Phe-mutation on platelet adhesion. This mutation can be found in approximately 50 % of ET-patients but the influence of the mutation on thrombosis risk is still debated (Tefferi & Elliott 2007). In our study, 40 % of patients were JAK2-positive but we

did not find any direct connection between JAK2-status and platelet adhesion. However, since we were also unable to detect any connection between platelet adhesion and disease history it is difficult to conclude anything regarding JAK2-status and thrombotic risk based on this study. Nevertheless, the finding that platelet adhesion was generally higher for ET-platelets compared to controls suggests that the adhesion assay is able to detect groups of patients with increased thrombotic risk. Also, the patients classified by us as having high adhesion were generally treated with more aggressive anti-platelet therapy independent of our results. This indicates that the assay is able to detect patients that are clinically classified as having high risk of thrombosis.

Influence of platelet inhibiting treatment on platelet function

In paper V we addressed the issue of drug resistance by measuring platelet adhesion in patients with unstable angina being treated with anti-platelet drugs. The study used a cross-over design enabling us to measure platelet activity under the influence of three different anti-platelet treatments for all patients. The anti-platelet regimens studied were ASA and clopidogrel combined as well as both drugs used alone. Initially, 33 patients and 30 healthy controls were enrolled in the study and a total of 4 patients and 1 control were lost during follow-up. Platelet activity was estimated through platelet adhesion as well as by flow cytometry and measurements of serum TXB₂-levels. Conditions employed in the static adhesion assay were primarily based on the results from Paper I and II. The soluble activators ADP, adrenaline and ristocetin were found to be able to increase platelet adhesion (Paper I) and were consequently included in Paper V. Surfaces of collagen and fibrinogen were included in the same paper because of their physiological importance. That study also included an albumin-coated surface since we found this surface to be especially interesting for investigation of synergistic effects (Paper II + previously unpublished results discussed above). The induction of a synergistic effect by two activators is interesting since this mimics the *in vivo* situation with several platelet activators being present simultaneously. Activating platelets with two activators simultaneously has actually been considered necessary in order to detect effects of anti-platelet drugs (Graff, *et al* 2004). One advantage with the adhesion assay is the use of microplates, which allow measurements of platelet adhesion during several conditions. This increases the probability of finding conditions that is sensitive for platelet-inhibiting treatment and this characteristic was therefore utilized for Paper V. However, a drawback of measuring a multitude of parameters simultaneously is that it is difficult for the

human mind to interpret the results. Because of this we analyzed the data from Paper V using Principal Component Analysis. This statistical method divides the variables into separate factors based on the correlations between the individual variables. Thus, variables that are well correlated are grouped into a distinct factor where all variables can be considered to measure aspects of the same phenomenon. Through this procedure it is possible to transform a large amount of variables into a few factors and thereby reduce the complexity of the data. Our Principal Component Analysis included all results, *i.e.* both the values obtained for the three separate treatments of patients as well as the two repeated measures on donors. By using this approach we were able to describe the 54 adhesion variables measured in Paper V as 8 distinct factors. We also managed to give the factors names that described the property that was measured by the factors. The names were as follows; *Absent adhesion*, *ADP-induced adhesion*, *Adrenaline-induced adhesion*, *Ristocetin-induced adhesion*, *Adhesion to fibrinogen*, *Ristocetin-induced adhesion to albumin*, *LPA-induced adhesion to albumin* and *Adhesion to collagen*. It is interesting that the factors distinguish basal platelet adhesion from platelet adhesion induced by platelet activators. Adhesion to fibrinogen and collagen formed distinct groups separated from groups including platelet activators. This indicates that different surfaces induce different platelet behaviour primarily when basal platelet adhesion is investigated. Instead, when a platelet activator such as ADP, ristocetin or adrenaline is added the adhesion is mostly dependent on the activator and not on the surface that is being investigated. Accordingly, future studies aimed at investigating the 54 variables used in Paper V must not analyse all 54 variables but investigating one representative variable from each factor should be enough to gather information regarding 8 different aspects of platelet adhesion. Two interesting methodological characteristics of the platelet adhesion assay was discovered in paper V. First, we found that some but not all variables measured were stable over time in healthy individuals. This indicates that it might be possible to use the assay in order to monitor platelet function over time in patients. The presence of variables that were unstable over time is not surprising since environmental factors are known to influence parameters connected to platelet function. This can be exemplified by seasonal variation of urinary levels of TXB₂ (Mustad, *et al* 1996) as well as changes in plasma levels of adrenaline induced by emotional or physical stress (Dimsdale & Moss 1980). Secondly, we found a relatively good correlation ($p < 0001$, $r^2 = 0.49$, $n = 92$) between ADP-induced platelet adhesion and ADP-induced platelet activation as measured by flow cytometry. This correlation with the flow cytometric data verifies the results obtained with the platelet adhesion analysis. Furthermore, the r^2 -value shows that approximately 50 % of the variation

observed with one assay cannot be explained by the variation in the other assay. This could be explained by the fact that platelet adhesion and flow cytometry measure two different aspects of platelet function. Adhesion measures the ability of platelets to attach to a surface, while flow cytometry measures the activity of platelets in solution.

The pharmacological drugs to be investigated in Paper V were clopidogrel and ASA. The easiest way of evaluating the effects of these drugs would be to measure aspects of platelet function that are directly dependent on these two signalling pathways. The direct effect of clopidogrel was aimed to be evaluated by using ADP as *in vitro* platelet activator in both the static adhesion assay and in the flow cytometric measurements. Both assays showed that ADP-induced platelet activation was decreased for patients treated with clopidogrel compared to ASA. This clearly showed that the assays were able to measure the pharmacological effect of clopidogrel. In the same way we chose to measure serum TXB₂-levels as a direct estimate of the effects of ASA. The results clearly showed that ASA-treated platelets produced less TXB₂ than platelets treated with clopidogrel. Consequently, measuring ADP-induced platelet activation by static adhesion or flow cytometry as well as serum TXB₂-levels would generate information regarding the pharmacological effects of clopidogrel and ASA respectively. However, it is not certain that such measures would estimate the *in vivo* effects of the drugs. Opinions about measuring metabolites of TXA₂ in order to evaluate the effects of ASA are diverged. Urinary levels of 11-dehydro-TXB₂ have been associated with clinical outcome in a study including 976 patients (Eikelboom, *et al* 2002). In opposite, a similar sized study including 682 patients and estimating ASA-effect by TXB₂-analysis only found 12 ASA-treated patients ($\approx 2\%$) to have residual TXB₂-synthesis above mean + two standard deviations (Frelinger, *et al* 2006). Also, Rao and Michiels (2007) reviewed their personal experiences on arachidonic acid-induced platelet aggregation, which also should be considered a direct measure of the ASA-effect. They found ASA-resistance to be extremely rare, exemplified by its occurrence in only one healthy donor in their laboratory over three decades (Rao & Michiels 2007). In accordance with this, we found all our patients to be responsive to ASA as measured by serum TXB₂. This is interesting since the cause of ASA-resistance is not known. One interesting theory claims that ASA-resistance is caused by platelet activation through signalling pathways not affected by ASA such as activation by ADP, collagen or adrenaline (Rao, *et al* 1986, Hurlen, *et al* 2000, Kawasaki, *et al* 2000, Macchi, *et al* 2002). Consequently, this kind of compensatory resistance is impossible to detect by direct measures of the pharmacological function of a drug. It can therefore be

suggested that more complex measures of platelet activity are needed in order to generate a measure of the *in vivo* situation. In this study this was accomplished by using several different activating stimuli *in vitro*. Finding a stimulus that is partly dependent on secreted ADP and/or TXA₂ might generate results more closely linked to the *in vivo* conditions. If adhesion to such a surface is normal or elevated despite treatment with clopidogrel and/or ASA one might suspect compensatory drug resistance (Figure 6). In our study we found adhesion to albumin induced by lysophosphatidic acid or ristocetin as well as SFLLRN-induced platelet activation measured by flow cytometry to be partly dependent on ADP. Collagen-induced adhesion seemed to be dependent on both ADP and TXA₂. However, it must be stated that the dependence on TXA₂ for adhesion to collagen was rather weak suggesting that further research should be performed in order to increase the ASA-sensitivity of this surface. Also, a future approach would be to combine the direct measures of the effects of clopidogrel and ASA with those indirect measures for more thorough investigations of platelet function. This could be a successive way for monitoring anti-platelet treatment.

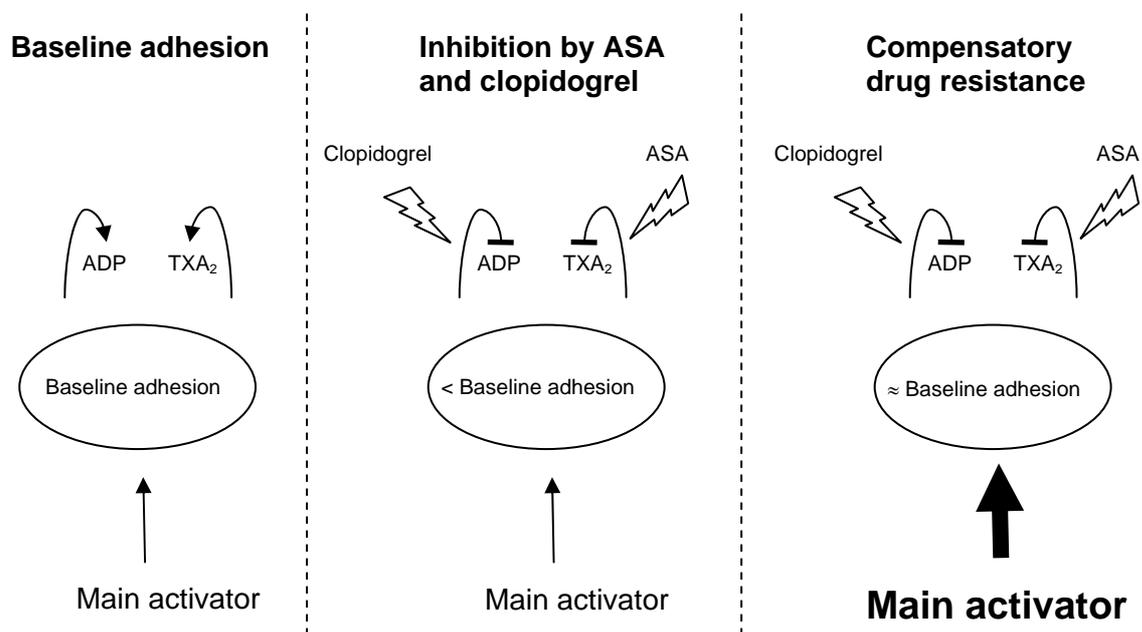


Figure 6. The left figure represent adhesion induced by a substance other than ADP or TXA₂ (Main activator). The main activator could be a surface such as collagen, a soluble activator such as adrenaline or a combination of both. Part of the adhesion induced by the main activator comes from secretion of ADP and TXA₂. When clopidogrel and ASA inhibit their respective targets, adhesion is partly but not totally decreased since the main activator acts through pathways both dependent and independent on secretion of ADP and TXA₂ (middle figure). The right figure shows compensatory drug resistance. In this case, clopidogrel and ASA effectively inhibit ADP- and TXA₂-signalling. However, the secretion-independent activating pathways induced by the main activator are up-regulated resulting in adhesion approximating baseline values.

Conclusions

The main conclusions from these studies can be presented as follows (Figure 7). Regarding experimental research we have shown that the adhesive interactions occurring in the assay are in line with general knowledge of platelet function. The $\alpha_2\beta_1$ -integrin was responsible for platelet adhesion to collagen while adhesion to fibrinogen was achieved by $\alpha_{IIb}\beta_3$. It was also found that most of the adhesive interactions were dependent on secreted ADP and/or TXA₂. These conclusions highlight the usefulness of the assay by showing that it measures known platelet functions. Also, the assay is able to detect $\alpha_{IIb}\beta_3$ -dependent adhesion of activated platelets to albumin-coated surfaces. This is an interaction that has not been thoroughly investigated before, but which might turn out to be of physiological importance. We further show that the assay might be particularly well-suited for investigations of synergistic platelet activation. This can be exemplified by the synergistically increased adhesion to albumin observed when combining adrenaline with LPA or ristocetin. This suggests that the assay can be used for studies aimed at investigating interactions between novel platelet activators. Concerning our clinical studies we have shown that the platelet adhesion assay is able to detect increased platelet activity in ET-patients. This is a group of patients with an inherent risk of thrombosis suggesting that the assay is able to mirror the *in vivo* platelet activity. Finally, the assay is able to detect the effect of *in vivo* treatment with clopidogrel. Although not as evident as for clopidogrel, *in vivo* treatment with ASA also seems to affect platelet function in the adhesion assay. In conclusion, our results show that the platelet adhesion assay is suitable for experimental research and that further studies should be performed in order to develop the assay into a clinically useful device.

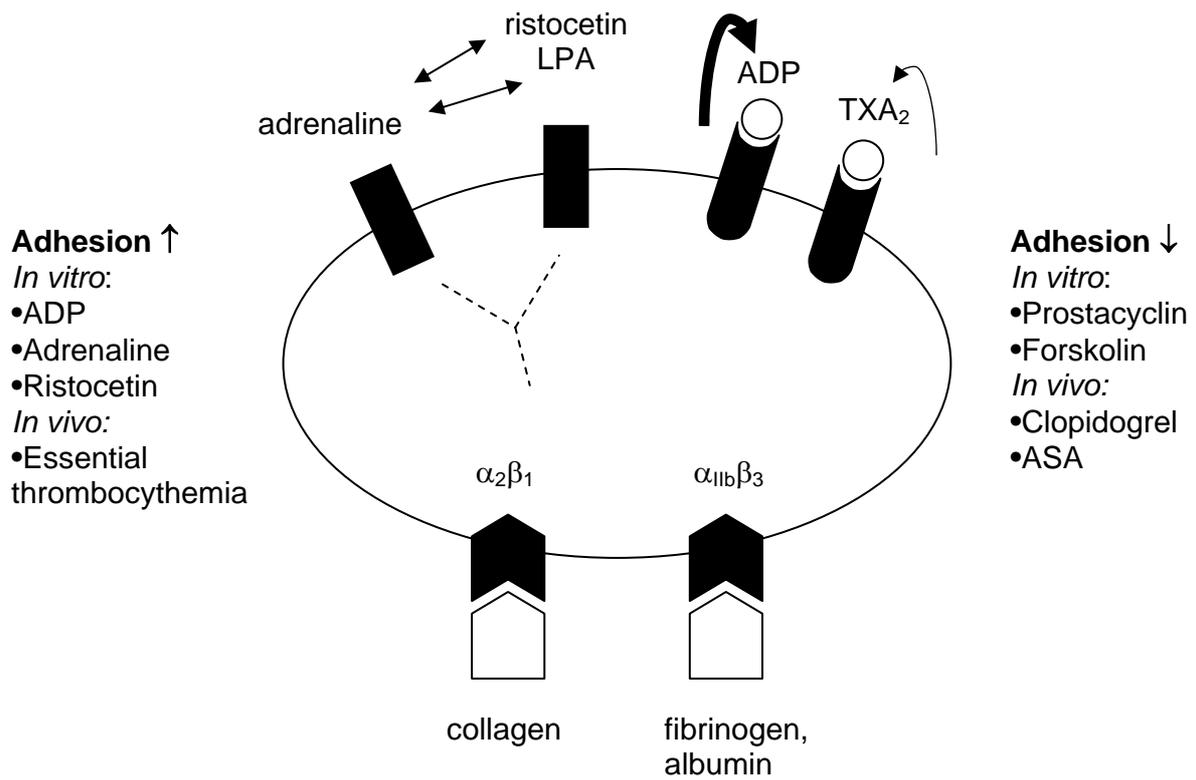


Figure 7. The main conclusions of this thesis. Based on the receptor dependency for adhesion and the importance of autocrine signalling, primarily by ADP but also by TXA₂, we conclude that the adhesion assay measures relevant aspects of platelet function. Increased as well as decreased platelet adhesion can be detected by *in vitro* addition of platelet activators and inhibitors. Likewise, increased *in vivo* activity because of essential thrombocythemia and decreased *in vivo* activity because of treatment with clopidogrel and ASA could also be observed. Finally, the assay seems suitable for investigation of synergistic effects between platelet activators. This is exemplified by synergistically increased adhesion to albumin occurring when combining adrenaline with LPA or ristocetin.

TACK

Självklart finns det ett antal personer som förtjänar att få ett officiellt tack från min sida.

Så jag säger Tack till:

- min handledare **Per Whiss** för allt stöd och all uppmuntran jag fått under dessa år. Du har definitivt lyckats med att ge mig gott om utrymme för att tänka självständigt samtidigt som du ingripit när det behövs. Jag är också väldigt tacksam över att vi inte bara diskuterat arbete utan även hittat på saker utanför jobbet (även om golfrundorna inte alltid gått min väg).

- ämnesföreträdaren **Rolf Andersson** som låtit mig spendera fem år på Farmakologen.

- mina kollegor på Farmakologen för den sköna stämning som ni har bidragit till och för att ni därmed fått det att kännas som ett nöje att gå till jobbet.

- **Liza Ljungberg** som lyckats med konststycket att vara en mycket god vän samtidigt som hon är min ärkefiende inom diverse tävlingssammanhang. Det viktigaste ÄR att vinna!

- **Caroline Skoglund** som är en grymt hjälpsam och schysst människa och som med glädje delar med sig av sina väsk- och sko-kunskaper.

- **Hanna Björck** som räddat mig från att jobba ihjäl mig genom att pressa in fikaraster i mitt schema.

- **Peter Gunnarsson** för alla givande diskussioner inom ämnesområdena K-1 och MMA samt för värdefulla tips om sevärd UFC-galor.

- **Torbjörn Bengtsson** och doktoranderna inom Cardiovascular Research Centre för alla våra intressanta forskningsmöten.

- **Ulrika Nilsson, Kourosch Lotfi, Lena Jonasson, Tomas Lindahl** och **Bo Hedbäck** som varit mina medförfattare i den här avhandlingens olika delarbeten.

- **Margareta Hedbäck** och **Kerstin Gustafsson** som båda gjorde enorma arbetsinsatser till arbete nr 5 i den här avhandlingen.

- **Lars Faxälv** som är en teknikens mästare och som varit en oundgänglig hjälp vid mikroskoperingen av mina mikroplattor.

- alla ex-jobbare och stipendiater som jag har haft nöjet att få lära känna och fått försöka handleda på labb.

- personalen på blodcentralen samt alla blodgivare och patienter som bidragit med blod till min forskning.

- min goda vän **Daniel Madan Andersson** för alla djupa ingående diskussioner om ... ALLT. Känns som om vi har lyckats täcka in det mesta från avancerad livsfilosofi till virus-infekterade odöda.

- min goda Tranås-polare **Peter Tureby** för alla roliga filmkvällar där vi kollat på meningslösa action-filmer med grabbar som Steven Seagal och Chuck Norris i huvudrollerna.

- **Joakim Henricson** för alla mer eller mindre sjuka videoklipp som du levererar till min mailbox.

- mina föräldrar som alltid varit intresserade av vad jag sysslar med och som stöttat mig i alla de val jag gjort i livet.

- företeelser såsom musik av tyngre karaktär och Guitar Hero II+III för deras förmåga att ladda om mina batterier efter långa dagar med jobb.

Finansiärer till denna avhandling:

- Cardiovascular Inflammation Research Centre vid Linköpings Universitet
- Östergötlands Läns Landsting
- Hjärt-Lung Fonden
- Svenska Sällskapet för Medicinsk Forskning
- Forskningsrådet i Sydöstra Sverige
- Vetenskapsrådet
- Hjärtfonden vid Linköpings Universitet
- Eleanore Demeroutis Fond för Kardiologisk Forskning vid Universitetssjukhuset i Linköping
- Stiftelsen för Gamla Tjänarinnor
- Lions Forskningsfond mot Folksjukdomar
- Familjen Janne Elgqvists Stiftelse

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