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Functional Relevance of HPA-1 and α_2 807C/T Platelet Receptor Polymorphisms Under Standardized In-Vitro Blood Flow Conditions

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1. Introduction

The scope of the problem of arterial thrombosis is staggering: at least 5 million adults in the United States alone suffer from its related symptoms. About 50% of the annual non-accidental deaths in the United States are caused by thrombi predominantly composed of platelets in coronary or cerebral arteries. Platelets adhere to subendothelial structures or deposed fibrinogen, become activated, in turn, aggregate causing a thrombus. Therefore, any genetic variation that might alter the expression or function of platelet receptors may lead to increased (or decreased) thrombus formation in pathological conditions. All these reactions are determined through specific platelet receptors and their interplay under (micro) environmental conditions.

To improve the prevention and therapy of arterial thrombotic disorders, it is imperative to elucidate the basic mechanisms of platelet thrombus formation in the arterial blood flow. In vitro systems, capable of modelling flow-mediated platelet adhesion and aggregation, have been developed to investigate the mechanisms by which platelet receptor polymorphisms as well as mechanical forces in related microenvironmental conditions affect a platelet thrombus formation.

1.1. Pathogenesis of arterial thrombosis

Ischemic heart disease and cerebrovascular disorders are leading causes of morbidity and mortality, independent of gender, in the Western world and are steadily increasing in the Third world (1,2). Epidemiological studies indicate that these diseases result from complex interactions between genetic susceptibility factors, chronic environmental influences (e.g. hormonal imbalance, smoking, obesity) and established, intercurrent disorders (e.g. diabetes, hypertension, dyslipidemia, or hyperhomocysteinemia). The most devastating complication of these disorders are acute myocardial infarction or stroke resulting from the formation of an occlusive thrombus at the site of a ruptured atherosclerotic plaque (1,2,3).

1.1.1. Central role of platelets in arterial thrombosis

A platelet-dependent process is the underlying mechanism of arterial thrombosis, and the critical role of platelets in this process is now widely accepted (1-5). Participation of platelets in arterial thrombosis is centered on the platelet's adhesive properties and the ability to respond to stimuli with rapid activation and, in turn, aggregation (4-6) - the same features that support the arrest of bleeding from wounds (Fig. 1). The normal function of platelets is, however, to arrest bleeding from wounds, which requires adhesion to altered vascular surfaces and rapid cellular activation ensuring the accumulation of circulating platelets and the formation of fibrin in the growing thrombus (1,7-9).

The main trigger for the hemostatic thrombus formation after traumatic vascular injury is the loss of the endothelial cell barrier between the extracellular matrix and flowing blood (4,8). Disruption of the endothelial lining by atherosclerotic plaque rupture or by external injury initiates a complex response (4,10). Circulating platelets operate simultaneously as injury sensors in the first line of defence to prevent blood loss and as triggers for the subsequent chain of events that involve other coagulation factors and a variety of cell types such as erythrocytes, monocytes, and endothelial cells terminating in platelet thrombus formation (4,10,11). Pathologic hemodynamic conditions, fibrinogen deposition onto atherosclerotic plaque, disruption of the endothelial lining by plaque rupture or by external injury all lead to exposure of collagens, immobilization of von Willebrand factor (vWF), and adhesion of circulating platelets to the damaged vessel wall (4,11,12). While platelets can adhere to the

damaged endothelial cells, their principle adhesion surface is the extracellular matrix (ECM), which becomes exposed in injured vessels and offers a panoply of ligands for platelet adhesion receptors. In this context, platelet integrin adhesion receptors play a critical role in platelet function as well as in arterial thrombogenesis (3,4,10-13) .

A progress in understanding the mechanisms of platelet adhesion, activation and aggregation under different rheological conditions may improve the ability to prevent thrombosis and, possibly, the manifestation and progression of atherothrombotic complication.

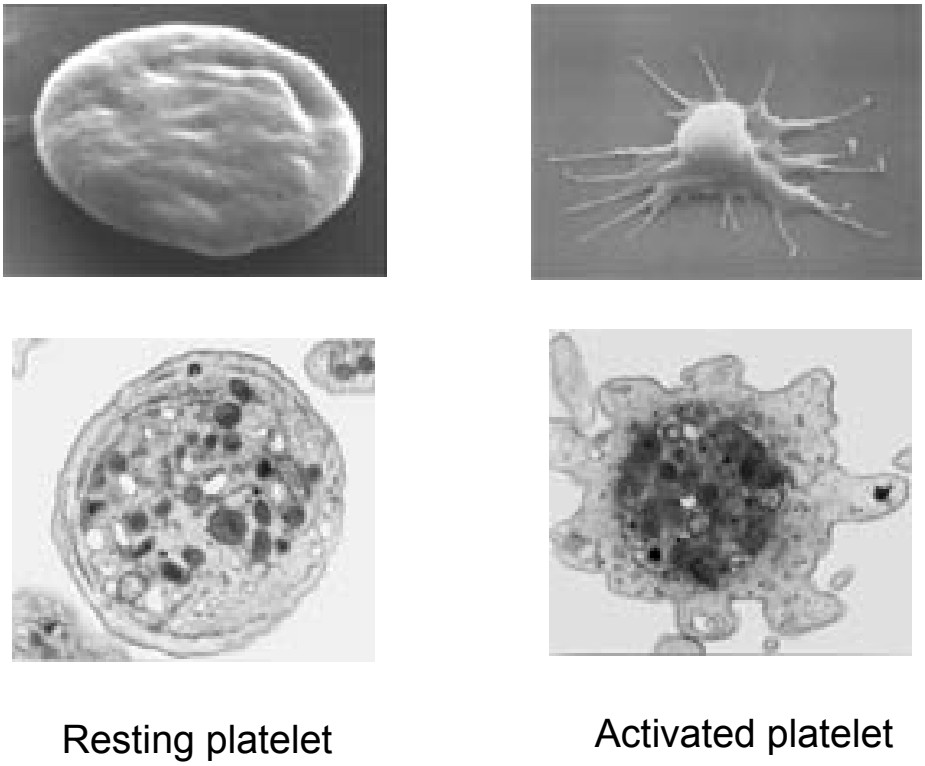

Figure 1. Electron scanning microphotography of resting (top and bottom left) and activated platelets (top and bottom right) using different magnifications.

1.1.2. Specific platelet receptor interplay during thrombus formation

Once the arterial vessel wall has been injured under high shear stress and arterial subendothelial structures have been exposed, platelets adhere onto the subendothelial extracellular matrix (4). This first adhesion (under high shear stress) is mediated by the contact between immobilized subendothelial von Willebrand factor and the platelet glycoprotein (GP) Ib/IX/V receptor complex (4, 8,14,15). This reaction

is initially reversible and allows platelets to tether and roll over the thrombogenic surface. Stimuli originating from the initial adhesion at the site of vascular injury act, within seconds, through the signaling networks to enhance the adhesive and procoagulant properties of the platelets tethered to the lesion or circulating in close proximity (4,8,14-18).

In the next step, the contact between platelets and subendothelium is tightened, stationary and stable adhesion with subsequent platelet activation initiates platelets covering the thrombogenic surface. This firm adhesion can be mediated by the binding of subendothelial collagen to the platelet integrin $\alpha 2\beta 1$ (also known as platelet receptor GPIa/IIa) or GPVI (19-21). Interestingly, both receptors are crucial for platelet adhesion and activation. Congenital deficiency of integrin $\alpha 2\beta 1$ causes a mild bleeding tendency, a failure of platelets to change shape and aggregate when stimulated with collagen types I and III and decreased adhesion to the subendothelium. It seems that GPVI alone is not able to impart effective platelet adhesion to collagen. Reduced platelet expression of GPVI also causes a mild bleeding tendency and a decrease in the adhesion to collagen (20,21). Recent data indicate that integrin $\alpha 2\beta 1$ (GPIa/IIa) is responsible for the adhesion and GPVI for the activation of the adhered platelets (22,23). Moreover, integrin $\alpha 2\beta 1$ shows a higher-affinity state for collagen after platelet activation, indicating a functional involvement beyond that of supporting initial contacts (23). Results indicate that platelet adhesion onto collagen involves both receptors, which also contribute to generating intracellular signals that mediate platelet activation (21). At this crucial stage of platelet activation, the platelets change shape. Typical phenotypic manifestations of activated platelets include an actin polymerization with cytoskeletal reorganization, secretion from storage granules and aggregation dependent on the modulation of soluble ligand binding to integrin $\alpha IIb\beta 3$ (GPIIb/IIIa) (4,24-27). Secretion itself leads to the release of granular components into the cytoplasm (e.g. calcium ions) and the extracellular space (e.g. vWF, growth factors, coagulation factors, and nucleotides), as well as the relocation of membrane proteins to the cell surface (e.g. P-selectin, active form of integrin $\alpha IIb\beta 3$); all these events enhance activation and aggregation (4,25,27).

The ultimate step in thrombus formation is platelet aggregation and the formation of a platelet-rich plug mediated by the binding of divalent or multivalent ligands, fibrinogen or von Willebrand factor, to the activated platelet integrin $\alpha IIb\beta 3$ (4,11,16-18).

Thrombin generated at the blood-plaque interface converts fibrinogen to fibrin, which stabilizes thrombus growth (11,25,28-31).

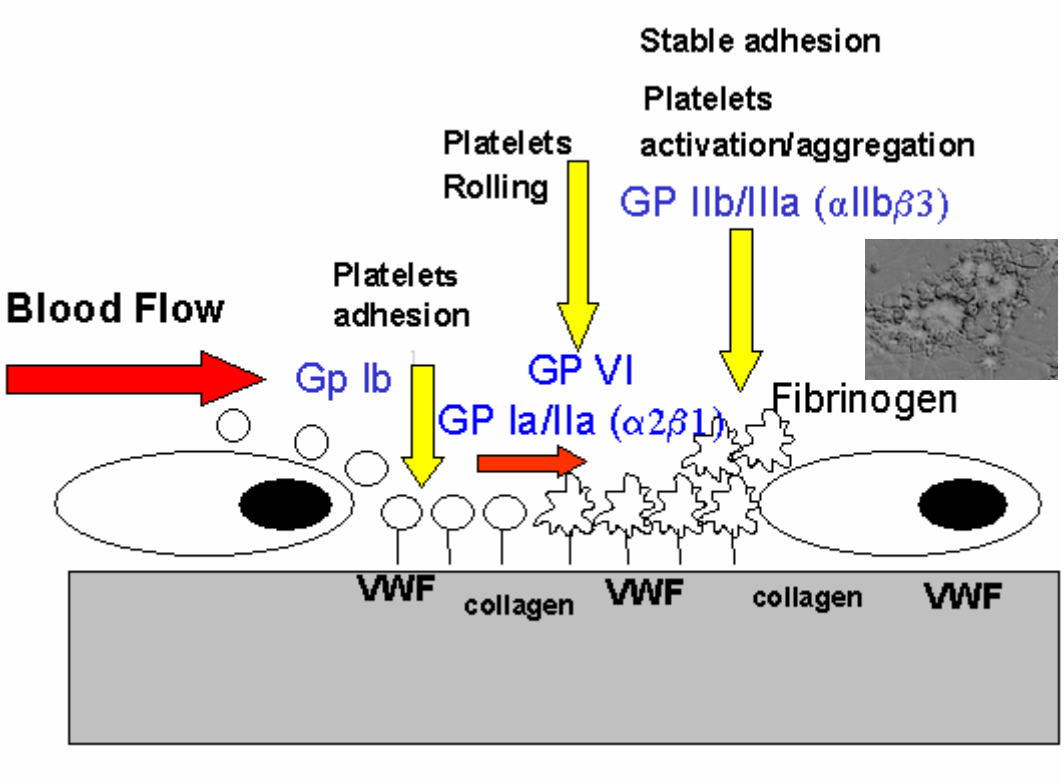

Figure 2. Schematic presentation of platelet receptors interplay during thrombus formation.

As presented above, four receptors (Fig. 2) play a pivotal role in the initiation of the platelet adhesion and consecutive aggregation onto the respected ligand: the glycoprotein (GP) Ib-IX-V complex (GPIb complex), integrin α2β1 (GPIa/IIa), GPVI and integrin αIIbβ3 (GPIIb/IIIa). Therefore, any genetic variation that might alter expression or function of these receptors may lead to the excessive bleeding or thrombus formation under pathological conditions.

There are two critical points in thrombus formation: 1) adhesion of inactivated (resting) platelets to subendothelial components such as collagen, deposed vWF or fibrinogen and 2) spreading of activated platelets with consecutive aggregation and thrombus formation. Both processes involve the platelet integrin receptors α2β1 and αIIbβ3 and both of them show certain polymorphism. Clinical studies conducted within the last ten years indicate that there could be a genetically determined

predisposition for hyperaggregability which might be mediated by polymorphic receptors including integrins α2β1 and αIIbβ3 involved in platelet adhesion and aggregation (32-40).

1.2. Integrin αIIbβ3 (GPIIb/IIIa)

Platelet integrin αIIbβ3, also known as the platelet fibrinogen receptor or GP IIb-IIIa, is a major integral platelet plasma membrane protein and belongs to the integrin superfamily of adhesion receptors (41). Integrin αIIbβ3 is complex compound of αIIb subunit with a molecular mass of 136 kD and β3 subunit with a molecular mass of 92 kD. αIIb subunit is composed of one heavy (114 kD) and one light chain (23 kD), linked by a single disulphide bond. Each subunit of the Integrin αIIbβ3 complex is produced by separate genes, located on the q21-22 region in the long arm of the human chromosome 17 (41,42).

Both components make a 1:1 stoichiometric Ca^{2+}-dependent noncovalently-associated complex form and are present in the platelet membrane. As major plasma membrane protein, the integrin αIIbβ3 complex represents 3% of total platelet protein and 17% of the platelet membrane protein mass (42).

Approximately 80 percent of the 80,000-100,000 copies of integrin αIIbβ3 are randomly distributed and exposed on the platelet's surface and have an important role in platelet adhesion and aggregation through the binding of a variety of ligands under different conditions of shear stress (42-44).

The remaining 20% of the integrin αIIbβ3 pool is located on the surface–connected canalicular system (invaginations of the plasma membrane) and in the inner membrane of the cytoplasmic α-granules. This cryptic αIIbβ3 pool becomes surface-expressed and function-capable upon platelet activation, change of shape, and dislocation as well as fusion of the α-granule membrane with the platelet surface (42-46). Through the described translocation of cryptic αIIbβ3, the number of functional receptor copies can be rapidly and significantly increased under action of different stimuli (42,46).

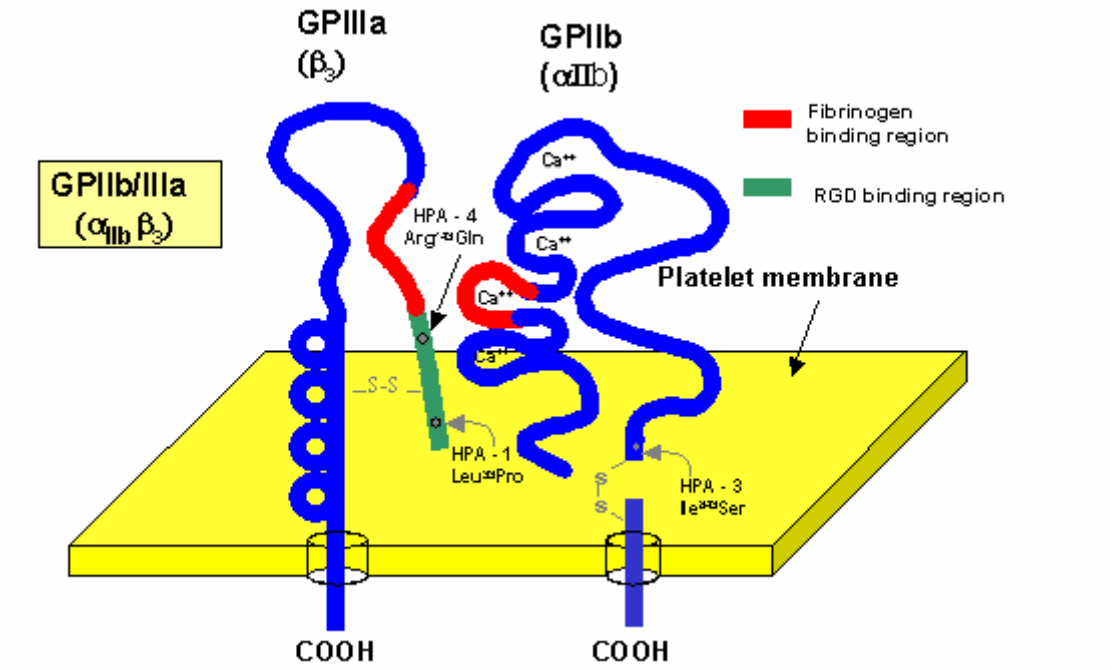

Figure 3. Schematic presentation of the platelet GPIIb/IIIa receptor (integrin αIIbβ3).

1.2.1. Structure and physiological role

A schematic model of integrin αIIbβ3 based on electron micro-photography showed that the membrane presented receptor has an extracellular, a transmembranous and a cytoplasmatic domain. The extracellular domain is the site to which fibrinogen and other adhesive ligands react (ligand binding domain) and it is formed by both subunits (41,43). Point mutation within either the αIIb (GPIIb) or β3 (GPIIIa) subunit can destroy the capacity of the receptor to interact with the ligand. Approximately 90% of the mature β3 and 60% of αIIb subunits are extracellular (41,42). The previously described chain of β3 subunit is entirely extracellular with disulfide bond linked to the light chain, which contains a single transmembrane region. The cytoplasmatic tail of the light chain is short (composed of 21 amino acids) and contains a high-affinity cation-binding site.

The β3 subunit is 90% extracellular with a dominant disulfide loop that links its amino-terminal parts to its midsection. Located near the membrane are four cysteine-rich tandem repeats which render this region resistant to the proteolysis. A single

transmembrane tail is followed by cytoplasmatic tail (potential phosphorylation site). The cytoplasmatic tails of both subunits interact with each other and form a cytoplasmatic domain that regulates αIIbβ3 activation and signalling (42,46).

The central role of platelet integrin αIIbβ3 is to serve as a platelet receptor for adhesive proteins and other macromolecular ligands (fibrinogen, von Willebrand factor, fibronectin, vitronectin, thrombospondin). This interaction determines two important roles of integrin αIIbβ3. Firstly, all ligands are constitutes of matrix and their interaction with αIIbβ3 promotes platelet adhesion. Secondly, integrin αIIbβ3 mediated platelet-platelet interaction is a conditio sine qua non for platelet aggregation as a crucial step in physiological and pathological hemostasis (43, 46,47). The main physiological role of platelet integrin αIIbβ3 is established through its binding capability for fibrinogen.

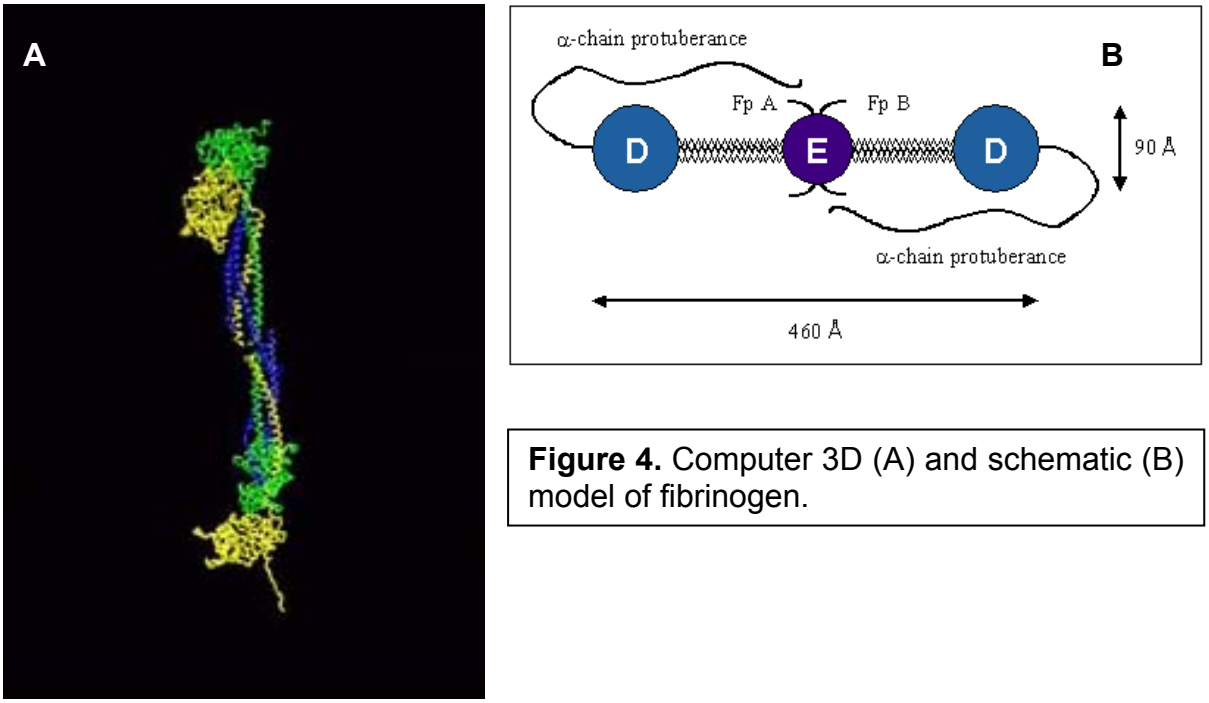

Figure 4. Computer 3D (A) and schematic (B) model of fibrinogen.

This binding activity of platelet integrin αIIbβ3 was observed at both immobilized and soluble fibrinogen. An interaction between immobilized fibrinogen and platelets does not require previous platelet activation (resting platelets) or conformational changes in platelet integrin αIIbβ3 and has been shown to occur even in the presence of platelet inhibitors (4, 11,47). Although the resting, unstimulated platelets do not interact with fibrinogen in the fluid phase, it was observed that resting platelets may interact with fibrinogen deposited on the damaged blood vessel or on the surface of

adjacent activated platelets and thus become incorporated in the growing hemostatic plug (4,11,47). In response to platelet stimulation, integrin αIIbβ3 also becomes competent (activated platelet) to bind soluble ligands. It also appears that under high shear stress conditions, the adhesion of platelets to von Willebrand factor via GPIb/V/IX-complex can induce intracellular signaling that activates integrin αIIbβ3 (4,47). However, when platelets are activated by various stimuli, platelet integrin αIIbβ3 undergoes a conformational change and provides a binding site for the soluble fibrinogen (Inside-Outside-Signaling).

Fibrinogen also acts as a bridging molecule between pairs of integrin αIIbβ3 molecules in adjacent activated platelets (47-51). This interaction is divalent cation-dependent with an initial dissociation constant of 300nM. The occupancy of integrin αIIbβ3 leads to the series of intracellular responses, referred to as outside-in signalling (42, 46).

Binding activity of platelet integrin αIIbβ3 to both fibrinogen phases (immobilized and soluble) is irreversible and selective to the γ-chain dodecapeptide site of fibrinogen. Examination of the interaction of αIIbβ3 with fibrinogen by electron microscopy showed a specific interaction of the nodular head of αIIbβ3 with distal domain of fibrinogen indicating that binding between receptor and fibrinogen predominantly involves the carboxy-terminus of the three constituent chains of fibrinogen. The γ-chain (the final six amino-acids of the γ-chain) are KQAGDV, and referred to collectively as γ-chain. The γ-chain sequence is found exclusively at fibrinogen, and it appears that the C-terminus of the γ-chains contain essential information for the receptor interaction with its ligand (41-43, 46).

In addition to its main role as fibrinogen receptor, integrin αIIbβ3 also has binding capability for other adhesive proteins including fibronectin, von Willebrand factor, thrombospondin, and vitronectin (41,42). The binding of these adhesive proteins to αIIbβ3 is partly mediated by amino acid sequence Arginine-Glycine-Aspartate (RGD-peptide), which is present in their primary structure. The binding of RGD-containing peptides to αIIbβ3 has many features in common with fibrinogen binding (specific to single site, saturable and Ca^{2+} dependent). In the presence of its C-terminus of the γ-chains, a fibrinogen competes with the RGD-containing peptides for binding to αIIbβ3 and is capable of inhibiting the binding of each of the other four adhesive proteins (fibronectin, von Willebrand factor, thrombospondin, vitronectin) to αIIbβ3 (46).

1.3. Integrin α2β1 (GPIa/IIa)

In 1985, Nieuwenhuis et al. described a patient with a mild bleeding disorder whose platelets showed no reactivity to collagen. The GPIa in her platelets was found to be decreased to 15-20% of the normal level (52). This was the first indication that GPIa is a physiologically active collagen receptor on platelets. Later, GPIa was indicated to be present as a complex with GPIIa, and the GPIa/IIa complex was found to be identical to the very late activation antigen-2 (VLA-2) on activated T-cells and the class II extracellular matrix receptor (ECMR-II) on fibroblasts (53). The complex has now been identified as a member of the integrin family and designated as integrin α2β1. Integrin α2β1 is widely distributed on various cell types, functioning as a collagen receptor (20,21,54). However, it appears to function as both a collagen receptor and a laminin receptor on endothelial cells, melanoma cells and epithelial cells (20). Expression of α2β1 on the platelet surface differs markedly (35) among normal subjects (< 1000 to 10000 molecules/platelet). The collagen binding integrin heterodimers are glycoproteins having masses of~220 kDa (α~130, β~90) for the polypeptides plus additional glycosylation (20). Human integrin subunits are encoded on a chromosome 12 as propeptides with N-terminal export signals and single transmembrane α-helices.

1.3.1. Structure and physiological role

The structures of integrin α2β1 were deduced from the corresponding cDNA sequences. Each subunit of the extracellular domain consists of the N-terminal head region and the leg or stalk region (Fig. 5). The α-subunit head regions are formed by a seven-bladed β-propeller domain with the αI-domain looping out between blades 2 and 3. The integrin α2-subunit is a single polypeptide chain that contains a I-domain insert next to its three metal binding domains, a characteristic common to integrin proteins. This I-domain is homologous to the collagen binding domain of vWF, and the critical involvement of the I-domain for the binding to collagen was indicated. The β-subunit head region includes the I-like βI-domain. The βI-domain interacts extensively with the β-propeller domain of the α-subunit. It appears that the βI-domain is a determinant of the integrin activation state and ligand affinity. The β1-subunit has a similar structure to other integrin β proteins and contains four cysteine-

rich repeats. Several antibodies are known to activate α2β1, and the epitopes for these antibodies were identified to be small regions of the β1-subunit (54,55).

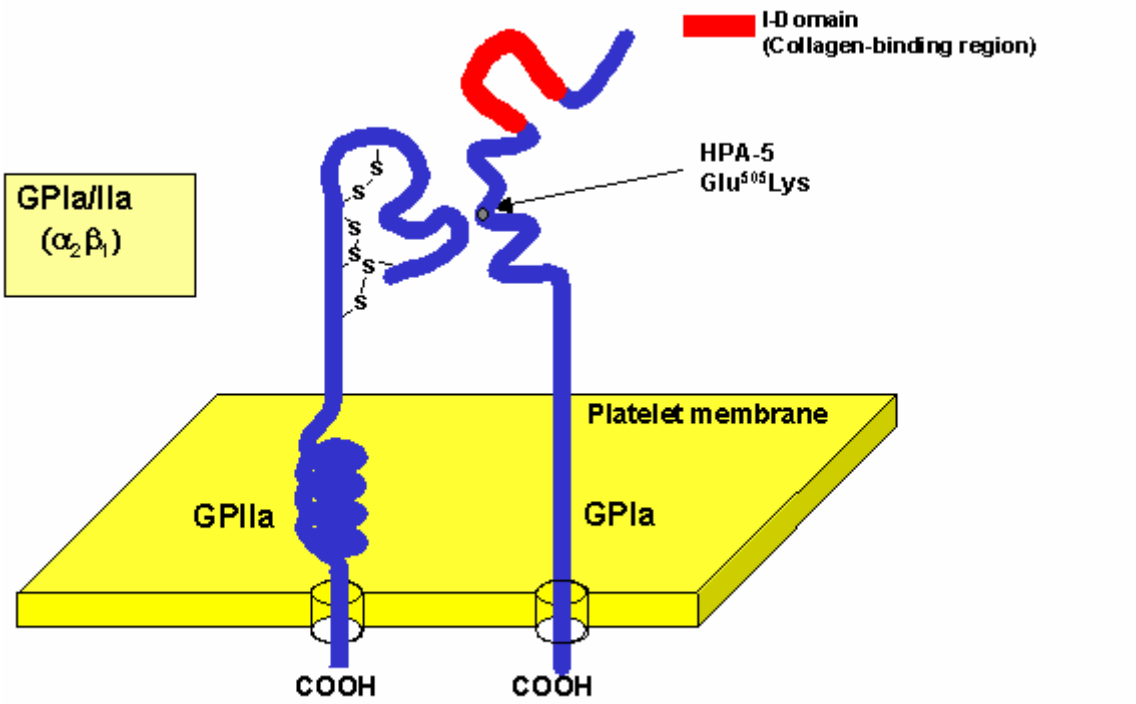

Figure 5. Schematic presentation of platelet GPIa/IIa receptor (integrin α2β1).

Some antibodies inhibiting α2β1 also recognize this region. The data suggests that this essential region of integrin β1 regulates the activity of α2β1 since the putative binding site for collagen is close to this region. Other epitope regions of the β1-subunit were also proposed for other activating antibodies. These regions are located near the transmembrane domain or inside a cysteine-rich domain, and all of these epitopes are located apart from the ligand binding site. Therefore, the binding of the latter activating antibodies to these regions would regulate the ligand binding characteristics at a region sterically apart from the ligand binding site by inducing a conformational change of the protein. The data suggests that activation of α2β1 would be induced when platelets are stimulated with agonists (21-23). Opposite to the main fibrinogen receptor, α2β1 is unable to bind a soluble collagen when platelets are in their resting state. Significant binding of soluble collagen was observed after platelets reacted with the related activating antibody or platelet agonists, thrombin, ADP or CRP (collagen-related peptide). The binding was Mg-ion dependent and inhibited by an anti-α2β1 antibody. PGI_2, an inhibitor of platelet function, potently inhibited the activation of platelets to bind a soluble collagen

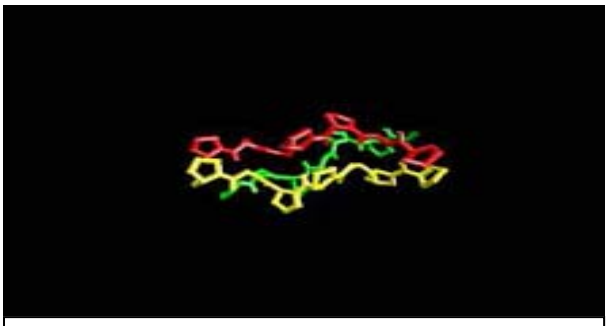

Figure 6. Computer 3D model of collagen.

induced by each of the tested agonists. These results suggest that integrin $\alpha 2\beta 1$ is activated when platelets are stimulated by agonists and then becomes able to bind a soluble collagen with high affinity. Thus, it appears that both integrins $\alpha IIb\beta 3$ and $\alpha 2\beta 1$ have similar activation mechanisms. The interaction of $\alpha 2\beta 1$ with collagen induces phosphorylation of many platelet proteins. In experimental studies, syk and phospholipase Cy2 were found to be tyrosine-phosphorylated in the presence of collagen, but not tyrosine-phosphorylated, when an anti-$\alpha 2\beta 1$ antibody was simultaneously presented. The interaction of integrin $\alpha 2\beta 1$ with collagen requires the presence of Mg^{2+}, whereas the presence of Ca^{2+}-ion is inhibitory (20,21). At present, 18 types of collagens have been identified and platelet adhesion onto collagen types I to VIII was observed. Collagen types I, II, III and IV react strongly with platelets, but types VI, VII and VIII react only weakly. The antibody to $\alpha 2\beta 1$ inhibited platelet adhesion to all collagen types, so that integrin $\alpha 2\beta 1$ would be able to bind with any of these types of collagen. A specific amino acid sequence in collagen that reacts with $\alpha 2\beta 1$ was studied, and a short DGEA sequence in the $\alpha 1$(I)-CB3 fragment appeared to be a recognition sequence of collagen. In contrast to these results, other groups identified the $\alpha 2\beta 1$ recognition sites in residues 520-528 of the $\alpha 1$ (III) collagen triple-helical chain (20). Since denatured collagen (gelatine), does not react with platelets at all and does not bind to $\alpha 2\beta 1$, integrin $\alpha 2\beta 1$ would interact with the collagen molecule by recognizing its steric structure.

1.4. Platelet receptor polymorphism

As already presented, both receptors play a crucial role in an initiation and consecutive thrombus growth and both of them are highly polymorphic. Until recently, the major clinical relevance of platelet receptor polymorphisms has been attributed to their capability to induce an immune response leading to the production of alloantibodies, causing immune-mediated platelet disorders (56). However, platelet GP polymorphisms might also affect the sensitivity of these important receptors, thus influencing platelet susceptibility to activating and aggregating stimuli. Several allelic

variants of key platelet glycoproteins are known to exist within the human gene pool, creating diversity in the expression, function and immunogenicity of these important adhesion receptors components (34,38,55). Historically, platelet GP polymorphisms have been of clinical interest because of their ability to elicit the formation of platelet specific alloantibodies (56). These antibodies cause enhanced platelet clearance and/or destruction leading to thrombocytopenia and/or hemorrhagic diatheses in three clinically defined conditions: neonatal alloimmune thrombocytopenia; post-transfusion purpura; and post-transfusion refractoriness. In addition to a few human platelet alloantigen (HPA) systems residing on the platelet receptors (Table 1), a number of low frequency alloantigens have also been described (34,38,55). Since the first report in 1996 (32) on the association of the PlA2 (HPA-1b) allele of the subunit β3 of the integrin αIIbβ3 as a risk factor for coronary thrombosis, human platelet alloantigens have received widespread and ever-increasing attention that extends beyond the field of platelet immunology. The hundreds of clinical and epidemiological studies have been performed worldwide to evaluate the role of several GP polymorphisms in arterial thrombosis. Table 1 (38) lists many known platelet polymorphisms that have been studied in thromboembolic disorders (32,57).

Table 1. Main platelet polymorphisms (from Corral et al. J Biol Regul Homeost Agents. 2004;18:166-171)

TABLE I - MAIN PLATELET POLYMORPHISMS AND OTHER POLYMORPHISMS ASSOCIATED WITH PLATELET FUNCTION THAT COULD BE INVOLVED IN THROMBOEMBOLIC DISEASES

Protein	Name	Location	Consequence	Prothrom[1]	Freq[2]
GP IIIa	HPA-1	T1565C	Leu33Pro	Pro33	0.15
GP IIb	HPA-3	T2622G	Ile843Ser	???	0.33
GP Ia	HPA-5	G1648A	Glu505Lys	???	0.12
			< expression		
	807	C807T	> expression	807T	0.35
GP Ibα	HPA-2	C1018T	Thr145Met	Met145	0.12
	VNTR	1-4 repeats	Size molecule	VNTR-B	0.11
	Kozak	T-5C	> expression??	-5C	0.10
GPVI		T13039C	Ser219Pro	Pro219	0.05
P-selectin		A8069423C	Thr756Pro	Thr756	0.860
		G8070818T	Val640Leu	Leu640	0.312
FcγRIIa		A507G	His131Arg	Arg131	0.46
TXR		T1915C	Thr924Cys	Cys924	0.19
		G179T	Arg60Leu	Leu60	0.003
GNAS1	VNTR	12-14 repeats	Conflictive	???	0.018
ANV	Kozak	C-1T	> expression	-1C	0.879
PSGL-1	VNTR	14-16 repeats	Adhesion	VNTR-A	0.860

[1] Suggested prothrombotic variant.
[2] Allelic frequency in Caucasian population of the suggested prothrombotic allele(s) or low frequency allele

Most of these polymorphisms are localized in the coding region of the affected gene, but others are located in regulatory regions involved in the control of transcription or translation. As expected, platelet receptor polymorphisms are mainly single

nucleotide polymorphisms (SNPs). However, polymorphisms involving a region that is repeated in tandem (Variable Number of Tandem Repeats: VNTR) have also been described in genes encoding different platelet proteins (3,35). Several SNPs changes result in the substitution of a single amino acid (missense modifications). In contrast, the VNTR polymorphisms have more relevant structural consequences affecting the entire molecule, and the distance between the functional binding domain from the platelet surface. Finally, other polymorphisms, specifically those located in regulatory regions, affect the level of expression of the GP on the platelet surface, which results in significant interindividual GP receptor density (35-38). These effects support the idea that platelet receptor polymorphisms might be associated with qualitative or quantitative differences in platelet adhesion and thrombus formation. Most polymorphisms are silent, but platelet polymorphisms of interest in thrombosis are those with functional consequences (57,58). In our study, we have been focusing on polymorphisms of the fibrinogen (HPA-1 polymorphism of the subunit β3 of the integrin αIIbβ3) and collagen (807C/T polymorphism of α-subunit of the integrin α2β1) platelet receptors.

1.4.1. HPA-1 polymorphism of αIIbβ3

The integrin αIIbβ3 is the most abundant receptor on the platelet membrane surface, at about 80,000 copies per platelet, and is known as the receptor for fibrinogen or von Willebrand factor that mediates platelet cohesion, i.e., the formation of platelet aggregates (42-44). This receptor is also characterized by several inheritable dimorphisms, such as four alleles of the αIIb α-subunit and eight alleles of the β3 β -subunit. The two most common and clinically important β3 alleles encode Leu33 (PlA1 or HPA-1a) and Pro33 (PlA2 or HPA-1b allele of the subunit β3 of the integrin αIIbβ3), with gene frequencies of 0.85 and 0.15, respectively, in the Caucasian population.

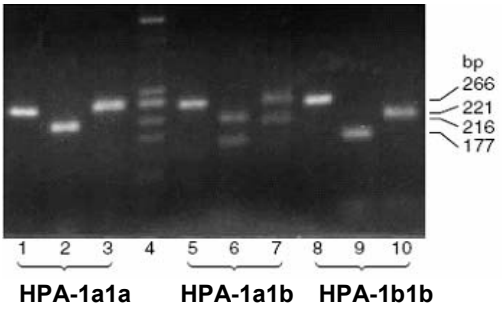

Figure 7. 3% argarose gel containing undigested PCR products (lanes 1,5,8) and PCR products digested with MspI (lanes 2,6,9) and NciI (lanes 3,7,10) corresponding to the tree possible allelic combinations: HPA-1a1a, HPA-1a1b and HPA-1b1b.

Weiss et al. (32) reported that the gene frequency of the HPA-1b allele was 3.6 times higher among younger patients (< 60 years of age) with myocardial infarction or unstable angina as compared to the age-matched controls (odds ratio 6.2). The impact of HPA-1b allele as genetic risk factor of ischemic vascular disease has been given credence by some, but not all, subsequent studies (33, 59). There are at least seven reports that confirm an association between the HPA-1b allele and the risk for myocardial infarction in younger individuals and at least eleven studies that have failed to find such an association. Despite the controversy surrounding these clinical correlations (32-36,59,60), current investigations have indicated that the HPA genotype might have a possible effect on platelet function supporting the contention that it is a clinical risk factor for thrombosis (33, 40,61-63). The HPA-1b genotype seems to confer a lower threshold for agonist-induced platelet responses, and Vijayan et al. demonstrated that the HPA-1b polymorphism alters αIIbβ3-mediated functions, such as an adhesion, spreading and clot retraction (64,65).

1.4.2. α2 807C/T polymorphism of α2β1

Integrin α2β1 is one of the most well characterized platelet collagen receptors (66). Interestingly, the expression of α2β1 on the platelet surface differs markedly among healthy subjects. Normal platelets contain approximately between 1000 and 10000 receptor copies of Integrin α2β1. This variation depends on the inheritance of linked, allelic polymorphisms within the coding sequence of the α2 gene (20,35,67,68). Four α2 alleles can be defined. The allele 1 (807T/1648G/2531C) is associated with increased levels of α2β1, while allele 2 (807C/1648G/2531C) and allele 3 (807C/1648A/2531C) are each associated with decreased levels of this receptor. The gene frequencies (35) of these three alleles in the Caucasian population are: 39% (allele 1), 52% (allele 2) and 3, 7.6% (allele 3). The very rare allele 4 (807C/1648G/2531T) has gene frequency <1%. Beside this variation depends on the inheritance of linked allelic polymorphisms, a inherited, single base substitutions are also found at two positions, C-52T and C-92G, within the proximal 5'-regulatory region of the human integrin α2 gene. The T-52 and G-92 sequences have a gene frequency of 0.35 and 0.15, respectively, in a typical Caucasian population, and the presence of either allele correlates with reduced expression of α2β1 on the platelet surface.

The role of the 807C/T polymorphism in the α-subunit of integrin α2β1 in arterial disease has been evaluated in several studies. The initial report described a correlation between 807TT genotype (high receptor density) and risk for myocardial infarction (35). In one larger study with 2237 male patients undergoing angiography, an association between 807TT genotype and myocardial infarction was observed in younger patients (70). More recently Roest et al. found that 807TT genotype was associated with increased vascular mortality in women who are heavy chronic smokers (71). In addition, a significant association was also found in younger patients with stroke and in patients with diabetic retinopathy (35,40). On the other hand, some studies were unable to establish an association between 807TT genotype and myocardial infarction (62,72). However, in two of these studies the frequency of the homozygous TT genotype among the study controls was higher than that reported among normal Caucasian or Oriental populations, respectively, and may have markedly influenced the conclusions. It is evident that a patient and control selection bias needs to be eliminated from clinical studies of this kind by careful consideration of ethnic and racial influences on allele frequencies.

1.5 Platelet receptor polymorphisms and their role in arterial thrombosis, clinical and epidemiological evidence

Pub-Med search using polymorphism and myocardial infarction, results in more than one thousand publications, which proves the interest in this field. Up to 80% of these reports are association studies. Polymorphism association studies compare the prevalence of a genetic marker in subjects with a given condition with the prevalence in controls. Surprisingly, these studies give different and, in some cases, contradictory results (32,33,40,74,75). Moreover, only 16% of initially identified associations were subsequently replicated. This is a consequence of the heterogeneity of these studies (variations in study design, patient demographics and clinical presentation) and complexity of the thromboembolic disease.

Firstly, the role of a polymorphism might depend on the population studied. Certainly, there are substantial differences in haplotype frequencies among racial/ethnic groups, particularly in the case of suggested, most prothrombotic platelet receptor polymorphisms: 807C/T polymorphism in the α-subunit of integrin α2β1 and HPA-1 polymorphism of the β subunit of integrin αIIbβ3. The most representative example is

the HPA-1 polymorphism. HPA-1b allele is present in 15% of Caucasian subjects, but almost absent in Asian population. Moreover, the genetic background and the influence of environmental risk factors (diet behaviour, exposition to occupational stress, incidence of estrogen replacement therapy by females, life style etc.) differ among populations. In accordance, the results of association studies performed in different populations can hardly be compared.

Secondly, the clinical endpoint designed in these studies is extraordinarily heterogeneous, which makes it difficult to compare. Certainly, the role of a common platelet polymorphism will probably differ in each specific thrombotic disease or under particular conditions. For example, the relevance of a polymorphism affecting the number of collagen receptors on the platelet surface might be different in cerebrovascular disease or coronary artery disease. But its role could be different in unstable angina or myocardial infarction, in old or young patients, in male or female patients. Cole et al. (75) conducted a straight controlled case-control study involving 180 stroke patients and 172 controls to determine whether the α2 807C/T polymorphism is associated with an increased risk of ischemic stroke. They found no statistically significant differences in the distribution of α2 807C/T in patients and controls overall or after stratification by etiological subtype. A significant role for the α2 807C/T polymorphism in the development of first-event ischemic stroke could not be supported. To the contrary, Rainer et al. suggested a possible association between α2 807C/T polymorphism and the risk of ischemic stroke, especially in young women (76). As a third example, Mannucci et al. ("Atherosclerosis, Thrombosis and Vascular Biology Italian Study Group") conducted one nationwide case-control study including 1210 enrolled patients who survived a first myocardial infarction at age < 45 years and 1210 healthy age-, sex- and geographical origin matched subjects. After carefully evaluation, the authors could not provide any evidence supporting an association between nine of the most frequently investigated polymorphisms of genes encoding hemostatic factors (including the α2 807C/T polymorphism) and the occurrence of myocardial infarction (62). Similarly, the platelet HPA-1b allele has been evaluated in more than 50 independent studies including more than 10,000 patients and controls and it is still unknown if it plays a significant role in thromboembolic diseases (77).

Moreover, the relevance of a common platelet polymorphism in thromboembolic diseases will be conditioned by environmental risk factors or medication(s) (smoker

or non-smoker, diabetics, etc.). The influence of microenvironmental conditions as local hemodynamic (shear stress), biochemical (hematocrit, hemostatic factors etc.) and vessel wall conditions (atherosclerotic status) could significantly restrict measurable allotment of single or combined polymorphisms (3,5, 34-38). It seems that in selected young patients, where the influence of environmental risk factors is lower than in old patients, the role of genetic risk factors could be more relevant (5,40). On other hand, a possible explanation for inconsistent results of clinical study could be found in the nature of platelet function.

After initial adhesive interactions (see Introduction), the agonists released or generated at the site of vascular injury as well as other local stimuli, act promptly, building up a signalling network within seconds and enhance the adhesive and procoagulant properties of the platelets tethered to the lesion. The activation begins as the binding of adhesive ligands and agonists to cognate receptors on the platelet membrane, and is propagated by intracellular signaling reactions that involve enzymes, substrates and co-factors engaged in specific protein−protein and protein−lipid interactions. A parallel action of inhibitory substances, such as prostacyclin, adenosine and nitric oxide, contributes to the limiting of thrombus formation within the area of vessel wall damage (4).

The variant alleles could determine variations in the function of the affected platelet receptors, resulting in functional consequences (reduced or increased binding efficiency, or different expression), which, in some cases, are hardly identifiable by standard methods to evaluate a platelet function. However, these changes neither abrogate the function nor facilitate the activation of the affected protein by reduced stimulation or in the absence of functional stimulus. In other words, platelets carrying allelic variants seem to respond similarly to the same stimuli (78). Therefore, under a strong stimulation such as that generated by plaque rupture, platelets with different allele variant respond properly or with minor differences. Otherwise, in standard test systems, which evaluate primary hemostasis (PFA-100, bleeding time in-vivo according to Ivy, platelet aggregation test) after application of standard agonists or stimulus, a similar response independent of genotype was observed.

In contrast, resting platelets carrying different allelic variants of GP due to polymorphisms are not able to trigger a whole platelet response in the absence of strong stimulation. These features, together with the complexity of thromboembolic disease, explain why none of the polymorphisms encoding platelet GP has been

consistently associated with an increased or decreased risk of complex and multifactorial diseases such as myocardial infarction or stroke. According to the heterogeneity of study results, there is actually general consensus that the relevance of a platelet receptor polymorphism in thromboembolic diseases will be conditioned by micro- or/and macro-environmental risk factors (35,38,77). Therefore, collaborative studies evaluating the combination of polymorphisms affecting platelets, coagulation, fibrinolysis and inflammation, together with their relationship with the environment or local risk factors should be performed. Thousands of patients and controls are required in order to identify such gene to gene and gene to environment interaction in the development of complex diseases such as myocardial infarction or stroke.

1.6. Shear force and shear stress in arterial thrombosis

For more than a century, hemodynamic forces have been proposed as a determinant regulating blood vessel structure and influencing the development of vascular pathology such as atherosclerosis and thrombosis (1,7-9). In this part of the introduction, I would like to present the basic mechanical (rheological) properties and the biological effects of shear stress and shear force in the human vasculature.

1.6.1. Basic rheological blood features

The mechanical forces produced in blood vessels can result from (i) a change of luminal pressure that causes a change in blood flow (which yields shear stress), or (ii) from a change of transmural pressure that causes circumferential deformation of the vessel wall during the cardiac cycle (caused by tensile stress).

The rheological regimes in the vascular system can be described by applying the principles of classical fluid dynamics (pressure-driven laminar flow of a Newtonian fluid through the cylindrical tube with constant cross-sectional area). According to this state, an axial velocity field is dependent on the radial position, main fluid velocity and vessel radius. An axial velocity reaches its maximum at the center of the tube where radius is equal to zero (Fig. 8).

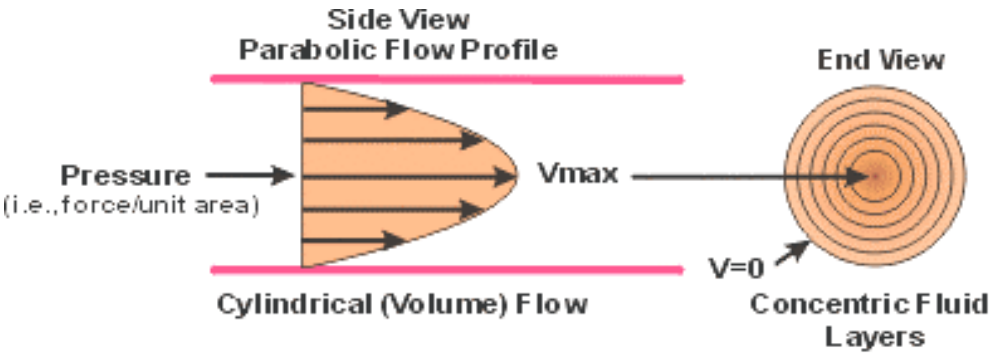

Figure 8. Schematic presentation of classical fluid dynamics (pressure-driven laminar flow).

Normally blood flow can be described by laminar flow which is characterized by concentric layers of blood moving in parallel down the length of a blood vessel. The highest velocity (V_{max}) is found in the center of the vessel and the lowest velocity (V_{min}) along the vessel wall. The flow profile is parabolic, once laminar flow is fully developed. This occurs in long, straight blood vessels, under steady-state flow conditions. Moving away from the vessel center, the velocity decreases and reaches a minimum at vessel wall (7,8,80).

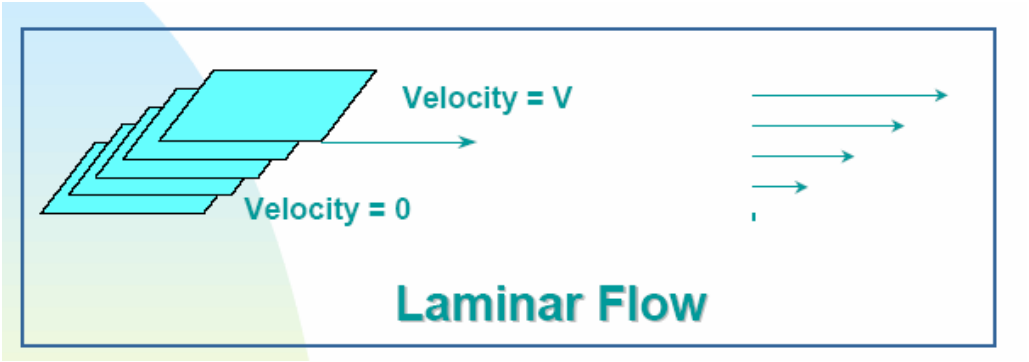

Figure 9. Schematic presentation of different fluid planes at laminar flow.

A fluid shear stress and fluid shear rate are commonly used to the described rheological conditions in the vascular system. The shear could be described as relative motion of the different fluid planes in the velocity profile (Fig. 9,10). The mechanical force most relevant to platelet-mediated hemostasis and thrombosis is shear stress. The shear stress is defined as “the force per unit area between laminae”; and blood flow can be described as an “infinite number of infinitesimal laminae sliding across one another, each lamina suffering some frictional interaction with its neighbours”. A fluid shear stress is the force per unit area generated by flow

of viscous liquid and usually expressed in dynes per square centimeter (dyn/cm^2) or Newton per square centimeter. A wall shear stress is the force per unit area applied by the flowing viscous blood to the vessel surface. The shear rate is a measure of the velocity gradient and indicates the rate at which fluid layers are sliding past one another. According to that, a relationship between a fluid velocity and velocity gradient (or shear rate) is inverse. Although the fluid velocity is greatest at the center, Shear stress and shear rate are commonly used to the described rheological conditions in the vascular system. The shear could be described as a relative motion of the different fluid planes in the velocity profile (Fig. 10). The mechanical force most relevant to platelet-mediated hemostasis and thrombosis is shear stress. Shear stress is defined as “the force per unit area between laminae”; and blood flow can be described as an “infinite number of infinitesimal laminae sliding across one another, each lamina suffering some frictional interaction with its neighbours”. Fluid shear stress is the force per unit area generated by flow of viscous liquid and usually expressed in dynes per square centimeter (dyn/cm^2) or Newton per square centimeter. Wall shear stress is the force per unit area applied by the flowing viscous blood to the vessel surface. The shear rate is a measure of the velocity gradient and indicates the rate at which fluid layers are sliding past one another. According to that, fluid velocity is inversely related to the velocity gradient (or shear rate). Although the fluid velocity is greatest at the center, the velocity gradient as well as shear rate is

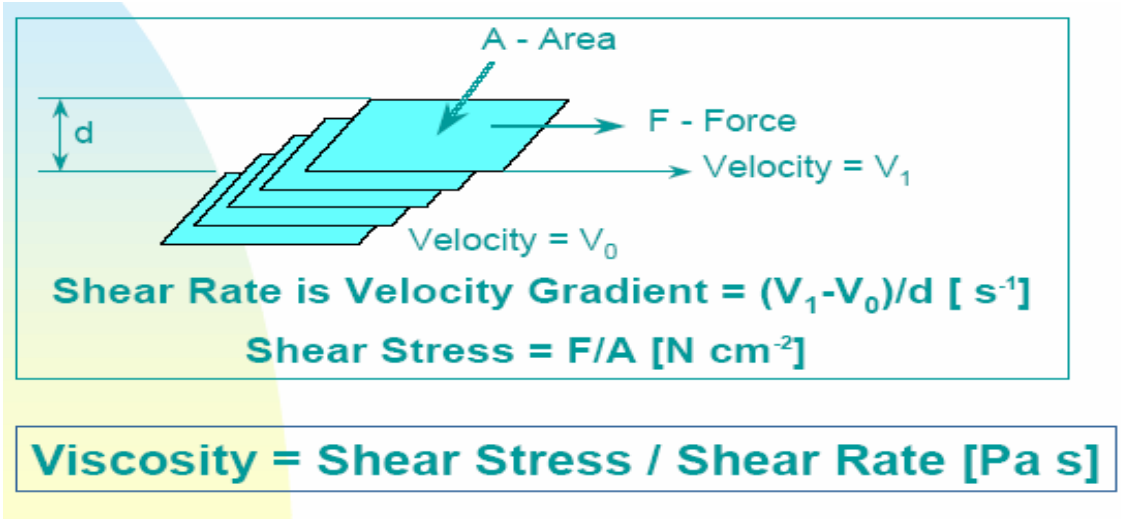

Figure 10. Relationship between shear rate and shear stress in laminar flow.

equal to zero (7,8). In contrary, at vessel wall at lowest fluid velocity, a velocity gradient and shear rate reaches the maximum. The shear rate is usually expressed as inverse seconds (s^{-1}).

Table 2. Typical ranges of wall shear rates and wall shear stress (from Kroll et al. Blood. 1996;5:1525-1541, ref. 8)

Blood vessel	Wall shear rate, s^{-1}	Wall shear stress, dynes/cm^2
Large arteries	**300-800**	**11.4-30.4**
Arterioles	**500-1600**	**19.0-60.8**
Veins	**20-200**	**0.76-7.6**
Stenotic vessels	**800-10000**	**30.4-380**

For Newtonian fluids, shear stress is directly proportional to the shear rate. Newtonian fluids are defined as having a constant viscosity at all shear rates, temperatures and pressures. For arterial vessels, with relatively high flow, blood can be considered a homogenous Newtonian fluid for shear rates greater than $100s^{-1}$. At these shear rates, the viscosity of blood remains constant and is determined by hematocrit, temperature and protein concentration. This means that shear stress (τ) in the tubular chamber, having radius r and flow direction z, can be represented by the mathematical formula: $\tau=\mu(dv/dr)$, where dv/dr is the local velocity gradient (or shear rate) and viscosity (μ) the proportionality constant.

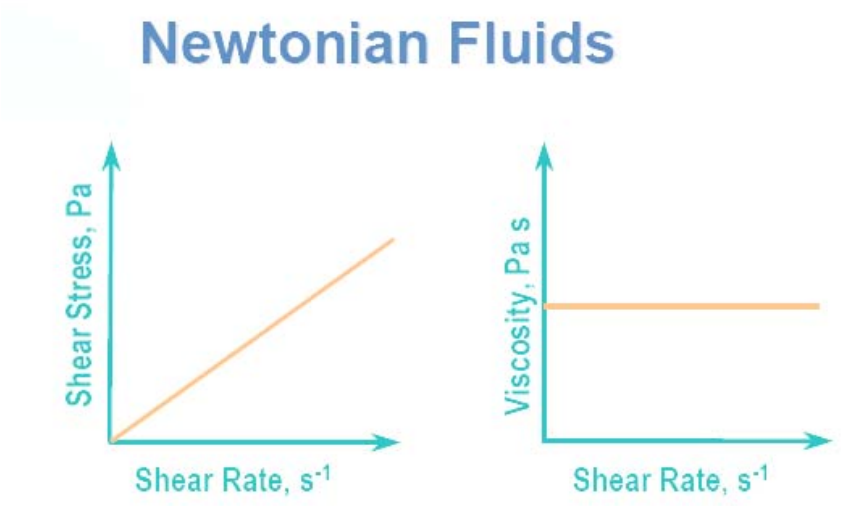

Figure 11. Relationship between shear rate and shear stress in blood anticipated as Newtonian fluid.

For Newtonian fluids, the relationship between shear stress and shear rate [τ =μ(dv/dr)] allows one to convert simply from shear stress to shear rate: shear rate(in cm/s per cm, or s^{-1}) = shear stress/viscosity. For example, in the whole blood with a

viscosity of 0.038 poise, a wall shear stress (τ) of 90 dynes/cm^2 results in a wall shear rate of 2368 s^{-1}. The wall shear stress (τ_w) of Newtonian fluids for flow in tubular vessels can be calculated as a function of volumetric flow rate: $\tau_w=4\mu Q/\pi r^3$, where Q is the volumetric flow rate and r the radius of the tubular chamber. This formula has been generally used to calculate shear forces in vivo. A steady Newtonian flow through a cylinder generates a parabolic velocity profile and a linear distribution of shear stresses, with liquid components (e.g., platelets) at the periphery of the cylinder (the wall) subject to maximal shear stress and liquid components at the center of the cylinder subject to zero shear stress (7,8,80) . A practical implication of parabolic laminar flow is that the orderly movement of adjacent layers of blood flow through a vessel helps to minimize energy losses in the flowing blood due to viscous interactions between the adjacent layers and the vessel wall. A disruption of laminar flow leads to turbulence and increased energy dissipation. Turbulence occurs when smoothly flowing laminar flow is disrupted. This occurs distal to stenotic heart valves or arterial vessels, at vessel branching points, and in the ascending aorta at high cardiac ejection velocities (e.g., during exercise). The onset of turbulence under ideal conditions can be predicted by calculating the Reynolds number (Re):

$$\mathrm{Re} = \frac{(\bar{v} \cdot D \cdot \rho)}{\eta}$$

v = mean velocity, *D* = 2r= vessel diameter, ρ = blood density, and η = blood viscosity.

There is a critical Reynolds number (Re) above which laminar flow is disrupted and turbulence occurs. Therefore, as blood flow velocity increases in a blood vessel or across a heart valve, there is a gradual increase in turbulence as the Reynolds number increases. Laminar flow will continue until a critical Reynolds number is reached. Under ideal conditions (e.g., long, straight, smooth blood vessels), the critical Re is relatively high. However, in branching vessels, or in vessels with atherosclerotic plaques protruding into the lumen, the critical Re is much lower, causing turbulence even at normal physiological flow velocities. It is important to note that as the vessel diameter decreases, there is a disproportionate increase in mean velocity because the velocity scales with $1/r^2$. This relationship is based on the relationship between flow, velocity, and cross-sectional area of a vessel. For example, if an arterial stenosis reduces the vessel diameter by 50%, the mean

velocity will increase 4-fold. The net effect will be the 2-fold increase in Re, bringing the Re closer to its critical value for the development of turbulence. Besides increasing Re, an increase in velocity also increases the kinetic energy of the flowing blood. This can lead to a decrease in potential energy (Bernoulli effect) and result in vessel collapse and flutter under some conditions. The turbulence causes an increased energy dissipation and a pressure drop.

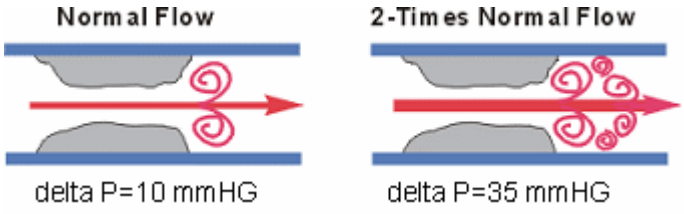

Figure 12. Relationship between pressure and flow across a stenotic lesion (e.g., stenotic valve or arterial segment) in which turbulence occurs.

For example, if blood flow is increased 2-fold across the stenotic arterial segment, the pressure drop across the stenosis may increase by 3 or 4-fold (Fig. 12). Therefore, turbulence alters the relationship between flow and perfusion pressure, as the relationship is no longer linear, as described by the Poiseuille relationship (pressure gradient is proportional to flow under laminar flow conditions). This implies that a greater perfusion pressure is required to propel blood at a given flow rate.

1.6.2. Biological effects of shear stress

However, shear stress has not only been shown to be a critical determinant of vessel calibre but has also been implicated in a vascular remodelling and vascular pathobiology. Local rheological conditions play an extraordinary role in the ontogenesis of the vascular system and in the development of arterial or venous thrombosis (4,7,8,17-19, 81,82).

Two contrary hypotheses explain the impact of shear stress on the development of atherosclerosis and arterial thrombosis. The first implicates that high shear stress (>400 dyn/cm^2) induces a direct endothelial injury. Caro et al. (83,84) established the second hypothesis designated a “low shear hypothesis”. According to the extensive angiography evaluation, Caro et al. concluded that predisposed localization of atherogenesis (condition sine que non for consecutive arterial thrombosis) is within a

low shear stress area. This association suggests that a physiological or elevated shear stress might have some protective effect against atherosclerosis (7).

Recent experimental studies have made a great advance in understanding the regulation of endothelial cell function and gene expression under shear stress (8). The modulation of endothelial phenotype by local hemodynamic shear stress is postulated to contribute to the focal geometric progression of atherogenesis in the setting of local and systemic risk factors that enhance the prothrombotic, proliferative, and pro-inflammatory components of different pathological processes (7). Other studies (85) have demonstrated that local mechanical forces can also alter the metabolism and gene expression of vascular cells (Table 3). Some of the more striking effects of flow concern the synthesis and release of important biologically active molecules like nitric oxide (NO) or prostacyclin (PGI_2), both of which are potent vasodilators and inhibitors of platelet activation. A shear stress increase from 1 dyne/cm^2 to 10 dynes/cm^2 is followed by an increased NO release by several orders of magnitude (7). A high shear stress is also associated with an enhanced antioxidant cell expression (Mn SOD, Cu SOD, Zn SOD) as well as reduced production of inflammatory mediators. Conditions of low shear stress are followed by a low NO and prostacycline production which, for example, promote local platelet adhesion (7,8,87). Shear stress also has a differential effect on the secretion of ET-1 by endothelial cells. However, the release of the vasoconstrictor endothelin-1 (ET-1) is increased under low shear conditions (5 dynes/cm^2) and decreased at higher shear stress (7). Recent studies conducted on canine models showed that shear stress can also been associated with the endothelial proliferative state (7). With increased levels of shear stress, cells were inhibited from entering the S-phase of the cell cycle and endothelial renewal decreased. During the S-phase endothelial cells replicate DNA. In contrary, endothelial cell proliferation is increased 18-fold within 48 hours of reduction of shear stress (7,87).

Table 3. Biological effects of hemodynamic shear stress (Modified from Malek et al. JAMA. 1999; 282: 2035-2042, ref. 7 and from Kroll et al. Blood. 1996; 1525-1541, ref.8)

	Hemodynamic shear stress	
Parameter	**Physiological arterial range τ >15 dyn/cm^2**	**Low arterial range τ < 4 dyn/cm^2**
Endothelial cell morphology	fusiform aligned	polygonal unaligned
Endothelial proliferation	↓	↑
Endothelial apoptosis	↓	↑
ACE	↓	↑
NO/NO syntase	↑	↓
PGI_2/PGI_2 syntase	↑	↓
COX-1,2	↑	↓
MN SOD	↑	↓
Cu/Zn SOD	↑	↓
PDGF-A,B	↓	↑
TGF-β	↑	↓
VCAM-1	↓	↑
t-PA	↑	↓

A decrease in shear stress is followed by an endothelial cell loss and desquamation, altered morphology with decreased elongation (Fig. 13), decrease in actin stress fibers, greater monocyte attachment to and migration across the endothelial layer and increased endothelial surface expression of vascular cell adhesion molecule. The increased endothelial cell loss in response to decreased shear has recently been suggested to be the result of apoptosis, which remains unabated until the shear has returned to an equilibrium value (7,8).

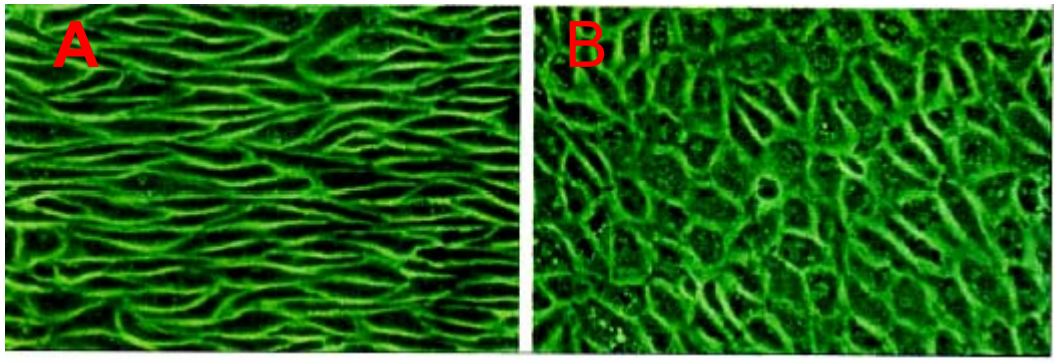

Figure 13. Bovine aortic endothelial cells (A) exposed to physiological shear stress (>15 dyn/cm^2) and (B) exposed to low shear stress (up to 4 dyn/cm^2). (Modified from Malek et al. JAMA. 1999; 2035-2042, ref. 7)

Generally, it can be concluded that shear stress impacts gene expression of vascular cells and plays a significant role in forming a stress-related endothelial phenotype (7). This phenotype could be defined as prothrombotic (shear stress < 4 dyn/cm^2) and antithrombotic (shear stress > 15 dyn/cm^2).

1.6.3. Experimental model of the vascular system

To identify the molecular mechanisms of thrombosis, the dynamic nature of the vascular system must be incorporated into the experimental model. This requires the application of engineering principles to develop physiological models as close as possible to the biological vascular system. These models are used to generate controlled mechanical forces in vitro, similar to those produced by hemodynamic forces in vivo. A flow chamber is a well-established in vitro system in which cell-surface interaction can be examined (88,89). In these systems, whole blood or a cell suspension is perfused through the chamber across the surface of immobilized cells, proteins and other matrices (4,8,12,14,17,18,47). Using different flow chamber geometries, different flow patterns can be simulated resembling laminar, turbulent or complex laminar flow regimes. Simultaneously, both arterial and venous shear conditions can be produced by change in the volumetric flow rate only (14,88,89). As shown in Fig. 14, the parallel plate flow chamber consisting of a polycarbonate slab, silicon gasket and glass coverslip. The glass coverslip forms the bottom side of the parallel plate flow chamber and cells or different ligands can be immobilized onto this surface. The thickness of the silicon gasket determines the height of the gap between coverslip and the polycarbonate slab. Fluid is pumped (or aspirated) across this gap through the chamber. The wall shear stress (τ) in this system depends on the height of the gap, width of the chamber, fluid viscosity and volumetric flow rate through the chamber. The system allows measurements of a dynamic and an endpoint platelet deposition on the related surface. Direct visualization of platelet deposition over time can be determined by combining epifluorescence, video-microscopy and digital image processing. In each case, platelets have to be labelled for fluorescence prior to perfusion. Using these methods, the total number of platelets deposited, thrombi morphology as well as the velocity of rolling platelets may can be evaluated. Shear forces generated by flowing blood significantly impact blood and vascular cell function. Thrombus formation depends on local flow conditions, different cell receptors and ligands supporting cell-cell and cell-ligand interaction(s) under arterial,

venous and pathological shear conditions. In our experimental study, a parallel plate flow chamber device (Figure 14) was used in an attempt to quantify the platelet adhesion to immobilized substrates.

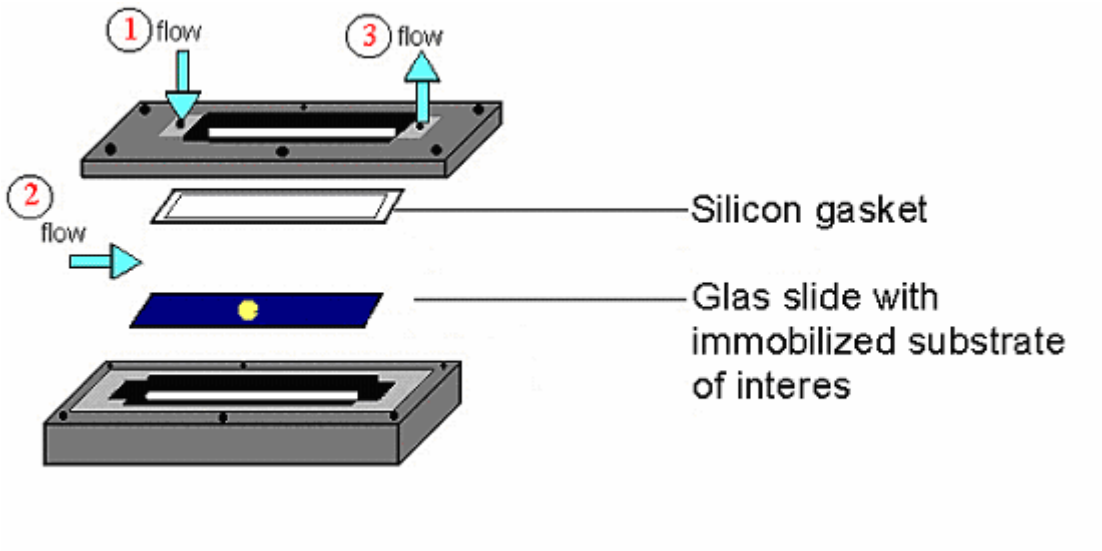

Figure 14. Schematic presentation of rectangular flow chamber.

In the device, a glass slide is placed on the bottom of the chamber, separated by a gasket of specified height (50μm), forming a small flow channel (slit). The top slide is transparent (plexiglas), allowing microscopy. The bottom slide contains the material of interest (adhered cells, fibrinogen, collagen), and the wall shear acting on the substrate is defined by the following expression:

$$\tau_{wall} = \frac{6 * \mu * Q}{w * h^2}$$

where μ is the blood viscosity, *w* is the channel width and *h* the channel height. By controlling the volumetric flow rate (Q) through the parallel plates, it is possible to change the wall shear stress acting on the slides. The wall shear stress at which cells adhere or detach from channel surface can then be quantified, providing an idea of the cell adhesion properties of the surface.

2. Goal of the study

Platelet adhesion and subsequent thrombus formation on a subendothelial matrix at the site of vascular damage play a crucial role in the arrest of posttraumatic bleeding but also in different pathological thrombotic events, such as acute coronary syndrome and stroke (1,2,3). Recently published studies have clearly demonstrated that platelet integrins αIIbβ3 and α2b1 are intimately involved in the occlusive thrombus formation at the site of endothelial damage (4,5). Therefore, any genetic variation in the expression of these receptors may lead to an excessive bleeding or excessive thrombus formation (31-40).

Since Weiss et al. (32) for first time in 1996 reported that the gene frequency of the HPA-1b allele of integrin αIIbβ3 was significantly higher among young patients with myocardial infarction compared with age-matched controls, numerous clinical studies were conducted in order to define a role of related polymorphisms in arterial thrombosis. Surprisingly, these studies gave different, and in some cases, contradictory results (32,33,40,74,75).

Taking into consideration the controversy surrounding these clinical correlations, the overall goal of this study is to test the functional relevance of HPA-1 and α2 807C/T platelet receptor polymorphisms under standardized microenvironmental conditions using a well-characterized in-vitro flow model described in chapter 1.6.3.

In order to achieve this goal an in-vitro flow model was modified and evaluated to investigate the mechanisms by which platelet receptor polymorphisms as well as mechanical forces modulate platelet adhesion and consecutive thrombus formation. A flow chamber model was used to generate controlled mechanical forces in vitro, similar to those produced by hemodynamic forces in vivo. Using a stable flow chamber geometry, arterial and venous hemodynamic conditions are simulated by change in the volumetric flow rate (14,88,89). Under strictly defined hemodynamic conditions the following basic questions will be evaluated: 1) adhesive properties of platelet fibrinogen (αIIbβ3) and collagen (α2β1) receptors; 2) specific interaction between the receptors and related ligands and 3) receptor interplay.

The main goal of the study, using the in-vitro flow model, concerns the evaluation whether and in which manner HPA-1 and α2 807C/T platelet receptor polymorphisms affect platelet adhesion and consecutive thrombus formation. In these experiments the following specific questions will be addressed: 1) whether HPA-1 polymorphism

of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1 influence platelet adhesion onto related ligand(s); 2) is the observed effect on adhesion dependent on shear rate and perfusion time; 3) which effect has the critical prothrombotic genotype combination (HPA-1b/1b and α2 807TT) on platelet adhesion and consecutive thrombus growth compared to “wildtype” variants of αIIbβ3 and α2β1 (HPA-1a/1a and α2807CC).

3. Subjects and methods

3.1. Subjects

The study was conducted on 88 healthy blood donors who were previously genotyped. The mean age was 42±12 years, 66 (75%) were men and 22 (25%) were women. None of donors had taken any medication in the preceding 14 days. Screening parameters of hemostasis as well as factors of coagulation were within the normal range. Excessive smokers, obese subjects and individuals with positive familiar history related to arterial or venous disease were excluded. Since the frequency distribution of platelet glycoprotein alleles differs with regard to race, only Caucasians were included in the study.

3.2. Blood collection and preparation

Venous blood obtained from the cubital vein was immediately anticoagulated with the thrombin inhibitor PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihidrochloride, final concentration 40 μM, Calbiochem, San Diego Ca, USA). Platelets were labelled with a fluorescence dye mepacrine (quinacrine dihydrochloride, final concentration10 μM; Sigma Chemical, 60 min at 22°C). It immediately accumulates in the delta granules of platelets but does not influence platelet physiology (47). Blood was used within two hours of withdrawal. Blood anticoagulated with EDTA and 3.4% sodium citrate was immediately processed for genotyping and for assessment of screening parameters and factors of coagulation, respectively.

3.3. Determination of HPA-1 and α2 807C/T genotypes

EDTA-anticoagulated blood was immediately centrifuged at 2500 rpm for 10 min at 4°C. Cells were further processed to assess genetic polymorphisms. Genomic DNA was extracted from whole blood with the QIAmp blood kit (Qiagen, Hilden, Germany). After amplification by polymerase chain reaction, genotypes were determined by allele-specific restriction enzyme analysis. Determination of the HPA-1 polymorphism

of the β subunit of αIIbβ3 and the C807T polymorphism of the α subunit of α2bβ1 was performed as previously described (90,91).

3.4. Assesment of screening parameters of hemostasis and factors of coagulation

Screening parameters and factors of coagulation were assessed with commercial hemostatic high-speed analyzers and kits for clotting, chromogenic and immunologic coagulation assays (BCT® and BCS®, Dade-Behring, Marburg Germany). Both systems measure coagulation capabilities of plasma from blood collected using the anti-coagulant 3.8% sodium citrate.

Prothrombin time (PT), activated partial prothrombin time (aPTT), thrombin time (TT) and the level of functional fibrinogen were analyzed employing recombinant human tissue factor (Innovin, aPTT test reagent, bovine thrombin (BC-Thrombin) reagent and fibrinogen test reagents (MultifibrinU, Dade-Behring, Marburg, Germany). The level of FXIII and AT was measured photometrically using a chromogenic substrate (Berichrom FXIII, Berichrom Anti-Thrombin III both from Dade Behring). All measurements were performed using BCS®-Analyzer from Dade-Behring.

Activity of factors II, V, VII, VIII:C, IX, X, XI, and XII were performed on a Behring Coagulation Timer (BCT®) using plasma, which was deficient for factor in question. von Willebrand Factor Antigen was measured using an immunoassay for determination of vWF-Antigen (vWF Ag, Dade Behring, BCT® analyzers). Additionally, the von Willebrand Factor Activity (vWF:RCo) was measured using BC von Willebrand Reagent, from Dade Behring on BCS®.

3.5. Platelet Functional Analyzer (PFA-100)

Using PFA-100 system (Dade-Behring, Marburg, Germany), we investigated the influence of examined platelet receptor polymorphisms on primary hemostasis (platelet adhesion and aggregation) under high shear rate conditions, similar to those encountered in a stenotic blood vessel (5000 s^{-1}–6000 s^{-1}). The end point is determined as the time required to occlude an aperture (closure time, CT in sec) in the membrane of a test cartridge, which is coated with platelet agonists (92).

Two PFA-100 test cartridges are used to measure the CT of citrated whole blood; either the collagen/ADP (PCA) or the collagen/ epinephrine (PCE). A sample (500 µl) of anticoagulated blood was placed in the reservoir of the test cartridge, which is maintained at 37°C. PFA-100s were carried out with anticoagulated blood within 1 h of sampling. According to the recommendations by the manufacturer, the PFA-100 results were considered abnormal when the occlusion time was higher than 160 s for PFA-EPI and 120 s for PFA-ADP.

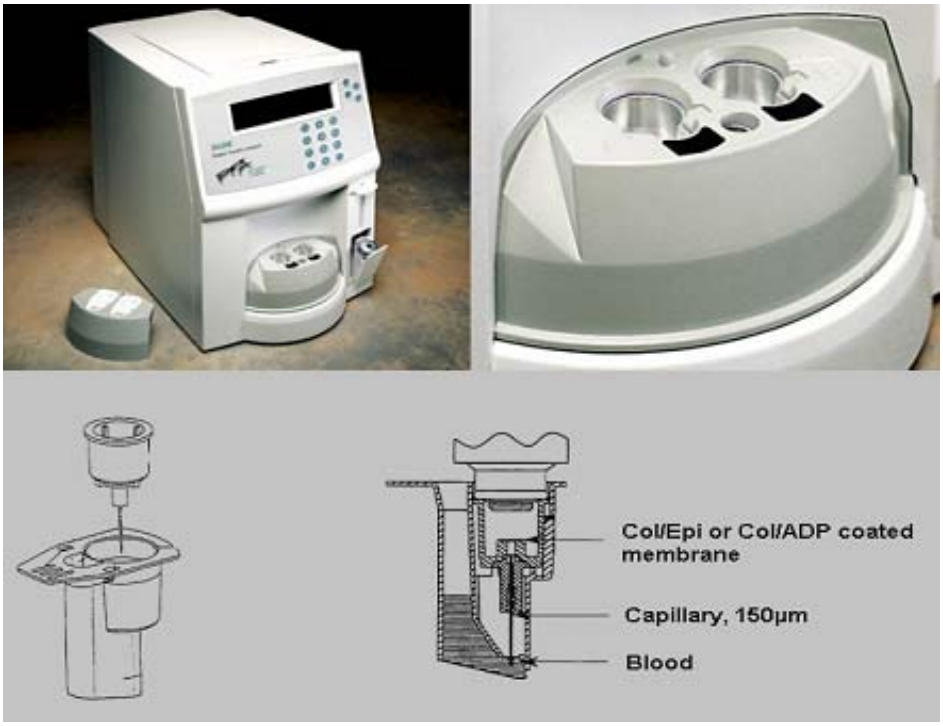

Figure 15. PFA-100 uses small membranes coated with either collagen and epinephrine (Col/Epi) or collagen and ADP(Col/ADP). Anticoagulated whole blood is passed through the membranes at a high shear rate to simulate the *in vivo* hemodynamics in the small capillaries. Platelets adhere to the membranes and gradually occlude a small aperture in the center of each membrane. The time, in seconds, for blood to completely occlude the aperture is referred to as the Closure Time (CT).

3.6. Preparation of fibrinogen-coated coverslips

A suspension of human fibrinogen (Sigma-Aldrich) was prepared as described previously (47). Glass coverslips (24x50 mm) were coated with 50µl of fibrinogen

solution (2,5 mg/ml) and the coverslip was placed in a humid environment (60 min at 37°C) to allow the protein to adhere to the glass surface. To remove non-adherent fibrinogen coated coverslips were rinsed with 10 ml of 50 mmol/l phosphate buffered saline (pH 7.35, Serag-Wiessner, Germany) and placed in a rectangular flow chamber. Fibrinogen density on the glass surfaces was calculated to be 0.13 µg/mm^2.

3.7. Preparation of collagen-coated coverslips

A suspension of collagen from bovine Achilles' tendon (95% type I and 5% type 5, 3 mg/mL protein in 0.1 N acetic acid) was obtained from Sigma-Aldrich. Glass coverslips (24x50 mm) were airbrushed with 50µl of collagen solution and the coverslip was placed in a humid environment (60 min at 37°C) to allow the protein to adhere to the glass surface. Coated coverslips were rinsed with 10 ml of 50 mmol/l phosphate buffered saline (pH 7.35, Serag-Wiessner, Germany) to remove non-adherent collagen and placed in the rectangular flow chamber (17). Collagen density on the glass surfaces was calculated to be 0.15 µg/mm^2.

3.8. Flow chamber, perfusion, laser-scan microscopy and data acquisition

Platelets adhesion rates onto fibrinogen and collagen-coated glass coverslips were measured in the rectangular flow chamber under linear shear rate of 50 s^{-1}, 500 s^{-1} and 1500 s^{-1}. A shear rate of 50 s^{-1} represents a venose system, 500 s^{-1} mimics a wall shear rate of larger arteries and 1500 s^{-1} represents a typical arteriolar shear rate as well as a shear rate by moderate arterial stenosis (7,8). Recent study indicated that mechanism of platelet adhesion and thrombus formation is shear rate dependent (4,47). Related shear stress (τ) was calculated using the following estimation $\tau = \frac{6 \cdot \mu \cdot Q}{w \cdot h^2}$, where μ is a fluid viscosity, Q volumetric flow, and w is the width of the chamber and h is the height of the chamber gap. According to the following estimation of $Q = \frac{\tau \cdot w \cdot h^2}{6}$, expected volumetric flow was calculated. In our experimental system blood flow of 1.2 ml/h, 11.5 ml/h und 34 ml/h generated a shear stress, expressed as the force per unit area (dynes per square centimeter, dyn/cm2)

of 1.9 dyn/cm^2, 19 dyn/cm^2 and 57 dyn/cm^2, respectively. Taking into consideration that blood was defined as Newtonian fluid, shear stress and shear rate are directly proportional. Those shear stresses correspond to desirable wall shear rates of 50 s^{-1}, 500 s^{-1} and 1500s^{-1}.

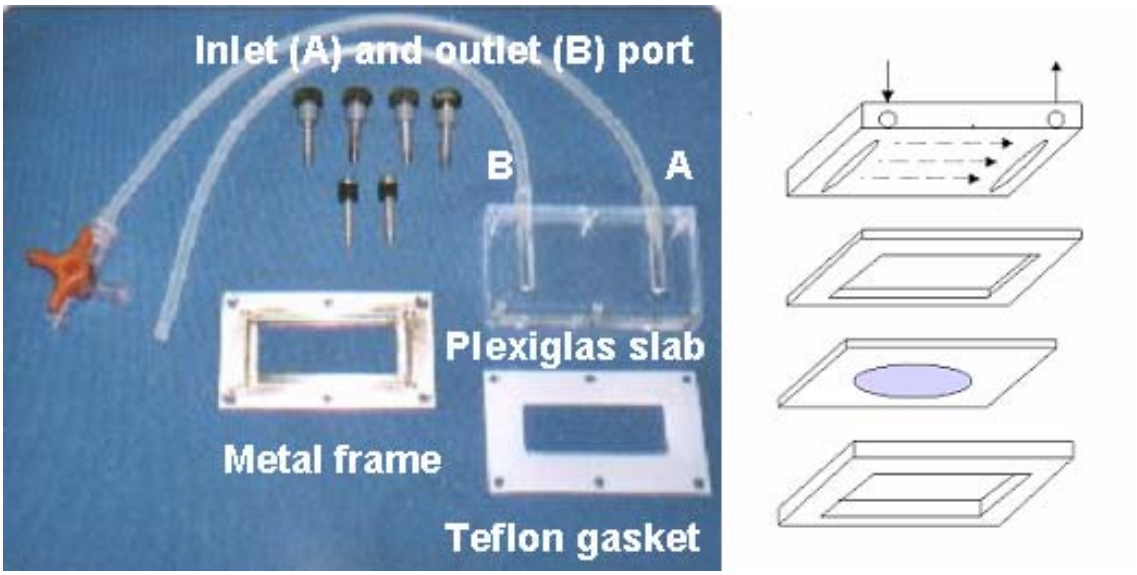

Figure 16. The chamber consists of plexiglas slab (transparency allows microscopy), a teflon gasket (50μm of thickness) determines the height of the chamber flow path between glass coverslips inserted onto a metal frame and a plexiglas slab. A syringe pump connected to the inlet port pumps blood across the gap through the chamber.

The bottom side of the parallel flow chamber was formed by fibrinogen/or collagen coated glass coverslips and a flow path height of 50 μm, determined by silicon gasket inserted between a metal frame and plexiglas slab. The assembled flow chamber was filled with phosphate buffered saline (pH 7.35).

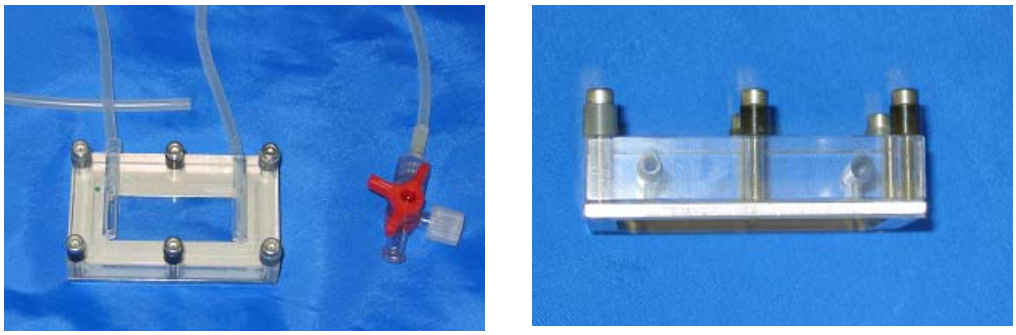

Figure 17. Assembled flow chamber.

According to Newtonian fluid axiom, a shear stress is constant and dependent upon the flow rate (Perfusor, B.Braun, Meslingen, Germany). Epifluorescence laser-scan microscope (Axiovert 100M, Carl-Zeiss, Jena, Germany) allowed real-time visualization of labelled platelets during perfusion through the chamber.

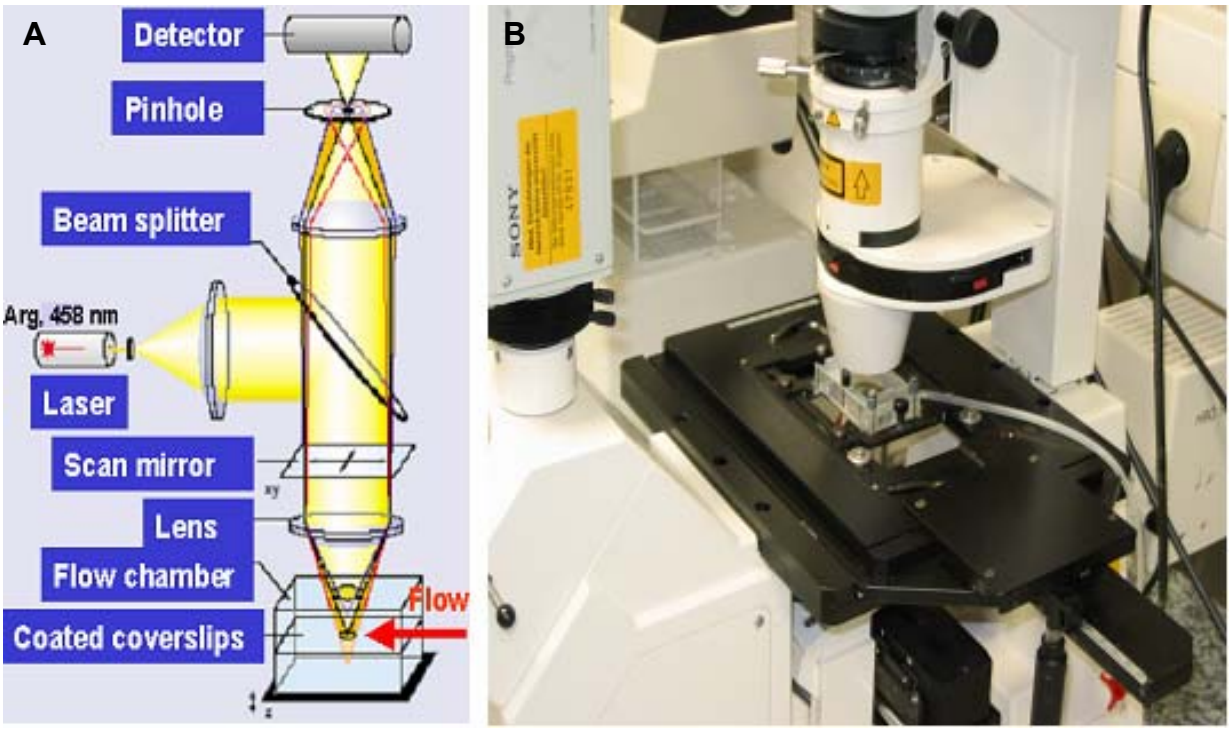

Figure 18. The schematic presentation of laser scan microscope (A) and assembled flow chamber mounted at microscope during one perfusion experiment (B).

To assess the time-course of platelet adhesion, a series of images (five images per series, 0,7 s per image) were recorded at 0, 1 and 5 minutes following perfusion. Time zero represents 15 second after start of the perfusion. Image analysis was performed using the ImageJ software (version 1.26t, NIH, USA). This program allows evaluation of platelet-surface interaction, consecutive aggregation and evaluation of thrombus generation at the defined area in each image. A single frame image corresponded to the area of 980x980 µm. The number of platelets exhibiting stable platelet adhesion to the surface was calculated as the number of platelets, which remain initial adhering position in the first and second image (time frame of 0.7 sec). Platelets were considered to move on the surface when exhibiting spatial displacement greater than the diameter of one platelet. To estimate motion of platelets, series of 5 images (time frame 0.7 sec) at one time point were recorded. Using ImageJ software, images were binarizied and a threshold was applied to

distinguish platelets from the background. The first two consecutive frames in series were superimposed using the logical “AND” function and the resulting image represented only the overlapping areas of a single platelet(s) at two different time points. Displacement of platelets was estimated as substraction of stable attached platelets from total adherent platelets in the first image.

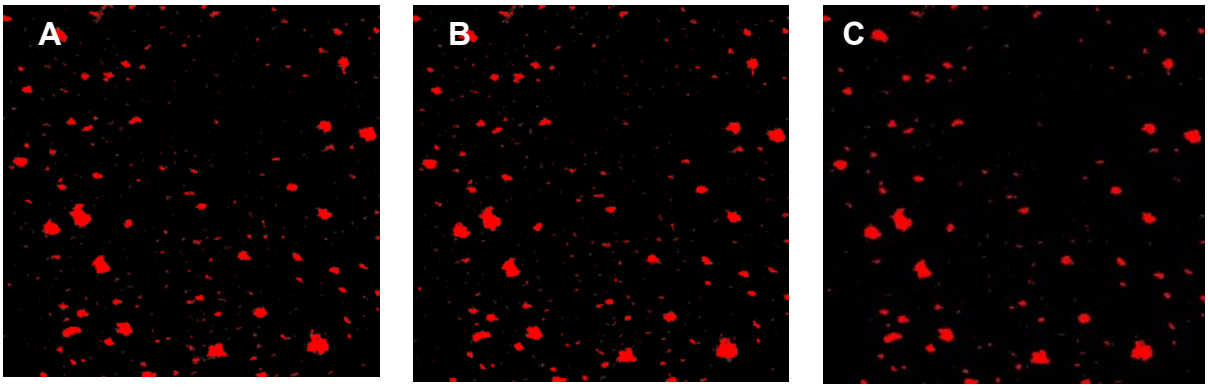

Figure 19. Representative microphotographs of platelet adhesion and aggregation onto immobilized collagen under arterial flow conditions (1500 s^{-1}, 5 min). A und B represents first and second image within one series (time frame 0.7 sec) and C microphotograph represents superimposing of A and B image reduced for movable platelets in respected recording time.

3.9. Specificity of platelet adhesion onto immobilized ligand(s)

Specificity of platelet adhesion onto immobilized fibrinogen was tested in flow experiments using glass coverslips co-coated with bovine serum albumin (BSA, 5µg/mm^2, n=3). Additionally, specific platelet adhesion onto immobilized fibrinogen was also tested in the presence of abciximab (c7E3, Centocor Inc. Leiden, Niederland). c7E3 Fab is a chimeric human/mouse Fab fragment derived from the murine monoclonal 7E3 antibody that blocks integrin αIIbβ3 (93). In three experiments, blood was additionally preincubated with abciximab (4µg/ml for 10 min, 37°C, n=3). Specificity of platelet adhesion onto immobilized collagen was tested using glass coverslips co-coated with bovine serum albumin (BSA, 5µg/mm^2, n=3). For control experiments three coverslips were coated with bovine serum albumin in final concentration of 5 µg/mm^2. Additional visualization of attached platelets was performed using scaning electron microscope. After perfusion experiments, fibrinogen- or collagen-coated coverslips were rinsed with phosphate buffer (10 ml)

and immediatelly fixed with paraformaldehyde. Respected parts of coverslips were coated with a very thin layer of gold and placed inside the microscope's vacuum column through the air-tight door.

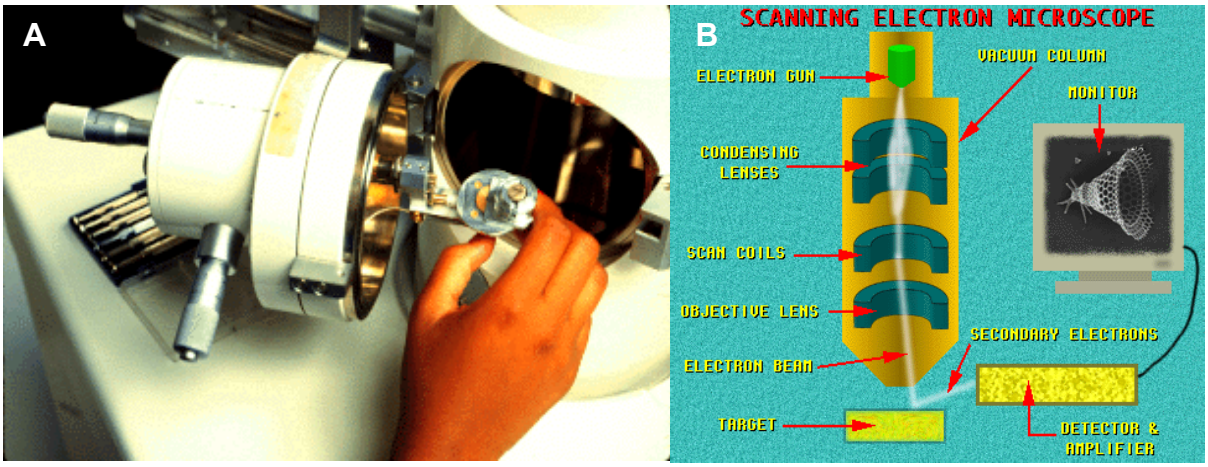

Figure 20. The electron scan microscope vacuum column (A) and schematic presentation (B).

After the air is pumped out of the column, an electron gun emits a beam of high-energy electrons. This beam travels downward through a series of magnetic lenses designed to focus the electrons to a very fine spot. Near the bottom, a set of scanning coils moves the focused beam back and forth across the specimen, row by row. As the electron beam hits each spot on the sample, secondary electrons are reflected from its surface. A detector counts these electrons and sends the signals to an amplifier. The final image is built up from the number of electrons emitted from each spot on the sample.

3.10. Estimation of activated platelets, experiments with PGE1

To test whether the experimental procedure activates platelets, in three experiments blood samples were drawn in duplicate. One sample of whole blood was directly collected into the tube supplemented with 10 μM PGE1 (47) and the second tube was collected according to the standard procedure. Blood containing PGE1 and standard treated blood were further processed according to the standard

experimental protocol. To test whether the number of activated platelets differs with regard to the exposition of shear rate, the expression of P-selectin (a marker of platelet activation, CD62) was assessed by FACS-analysis of probes before and after perfusion experiments (blood collected at outlet).

3.11. Interaction between platelet adhesion and factors of coagulation

In each participant factors of coagulation were assessed immediatelly after blood withdrawal. Plasma activity or concentration of measured parameters were carefully evaluated and related to platelet adhesion with regard to the shear rate and time of perfusion. Based on the correlation between related factor of coagulation and platelet adhesion, the influence of factors of plasmic hemostasis onto platelet adhesion and consecutive thrombus formation was evaluated.

3.12. Estimation of platelet adhesion onto immobilized fibrinogen and collagen with regard to the shear rate

Influence of shear rate onto platelet adhesion was measured in three flow chamber experiments (per each immobilized ligand) from three randomly selected subjects. Blood preparation and flow experiments were done as described previously. Flow experiments with immobilized fibrinogen were conducted under shear rates ranged from 50 s^{-1} to 10,000 s^{-1}. In experiments with immobilized collagen applied shear rates ranged from 50 s^{-1} to 25,000 s^{-1}. Perfusion time at each shear rate was 5 min. Further image acquisition and evaluation were described in the chapter 3.8.

3.13. Interaction between HPA-1 polymorphism of the β subunit of αIIbβ3 and the 807C/T polymorphism of the α subunit of integrin $\alpha_2\beta_1$: abciximab experiments

Blood from subjects with related genotype combination with regard to the HPA-1 and α2 807C/T polymorphisms was processed according to the standard experimental protocol. Flow experiments were conducted in the flow chamber with collagen-coated coverslips. The influence of the HPA-1 polymorphism of the β subunit of αIIbβ3 was assessed in experiments with blood preincubated with abciximab (4 μg/ mL, 10 min, 37°C).

4. Statistics

Data in text are given as mean values ± SD. Absolute fluorescence was expressed as arbitrary units (pixel units) and represents the sum of fluorescence of each thrombus or individual adherent platelet in one defined area. Only platelets, which showed stable adherence during one image series, were taken into calculation. Platelet adhesion was calculated using a logic function of the applied software (ImageJ) and represents stable platelet adhesion between first and second image. To reduce the influence of interindividual variation, data were normalized (absolute fluorescence recorded after five minute of perfusion was divided by recorded fluorescence after 1 minute) and expressed as a relative adhesion. The relative adhesion represents the increase of absolute fluorescence in function of time.

Differences between experimental groups were tested by Student's t-test (two-sided). Regression analyses were based on individual measurements using Spearman's rank correlation coefficient. Statistical analyses were performed using SPSS for Windows, version 6.0.1. *P*-value of less than 0.05 (two-sided) was used to indicate a significant difference.

The observed heterogeneity of the platelet adhesion rate represents the composite of methodical heterogeneity and a true biological heterogeneity. This may be expressed, using separate variation coefficients, by the equation $CV_{obs}^2 = CV_{biol}^2 + CV_{analyt}^2$ (94). To determine the contribution of the methodological variation (CV_{analyt}) onto observed variation (CV_{obs}), a representative sample was subdivided into ten samples. Each sample was then separately processed and analyzed. From these individual measurements, CV_{analyt} was calculated. True biological heterogeneity was then calculated as $CV_{biol} = (CV_{obs}^2 - Cv_{meth}^2)^{1/2}$.

5. Results

5.1. Baseline characteristics of the study participants

The baseline characteristics of the 88 study participants are summarized in the Table 4. Sixty-six (75%) were male and twenty-two (25%) were females. Mean age was 42 ± 12 years and did not significantly differ between males and females (41 ± 10 vs. 46 ± 12 years, p>0.05), respectively.

Table 4. Descriptive statistical data related to participant gender, age, hemostatic and biochemical parameters

Parameter	All	Female	Male	p
Age (years)	42 ± 12	46 ± 12	41 ± 10	ns
Hct (%)	40 ± 3	37 ± 3	41 ± 2	0.014
Fibrinogen (mg/dl)	255 ± 61	285 ± 71	231 ± 60	ns
FII (%)	130 ± 28	146 ± 20	124 ± 20	0.037
FV (%)	129 ± 25	141 ± 22	125 ± 27	ns
FVII (%)	120 ± 35	140 ± 46	116 ± 32	ns
FVIII (%)	174 ± 109	174 ± 109	119 ± 34	ns
VWF-Activity (%)	179 ± 105	179 ± 105	172 ± 103	ns
VWF-Ag (%)	164 ± 69	164 ± 69	133 ± 74	ns
FIX (%)	117 ± 22	126 ± 24	116 ± 27	ns
FX (%)	125 ± 28	138 ± 34	122 ± 24	ns
FXI (%)	103 ± 23	111 ± 25	101 ± 32	ns
FXII (%)	102 ± 16	113 ± 16	98 ± 8	0.029
FXIII (%)	118 ± 20	127 ± 16	108 ± 19	0.04
Protein C (%)	112 ± 22	120 ± 21	112 ± 16	ns
Protein S (%)	101 ± 19	92 ± 18	105 ± 15	ns
Plasminogen (%)	110 ± 13	110 ± 14	108 ± 12	ns
Triglyceride (mg/dl)	175 ± 97	156 ± 99	197 ± 110	ns
Cholesterol (mg/dl)	194 ± 30	199 ± 36	190 ± 37	ns
LDL (mg/dl)	118 ± 26	124 ± 28	108 ± 25	ns
HDL (mg/dl)	42 ± 13	52 ± 18	39 ± 10	ns
CRP (mg/dl)	0.5 ± 0.5	0.6 ± 0.5	0.5 ± 0.6	ns
Fe (µg/dl)	96 ± 65	60 ± 34	101 ± 49	ns
Ferritin (ng/ml)	31± 37	11 ± 7	38 ± 41	ns
Homocysteine (µM)	10± 3	9 ± 4	10.5 ± 4	ns
ATIII (%)	99 ± 9	104 ± 8	99 ± 7	ns
Platelet counts/nl	263 ± 52	304 ± 50	243 ± 51	0.019

p=level of statistical significance (two-side)
ns= not significanct

From Table 4 is evident that non-specific infection parameters, clinical chemistry, tests of hemostasis as well as factors of coagulation were within the normal range according to the international reference ranges.

With respect to the sex differences, males show significantly higher hematocrit values and females had higher platelets counts. A significant difference between males and females was observed for FXII. Even the gender distribution is independent from examined polymorphism, in further analysis, data were normalized (adjusted) and additional statistical analysis excluded any significant influence of noted parameters on interpretation of the results. Is it of importance to note that a value of tested parameters was distributed independently from examined receptor polymorphism and vice versa.

5.1.1. Frequency distribution of the HPA-1 polymorphism of the β subunit of integrin αIIbβ3 and of the α2 807C/T polymorphism of integrin α2β1

The frequency distribution of the HPA-1 polymorphism of the β subunit of integrin αIIbβ3 and of the α2 807C/T polymorphism of integrin α2β1 in our collective of healthy blood donors (3000 genotyped subjects) was in accordance with previously published data (35,38). Distribution of assessed polymorphisms is presented in Table 5 and it is representative for Caucasian Middle Europe population.

Table 5. The frequency distribution of the HPA-1 polymorphism of β subunit of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1 in our collective of healthy blood donors (n=3000).

Polymorphism		**Genotype**	
HPA-1	HPA1a/1a	HPA1a/1b	HPA1b/1b
n (%)	2280 (76%)	660 (22.2%)	54 (1.8%)
807 CT	807 CC	807CT	807TT
n (%)	1110 (37.2%)	1350 (45.6%)	510 (17.2%)

A careful selection of subjects with related genotypes was conducted according to the criteria listed in the Methods. Finally, we enrolled 88 healthy blood donors. Table 6 shows the allele distribution of HPA-1 polymorphism of β subunit of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1 in the selected population.

Table 6. Descriptive statistical data related to the HPA-1 polymorphism of β subunit of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1 of all subjects enrolled into study (n=88).

Polymorphism		**Genotype**	
HPA-1	HPA1a/1a	HPA1a/1b	HPA1b/1b
n	54	22	12
α2 807CT	807CC	807CT	807TT
n	26	42	20

Distribution of the assessed polymorphism as well as their combination among subjects enrolled into the study were presented in Table 7.

Table 7. Incidence of combined mutation (HPA-1 and α2 807C/T) of the study population.

Combined mutation	n=88
HPA-1a/1a - α2 807CC*	22
HPA-1a/1a - α2 807CT	19
HPA-1a/1a - α2 807TT	13
HPA-1a/1b - α2 807CC	6
HPA-1a/1b - α2 807CT	16
HPA-1a/1b - α2 807TT	1
HPA-1b/1b - α2 807CC	3
HPA-1b/1b- α2 807CT	4
HPA-1b/1b- α2 807TT**	4

*"wild type", ** "prothrombic type"

The influence of the described platelet receptor polymorphisms on platelet adhesion in flow chamber experiments was evaluated in 49 subjects (25 subjects with HPA-1a/1a, twelve with HPA-1a/1b and twelve with 1b1b genotype). According to α2 807CT polymorphism 12 subjects were with α2 807 CC genotype, twenty-five with α2 807 CT and twelve with α2 807 TT genotype.

5.2. Perfusion experiments

During perfusion, a significant linear increase in platelet adhesion onto fibrinogen- and collagen-coated surfaces, in function of time and in shear rates, was observed.

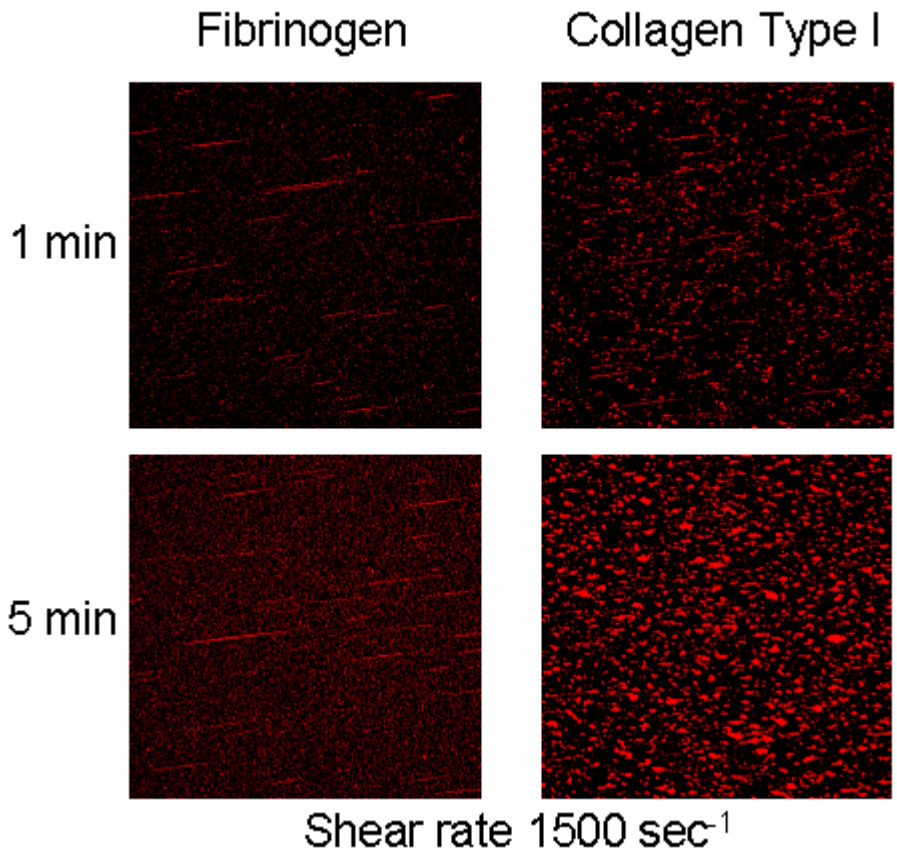

Figure 21. Microphotographs of platelets adhesion onto immobilized fibrinogen and collagen after one and five minutes of perfusion at shear rate of 1500 s^{-1}.

Figure 21 shows a representative microphotographs of platelet adhesion onto immobilized fibrinogen and collagen after one and five minute of perfusion. A significant quantitative and qualitative difference with regard to platelet adhesion onto different ligands was evident. While platelet adhesion to fibrinogen revealed a monolayer covering the formation of small thrombi was already evident after 1 min of perfusion over immobilized collagen.

In 49 individual experiments, mean platelet adhesion onto fibrinogen-coated coverslips at a shear rate of 50 s^{-1}, increased from 0 to 5 minutes 3.47 fold (from 3006 ± 1936 AU to 10449 ± 4850 AU), at 500 s^{-1} 9.54-fold (from 4074 ± 1986 AU to 38900 ± 13290 AU) and at 1500 s^{-1} platelet adhesion increased 14.5 fold (from 3660 AU ± 1917 U to 54500 U ± 22400 AU), respectively (Fig. 22A).

Similarly, a significant increase as function of time and shear rate was also observed during perfusion onto collagen-coated surfaces (Fig. 22B). At a shear rate of 50 s^{-1}, a platelet adhesion onto collagen-coated coverslips increased from 0 to 5 min 4.8 fold (from 1926 ± 1569 AU to 9296 ± 3000 AU), at 500 s^{-1} 13.1-fold (from 1843 ± 1696 AU to 24318 ± 9789 AU) and at 1500 s^{-1} platelet adhesion increased 27 fold (from 1855 AU ± 1463 AU to 50138 U ± 19700 AU), respectively.

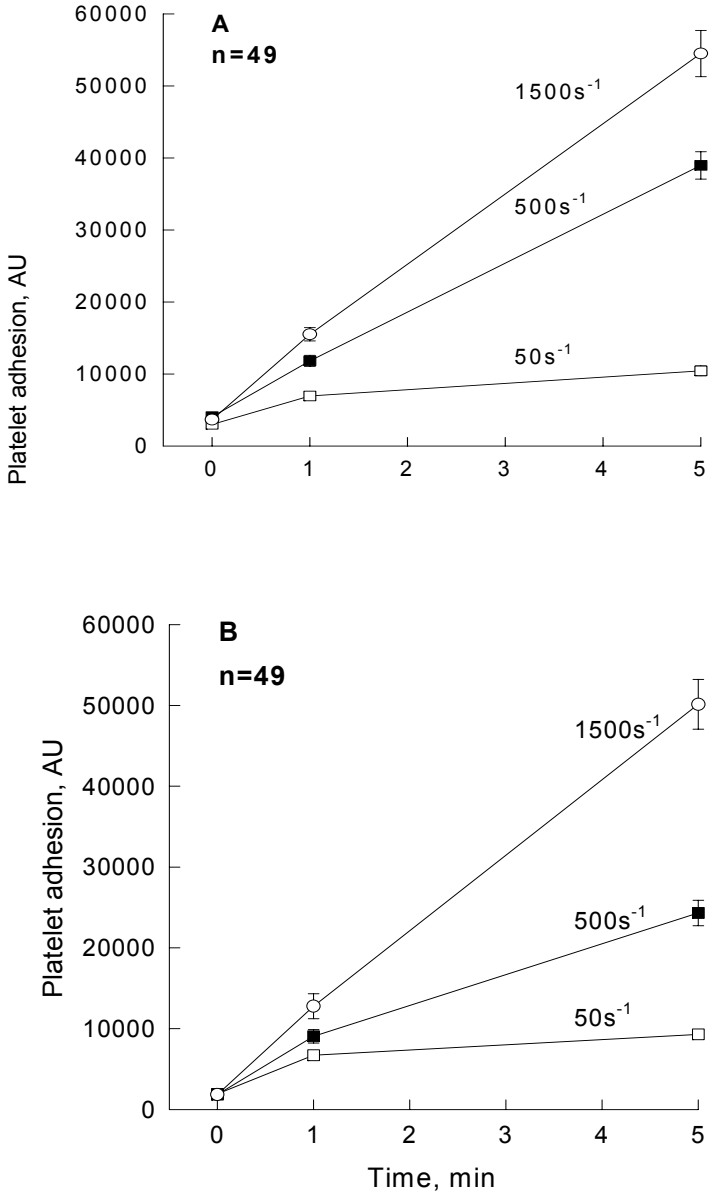

Figure 22. Platelet adhesion onto immobilized fibrinogen (A) and collagen (B) during perfusion under different shear rates. n represents the number of tested subjects. Per each subject 3 flow experiments were conducted (50s^{-1}, 500 s^{-1} and 1500 s^{-1}). At each time point of perfusion (15 sec, 1min and 5 min) a stack of 5 images was collected and analyzed. Finally 294 perfusion experiments were conducted and 1470 images were analyzed.

The coefficient of variation (CV) showed shear rate and time dependency during perfusion. The highest CV was observed at venous flow and declined with increase of shear rate. Simultaneously, at all shear rates CV were significantly higher at start of perfusion (15 sec) compared to the end of it (5 min) 54±8 vs. 40±6 for fibrinogen and 83±7 vs. 37±4 for collagen, respectively.

5.2.1. Relationship between platelet adhesion and shear rate

To tested the relationship between platelet adhesion onto respected ligands and shear rate, blood from three different blood donors, independent of HPA-1 polymorphism of the β subunits of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1, was exposed to different shear rates.

The initial shear rate was 50s^{-1}. In experiments with fibrinogen-coated coverslips, shear stress was ranged between 50 s^{-1} and 7500s^{-1}. Fig. 23 shows that platelet adhesion onto immobilized fibrinogen correlated positively to the shear rate up to the 1500s^{-1}. The estimated plateau was reached at 1800 s^{-1}. At shear rates above 2000 s^{-1} platelet adhesion showed an inverse correlation with a shear rate, and reached a minimum adhesion at 7500 s^{-1}. Platelet adhesion onto immobilized collagen correlated with shear rate positively up to the shear rates of 9000 s^{-1}. Above shear rates of 9000 s^{-1}, platelet adhesion start to decline.

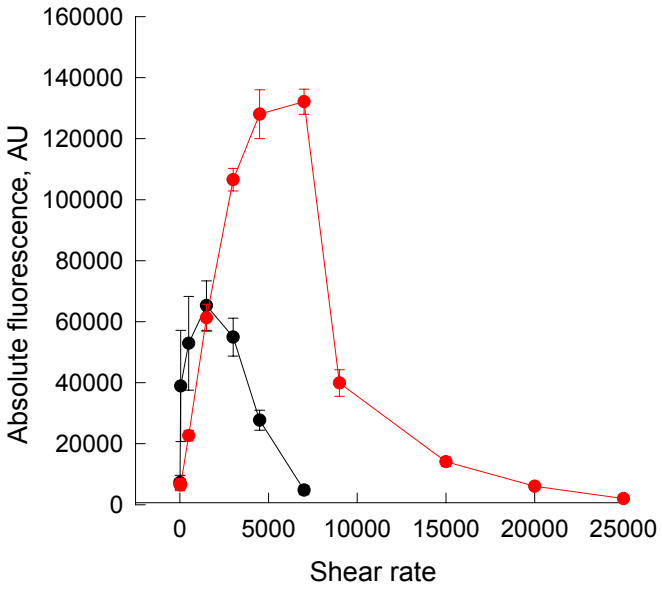

Figure 23. Platelets adhesion onto immobilized fibrinogen (black circle) and immobilized collagen (red circle), each n=3, in function of shear rates after 5 min of perfusion. Finally 51 perfusion experiments were conducted and 255 images were analyzed.

5.2.2. Initial and late platelet adhesion

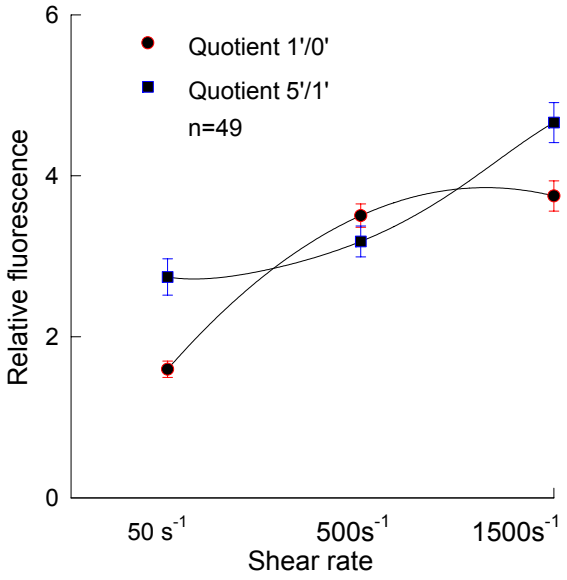

Figure 24. Relationship between initial and late platelet adhesion after 5 min perfusion onto immobilized fibrinogen (n represents the number of tested subjects). Per each subject 3 flow experiments were conducted ($50s^{-1}$, 500 s^{-1} and 1500 s^{-1}) and one stack of 5 images (at 15 sec, 1min and at 5 min of perfusion) was collected and analyzed. Finally 147 perfusion experiments were conducted and 735 images were analyzed.

The increase of stable adherent platelet was evaluated in regard of perfusion time. Dynamic of platelet adhesion between 15 second and 1 min of perfusion represented initial platelet adhesion and dynamic of platelet stable adhesion between the first and fifth min was designed as late platelet adhesion. Figures 24 and 25 represent the relationship between initial and late platelet adhesion onto immobilized fibrinogen and collagen in regard of shear rate and time. The dynamic of stable platelet adhesion shows a significant quantitative difference with regard to the applied immobilized ligands and shear rates. Under low flow conditions ($50s^{-1}$), the initial platelet adhesion is similar for both ligands but a late platelet adhesion onto collagen is significantly higher, compared to fibrinogen. Under arterial flow conditions (500 and 1500 s^{-1}) adhesion of platelet (initial and late phase) were significantly higher when collagen was applied. Careful analysis of both initial and late platelet adhesion onto immobilized fibrinogen (Fig. 25) indicated that the majority of platelets, which within first 45 sec under $500s^{-1}$ adhere onto immobilized fibrinogen, also remain stable adherent. The difference between initial and late dynamic of platelet adhesion at 1500 s^{-1} indicated that under high shear rate some platelets, after initial contact and tethering over fibrinogen, could not reach stable adhesion. Shear force started to be a dominant, compared to the adhesion force between fibrinogen receptor and fibrinogen. Further increase of shear rate (up to 1800 s^{-1}) additionally reduces initial adhesion, compared to the late adhesion. At shear stress exceeding 1800 s^{-1}, platelet

adhesion onto immobilized fibrinogen was inverse. A similar phenomenon was observed during platelet adhesion onto immobilized collagen. The majority of platelets, which within first 45 sec under 1500s^{-1} adhere onto immobilized collagen, also remain stable adherent

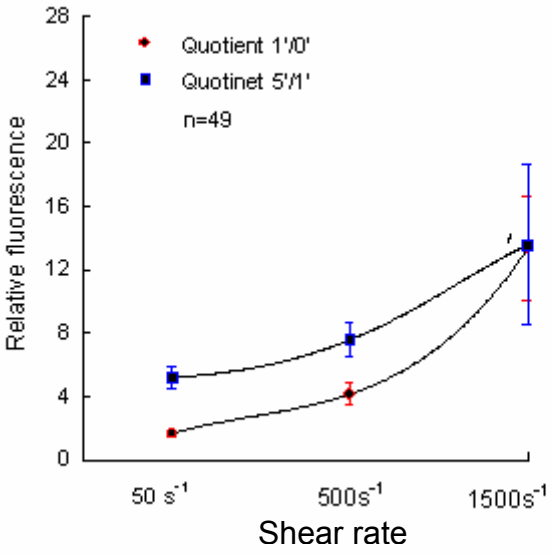

Figure 25. Relationship between initial and late platelet adhesion onto immobilized collagen after 5 min of perfusion (n represents the number of tested subjects). Per each subject 3 flow experiments were conducted (50s^{-1}, 500 s^{-1} and 1500 s^{-1}) and one stack of 5 images (at 15 sec, 1min and at 5 min of perfusion) was collected and analyzed. Finally 147 perfusion experiments were conducted and 735 images were analyzed.

5.3. Specificity of binding of platelets to immobilized fibrinogen and collagen

Binding specificity of platelets to immobilized fibrinogen was tested bi-directionally with two additional experimental designs. In three experiments blood was incubated with abciximab (4µg/ml for 60 min, 37°C). Perfusion of preincubated blood over fibrinogen-coated coverslips did not show a significant platelet adherence (absolute fluorescence of stable adherent platelet: 168 ± 35 AU vs. 53000 ± 19000 AU in control experiments, $p<0.05$).

Similarly, in experiments with perfusion of labelled platelets in whole blood using BSA-coated glass coverslips, no significant adhesion compared to fibrinogen and/or collagen-coated coverslips was found. Figure 21 shows representative micro-photographs of experiments conducted with standard experimental design. In Figure 26 experiments with abciximab (c7E3) (A) and experiments with BSA-coated glass coverslips (B) are presented.

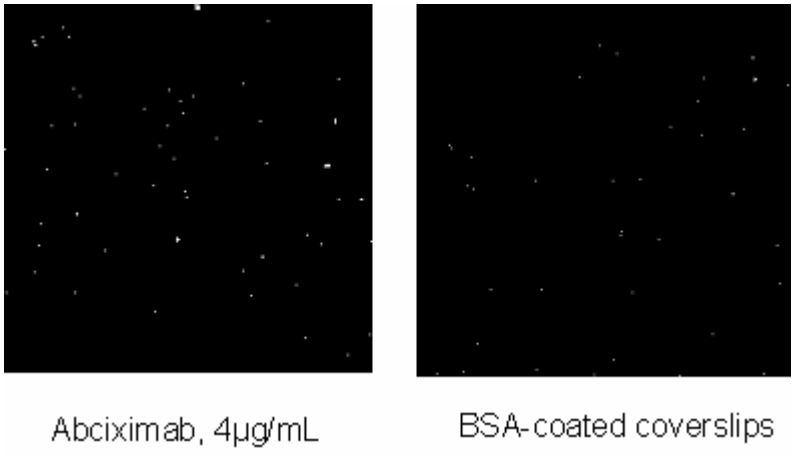

Figure 26. Specificity of platelet adhesion onto immobilized fibrinogen was tested using blood preincubated with abciximab (4µg/ml for 10 min, 37°C, n=3) and using glass coverslips co-coated with bovine serum albumin (BSA, 5µg/mm^2, n=3).Specificity of platelet adhesion onto immobilized collagen was tested using glass coverslips co-coated with bovine serum albumin (BSA, 5µg/mm^2, n=3).

Additional analysis by electron scanning microscopy showed a high specificity of platelet adhesion onto the related ligand (Figure 27).

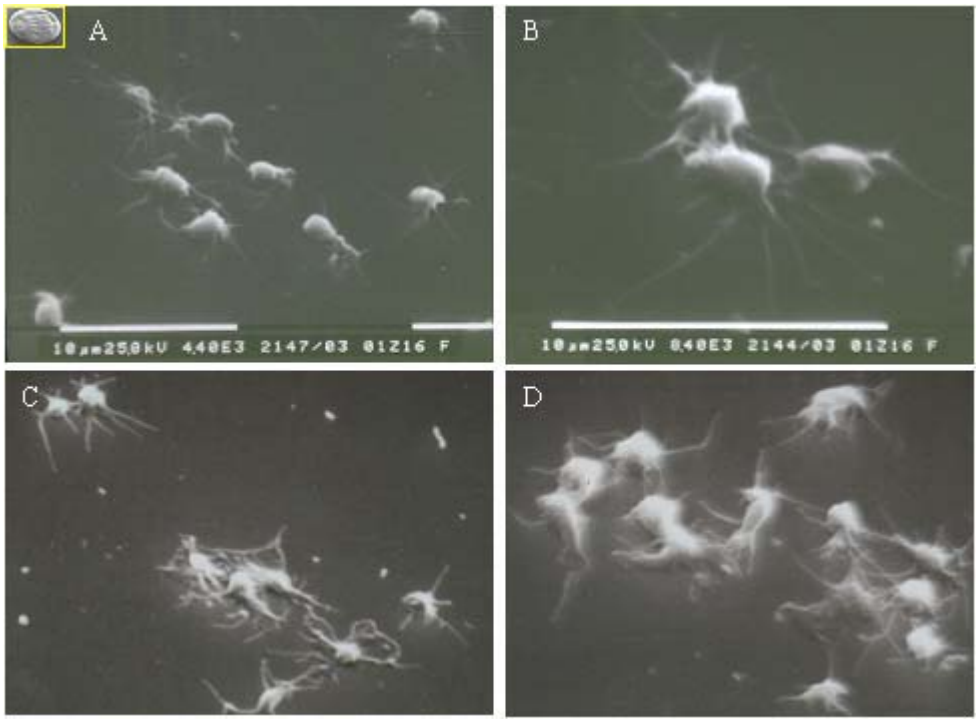

Figure 27. Scanning electron microscopy attitudes specificity of platelet adhesion onto immobilized fibrinogen (A,B) and collagen (C,D) under experimental conditions described in Methods.

The Figure 27 (A and B) shows a monolayer of adherent platelets onto immobilized fibrinogen after 1 min of perfusion (1500s^{-1}). There is the evidence that already in the

initial phase of adhesion activating platelets loose their spherical form and pseudopodia appear. In the yellow frame, in the left upper corner of figure A, the electron microscopy image of resting platelet is present. Figure C and D show platelet adhesion onto immobilized collagen after 15 sec of perfusion ($1500s^{-1}$). A significant difference in platelet morphology in regard to the different ligands was evident.

5.4. Resting versus activated platelets

To test whether platelet are activated during experimentation three additional experiments were conducted. Whole blood was aliquoted and one part was preincubated with a strong inhibitor of platelet activation (10 µM PGE1, 37°C,15 min). Pre-treated and control blood was further processed following standard experimental procedures. A significant difference in platelet adhesion onto immobilized fibrinogen between PGE1 pre-treated and untreated blood was not observed, independent of shear rate and perfusion time. Absolute fluorescence after 5 minutes of perfusion at 500 s^{-1} was 17500 ± 1397 AU vs. 22400 ± 4657 AU and at $1500s^{-1}$ 46000 ± 8860 AU vs. 57000 ± 11130 AU, respectively ($p>0.05$). To test whether the number of activated platelets differs with regard to the exposition to shear rate, expression of P-selectin (marker of platelet activation, CD62) a FACS-analysis before and after experiment (blood collected at outlet) was performed. Samples were incubated with

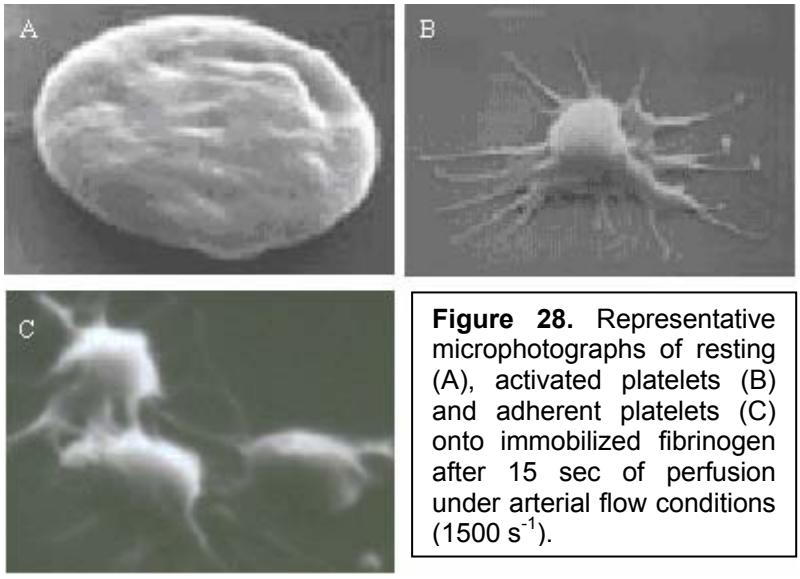

Figure 28. Representative microphotographs of resting (A), activated platelets (B) and adherent platelets (C) onto immobilized fibrinogen after 15 sec of perfusion under arterial flow conditions (1500 s^{-1}).

FITC-CD62 (P-selectin, Serotec GmbH, Germany) and analyzed with FACS. Percent of activated platelet was 22% and 20% in experiments with 500 s^{-1} and 1500 s^{-1}, respectively, $p>0.05$. Three fold increase in shear rate did not have a significant

influence on platelet activation. This surprisingly observation could be explained by very short chamber transition time and/or short exposition to related shear rate (max. 2 seconds). A similar allotment of activated platelets between blood analyzed before perfusion and blood collected after perfusion (under 1500 s^{-1}) did not show a significant difference (22%). Additional analysis by electron scanning microscopy indicated that over 80% of platelets prior to perfusion and in blood collected after perfusion were in the resting state.

5.5. Flow experiments and HPA-1 polymorphism of integrin αIIbβ3

To test the influence of the HPA-1 polymorphism of integrin αIIbβ3 on platelet adhesion during perfusion over fibrinogen-coated coverslips blood obtained from 49 blood donors of known HPA-1 genotype (25 with 1a1a, 12 with 1a1b and 12 with 1b1b genotype) was used in our flow experiments.

Platelet adhesion onto immobilized fibrinogen under venous and arterial flow ($50s^{-1}$-$1500s^{-1}$) was analyzed after 1min and 5 min of perfusion and expressed as absolute fluorescence in arbitrary units (AU) (Figures 29 A and B show platelet adhesion onto

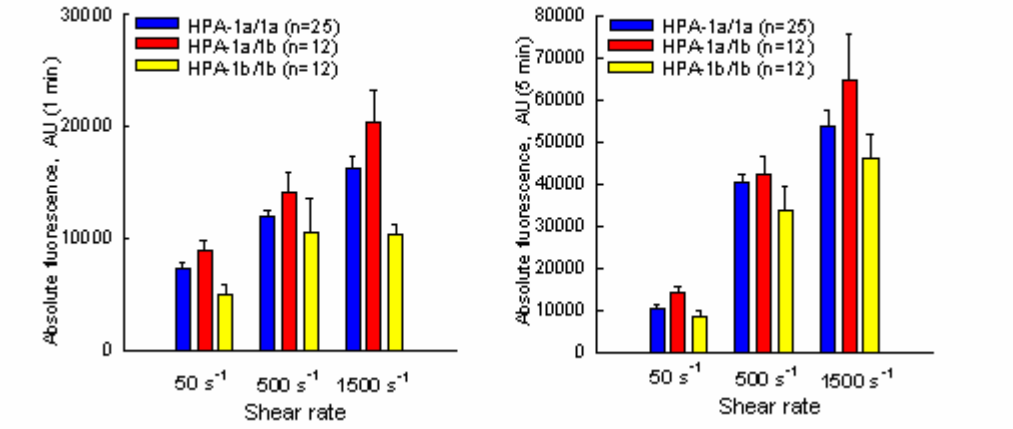

Figure 29. Relationship between platelet adhesion after 1 min (A) and 5 min (B) of perfusion over immobilized fibrinogen under different shear rates ($50s^{-1}$, $500s^{-1}$ and $1500s^{-1}$) with regard to the HPA-1 polymorphism (each bar represents mean value ± SE).

Interestingly, HPA-1b/1b platelets did not show enhanced adhesion capacity compared to the other HPA-1 genotypes. By contrast, platelets with HPA-1a/1a and HPA-1a/1b genotypes showed higher adhesion activity compared to platelets with the HPA-1b/1b genotype. The significantly higher adhesion activity of platelets with

the HPA-1a/1a genotype was observed after 1 min of perfusion at $50s^{-1}$ (HPA-1a/1a 7402 ± 2542 AU, HPA-1a/1b 8910 ± 2896 AU and HPA-1b/1b 4947 ± 2699 AU) and at $1500s^{-1}$ (HPA-1a/1a 16324 ± 5515 AU, HPA-1a/1b 20474 ± 8547 AU and HPA-1b/1b 10384 ± 2516 AU), $p<0.05$. A similar trend was also observed at $500s^{-1}$ but without statistical significance.

Interestingly, after 5 min of perfusion, a significant difference between platelet adhesion activities with regard to the HPA-1 polymorphism was found only under venous flow (50 s^{-1}) between HPA-1a/1b and HPA-1b/1b genotypes (14224 ± 4718 AU vs. 8436 ± 4660 AU, $p<0.05$). Even higher platelet adhesion activity of HPA-1a/1a and HPA-1a/1b genotype was also observed under arterial flow, no significant difference was observed neither at 500 s^{-1} (HPA-1a/1a 40319 ± 11255 AU, HPA-1a/1b 42424 ± 12344 AU and HPA-1b/1b 33581 ± 18756 AU) nor at $1500s^{-1}$ (HPA-1a/1a 53578 ± 19251 AU, HPA-1a/1b 64548 ± 32963 AU and HPA-1b/1b 46154 ± 17221 AU), $p>0.05$.

Taking into consideration individual and methodical variation, our experimental system was validated. Analytical variation (CV_{analyt}), tested through 10 repeated measurements at $1500s^{-1}$ (5 min of perfusion) of one individual sample, indicated that our analytical variation was 15%; a mean observed variation at $500s^{-1}$ was 0.36 and at 1500 s^{-1} was 0.40, respectively. Observed variation was corrected to CV_{analyt} and real biological variation (CV_{biol}) at $500s^{-1}$ and 1500 s^{-1} in all examined subjects was 0.32 and 0.37 ($p>0.05$), respectively. Related to the polymorphism, no significant difference in CV_{biol} between different genotypes was observed, neither at $500s^{-1}$ nor at $1500s^{-1}$, $p>0.05$. To omit the influence of individual and methodical variation, data were normalized and expressed as a "relative adhesion" (see the Statistics). According to the normalized data, an adhesion rate (adhesion between 15 sec and 5 min of perfusion) of platelets with HPA-1b/1b genotype was significantly higher in relation to HPA-1a/1a genotype (Fig. 30A). A relative adhesion at shear rate of $1500s^{-1}$ was 23.17 ± 9.9 for 1b/1b genotype versus 14.8 ± 6.5 for 1a/1a genotype, $p<0.05$, respectively. A marginal statistical significance was also observed at shear rate of $500s^{-1}$ (HPA-1b/1b 14.45 ± 6.4 vs. 10.66 ± 5.0 for HPA-1a/1a, $p=0.07$).

The time course of platelet adhesion onto immobilized fibrinogen, in regard to the shear rate and genotype was further analyzed. Thrombus growth was analyzed within two different periods of perfusion, at “initial phase” (Fig. 30B) and at “late phase” (Fig. 31). The time frame between start of perfusion (15 sec, point zero) and

one min of perfusion was designated as initial phase and the time frame between min 1 and min 5 as late phase. A statistically significant difference in platelet adhesion with regard to the HPA-1 polymorphism was observed. Surprisingly, the observed difference between different genotype was already present in the initial phase of platelet adhesion. The relative adhesion at shear stress of $500s^{-1}$ was 5.62 ± 2.4 for HPA-1b/1b versus 4.32 ± 1.25 for HPA-1a/1a genotype, $p<0.05$, respectively. At 500 s^{-1}, a similar trend was observed but without statistical significance (HPA-1b/1b 4.22 ± 2.5 vs. 3.13 ± 1.34 for HPA-1a/1a, $p>0.05$). In the

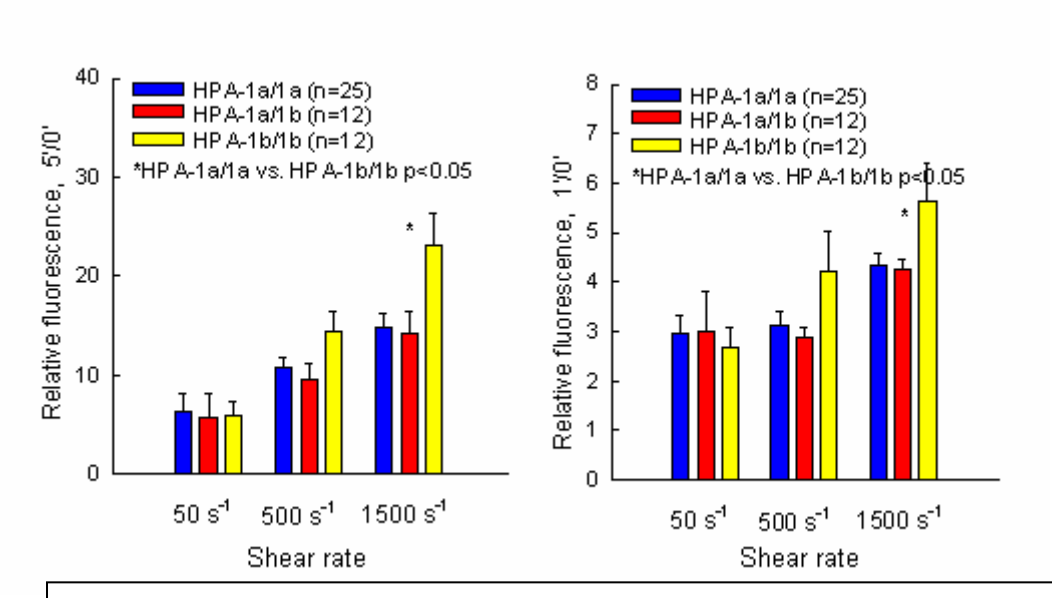

Figure 30. Increase of platelet adhesion onto immobilized fibrinogen under different under different shear rates ($50s^{-1}$, $500s^{-1}$ and $1500s^{-1}$) with regard to the HPA-1 polymorphism within 5 min (Fig. A) and within 1 min (Fig. B, initial phase) of perfusion (each bar represents mean value ± SE).

late phase (quotient between min 1 and min 5), a relative adhesion at shear rate of $1500s^{-1}$ was 4.42 ± 1.21 vs. 3.35 ± 1.17 for 1a/1a genotype, $p<0.05$, respectively. A significance was also observed at venous shear rate 2.06 ± 1.32 for HPA-1b/1b vs. 1.39 ± 0.32 for HPA1a/1a, $p>0.05$. At 500 s^{-1}, a similar trend was observed but without statistical significance (HPA-1b/1b 3.72 ± 1.1 vs. 3.29 ± 0.69 for HPA-1a/1a. Between heterozygous (HPA-1a/1b) and homozygous (HPA-1a/1a) genotype, no significant difference in platelet relative adhesion was observed.

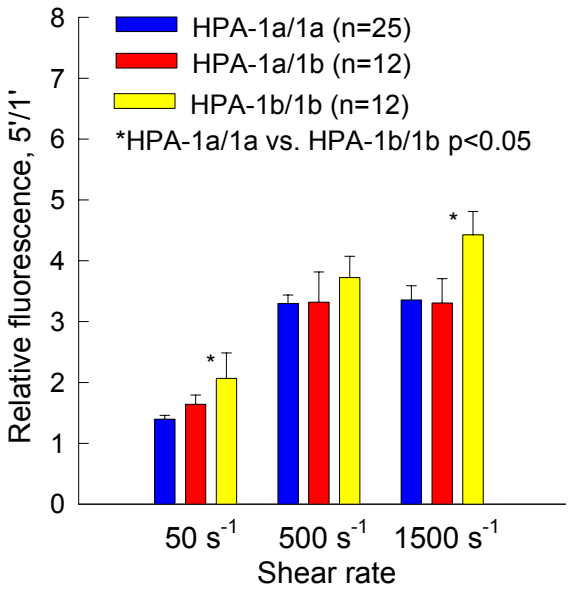

Figure 31. Increase of platelet adhesion onto immobilized fibrinogen under under different shear rates ($50s^{-1}$, $500s^{-1}$ and $1500s^{-1}$) with regard to the HPA-1 polymorphism between 1 and 5 min of perfusion ("late platelet adhesion"). Each bar represents mean value ± SE.

5.6. Flow experiments and α2 807C/T polymorphism of integrin α2β1

Similarly as in fibrinogen experiments, during perfusion through a rectangular chamber platelet adhesion linearly increased with regard to the exposition time at each tested shear rate. At a shear rate of $50s^{-1}$, platelet adhesion from 0 (15 sec of perfusion) to 5 minutes increased in average 8.3 fold (from 1926 ± 1564 AU to 9296 ± 3000 AU), at 500 s^{-1} 23.2 fold (from 1843 ± 1696 AU to 24318 ± 9389 AU), and at 1500 s^{-1} 38.3 fold (from 1859 ± 1463 AU to 50135 ± 19740 AU), respectively.

To test the influence of α2 807C/T polymorphism of integrin α2β1 on the platelet adhesion during perfusion over collagen-coated coverslips, 12 blood donors with 807CC, 25 with 807CT and 12 with 807TT genotype were included in our study.

After 5 minutes of perfusion, platelet adhesion ,expressed as absolute fluorescence, duffers significantly related to the α2 807C/T polymorphism (Fig. 32) at 500 s^{-1} (807CC 21714 ± 7786 AU vs. 807TT 32605 ± 11568 AU), $p<0.05$. Under a flow rate of $1500s^{-1}$, a similar trend was observed (807CC 47480 ± 19223 vs. 807TT 58582 ± 21794), $p=0.08$.

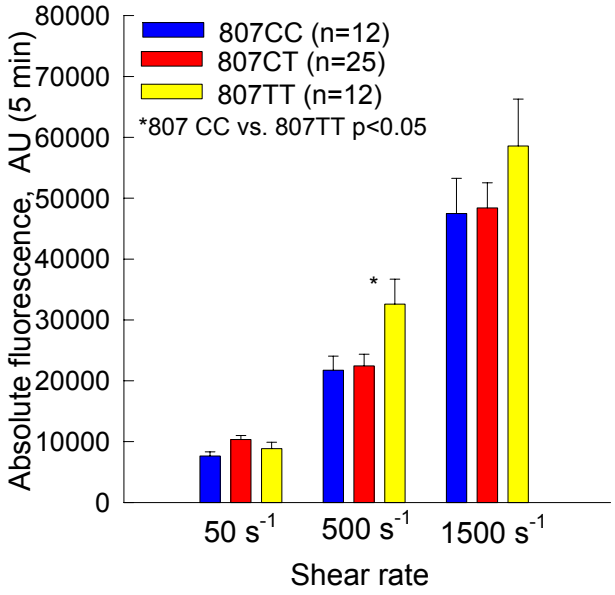

Figure 32. Platelet adhesion onto immobilized collagen under different flow conditions (shear rate between 50 s^{-1} and 1500 s^{-1}) in regard to the α2 807C/T polymorphism of integrin α2β1 (each bar represents mean value ± SE).

Under venous hemodynamic condition, no significant differences between platelet adhesion onto immobilized collagen with the regard to the α2 807C/T polymorphism were observed. A careful analysis of the time course of platelet adhesion onto immobilized collagen indicated that platelet adhesion, after 15 sec of perfusion (Fig. 33 A), was significantly modulated through the α2 807C/T polymorphism. Platelets with the T-allele (807CT and 807TT genotype) showed significantly higher adhesion activity compared to 807CC genotype. At 50s^{-1}, 807CC reached 1081 ± 589 AU vs. 2111 ±1635 of 807CT (p=0.05) and vs. 2579 ± 1959 AU of 807TT (p=0.02). Under arterial flow (500s^{-1}), a higher adhesion activity of platelets with 807CT or 807TT genotype compared to 807 CC genotype, was also evident (CC 909 ± 663 AU vs. 2289 ± 845 AU (p=0.037) and vs. 2012 ± 1384 (p=0.033), respectively. At 1500 s^{-1}, a similar trend was observed but without statistical significance.

Figures 33 A and B show platelet adhesion onto immobilized collagen within the first min of perfusion. Similarly as after 15 sec of perfusion, platelets with 807CT or 807TT genotype, showed a significantly higher adhesion activity compared to the platelets with 807CC genotype.

Interestingly, evaluation of normalized data did not show a significant difference with regard to the α2 807C/T polymorphism. This could indicate that platelet adhesion

onto collagen is modulated through the α2 807C/T polymorphism of integrin α2β1 but this polymorphism plays a secondary role in thrombus growth.

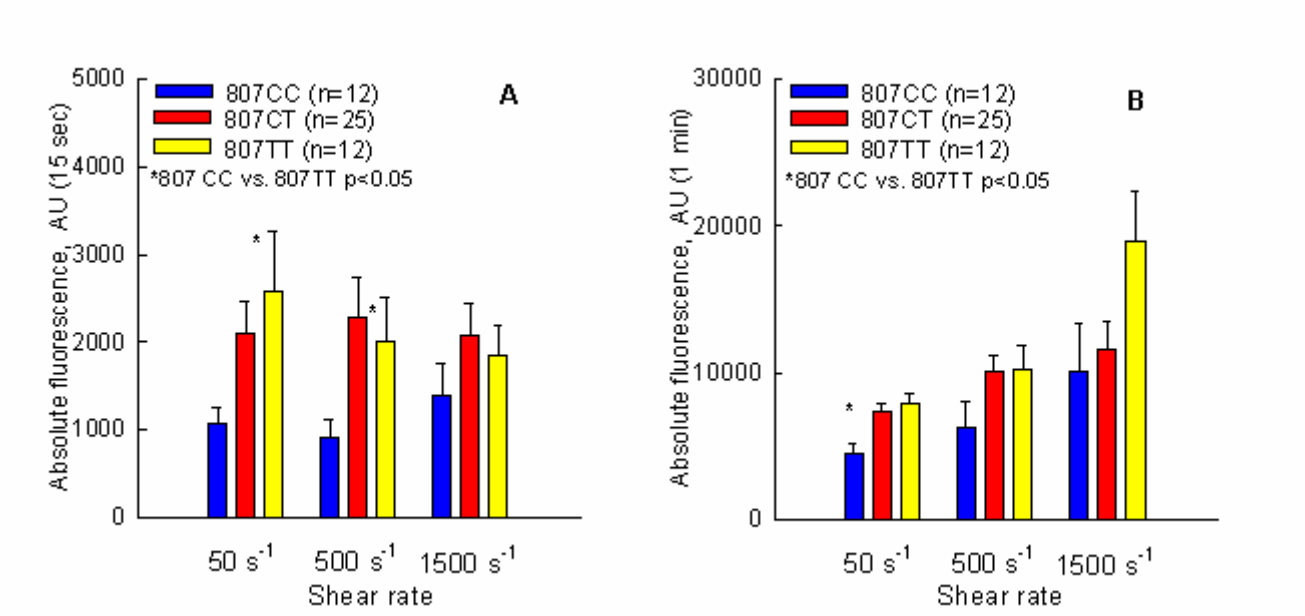

Figure 33. Initial platelet adhesion (within first min) onto immobilized collagen under different flow conditions (shear rate between 50 s^{-1} and 1500 s^{-1}) in regard to the 807 C/T polymorphism of integrin α2β1. Each bar represents mean value ± SE.

5.7. Influence of integrin αIIbβ3 onto platelet collagen adhesion and consecutive thrombus growth

To quantify a general contribution of integrin αIIbβ3 on thrombus growth, after initial adhesion onto immobilized collagen, additional experiments with abciximab were performed. Figure 34 shows that thrombus growth between min 1 and min 5 of perfusion is significantly inhibited after preincubation of blood with abciximab as a specific αIIbβ3 antagonist. The results indicated that approximately 30% of thrombus growth was modulated through integrin αIIbβ3. This contribution increases from 20% at venous flow condition to 35% under arterial flow (Fig. 34). This could implicate that HPA-1 polymorphism, under related conditions, influences a platelet adhesion onto immobilized collagen and consecutive thrombus growth. To test this hypothesis, subjects with respected genotype combination (HPA-1 and 807CT polymorphism)

were selected and flow experiments using immobilized collagen were conducted (Fig. 35).

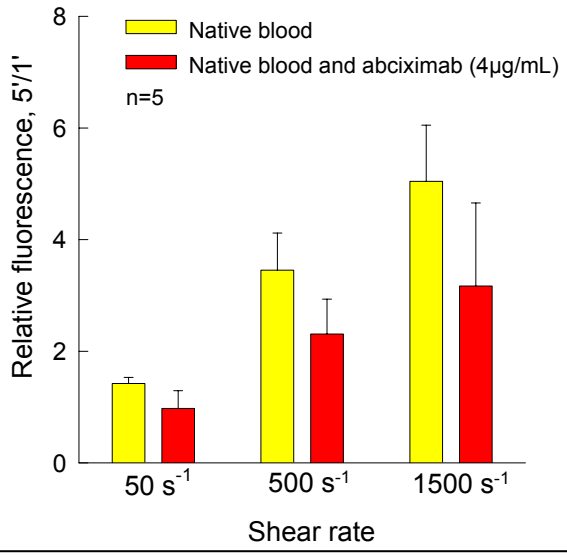

Figure 34. Preincubation of blood with abciximab reduces thrombus growth after initial adhesion onto immobilized collagen. The observed inhibitory effect of abciximab is independent of flow rate.

Figure 35 indicates that HPA-1 polymorphism of β3 subunit of integrin αIIbβ3 enhanced platelet adhesion and consecutive thrombus growth during perfusion experiments. According to our results, contribution of HPA-1b/1b genotype under arterial flow reached maximum after 1 min of perfusion (73%).

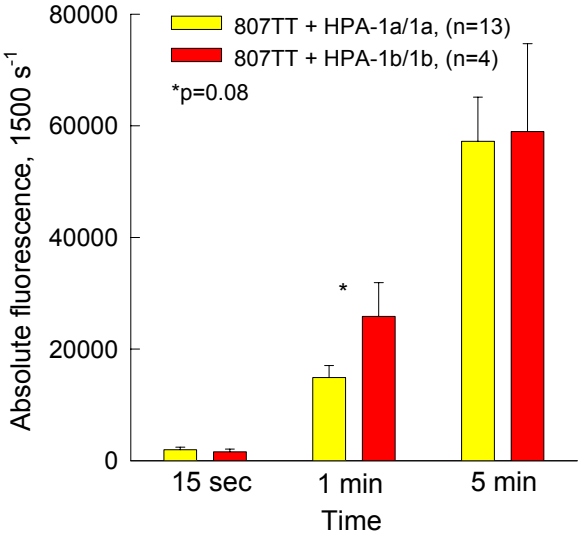

Figure 35. HPA-1 polymorphism modulates thrombus growth after initial adhesion onto immobilized collagen.

5.8. Influence of combined HPA-1 polymorphism of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1 on platelet adhesion

Synergism of the combination of HPA-1 and α2 807C/T polymorphism on platelet adhesion was evaluated using blood from carriers with defined combination of polymorphisms. Of special interest was to test the relationship between critical (prothrombotic) vs. uncritical "wildtype" variants of αIIbβ3 and α2β1, respectively. Blood preparation and flow experiments using collagen as specific ligand were outlined in the Methods. Critical genotype combination (HPA-1b/1b and α2 807TT) showed a higher platelet adhesion at each time point of perfusion compared to the wild genotype combination (HPA-1a/1a and α2 807 CC). Although statistical significance was not reached (p=0.8, Fig. 36), our results clearly indicate that platelet adhesion could be enhanced by critical prothrombotic genotype combination in the range from 20%-106% (Table 8). A maximal influence was observed after one min of perfusion.

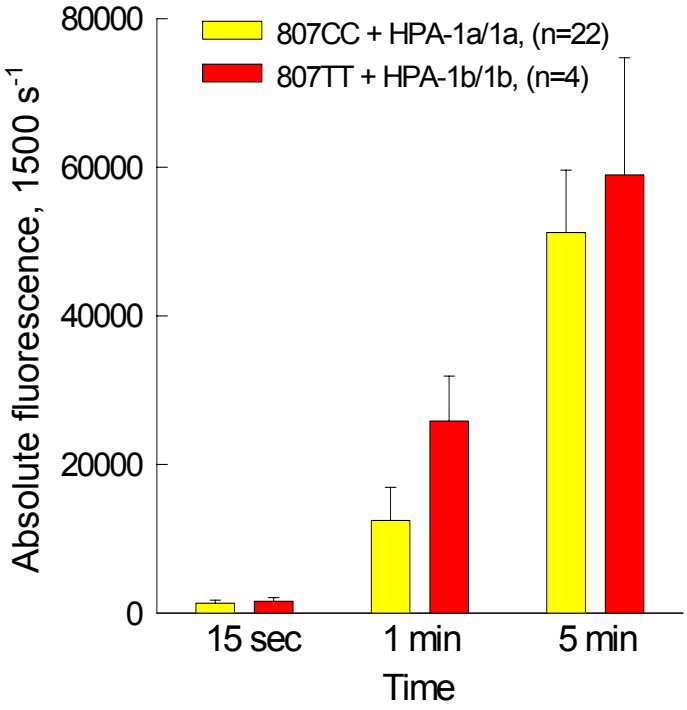

Figure 36. Effect of the combined "prothrombogenic" genotype combination on platelet adhesion onto immobilized collagen.

Table 8. Time course of platelet adhesion onto immobilized collagen with regard to the defined genotype combination.

Perfusion time	HPA-1a/1a+α2 807CC	HPA-1b/1b+ α2 807TT	Difference in %
15 sec	1317±1117 AU	1592±983 AU	20
1 min	12497±7650 AU	25855±12070 AU	106
5 min	51205±22255 AU	58967±27306 AU	15

5.9. HPA-1 and the α2 807C/T polymorphisms in primary hemostasis

Using the PFA-100 system (Dade-Behring, Marburg, Germany), the influence of examined platelet polymorphisms on primary hemostasis (platelet adhesion and aggregation) was also investigated under high shear rate conditions (n=49), similar to those encountered in a stenotic blood vessel (5000 s^{-1}–6000 s^{-1}). An end point is determined by the time required for whole blood to occlude the membrane aperture of the test cartridge, which is coated with platelet agonists. An average closure time did not show any statistical difference between different genotypes with regard to the HPA1 and 807CT polymorphism, neither with PFA-ADP nor with PFA-EPI cartridge (Table 9). Combined HPA-1 and α 2807C/T polymorphism was also tested using PFA-100. A critical “prothrombotic” genotype combination (HPA-1b/1b and 807TT) show no significant difference in closure time compared to the non-thrombogenic genotype combination (HPA-1a/1a and 807 CC), $p>0.05$.

Table 9. PFA-closure time with regard to the HPA-1 polymorphism of β3 subunit of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1.

	HPA-1a/1a	HPA-1b/1b	α2 807CC	α2 807TT
Coll/Epi, sec	128±17	120±23	124±19	122±17
Coll/ADP, sec	88±15	92±12	94±13	91±16

5.10. Influence of age and gender on platelet adhesion

In order to evaluate a potential influence of age and gender on platelet adhesion, the parameters were related with regard to belonging platelet adhesion under different

hemodynamic conditions. Our findings show no change of platelet reactivity with aging either in men or women. According to flow experiments, analysis of the platelet adhesion onto the immobilized ligands did not differ significantly related to the age and/or gender, neither at 50 s^{-1} nor at 500 s^{-1} or 1500 s^{-1} ,$p>0.05$.

5.11. Influence of factors of plasmic hemostasis on platelet adhesion

As described in the Subjects and Methods, in each participant, screening tests of coagulation and factors of plasmic hemostasis were determined. To test a influence of coagulation factors on platelet adhesion each of the tested coagulation factors was related with results of platelet adhesion on fibrinogen and collagen. Significant difference between examined parameters with regard to the HPA-1 polymorphism of β subunit of integrin αIIbβ3 and/or α2 807C/T polymorphism of integrin α2β1 under described experimental conditions was not observed.

6. Discussion

Platelet adhesion and subsequent thrombus formation on a subendothelial matrix at the site of vascular damage play a crucial role in the arrest of posttraumatic bleeding but also in different pathological thrombotic events (e.g. acute coronary syndrome and stroke) (1,2). Recently published studies have clearly demonstrated that integrins, especially integrin αIIbβ3 (GPIIb-IIIa) and α2β1 (GPIa-IIa), are intimately involved in occlusive thrombus formation at the site of an endothelial damage (4). Any genetic variation in the expression or function of these receptors may modulate integrin function. Weiss et al. reported in 1996 that the gene frequency of the HPA1b allele of integrin αIIbβ3 was significantly higher among young patients with myocardial infarction compared with age-matched controls (32). In 1998, Kunicki discovered that polymorphisms within the α2GPIa gene are associated with variations in platelet α2β1 expression levels (67). Platelets from individuals bearing the 807T allele express higher levels of α2β1; whereas, individuals who carry the 807C allele exhibit a lower density of the platelet integrin (68). One year later, the same group showed an association of the platelet α2 807C/T gene polymorphism with nonfatal myocardial infarction in younger patients. Since then, hundreds of case-control and association studies have been performed worldwide in order to define the impact of noted polymorphisms in clinical settings. These studies yielded different and, in some cases, contradictory results. This has been attributed to the heterogeneity of these studies, the complexity of the thromboembolic disease, the nature of platelet function, and the relevance of common polymorphisms under related microenvironmental conditions.

In this study, we now provide experimental evidence that platelet adhesion to immobilized ligands (fibrinogen and collagen) is modulated by related platelet receptor polymorphism. This modulation is determined by the specific receptor-ligand interaction, shear rate, and receptor interplay. In this sense, we provide experimental support for the epidemiological association of the related platelet-receptor polymorphism and arterial thrombosis.

6.1. Adhesive properties of platelet fibrinogen (αIIbβ3) and collagen receptors (α2β1) in relation to local shear stress

A significant linear increase in platelet adhesion, related to time and shear rate, was observed in our experimental system. At the constant shear rate of 50 s^{-1}, platelet adhesion onto fibrinogen-coated cover slips between 0 min and 5 min of perfusion increased 3.47 times and at shear rate of 1500 s^{-1} platelet adhesion increased 14.5 times. Similarly, a significant increase in platelet adhesion related to time and shear rate was also observed during perfusion onto collagen-coated surfaces. At shear rates of 50 s^{-1} and 1500 s^{-1}, platelet adhesion onto collagen-coated cover slips between 0 min and 5 min increased 4.8 times and 27 times, respectively. The different kinetic patterns of platelet adhesion, in regard to the shear rate, are determined by specific receptor-ligand interactions (Fig. 22, 23).

The role of shear rate on the adhesion capacity of αIIbβ3 receptor to immobilized fibrinogen is shown in Figure 23. An analysis of absolute fluorescence shows that shear rate, like chemical agonists, has “dose”-response and time-response characteristics. The adherent platelets formed a single layer on fibrinogen-coated surfaces, and a significant linear increase in platelet adhesion was observed, up to wall shear rates of $1500s^{-1}$. Kroll et al. (8), Kulkarni et al. (16), and Goto et al. (95) have also reported similar results. A maximal state of platelet adhesion is reached between shear rates of 1800 s^{-1} and 2000 s^{-1}. Increased detachment of adherent platelet (or reduced platelet adhesion) above a shear rate of $2500s^{-1}$ could be due to interruption of the fibrinogen αIIbβ3 receptor binding by the strong hemodynamic forces acting on rapidly flowing platelets. A threshold for αIIbβ3 binding potential for fibrinogen is reached at a shear rate of 7500 s-1. It was observed that platelets with a surface area of 4 μm^2, exposed to a shear force of 10.66 dynes/mm^2, under conditions of normal hemostasis, without pre-activation, and with intact vessel wall cannot adhere to the wall. This estimated force is 1332 times higher than adhesive forces between polystyrene microspheres and glass surfaces (89). The results of experiments conducted with immobilized collagen revealed different kinetic properties of platelet adhesion compared to immobilized fibrinogen. With immobilized collagen, the maximal adhesion was observed at $8000s^{-1}$, and the steady state between adhesive and dissociation forces was between $8000s^{-1}$ and $9000s^{-1}$. This is likely to be important for normal hemostasis yet even more prominent in situations relevant to arterial thrombosis. This equilibrium between adhesion and dissociation forces is

quite important because this is a limiting factor, which determines a maximal grade of arterial occlusion (7). A variation of occlusion grade could be explained by the influence of other microenvironmental conditions (plaque consistence, local hemostatic potential, etc). With a further increase of the shear rate, platelet adhesion decline and at 25000s^{-1} was not observed. These results clearly demonstrated that different receptor-ligand interactions have different kinetic properties. In other words, a specific receptor-ligand interaction is determined through their specificity per se and by local hemodynamic conditions.

In additional experiments (data not shown), a different shear rate threshold for the β3 polymorphism of integrin αIIbβ3 and 807CT polymorphism of α2β1, was not found. This result accords with the assumption that platelets carrying allelic receptor variants respond similarly or with minor modifications in the presence of a strong functional stimulus.

6.2. Specific interactions between platelet receptors and related ligands

Specific reactions between platelet receptors and related ligands were also evaluated Figures 26 and 27 present these experiments. A perfusion onto pure glass cover slips or onto immobilized BSA showed only marginal, unspecific interaction between perfused platelets and surface, despite related platelet receptors. The application of a specific antibody that binds selectively to αIIbβ3 (preincubation with abciximab, 5 min of perfusion at shear rate of 1500s^{-1}) dramatically (>90%) reduced platelet adhesion onto immobilized fibrinogen. The absence of inhibitory abciximab effects in experiments with BSA coated coverslips or pure glass confirmed the non-specificity of these reactions.

6.3. Platelet receptor interplay is dependent on ligand, shear stress, and time

Blocking of αIIbβ3 with abciximab reduced the formation of stabile platelet aggregates following initial platelet adhesion onto immobilized collagen by approximately 30%. This information clearly confirms that after initial platelet adhesion onto immobilized collagen (dependent on integrin α2bβ1), further spreading and consecutive thrombus growth are mediated through integrin αIIbβ3. Interestingly, the contribution of integrin αIIbβ3 to consequent thrombus growth onto immobilized collagen was constant at all ranges of applied shear rates (from 50s^{-1}up to 1500s^{-1}).

Under venous flow conditions, thrombus growth onto immobilized collagen was modulated through the integrin αIIbβ3 by approximately 32%. A thirty-fold increase of shear rate (from $50s^{-1}$ up to $1500s^{-1}$) induces an additional 15% increase of the contribution of αIIbβ3 to the thrombus growth. In contrast, Fig. 23 shows an almost linear increase in platelet adhesion onto immobilized collagen and consecutive thrombus growth for shear rates up to $8000s^{-1}$. These distinct properties could be explained by shear-rate-dependent activation of integrin αIIbβ3. In order to investigate the mechanism of activation of platelet by shear stress, Li et al. exposed whole blood to shear rates of 100, 150, 1000, and 3000 s^{-1} (96). The authors observed that the expression rate of CD62P is time dependent and shear-rate dependent. After exposure to a shear rate of 3000 s^{-1} for 7 minutes, the expression rate of CD62P started to increase and reached 26.4%. This result is comparable to our data, where the expression rate of CD62P is about 22% under arterial flow conditions after 5 min of perfusion. When the shear rate increases, the expression rate of GPIb/IX increased within first minute, then continually decreases (96). Similarly, αIIbβ3 expression increases quickly in a short time but began to decrease within 7 minutes. It seems that platelet spreading after initial adhesion onto immobilized collagen under high shear stress conditions is dependent on the degree of the integrin αIIbβ3 activation and modulated by additional soluble ligands as fibrinogen and/or von Willebrand factor. According to Coller et al. (31) and Ruggeri et al. (4,18), fibrinogen can support platelet-surface and platelet-platelet interactions, adhesion, and aggregation, by binding to integrin αIIbβ3. This interaction is fully efficient only at wall shear rates below $1800s^{-1}$, as shown in our experiments and also documented by Savage (29,30). Under higher shear stress, because of a relatively slow rate of bond formation or low resistance to tensile stress, a predomination of dissociation force compared to the adhesive forces was observed. On the other hand, it appears that glycoprotein Ibα has a faster association and bond formation with deposited von Willebrand factor, as well as high resistance to tensile stress (47). This implies a slow movement (deceleration, tethering) of platelets over collagen-coated surfaces and an interaction with collagen α2bβ1 receptor (platelet adhesion). At the same time, these new microenvironment conditions allow interaction between activated integrin αIIbβ3 with deposited von Willebrand factor and consecutive platelet-platelet interaction via soluble fibrinogen (platelet aggregation and spreading). Experiments conducted with the monoclonal LJ-Ib1 antibody (data are

not shown) directed against GPIbα showed dramatically reduced platelet adhesion onto immobilized collagen under arterial shear rate and no effects on platelet interaction with immobilized fibrinogen. The application of abciximab as a functional inhibitor of αIIbβ3 significantly reduced platelet adhesion onto immobilized fibrinogen and partially influenced platelet adhesion onto collagen (exclusively under physiological shear stress conditions, up to 1500 s^{-1}). Matzdorff and Voss have recently provided a possible explanation (97). It is well known that platelets contain an internal pool of αIIbβ3 receptor copies, which externalize upon activation and support platelet adhesion. During activation with thrombin receptor activating peptide (TRAP, 5 µM) or ADP (2 µM) the authors observed that the mean number of αIIbβ3 receptors increases by approximately 35% per platelet compared to receptor counts before platelet activation. Experiments with abciximab show that αIIbβ3 antagonists do not block these newly externalized receptors sufficiently to prevent platelet adhesion and consecutive aggregation. In summary, these data demonstrate the complexity of platelet adhesion onto immobilized fibrinogen or collagen. This complexity is determined by the biomechanical properties of different receptors under various hemodynamic conditions, activation status, disposable ligands, and the influence of the interplay of related receptors (e.g. between GPIb/IX, α2bβ1, and αIIbβ3 receptors). All these parameters are required to provide stabile platelet adhesion under all conditions of shear stress in the circulation.

This information is also quite relevant for our experimental design. In order to evaluate the functional relevance of HPA-1 and 807 C/T platelet receptor polymorphisms onto platelet adhesion, experimental shear rate must be below a threshold that causes platelet activation and ensures the maximal measurable adhesion of resting platelets. As the effect of HPA-1 and 807C/T polymorphism on platelet adhesion and consecutive thrombus formation could also depend on the duration of perfusion, images were acquired and analyzed at three perfusion times (15 sec, 1 min, and 5 min). Accordingly, our flow experiments were conducted under a maximal shear rate of $1500s^{-1}$, with a maximal exposition of 5 minutes.

6.4. HPA-1 polymorphism of integrin αIIbβ3 and α2 807C/T polymorphism of integrin α2β1 modulated platelet adhesion onto immobilized fibrinogen and collagen

Blood from 49 subjects was collected for use in flow experiments, in order to test the influence of β-subunit polymorphism of integrin αIIbβ3 on platelet adhesion to immobilized fibrinogen. Arterial flow was simulated with shear rates ranging from 500 s^{-1} to 1500 s^{-1} (shear stress from 19.0-60.8 dyn/cm^2) and venous flow was simulated with a shear rate of 50s^{-1} (shear stress of 1.9 dyn/cm^2). During perfusion, the shear rate expresses "dose"-response and time-response characteristics. The adherent platelets formed a single layer on fibrinogen-coated surfaces. A significant linear increase was observed in platelet adhesion as a function of time, at all shear rates. Image analysis after 1 and 5 minutes of perfusion clearly indicated that the platelets carrying the HPA-1a allele (HPA-1a/1a and HPA-1a/1b genotypes) have higher adhesion activity than platelets carrying the HPA-1b/1b genotype. This quantitative difference is continuously detectable at each shear rate applied and at each time point (Figure 29 A and B) but quantitatively dependent on perfusion time and shear rate. These results are in agreement with the results described by Cadroy et al. (98). In their study conducted with non-anticoagulated blood, blood was drawn directly from volunteers' cubital veins through a parallel flow chamber. Even though the experimental design differs significantly from ours (shear rates of 650s^{-1} and 2600s^{-1}, perfusion time of 2 and 4 min) one common result is evident: platelets with the HPA-1a/1a genotype show higher adhesion activity than platelets with the HPA1b allele. Carlsson et al. also recently reported similar results (99). The results obtained in a static microtitre-plate system coated with fibrinogen indicated that the adhesion activity of platelets with the HPA-1b/1b genotype is significantly lower compared to the HPA-1a/1a genotype. These unexpected experimental results (combined with the inconsistency of epidemiological studies) have created confusion but also led to additional analysis of our data. .

As shown above, HPA-1a/1a quantitatively enhances platelet adhesion activity, dependent on perfusion time and shear rate. This also accords well with Cadroy et al. (98), who reported that enhanced adhesion activity of platelets with the HPA1a allele observed at 650s^{-1} is abolished at shear rates of 2600s^{-1}. In our experiments, the adhesion activity of platelets with the HPA1a allele is constantly higher under venous

flow conditions than HPA-1b/1b but varies significantly in regards to perfusion time in experiments at shear rates of $1500s^{-1}$. After 1 and 5 min of perfusion under arterial flow conditions, platelets with HPA-1b/1b genotype reached 64% and 86% of the adhesion activity of platelets with the HPA-1a/1a genotype. This observation has initiated an assumption that platelets carrying the HPA-1b/1b genotype have a higher reactivity threshold for initial interaction of αIIbβ3 and fibrinogen but under the higher shear rate (in regard to time of perfusion) also realize more stable bonds with fibrinogen than platelets with the HPA-1a/1a genotype. To test this hypothesis and to omit some interindividual and methodical variation, data were “normalized” (see Methods), and a time course of platelet adhesion onto immobilized fibrinogen was analyzed with regard to the shear rate and HPA-1 polymorphism.

As shown in the Result, platelets carrying the HPA-1b/1b genotype demonstrated significantly higher adhesion rates between 15 sec and 5 min of perfusion than platelets carrying the HPA-1a/1a genotype (Fig. 30A). Relative adherence at a shear rate of $1500s^{-1}$ was 1.64 times higher compared to the HPA-1a/1a genotype and reached statistical significance. Although a similar trend was also observed at $500s^{-1}$, no statistically significant difference was reached (14.45 ± 6.4 vs. 10.66 ± 5.0 for HPA-1b/1b vs. HPA-1a/1a, $p=0.07$). At a venous shear rate, this enhanced effect of HPA-1b/1b genotype onto stable platelet adhesion was not observed. An analysis of the platelet adhesion rate of HPA-1b/1b genotype as a function of time (initial and late phase, see Results) showed surprisingly that the observed difference was present both in the initial phase of the platelet adhesion and in the late phase (Fig. 30B and Fig. 31).

Based on this observation, it can be concluded that the platelets with the HPA-1b/1b genotype develop stronger adhesive forces than the platelets with the HPA-1a/1a genotype. Statistically significant higher adhesion rates of the 1b/1b genotype could be related to the two determinants: 1) steadier platelet / solid surface interactions and 2) reduced displacement of platelets subjected to the shear stress. This results in the formation of thrombi that are more stable than the thrombi formed from HPA-1a/1a. Cadroy also indicated that clot retraction is enhanced by the involvement of platelets with the HPA-1b/1b genotype and that thrombi formed from HPA-1b/1b platelets are more resistant to thrombolysis than the thrombi formed from HPA-1a/1a platelets (60).

An interesting observation was made in light of the behavior of the heterozygous HPA-1a/1b genotype. According to our results, (Fig. 29 A and B and Fig. 30 A and B), it appears that platelets carrying “a” and “b” alleles (HPA-1a/1b) have a functional behavior similar to the HPA-1a/1a platelets. Absolute and relative adhesion are comparable with platelets that are homozygous for the “a” allele. Similar to homozygous HPA-1a/1a, our results indicated that platelets carrying the HPA-1a/1b genotype have a lower threshold for adhesion onto immobilized fibrinogen (Fig. 29 A and B), but they are not capable of producing a stable platelet adhesion as compared to platelets with the HPA-1b/1b genotype. It may be concluded that the functional characteristic of platelets with the HPA-1a/1b genotype is predominantly determined by the “a” allele. This could explain the inconsistency of results between some epidemiological studies in which subjects with different platelet genotype were pooled (for example: HPA-1a/1a vs. HPA-1a/1b plus HPA1b/1b).

In experiments conducted under hemodynamic conditions (shear rate ranging from $25s^{-1}$ to $125s^{-1}$) on stabile cell lines (Chinese hamster ovary cells, CHO) with over expression of HPA-1a/1a and HPA1b/1b genotypes, Vijayan et al. (100) also found significant differences in the cell adhesion onto immobilized fibrinogen in regard to the β3 polymorphism. By comparing these results with the results obtained under static conditions (64,65), the authors concluded that a thrombotic potential of respected platelet polymorphism could be underestimated when tested under static conditions. The author provided two indications for a possible explanation for significant differences in adhesion between the two tested cells lines. First, using rhodamine-phalloidin-stained actin, more F-actin was observed at the periphery of the cells with the 1b1b genotype compared with the 1a1a genotype, and second, cells with the 1b1b genotype 1b1b showed a more robust reorganization of actin. The application of cytohalasin D abolished the differences in cell adhesion in regard to the HPA-1 genotype. Although the experimental design significantly differs compared to our experimental protocol, this report represents one additional experimental proof that HPA-1 polymorphism significantly modulates platelet adhesion and that this modulation is also dependent upon local hemodynamic conditions.

In contrast to the experiments related to the HPA-1 polymorphism of integrin αIIbβ3, experiments conducted in our flow system clearly indicated that α2 807 C/T polymorphism of integrin $\alpha 2\beta 1$ influences platelet adhesion onto immobilized collagen. A careful data evaluation showed that this impact was influenced by flow

rate and perfusion time. After 5 min of perfusion under the arterial flow conditions, platelets carrying the TT genotype showed a higher adhesion activity. A statistically significant difference was reached under typical arterial shear rate conditions of $500s^{-1}$ and 1500 s^{-1}. Under the venous shear rates, a positive trend was observed in platelets carrying the TT genotype, which did not significantly enhance platelet adhesion compared to CC platelets. A separate analysis of the platelet adhesion after 15 sec and after 1 min of perfusion indicated that in the initial phase, α2 807 C/T polymorphism of integrin $\alpha 2\beta 1$ also significantly modulated platelet adhesion under venous shear rates. It seems that influence declined with the time of perfusion (Fig. 33 A and B). After 15 sec of perfusion, the quotient between 807TT and 807CC platelet was 2.45; after 1 min it was 1.38, and after 5 min of perfusion it was marginally higher. It appears that the discrete influence of α2 807C/T polymorphism under the venous shear rate as observed in the initial phase of the platelet adhesion could be mimicked during prolonged perfusion time through additional factors (e.g. receptor interplay, microenvironmental hemodynamic condition, etc). Otherwise, a constantly higher platelet adhesion under the arterial shear rate was observed. This higher platelet adhesion activity was statistically significant at shear rate of 500 s^{-1} and marginally significant at a shear rate of $1500s^{-1}$. Our assumption was that under shear rate of $1500s^{-1}$, the effect of α2 807C/T polymorphism is mimicked through newly activated platelets and/or platelet receptor interplay (for example, involvement of activated integrin $\alpha IIb\beta 3$). Cadroy et al. (98) tested four platelet membrane glycoprotein polymorphisms including α2 807C/T. In experiments with immobilized collagen conducted in a similar manner as described above, they found a significant difference in platelet adhesion in regard to the α2 807C/T polymorphism at a shear rate of $2600s^{-1}$. Even though some differences in experimental design those results are in good accordance with our conclusion that the α2 807C/T polymorphism affected platelet adhesion onto immobilized collagen under arterial flow conditions. In the case of the α2 807C/T polymorphism of integrin $\alpha 2\beta 1$, a dose-related effect could be a possible explanation for difference in the platelet adhesion activity. The α2 807C/T polymorphism of integrin $\alpha 2\beta 1$ is the determinant of the receptor surface density. The 807CC genotype has been associated with the lowest $\alpha 2\beta 1$ receptor density, and the α2 807TT genotype has been associated with high $\alpha 2\beta 1$ receptor density. According to our results, the increase in density of this platelet receptor may predispose the platelets to adhere more readily to thrombogenic surfaces and may

therefore augment the thrombotic tendency, especially at high shear rates. Interestingly, heterozygous platelets (α2 807CT) showed an enhanced adhesion activity only in the initial phase of the platelet adhesion (within 15 sec).

Furthermore, contrary to Cadroy et al (98), we found in our experiments a certain functional linkage between HPA-1 and the α2 807C/T polymorphism. The critical "prothrombotic" genotype combination (HPA-1b/1b and α2 807TT genotypes) enhance platelet adhesion activity at each time point, under arterial flow conditions, compared to the "wildtype" variants of α2bβ3 and α2β1 (HPA-1a/1a and α2 807CC genotype). This difference with the results of Cadroy et al. could be due to a difference in the experimental design. Cadroy composed both forms of β3 mutation (heterozygous HPA-1a/1b and homozygous HPA-1b/1b) and compared them to the HPA-1a/1a genotype.

Even through we have provided evidence that platelet adhesion onto fibrinogen and collagen is influenced through related platelet receptor polymorphism and their interplay, platelet adhesion is also strongly dependent on local hemodynamic conditions. The effect of single genetic factors on the risk of arterial vascular disease may be weak and could be seen only in the context of other common genetic or environmental cardiovascular risk factors.

7. Conclusions and Summary

- Platelet adhesion onto immobilized fibrinogen under arterial flow conditions is mediated by integrin αIIbβ3.
- This specific interaction is modulated by the HPA-1 polymorphism of the β-subunit of integrin αIIbβ3.
- The HPA-1b/1b genotype is associated with an increased platelet adhesion.
- This increased adhesion of HPA-1b/1b platelets is already evident within 1 min following the interaction of platelets with immobilized fibrinogen.
- Specific platelet adhesion onto immobilized collagen is shear-rate dependent and time dependent and modulated through the α2β1 polymorphism (α2 807 C/T).
- Platelets with the α2 807TT-genotype showed significantly higher adhesion rates compared to platelets with the α2 807CC-genotype.
- After an initial platelet adhesion onto immobilized collagen, a further platelet adhesion (spreading and consecutive thrombus growth) is modulated through the HPA-1 polymorphism of the β-subunit of integrin αIIbβ3.
- The critical "prothrombotic" genotype combination (HPA-1b/1b and α2 807TT genotypes) enhances platelet adhesion under arterial flow conditions, compared to "wildtype" variants of αIIbβ3 and α2β1 (HPA-1a/1a and α2 807 CC genotype).

Our data support the contention that genetically determined variants of platelet integrins αIIbβ3 and α2β1 could play a role in arterial thrombogenesis and thus confirm the hypothesis derived from epidemiological studies. However, the risk attributable to only a single polymorphism in a complex disease such as stroke or myocardial infarction is probably low. Thus, a single polymorphism may have no clinical significance if evaluated independently, but specific combinations of common polymorphisms and the combination of related polymorphisms might play a relevant role in these diseases. In other words, the thrombotic effect of platelet receptor polymorphisms (single or combined) might be restricted to a combination with microenvironmental risk factors as demonstrated in our study. Understanding of the functional roles of platelet receptor polymorphism may increase our ability to further stratify patients according to their genetically determined thrombosis risk and may

give us the opportunity to design and develop individually based therapeutic strategies in the prevention of arterial thrombosis.

8. Schlussfolgerungen und Zusammenfassung:

- Die Plättchenadhäsion an immobilisiertem Fibrinogen wird unter arteriellen Flussbedingungen durch das Integrin αIIbβ3 vermittelt.
- Die spezifische Interaktion wird durch den HPA-1-Polymorphismus der β - Untereinheit des Integrins αIIbβ3 moduliert.
- Der HPA-1b/1b-Genotyp ist mit einer signifikant erhöhten stabilen Adhäsionsaktivität assoziiert.
- Die vermehrte Adhäsion von HPA-1b/1b – Plättchen zeigt sich bereits während der ersten Minute der Interaktion von Plättchen und immobilisiertem Fibrinogen.
- Die Plättchenadhäsion an immobilisiertem Kollagen ist Scherraten- und zeitabhängig und wird durch den α2β1-Polymorphismus (α2 807C/T) moduliert.
- Plättchen mit α2 807TT-Genotyp zeigten gegenüber solchen mit α2 807CC-Genotyp eine signifikant höhere Adhäsionsrate.
- Nach der initialen Plättchenadhäsion an immobilisiertem Kollagen wird die weitere Plättchenadhäsion (spreading und konsekutives Thrombuswachstum) durch den HPA-1-Polymorphismus der β-Untereinheit des Integrins αIIbβ3 moduliert.
- Die kritische prothrombotische Genotypkombination (HPA-1b/1b and α2 807TT) erhöht die Aktivität der Plätttchenadhesion zu jedem Meßzeitpunkt unter arteriellen Flussbedingungen, verglichen mit der Wildtypvariante (HPA-1a/1a and α2 807 CC)

Unsere Ergebnisse stützen die Vorstellung, dass genetisch determinierte Varianten des Plättchenintegrins α2bβ3 and α2β1 eine Rolle in der arteriellen Thromobogenese spielen können. Dies bestätigt eine Hypotese, die aus verschiedenen epidemiologischen Studien abgeleitet werden konnte.
Allerdings, scheint das einem einzelnen Polymorphismus zuschreibbare Risiko in einem komplexen Geschehen, wie beispielsweise dem ischämischen Hirninfarkt oder dem Myokardinfarkt gering zu sein. Einzeln Polymorphismen dürfen nur eine geringe

klinische Signifikanz besitzen, allerdings könnten spezifische Kombinationen häufiger Polymorphismen eine relevante Rolle in solchen komplexen Erkrankungen spielen. Möglicherweise ist ein prothrombogener Effekt von Plättchenrezeptor-Polymorphismen (einzeln oder kombiniert) auf eine Koinzidenz von weiteren Risikofaktoren oder Flußbedingungen beschränkt, wie wir es in unserer Studie zeigen konnten. Ein tieferes Verständnis der funktionellen Rolle von Plättchenrezeptor-Polymorphismen könnte zu einer besseren Stratifizierung des genetisch determinierten Thromboserisikos führen und damit die Grundlage für eine individuelle therapeutische Präventionsstrategie legen.

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