

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**PLATELET ACTIVATION MARKERS:  
IN VITRO EXPERIMENTS AND STUDIES  
IN PROTHROMBOTIC DISORDERS**

Béla Nagy Jr, MD



Research supervisor: János Kappelmayer, MD, PhD, DSc

Program supervisor: László Muszbek, MD, PhD, DSc, MHASc

UNIVERSITY OF DEBRECEN  
KÁLMÁN LAKI DOCTORAL SCHOOL  
THROMBOSIS, HEMOSTASIS AND VASCULAR BIOLOGY PROGRAM

Debrecen, 2009

## CONTENTS

Abbreviations .....	3
Introduction .....	5
The aim of the dissertation .....	8
Previous studies .....	9
Subjects and materials .....	20
Methods .....	23
Results .....	29
Summary .....	48
Összefoglalás .....	49
References .....	50
List of original publications related to the dissertation .....	60
List of original publications not closely related to the dissertation .....	61
List of oral presentations at international congresses.....	62
List of poster presentations at international congresses .....	63
List of oral and poster presentations at national congresses.....	64
Keywords.....	65
Acknowledgements .....	66
Appendix .....	67

## **ABBREVIATIONS**

ADP: adenosine diphosphate

BMI: body mass index

BSA: bovine serum albumin

CAD: coronary artery disease

CVD: cardiovascular disease

DM: diabetes mellitus

EGF: epidermal growth factor

ELISA: enzyme-linked immunoassay

FITC: fluorescein-isothiocyanate

FSC: forward scatter

FXIII: blood coagulation factor XIII

FXIII-A: "A" subunit of FXIII

FXIIIa: activated form of FXIII

FXIII-A<sub>2</sub>: cellular form of FXIII

FXIII-A<sub>2</sub>B<sub>2</sub>: plasma form of FXIII

GMP-140: granule membrane protein, 140 kDa

GP: glycoprotein

GPCRs: G protein-coupled receptors

GT: Glanzmann thrombasthenia

MFI: mean fluorescence intensity

MI: myocardial infarction

MP: microparticle

PAD: peripheral artery disease

PADGEM: platelet activation-dependent external granule membrane

PBS: phosphate-buffered saline

PCI: percutaneous coronary intervention

PCR: polymerase chain reaction

PE: phycoerythrin

PFA: paraformaldehyde

PGE<sub>1</sub>: Prostaglandin E<sub>1</sub>

PMP: platelet-derived microparticle

PPP: platelet-poor plasma

PRP: platelet-rich plasma

PS: phosphatidylserine

PSGL-1: P-selectin glycoprotein ligand-1

PTCA: percutaneous transluminal coronary angioplasty

RANTES: regulated on activation normal T cell expressed and secreted

RFLP: restriction fragment length polymorphism

RGDS: Arg-Gly-Asp-Ser tetrapeptide

RT: room temperature

SSC: side scatter

TF: tissue factor

TRAP: thrombin-receptor activating peptide

TXA<sub>2</sub>: thromboxane A<sub>2</sub>

vWF: von Willebrand factor

## INTRODUCTION

Platelets are anucleated blood cells that originate from the cytoplasm of megakaryocytes in the bone marrow and circulate to maintain the integrity of the vascular system. In physiological conditions, this function contributes to hemostasis, the process that arrests blood loss after vessel injury. On the other hand, in diseases with several risk factors for atherosclerosis and atherothrombosis such as coronary artery disease (CAD), ischemic stroke or type 2 diabetes mellitus (DM), platelet function may lead to thrombotic occlusion of the vessel with obstruction of blood flow and subsequent tissue damage. Platelets are involved in both arterial and venous thromboembolism; however, they are more relevant to the process of thrombus formation in arteries. The main trigger for activation of platelets is the loss of endothelial cell barrier between extracellular matrix components and flowing blood resulting in the exposure of subendothelial collagen and von Willebrand factor (vWF). In addition, plaque rupture with tissue factor (TF) expression also induces platelet activation as a complication of atherosclerosis. There is a congenital disease called “sticky platelet syndrome”, when so called “hyperactive” platelets are present in the circulation without being triggered by the atherosclerotic lesion. These activated platelets have a key pathological role in this disease causing both arterial and venous thrombosis. The response of platelets to the exposure of subendothelial matrix proteins leads to three overlapping phases of platelet activation process called as initiation, extension and perpetuation. In the initiation stage, circulating platelets are activated and captured by exposed collagen and vWF causing the adhesion of a monolayer of platelets. Thrombin is generated on cell surface with high levels of phosphatidylserine (PS) after platelets are exposed to TF from vessel wall initiating the coagulation cascade and later platelet aggregation. Integrin receptors on the platelet surface are the important factors of these early events. Integrin  $\alpha_2\beta_1$  and glycoprotein (GP) VI bind collagen directly, while GPIb $\alpha$  and GPIIb/IIIa ( $\alpha_{IIb}\beta_3$ ) receptors bind vWF and thus collagen resulting in intracellular signal transduction and an increase of intracellular  $Ca^{2+}$ . In diseases with thrombosis and inflammation, platelets may also be primarily activated by thrombin via protease-activated receptors (PAR1 and PAR4) of G protein-coupled receptors (GPCRs). During the extension phase, additional platelets are recruited, activated and stick to each other on the platelet monolayer, when adenosine diphosphate (ADP) is secreted from dense granules, and  $\alpha$ -granule contents like P-selectin are expressed with many other

biomarkers. The surface level of exposed P-selectin increases rapidly in order to mediate different cell-cell heterotypic interactions between platelets and leukocytes or endothelial cells. Simultaneously, thromboxane  $A_2$  (TXA<sub>2</sub>) is *de novo* generated from arachidonic acid and then also released. ADP and TXA<sub>2</sub> along with accumulated thrombin activate platelets through GPCRs-mediated pathways. These events support more secretion, and the GPIIb/IIIa receptor activation as a result of inside-out signaling, and thus fibrinogen binding to platelets that is essential in full platelet aggregation and stable plug formation. PS- and TF-positive microparticles (MPs) with the size of 0.1-1  $\mu$ m are shedded from activated platelets by a budding process to facilitate coagulation. Perpetuation is the late stage of platelet activation, when the platelet plug is stabilized by close contacts between platelets via outside-in signaling induced by receptor-bound ligands. These junctions thus prevent the dissolution of the plug by supporting the accumulation of secreted agonists and shedded adhesive receptors like soluble P-selectin into the gaps between platelets. Finally, clot retraction occurs to narrow the interplatelet spaces resulting in a smaller, shrunk volume of the stable plug.

Accordingly, activated platelets are the key components in many vascular disorders with arterial thrombosis: in the different forms of CAD such as myocardial infarction (MI) or stable/unstable angina, in addition to ischemic cerebrovascular diseases, and peripheral artery disease (PAD). Moreover, in numerous other states like obesity, type 2 DM, or after invasive cardiological vascular intervention (e.g. stent implantation) in patients with CAD, activated platelets are present in the circulation resulting in further thrombotic and inflammatory complication. The fast and efficient investigation of platelet activation markers - by using different technical approaches - is essential in the detection and the follow-up of short-term and long-term consequences of the increased platelet activation status. Moreover, it is also useful to monitor the effect of different antiplatelet regimens in patients as a screening test.

In the last two decades, the surface expression of P-selectin receptors has been considered as one of the most sensitive, “gold standard” activation-dependent events investigated by flow cytometry. Its analysis can be readily used for routine diagnostic purpose in addition to the soluble form of P-selectin after shedding into the plasma measured by enzyme-linked immunoassay (ELISA). Along with alternative activation markers like high MP levels as well as elevated amount of platelet-leukocyte heterotypic aggregates, all these data can be applied for a comprehensive analysis of platelet

activation in prothrombotic disorders from *ex vivo* clinical samples. Furthermore, investigation of *in vitro* stimulated platelets by platelet agonists may help to introduce the testing of additional activation markers and provide new insights into the mechanism of these complex events.

## THE AIM OF THE DISSERTATION

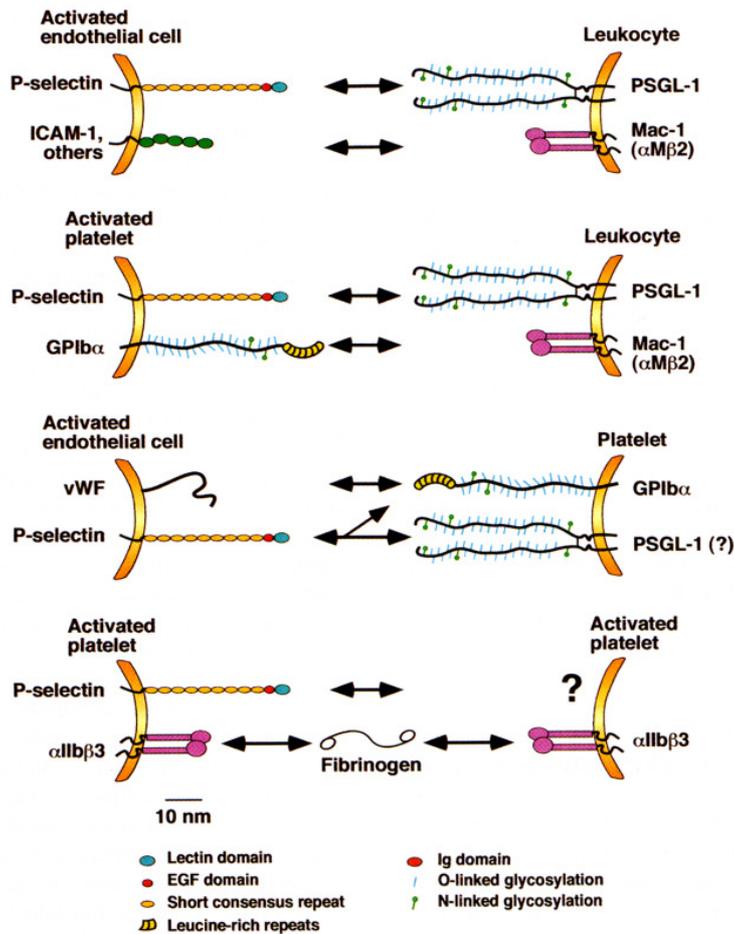
Our aim was to investigate the levels of distinct markers of increased platelet activation in *ex vivo* studies from patients who suffered from different clinical diseases with high risk for prothrombotic complications, and also in stimulated platelets by *in vitro* experiments.

For this purpose we decided

1. to measure the levels of platelet and soluble P-selectin in obese and type 2 DM patients versus healthy subjects, and to establish the effect of the most intensively examined variant of P-selectin gene polymorphism (Thr715Pro) on soluble P-selectin levels in these patients and controls,
2. to investigate the direct platelet-activating effect of stent implantation via studying the levels of platelet-derived MPs (PMPs) and other platelet activation markers (platelet and soluble P-selectin, platelet-leukocyte heterotypic aggregates) at an early (15 minutes) time point after stenting with bare metal stent in patients with CAD, and these results were compared to data obtained from subjects with diagnostic catheterization alone,
3. to study *in vitro* the binding of blood coagulation factor XIII (FXIII) to activated platelets by measuring the FXIII-A surface positivity of whole blood and washed human platelets stimulated with thrombin-receptor activating peptide (TRAP) in healthy subjects and a type I Glanzmann thrombasthenia (GT) patient as a control, and analyzed these samples by flow cytometry,
4. to observe whether the non-active FXIII-A<sub>2</sub>B<sub>2</sub> binds directly (to GPIIb/IIIa receptors) on stimulated platelets, or this binding is mediated via GPIIb/IIIa receptor-bound fibrinogen with the  $\gamma'$ - or  $\gamma$ A-chains.

## PREVIOUS STUDIES

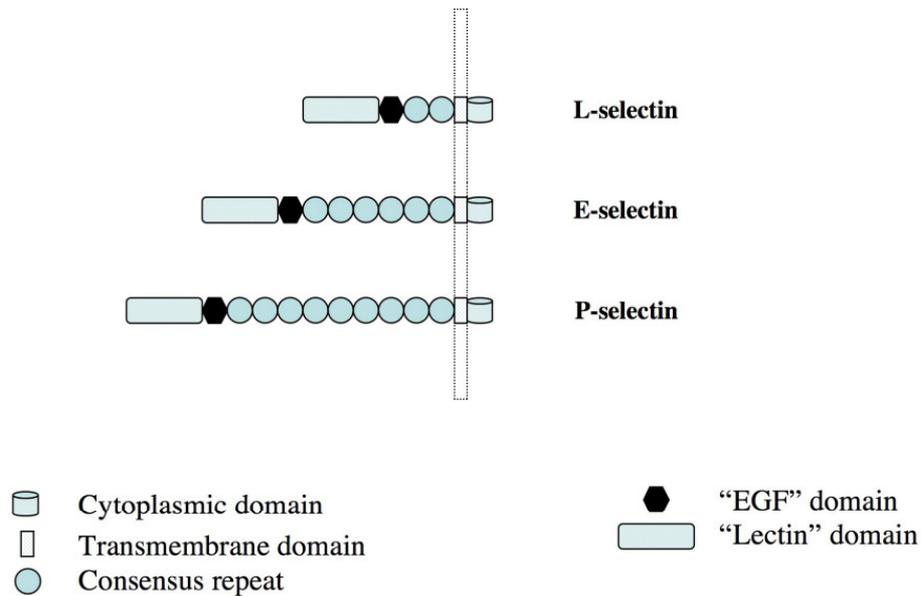
Activated platelets play a key role in the development of atherosclerosis and related thrombotic complications in CAD, DM and several other diseases [1,2]. The initial step for the development of atherosclerosis is associated with the dysfunction of endothelial cells as a result of the deposition of lipids in the vessel wall and the increased oxidative stress. These early events result in the inhibition of nitric oxide production, increased adhesiveness of endothelial cells for platelets and leukocytes due to the higher prothrombotic activity and the secretion of proinflammatory molecules. Platelets become activated induced by the metabolic alterations (hypercholesterolemia, hyperglycemia) [3,4] and the increased levels of adhesive molecules (ICAM-1, endothelin-1) as well as cytokines (interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ ) released from endothelial cells and leukocytes [1,2]. Different interactions between platelets and endothelial cells generate signals to recruit inflammatory cells to the site of vascular injury and promote the extravasation of more leukocytes to the inner layers of the vessel wall [1,2,5]. Adhesion of activated platelets to atherosclerotic lesion alters plaque progression and may induce prothrombotic conditions by the release of further adhesive proteins like P-selectin. Binding of several plasma molecules (e.g. fibrinogen) on the activated platelet surface, the generation of PS and TF-positive PMPs, and the secretion of cytokines, such as CD40L and RANTES (regulated on activation, normal T cell expressed and secreted) [2,5] occur simultaneously. Accordingly, active platelets have a central role in the propagation of these inflammatory and prothrombotic events via several cell-cell interactions, which are mediated typically through the association of P-selectin with its counter-receptors on different cell types (Figure 1). The significance of the distinct roles of P-selectin in hemostasis is even more evident from the studies in P-selectin-deficient mice, which demonstrated a number of defects in leukocyte function (adhesion, rolling, recruitment) [6], and platelet function caused prolonged bleeding time, and smaller instable thrombus formation [7,8].



**Figure 1.** Multicellular adhesive interactions among activated platelets, leukocytes, and endothelial cells. (Adapted from McEver RP. P-selectin/PSGL-1 and other interactions between platelets, leukocytes, and endothelium. In: Michelson AD, ed. Platelets. Academic Press, 2007: 231-249.)

P-selectin receptor (formerly called as Granule Membrane Protein, {GMP-140}; Platelet Activation-Dependent External Granule Membrane, {PADGEM}) is a 140-kDa integral membrane glycoprotein located in the  $\alpha$ -granules of platelets and the Weibel-Palade bodies of endothelial cells [9]. This receptor is one of the members of the selectin family, which contains P-, E-, L-selectin that bind to a dimeric mucin receptor (P-selectin glycoprotein ligand-1, {PSGL-1}) expressed constitutively on the surface of leukocytes [10] and platelets [11]. Selectins consist of a lectin domain followed by an epidermal growth factor (EGF)-like module, varying number of short consensus repeat, a transmembrane domain and a short cytoplasmic tail [12] (Figure 2). In addition, E-selectin

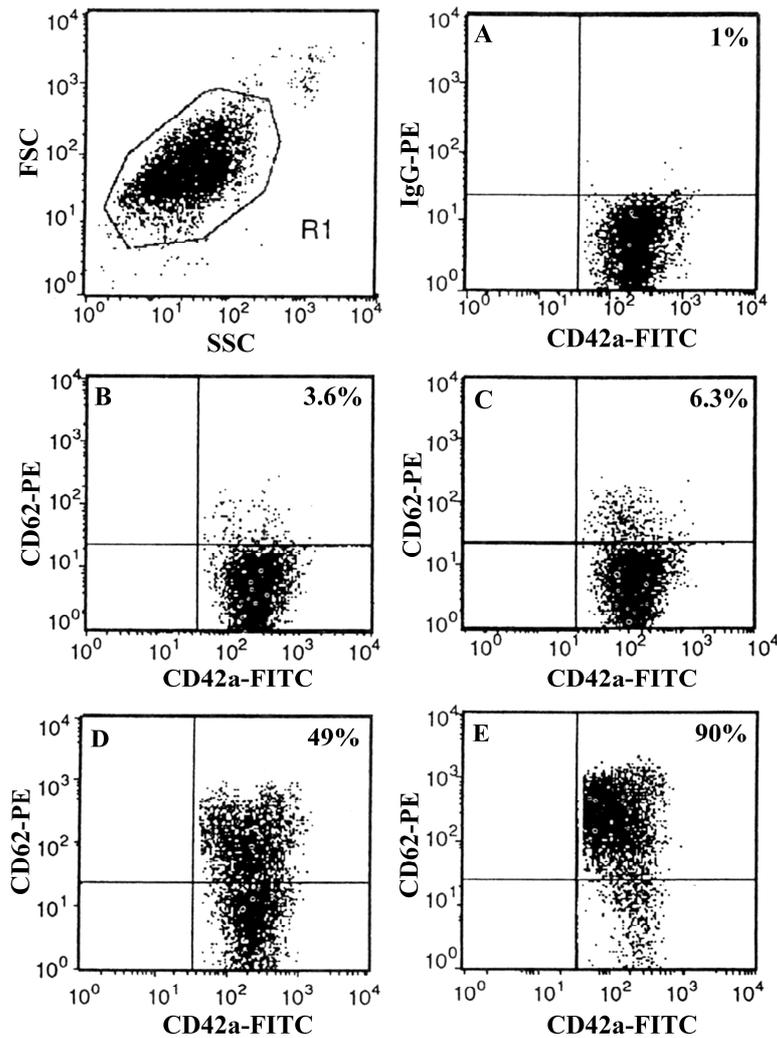
is present only on activated endothelial cells, while L-selectin is constitutively expressed on the leukocyte surface [13].



**Figure 2.** The domain structure of selectins.

P-selectin-PSGL-1 interaction is involved in leukocyte adhesion to endothelial cells and platelets [11,14], when P-selectin is expressed on the cell surface upon cell activation [15,16] (see Figure 1). Flow cytometry is the most advantageous technique for the analysis of platelet receptors especially for P-selectin exposure [17,18]. Whole blood flow cytometric assay for platelet activation using a fixed specimen has been increasingly used over recent years to assess platelet function in clinical research settings. This approach offers several advantages over other clinical tests of platelet function including relative simplicity, minimal manipulation of samples, thus preventing artificial *in vitro* platelet activation, analysis in the physiological milieu of whole blood, high sensitivity for detection of platelet subpopulations, and the need for only minimal blood volumes. The preanalytical conditions are extremely important in the analysis of platelet activation markers. In addition, rapid fixation of blood after venepuncture is also critical. We found that the preparation of platelet-rich plasma (PRP) [19], or even washed platelets resulted in a “false” up-regulation of P-selectin expression resulting in higher control values compared to whole blood analyses (see Results). Most authors in different *ex vivo* clinical

studies reported less than 1-2% positivity for platelet P-selectin surface level in whole blood in healthy subjects [4, 20-23]. Values above this rate mean an enhanced platelet activation status, and may predict a higher risk for prothrombotic events. According to our *in vitro* studies, platelets showed increasing levels of P-selectin up to 90% in agonist concentration-dependent manner (Figure 3).



**Figure 3.** Dot plots of platelet P-selectin (CD62) expression on unstimulated (B) and  $\alpha$ -thrombin-stimulated (0.01-0.1 U/ml) platelets in whole blood (C-E). Cells were identified according to their forward scatter (FSC) and side scatter (SSC) parameters and then gated into R1. The gated events were labeled with fluorescein-isothiocyanate (FITC)-conjugated anti-GPIX (CD42a) and phycoerythrin (PE)-labeled anti-CD62 antibodies. Stimulation was done in the presence of RGDS peptide in order to prevent fibrinogen binding to GPIIb/IIIa receptors and thus clot formation. (A) Isotype control sample was used to set the quadrants during analysis.

A theoretical disadvantage of flow cytometry relates to the possibility that activated platelets may display a decreased survival in circulation resulting in modest or non-evident platelet activation by flow cytometric analysis [24]. However, convincing experimental data support that activated platelets continue to circulate [25,26]. Thus, platelet activation is not necessarily the ultimate stage of platelet life.

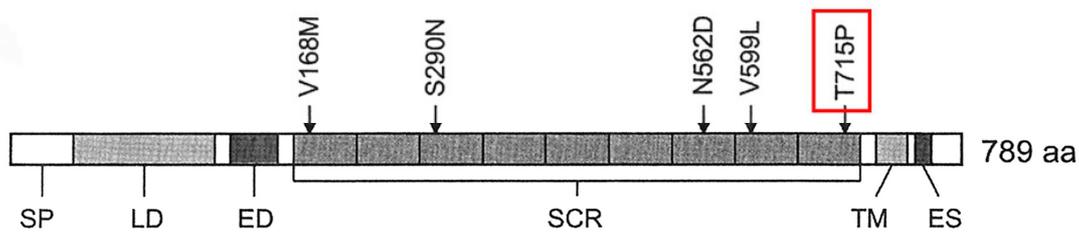
The soluble form of P-selectin receptor has been identified in plasma as an alternatively spliced molecule without the transmembrane domain released by the cleavage of the membrane-bound form from the cell surface [27]. In normal plasma, soluble P-selectin concentration showed difference between genders being higher in males [28,29]. The primary contributors to the formation of soluble P-selectin in physiological state are platelets, and thus baseline concentrations are mostly determined by platelet P-selectin shedding. The positive correlation between platelet count and the level of soluble P-selectin also supports this conclusion. In patients with bone-marrow aplasia, the soluble P-selectin concentration was reduced, but was not completely absent. The remaining soluble P-selectin in bone marrow aplasia could be derived from a few persisting megakaryocytes and/or platelets or from endothelial cells. Therefore, a minimal release of that from endothelial cells within normal conditions cannot be excluded [30]. Verhaar *et al.* found that the progression of endothelial damage in essential, renovascular and malignant hypertension was associated with a rise in circulating levels of P-selectin, which also confirmed the link between soluble P-selectin and endothelial cells as a source of this protein [31]. The shedding mechanism of P-selectin has not been exactly elucidated. Though no direct proofs have been reported, some metalloproteinases might be candidates to cause P-selectin shedding as has been found for L-selectin [32,33].

Significantly elevated levels of platelet and soluble P-selectin have been found in a number of different diseases like unstable angina, ischemic stroke, PAD, atrial fibrillation, or DM compared to healthy control subjects (Table 1) [4, 20-23,34-40]. Moreover, the increased soluble P-selectin levels might serve as a predictive marker for future cardiovascular events, such as MI and stroke [41].

References	Type of vascular disorder	Number of patients	Number of controls	P-selectin ratio	
				Platelet	Soluble
Blann AD et al. [34]	peripheral artery disease	170	119		1.5
Furman MI et al. [20]	stable coronary artery disease	19	19	1.4	
Gurbel PA et al. [35]	myocardial infarction	23	10	1.4	2.6
Ikeda H et al. [36]	unstable angina	23	15		2
Jilma B et al. [37]	type 1 diabetes	42	42		1.2
Kopp HP et al. [38]	type 1 diabetes	18	18		1.9
Marquardt L et al. [21]	ischemic stroke	50	30	2	
Minamino T et al. [39]	atrial fibrillation	25	25	1.6	
Tschoepe D et al. [4]	type 1 diabetes	21	50	5	
Tschoepe D et al. [22]	type 1 diabetes	19	50	3.2	
Yamazaki M et al. [23]	cerebral infarction	254	30	3	
Zeiger F et al. [40]	peripheral artery disease	50	50	1.4	
		<b>total: 714</b>	<b>458</b>	<b>mean: 2.4</b>	<b>1.8</b>

**Table 1.** Increase (relative ratio) in the levels of platelet and soluble P-selectin in different vascular disorders according to former clinical studies.

The P-selectin gene is located on chromosome 1q21 to 1q24 and it is highly polymorphic [42]. Among the 13 gene missense polymorphisms, the Thr715Pro variant has been the most intensively studied [43-48], while it may have considerable effect on the function of P-selectin. It is located in the last consensus repeat region of the P-selectin molecule (Figure 4), and it is possible that the substitution of threonine for proline may induce a conformational change in the precursor protein, which may influence its intracellular transportation and secretion [45]. Contradictory data have been published about the association of Thr715Pro polymorphism with cardiovascular disease [49]. The presence of Pro715 allele has been shown to have a “protective” effect on MI in two extensive studies [42,43], but some authors reported no such effect [44,45]. Anyway, Thr715Pro genotype seemed to have a substantial effect on the soluble P-selectin levels as significantly lower level of soluble P-selectin was measured in healthy controls with AC and CC genotype for this polymorphism versus wild type (AA) individuals [45,46,48]. It is still unknown whether the Pro715 allele is associated with the reduction in the level of membrane bound form of P-selectin [45], or the decreased shedding of distal fragments of the receptor [50].



**Figure 4.** Schematic figure on the most important polymorphisms of P-selectin receptor. Red quadrant signs the most studied variant (Adapted from ref. [51]).

Other polymorphisms (e.g. Ser290Asn, Asn562Asp) of this gene carried by the same haplotype were associated with an increased risk for MI in a study investigating French and Northern Irish populations [52]. On the other hand, the Thr715Pro phenotype together with Ser290Asn and Asn562Asp polymorphisms were excluded to be as major contributors to macrovascular complications in type 2 DM [50]. Moreover, several additional polymorphisms have also been described in the promoter region of the P-selectin gene, and no significant association was seen between -1817 T/C, -1969 G/A, and -2123 C/G polymorphisms and the soluble P-selectin levels [45]. Thus, the exact association between the presence of mutated alleles or specific haplotypes of P-selectin gene and their impact related to soluble P-selectin levels in several diseases, was still unclear.

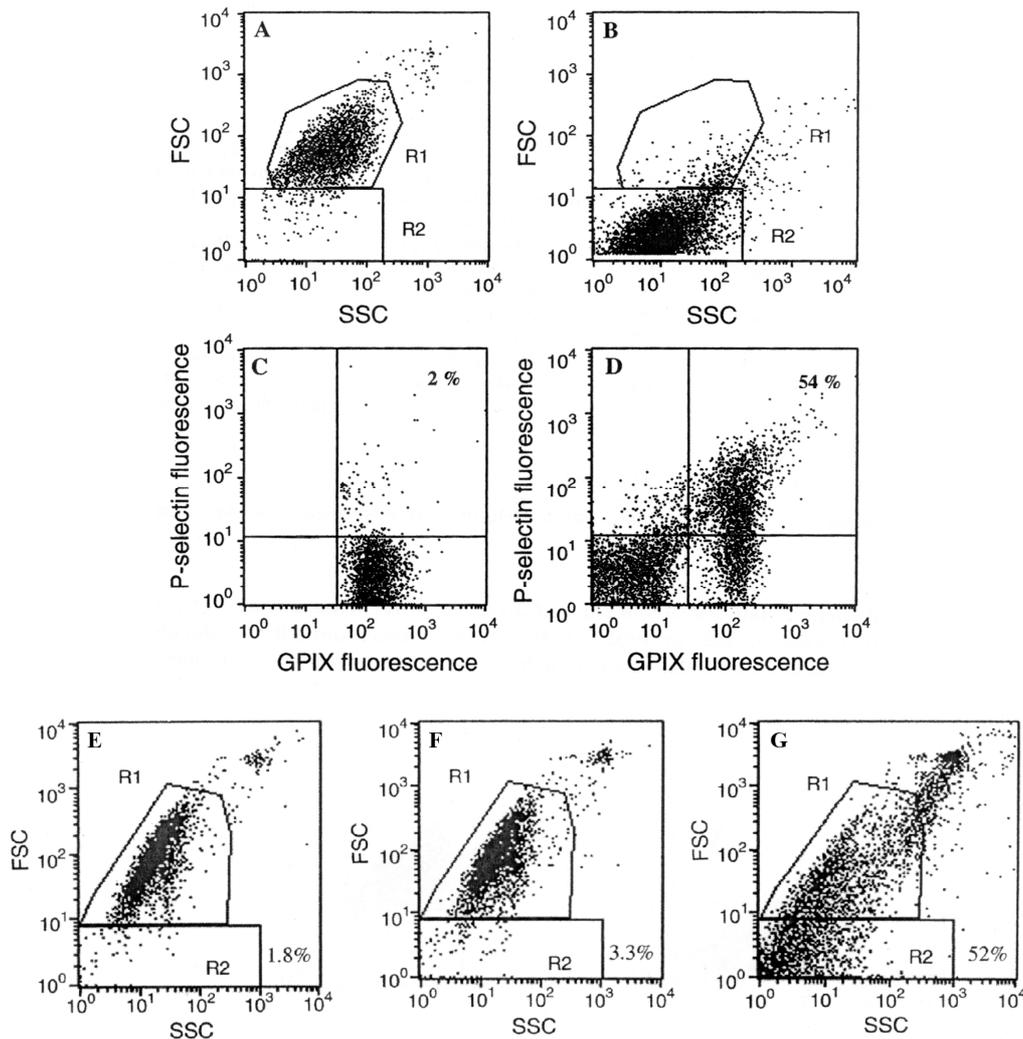
Degranulated platelets interact mostly with monocytes and neutrophils via P-selectin and PSGL-1 receptor interaction. The levels of these heterotypic aggregates can be investigated by flow cytometry when platelet-specific antigens (e.g. CD41a {GPIIb} or CD42a {GPIX} positivity) are analyzed in the subpopulations of leukocytes to measure indirectly their interactions with platelets (see Figure 6 in Methods). The presence of these cell-cell complexes has been shown to be a superior indirect marker of platelet activation in experiments *in vitro* and in *ex vivo* patient samples [53,54]. The half-life of detectable circulating monocyte-platelet aggregates was much longer (cc. 30 minutes) than that of P-selectin-positive platelets (cc. 5 minutes) because of the rapid loss of P-selectin expression *in vivo* [53]. However, in our preliminary *in vitro* studies, platelet P-selectin sensitively reflected the status of platelet activation compared to other platelet markers at low platelet agonist concentrations (e.g.  $\alpha$ -thrombin) (Table 2). Anyway, the analysis of platelet

activation markers at an early time point is necessary to detect elevated activation of platelets effectively.

Thrombin (U/mL)	P-selectin (%)	P-selectin MFI	GPIX MFI	Microparticle (%)
0	2	60	182	2
0.01	8	65	163	2
0.02	20	69	171	2
0.03	48	116	109	2
0.05	66	142	124	3
0.1	85	168	110	9

**Table 2.** Different sensitivity of direct platelet activation markers upon stimulation with increasing concentrations of  $\alpha$ -thrombin *in vitro*. MFI: mean fluorescence intensity.

MPs are mostly derived from activated platelets, since PMPs constitute the largest subpopulation of all MPs [55-60]. When MPs are isolated from normal human plasma, most vesicles express platelet-specific markers (e.g. GPIX), and also P-selectin as being activated (Figure 5D). *In vitro*, platelets were fragmented at high thrombin concentrations (0.5 U/ml) that were easily detectable by FSC and SSC analysis. P-selectin expression increased gradually by elevating concentration of  $\alpha$ -thrombin (Figure 3), while significant increase of the event count in the MP gate was only evident at high  $\alpha$ -thrombin stimulation (Figure 5G).



**Figure 5.** P-selectin expression is different on the surface of intact platelets and MPs. (A,C) In unstimulated whole blood, P-selectin level is only 2% on platelets (R1). (B, D) However, the majority of MPs (R2) separated from the same sample was of platelet origin (GPIX-positive) and 54% of them expressed P-selectin. The level of MPs (R2) increased in thrombin concentration-dependent manner, while the events of platelets (R1) decreased simultaneously due to their fragmentation (E-G).

However, these vesicles may also be released from endothelial cells, erythrocytes, leukocytes, and even megakaryocytes [61-63]. MPs are procoagulant because of high level of PS with almost 3 times higher density than on activated platelets, to where Annexin V protein binds specifically in the presence of  $Ca^{2+}$ . In addition, a smaller portion of MPs is highly procoagulant by expressing not only PS, but also TF [64]. P-selectin on activated platelets and endothelial cells and shedded soluble P-selectin bind to PSGL-1 receptors on monocytes, and this event induces TF-positive MP generation [65]. Furthermore, P-

selectin on platelets in thrombi promotes the recruitment of these MPs into the thrombus by binding to PSGL-1 on the MPs. This leads to increased thrombin generation at the site of injury [65].

Especially in the last decade, the pathological role of elevated PMP levels as a long-term consequence of platelet activation has been investigated in several diseases such as in the different forms of CAD, hypertension, paroxysmal nocturnal hemoglobinuria, chronic renal failure or in cancer reviewed in Refs [62,66]. At least five different variables may contribute to the results obtained in normal and pathological samples: the gauge of needle, the type of anticoagulant, type of sample (MP suspension or MPs in platelet-poor plasma {PPP}), conditions for centrifugation and storage conditions [67]. The source of sample (intracardiac catheter or peripheral blood) may also affect the findings, however only a few studies investigated blood samples drawn directly from the stenting area [57,59]. Thus, the total number of PMPs varies widely among different reports. Comparison of these data is difficult, since studies reported MP results in various units, and the MP distribution originating from other cells than platelets as well as the time course of MP clearance were also quite variable. Some former studies measured much higher mean PMP levels in PPP with larger variability ( $1137 \pm 790/\mu\text{l}$  plasma) compared to those, which analyzed PMPs in MP suspension ( $262 \pm 146/\mu\text{l}$  plasma). Cellular contamination may contribute to the very high PMP levels in PPP samples [67], hence, we also used MP suspension in our experiments.

Percutaneous coronary intervention (PCI) with coronary stenting is a widely administered invasive cardiological procedure in acute and elective cases of CAD with occluded or stenotic arteries in patients with stable or unstable angina or myocardial infarction [68]. This invasive intervention may result in endothelial injury and inflammation resulting in high levels of activated platelets with significantly increased levels of PMPs [55-60]. Such complex events may cause subacute and sometimes late stent thrombosis when platelets and PMPs adhere to and thus occlude the stent area. This is one of the major limitations of invasive cardiological interventions with the possibility of life-threatening complication. Thus, the measurement of PMP levels at a very early time point of sampling could make it possible to gain a new insight into the direct platelet-activating effect of stenting in these conditions.

As described above, platelets contain several biomarkers in their granules and release them upon activation such as P-selectin, thromboglobulin, and thrombospondin

from  $\alpha$ -granules, and lysosome-associated membrane protein {CD63} from lysosomes [4]. In addition, other types can be shown in the cytosol like CD40L [69], which is also expressed by activated platelets after being secreted. Coagulation FXIII is also found in platelet cytoplasm in large quantity [70], but its fate and role are not fully understood. FXIII is a protransglutaminase that is essential for maintaining hemostasis as a key regulator of fibrinolysis [70]. Plasma FXIII is a heterotetrameric zymogen (FXIII-A<sub>2</sub>B<sub>2</sub>) that consists of two potentially active A subunits (FXIII-A) and two carrier/inhibitory B subunits (FXIII-B) [71]. The cellular form of FXIII (FXIII-A<sub>2</sub>) that is a dimer of two A subunits, is present in monocytes, macrophages and the platelet cytosol [71,72]. Platelet-bound FXIII is targeted and concentrated at the site where platelet-rich thrombi are formed [71]. FXIII increases the fibrinolytic resistance of platelet-rich thrombi through the cross-linking of  $\alpha_2$ -antiplasmin to fibrin [73,74] and the presence of platelets accelerates the cross-linking process [74]. The interaction between activated FXIII (FXIIIa) and platelets has been investigated in a few former studies [75-77], and a saturable and specific binding of FXIIIa to thrombin-stimulated, but not to resting platelets was demonstrated. However, data on the binding site of FXIIIa were contradictory. In the first study, GPIIb/IIIa receptor and platelet-bound fibrin(ogen) were ruled out as binding site for FXIIIa; RGD peptide did not influence the interaction between FXIIIa and platelets, and FXIIIa binding to platelets from two patients with severe Glanzmann thrombasthenia (GT) was normal [75]. The binding of FXIIIa to thrombin-activated platelets was inhibited by plasmin [76]. In a later study, however, the GPIIb/IIIa receptor was shown to mediate the binding of FXIIIa to thrombin-stimulated platelets, and an indirect binding of FXIIIa through fibrinogen associated with its receptor was also demonstrated [77]. However, it was not studied whether FXIII in non-activated (A<sub>2</sub>B<sub>2</sub>) form can also bind to activated platelets directly, or via  $\gamma$ A/ $\gamma'$  and  $\gamma$ A/ $\gamma$ A fibrinogens only, and then on the surface of activated platelets it becomes easily activated by locally formed thrombin and exerts its cross-linking action.

## **SUBJECTS AND MATERIALS**

### ***Obese, type 2 DM patients and healthy controls***

In the study to investigate the effect of the Thr715Pro P-selectin polymorphism on soluble P-selectin levels, we recruited 119 type 2 DM patients (diagnosed according to WHO criteria), 57 healthy volunteers with body mass index (BMI) <25 kg/m<sup>2</sup>, and a BMI-matched non-diabetic study group consisting of overweight and obese subjects (n=48) (Table 3). Patients were enrolled from the Outpatients Clinic of the 1<sup>st</sup> Department of Internal Medicine, University of Debrecen. Type 2 DM patients were treated as required by antihypertensive, oral hypoglycaemic agents or diet, and none of them had thromboembolism. Only 21% of DM patients were administered on aspirin as antiplatelet medication except for 1 patient who took clopidogrel, while one of the exclusion criteria was severe symptomatic vascular disease such as angina. However, healthy and obese subjects took no such drugs, as did not suffer from cardiovascular or inflammatory disease. Other exclusion criteria were severe intermittent claudication, transient ischemic attack, malignancy, pregnancy, impaired liver or renal function and infectious diseases. Healthy controls did not suffer from any vascular, neoplastic, metabolic disease, as observed by careful examination and routine laboratory tests. Overweight and obese subjects were without history or clinical evidence of diabetes, however 6 individuals displayed impaired glucose tolerance and 15 subjects had mild hyperlipoproteinemia. An additional classification of type 2 DM patients was also made, that allowed a pairwise comparison of a subgroup of diabetic patients (age-matched DM group, n=57) with healthy controls (see in Results).

### ***Patients with stable angina underwent stenting or catheterization***

We studied the direct effect of PCI on platelet activation via measuring the level of PMPs. 25 patients (20 men and 5 women; mean age: 61.69 ± 12.65 years) with stable angina were recruited, who underwent stenting with (non-drug eluting) bare metal stent in the Department of Cardiology, University of Debrecen. Results were compared to 20 age-matched control individuals (15 men and 5 women; mean age: 59.6 ± 6.65 years) suffered from stable angina, but with no eligible coronary stenosis for stent implantation, who underwent diagnostic coronary catheterization only, and were not stented. All participants

were on aspirin (100 mg/day) monotherapy prior to the intervention, with no other antiplatelet medications. All individuals avoided taking other drugs that might have affected platelet function for 7 days before sampling. No significant difference in baseline laboratory parameters was found between the two study groups (data not shown). Diagnostic catheterization was carried out in all patients and stenting was processed in case significantly stenotic or occluded coronary artery was found. During the intervention an intravenous bolus of 100 IU/kg Na-heparin was administered to all patients. Since there was no difference in the medication of the individuals in these groups, we could readily compare the acute impact of the two distinct invasive procedures in the same disease states. No bleeding or thrombotic complications were recognized during and after the procedures for 30 days. Investigated subjects gave informed consent to the study, which was in accordance to the Declaration of Helsinki.

#### ***A patient with Glanzmann thrombasthenia***

We analyzed the binding of non-activated FXIII to whole blood and washed platelets stimulated with TRAP in healthy individuals. In order to test the role of GPIIb/IIIa receptor in FXIII binding, platelets with a severe type I GT (GPIIb<200 receptors) were obtained from an 8-year-old patient treated at the Department of Pediatrics, University of Debrecen [78].

#### ***Reagents***

Apyrase, prostaglandin E<sub>1</sub> (PGE<sub>1</sub>), bovine serum albumin (BSA), TRAP, purified fibrinogen from human plasma containing FXIII (6.2 µg/mg fibrinogen), RGDS (Arg-Gly-Asp-Ser) tetrapeptide, paraformaldehyde (PFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Eptifibatide (Integrilin<sup>®</sup>) was obtained from Schering-Plough (Kenilworth, NJ). Highly purified human FXIII-A<sub>2</sub>B<sub>2</sub> was prepared from the plasma of healthy volunteers according to Lorand *et al.* [79]. Human plasma fibrinogen variants γA/γA and γA/γ' (peak 1 and peak 2) were distinguished by DEAE-cellulose gradient elution chromatography [80] and were obtained from Enzyme Research Laboratories

(South Bend, IN, USA). Peak 1 and peak 2 fibrinogens were essentially FXIII free (FXIII content was less than 0.1  $\mu\text{g}/\text{mg}$  fibrinogen). FXIII content of all fibrinogen variants was measured by one-step sandwich ELISA (R-ELISA FXIII, Reanal-Labexpert, Budapest, Hungary), which is specific for the tetrameric plasma FXIII [81]. The monospecificity of FITC-labeled anti-human-FXIII-A antibody was tested by intracellular staining of FXIII content in permeabilized platelets (data not shown). PE-labeled anti-human-P-selectin (CD62), non-immune mouse IgG<sub>1</sub> (as isotype control) and PerCP-conjugated monoclonal antibody to GPIX (CD42a) antibodies were purchased from Becton Dickinson (San Jose, CA, USA). All other substances were of reagent grade.

## METHODS

### *Blood drawing and sample preparation*

Venous blood from patients and their controls was obtained into Vacutainer tubes containing 0.105 M sodium citrate (Becton Dickinson) by atraumatic venepuncture. Blood sampling conditions were designed to avoid artefactual activation of platelets during phlebotomy. In whole blood experiments within 2 hours after collection, 40  $\mu$ l blood from patient and control samples was fixed in 1 ml 1% PFA and kept at room temperature (RT) for minimum 1 hour. Platelet number was determined in each case by Advia 120 Hematology System (Bayer Diagnostics, Tarrytown, NJ). Fixed whole blood samples were centrifuged at 1300 x g for 15 minutes at RT. The pellet was washed in 1 ml phosphate-buffered saline (PBS) buffer, then centrifuged as above and finally resuspended in PBS.

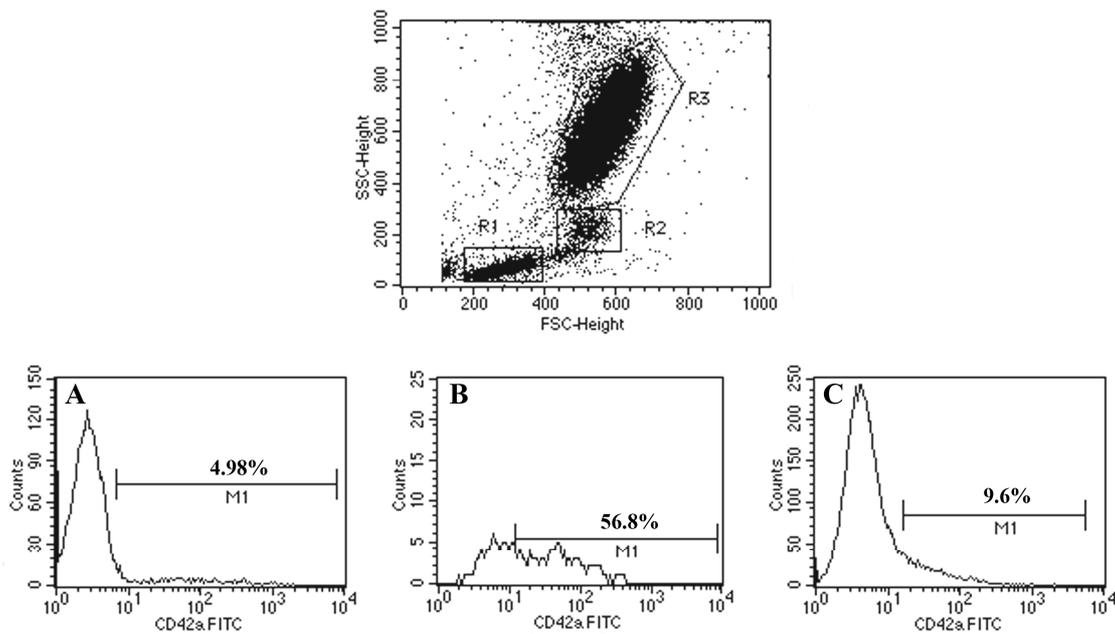
In order to study the amount of PMPs, PPP was obtained from whole blood anticoagulated with sodium citrate by centrifugation at 1550 x g for 20 minutes at RT. Five hundred  $\mu$ l of PPP was spun down at 13000 x g for 2 minutes to get rid of platelet debris, and then centrifuged at 16100 x g for 30 minutes at RT to isolate MPs as described in previous publications [82,83]. We used their protocol with some minor modifications.

In the *in vitro* experiments, blood was dropped into Vacutainer tube containing ACD (38 mM citric acid, 75 mM sodium-citrate, 136 mM glucose) and 300 ng/ml PGE<sub>1</sub> was added before any further procedure to achieve minimal platelet activation during upcoming sample manipulation. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 x g for 15 minutes at 37°C and then platelets were sedimented by centrifugation at 1200 x g for 15 minutes at 37°C. The platelet pellet was washed three times in a modified HEPES-Tyrode's buffer (140 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1% glucose, 0.36% BSA, 10 mM HEPES, pH 7.4) in the presence of 1 U/ml apyrase and 300 ng/ml PGE<sub>1</sub>. After washing, platelets were finally resuspended in the HEPES-Tyrode's buffer without apyrase and PGE<sub>1</sub>.

### Flow cytometric analysis of clinical samples

Platelets were identified by a monoclonal antibody to GPIIb/IIIa (CD42a-FITC), while their activation status was detected by using anti-P-selectin antibody (CD62-PE). Fixed platelets were incubated with saturating concentrations of antibodies for 20 minutes in the dark at RT. As a control for immunolabeling with anti-CD62, platelets were incubated with PE-coupled non-immune mouse IgG<sub>1</sub> antibody, and 10 000 dual-color labeled platelet events were acquired on a FACSCalibur flow cytometer by using the CellQuest 3.2 software (Becton Dickinson).

The levels of platelet-leukocyte aggregates were investigated from whole blood when CD42a positivity on cell surface was analyzed in the different subpopulations of leukocytes to measure indirectly their interactions with platelets (Figure 6).



**Figure 6.** Flow cytometric analysis of the different leukocyte populations (R1: lymphocytes, R2: monocytes, R3: granulocytes), which were identified and gated according to their FSC and SSC characteristics, and then platelet-specific CD42a positivity was observed on cell surface. Representative histograms display the percentage of CD42a positivity on lymphocytes (A), monocytes (B), and granulocytes (C) after being set with isotype control sample for each.

In the MP study, we used and compared two sets of fluorescent beads TruCOUNT<sup>®</sup> (Becton Dickinson) and CytoCount<sup>®</sup> (Dako, Glostrup, Denmark) with

standard size and bead number for enumeration of MPs. Beads were not added directly into samples during the analysis, since in our preliminary experiments we had found a decrease in MP number due to the possible attachment of MPs to some beads. Thus, tubes with beads were first processed, and then clinical samples were measured within a standard collection time (30 seconds). The numbers of MPs were calculated based on the event count from the bead tube collected for the same time period. MP preparation was processed at exactly 120 minutes after sampling when separation was feasible in each patient. MPs were gated into a restricted area by FSC and SSC parameters, and then PMPs were identified by their Annexin V and CD41a positivities. Furthermore, anti-CD62 antibody was also used to measure the P-selectin expression on PMP surface as the marker of their activation status.

### ***Laboratory assays***

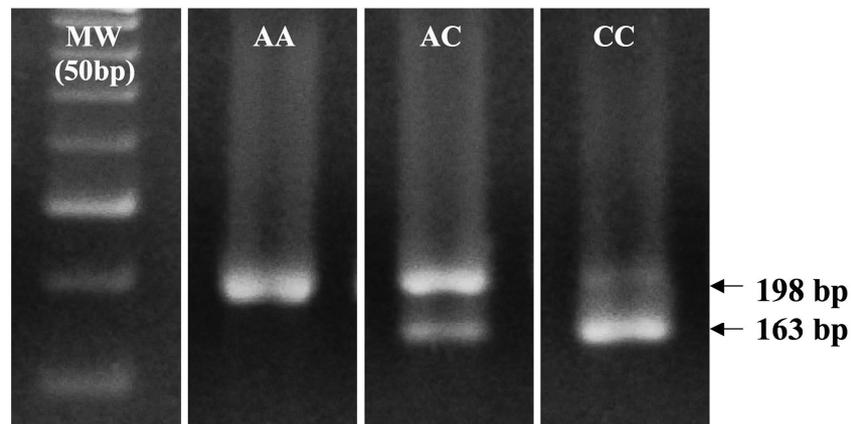
Soluble P-selectin levels in plasma were analyzed by ELISA (R&D Systems, Minneapolis, MN) commercial kits following the manufacturer's instructions. All plasma samples were centrifuged immediately at 2000 x g for 15 minutes at RT, aspirated and stored at -70°C until analysis. This procedure was completed within 30 minutes of blood drawing.

Blood glucose, total cholesterol, and triglyceride values in patients and their controls were measured on Hitachi analyzer (Roche, Mannheim, Germany) and LDL-cholesterol levels were calculated by the Friedewald formula. HbA1c was measured by HPLC (BioRad, Hercules, CA) and the fibrinogen levels were determined by the Clauss-method on Stago Compact (Stago, Asnières, France). CRP was measured by a turbidimetric assay on Integra 400 analyzer (Roche).

### ***Genetic analysis of Thr715Pro P-selectin polymorphism***

It was performed as previously described [46] with some minor modifications. Genomic DNA was extracted from whole blood anticoagulated with sodium citrate by QIAamp DNA blood kit (Qiagen, Hilden, Germany). Primers were designed by Primer3 and used to amplify exon 13 of P-selectin gene. The sequences of the oligonucleotide primers were 5'-TTTCTGCAGCTGTGAAATGC-3' and 5'-ATTGTACCTTGCCAGGTT

GG-3'. Polymerase chain reaction (PCR) was performed in a total volume of 50  $\mu$ l containing 100 ng of DNA, 10 pmol of each primer, 200  $\mu$ M dNTPs, 1.5 mM MgCl<sub>2</sub>, 10% DMSO and 2 units Taq DNA polymerase (Roche). After the initial denaturation at 94°C for 5 minutes, amplification was carried out for 40 cycles of 94°C for 30 seconds, 60°C for 60 seconds and 72°C for 60 seconds, and the final extension at 72°C for 10 minutes. In restriction fragment length polymorphism (RFLP) analysis, the PCR product (198 bp) was digested by Eco91I (Fermentas, Vilnius, Lithuania) and the digested products were run on a 3% agarose gel and visualized under UV light by ethidium bromide staining. In the presence of Thr715Pro mutation, a new (163 bp) DNA product could be detected during analysis (Figure 7).



**Figure 7.** Representative agarose gel detecting the DNA products with different size after RFLP. MW: molecular weight ladder (50 bp); AA: wild type, AC: heterozygous, CC: homozygous mutant for Thr715Pro.

#### ***Analysis of FXIII binding to TRAP-stimulated platelets***

First, FXIII binding to platelets was observed in whole blood. Citrated whole blood was stimulated with TRAP (0-40  $\mu$ M) for 15 minutes at 37°C in HEPES-Tyrode's buffer containing 5  $\mu$ l anti-human-FXIII-A antibody (1  $\mu$ g/ml). Platelets were identified by their CD42a positivity. Platelet activation was detected using anti-P-selectin (CD62) antibody in a three-color-labeling during flow cytometric analysis. As a control for immunolabeling, platelets were incubated with non-immune mouse IgG<sub>1</sub> antibody. After

activation, samples were processed as described in “Blood drawing and sample preparation”.

To investigate the plasma or cytosol origin of FXIII bound to stimulated platelets in whole blood, washed human platelets were also stimulated and labeled under the same conditions as above. Results were expressed as percentage of FXIII-A positivity of platelets. In order to evaluate the FXIII-A and CD62 positivity on platelets with different size, the whole platelet population was divided into three equal-sized subgroups according to their FSC signal. Large, medium and small platelets were individually analyzed at the same cut-off of fluorescence intensity.

### ***Measuring the effect of GPIIb/IIIa antagonists on FXIII binding***

To test the role of the GPIIb/IIIa receptor in the binding of non-active FXIII in whole blood, we used two pharmacological approaches. GPIIb/IIIa receptor antagonist eptifibatide (Integrilin<sup>®</sup>) was used as an agent with high specificity that blocks the final common pathway in platelet aggregation by inhibiting the binding of fibrinogen to the receptor on the surface of activated platelets, which then decreases cross-linking of platelets and thus platelet aggregation [84]. Furthermore, the effect of tetrapeptide containing RGD-sequence (RGDS) was also examined, which interferes directly with the interaction of fibrinogen and its platelet receptor [85]. Thus, citrated whole blood samples were preincubated with eptifibatide (2 µg/ml) or RGDS peptide (5 mM) for 15 minutes at 37°C. After preincubation, platelets were stimulated with different concentrations of TRAP and studied by flow cytometry under the same conditions as described earlier.

### ***Studying FXIII binding in Glanzmann thrombasthenia***

We confirmed the involvement of GPIIb/IIIa receptor in plasma FXIII binding in further experiments using whole blood sample from a patient with severe type I GT with very low level of platelet GPIIb/IIIa receptors [78]. Citrated whole blood was stimulated with increasing concentrations of TRAP for 15 minutes at RT. Results from this sample were compared to the platelets of healthy controls according to the FXIII-A % positivity.

### ***Analysis of the mechanism of non-active FXIII binding to activated platelets***

To determine if plasma FXIII binds directly to GPIIb/IIIa receptor or receptor-bound fibrinogen is essential for its binding to activated platelets, we studied platelet FXIII-A surface positivity when FXIII-A<sub>2</sub>B<sub>2</sub> (25 µg/ml) with/or without γA/γ' fibrinogen (2.5 mg/ml) or γA/γA fibrinogen (2.5 mg/ml) were added to unstimulated and TRAP-activated (40 µM) washed human platelets for 15 minutes at 37°C in the presence of CaCl<sub>2</sub> (1 mM). Native human plasma fibrinogen containing well detectable amount of FXIII was used as a positive control to demonstrate FXIII-A positivity on TRAP-stimulated platelets. After incubation, samples were fixed in 1% PFA and then unbound FXIII-A<sub>2</sub>B<sub>2</sub> with the excess of antibodies were removed by washing in PBS prior to analysis.

### ***Statistical analysis***

Unless otherwise indicated, data were expressed as mean ± SEM and analyzed by unpaired Student's *t* test. The *p* values less than 0.05 were considered significant.

In the DM study, Kolmogorov-Smirnov test was used for the evaluation of the normality of the data. Most outcome continuous parameters were non-normally distributed; therefore analyses were performed on log-transformed data for Student's independent *t* test analysis. Differences in various parameters among study groups were tested using analysis of variance and chi-square test as appropriate. Deviations from the Hardy-Weinberg equilibrium were analyzed using the chi-square test in each group. Multiple regression analysis was computed for checking the association of baseline characteristics with soluble P-selectin level. Univariate analysis of variance was used to adjust for significant variables and check for differences in soluble P-selectin levels within the different genotypes (see Table 4 below). Statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

### *Significantly increased platelet and soluble P-selectin levels in obese and type 2 DM patients compared to healthy individuals*

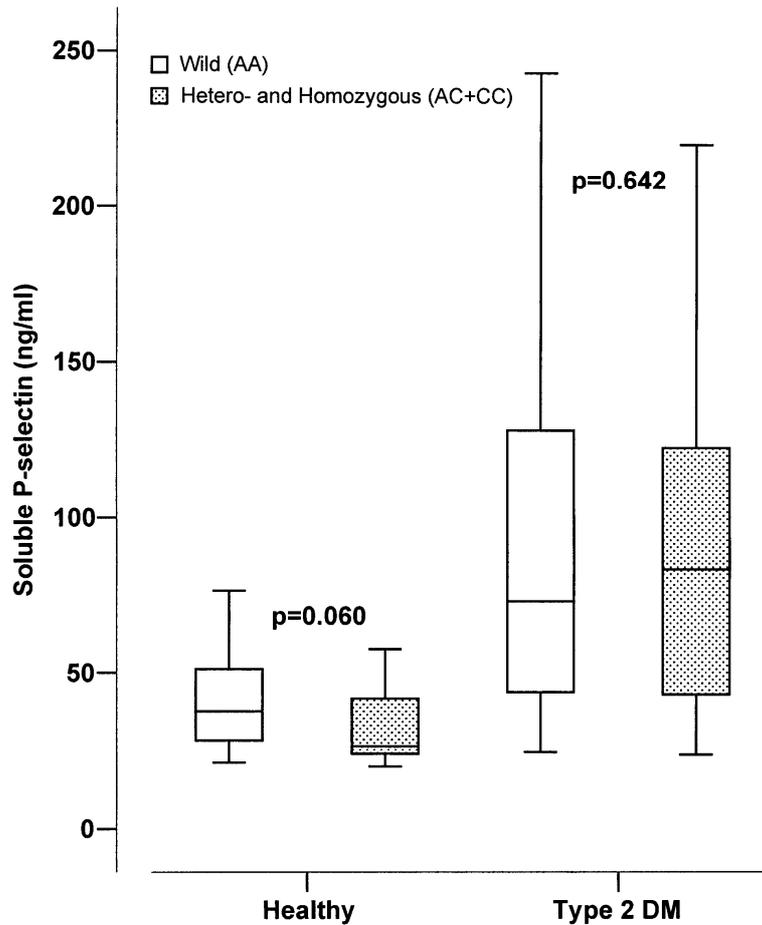
We investigated only the Thr715Pro P-selectin polymorphism; since it has been proved to be the most relevant to influence soluble P-selectin levels due to its localization [45,46,48]. Regarding to all study individuals, there were marked differences in the levels of baseline demographical and laboratory parameters and platelet activation markers between the study groups (Table 3). Beside BMI, age also differed significantly between DM patients and healthy controls, since we could only enroll younger volunteers without having any chronic disease or long-term medication. Unfortunately, those older normal subjects who would have been more comparable to DM patients, were excluded because of suffering from other disorders. Both the plasma levels of soluble P-selectin and the percentage of platelet P-selectin were significantly increased in the BMI-matched non-DM obese subjects and in the type 2 DM group upon comparison with healthy individuals (Table 3). Enhanced platelet reactivity was earlier demonstrated in both types of DM suggesting the causal relationship between severe angiopathy and activated platelets in children and adults [86].

	Healthy (n= 57)	Type 2 DM (n = 119)	BMI-matched non-DM (n = 48)	p value
<i>Demographic parameters (median, range)</i>				
<b>Male (%)</b>	35.1	65.5	37.5	<0.001
<b>Age (years)</b>	38 (22-61)	54 (18-76)	45.5 (20-63)	<0.001
<b>BMI (kg/m<sup>2</sup>, mean <math>\pm</math> SD)</b>	22.1 $\pm$ 1.9	31 $\pm$ 6.4	29.8 $\pm$ 2.8	<0.001
<b>Duration of DM (years)</b>	0	8 (1-32)	0	<0.001
<b>Never smokers (%)</b>	95	76	69	<0.05
<b>Current smokers (%)</b>	5	24	31	<0.05
<b>Hypertensive (%)</b>	0	82	54	<0.001
<i>Laboratory parameters (mean, 1st and 3rd quartile)</i>				
<b>Fasting glucose (mmol/l)</b>	4.6 (4.25-4.95)	9.4 (6.7-12)	5.1 (4.8-5.4)	<0.001
<b>HbA1c (%)</b>	5.1 (5-5.7)	7.8 (6.7-8.9)	5.6 (5.2-5.8)	<0.001
<b>Total Cholesterol (mmol/l)</b>	4.7 (4.1-5.4)	5.4 (4.4-6)	5.4 (4.6-6.2)	<0.001
<b>LDL-Cholesterol (mmol/l)</b>	1.9 (1.4-2.3)	2.8 (2.2-3.4)	2.4 (1.4-3.5)	<0.001
<b>Triglyceride (mmol/l)</b>	0.9 (0.6-1.1)	3.3 (1.3-3.2)	1.7 (0.9-2)	<0.001
<b>Fibrinogen (g/l)</b>	3.0 (2.5-3.3)	4.1 (3.2-4.8)	3.6 (2.9-4.1)	<0.001
<b>CRP (mg/l)</b>	1.8 (0.6-2.1)	5.8 (1.7-7)	4.9 (1.6-4.2)	<0.001
<i>Flow Cytometric parameters (mean, 1st and 3rd quartile)</i>				
<b>Surface P selectin (%)</b>	1.0 (0.4-1.2)	1.8 (0.7-2.5)	1.9 (0.6-2.3)	<0.001
<b>Soluble P selectin (ng/ml)</b>	39.4 (25.6-49.1)	91.3 (43.8-126.9)	54.4 (42-61)	<0.001

**Table 3.** Characteristics of type 2 DM patients, BMI-matched non-DM (obese) subgroup, and healthy controls. Non-normally distributed data were log-transformed for independent Student's *t* test analysis. Differences in various parameters among study groups were tested using analysis of variance and chi-square test.

***Thr715Pro has minor effect on soluble P-selectin levels in the two patient groups***

The frequency of Thr715Pro P-selectin genotype did not vary significantly between study groups. Healthy: 77.2% (AA, n=44), 22.8% (AC, n=13); BMI-matched non-DM obese: 81.3% (AA, n=39), 18.7% (AC, n=9); type 2 DM: 74.8% (AA, n=89), 23.5% (AC, n=28), 1.7% (CC, n=2). All groups were in Hardy-Weinberg equilibrium. There was no subject with CC genotype in the healthy and obese groups. The CC genotype was rare in DM group, thus these subjects were pooled into the subgroup of patients with the AC genotype. In type 2 DM patients, the levels of soluble P-selectin were significantly increased compared to controls, but no difference ( $p=0.642$ ) was observed between the AC+CC and AA genotypes (Figure 8). In addition, contrary to previous studies, markedly lower soluble P-selectin levels were measured in healthy Pro715 allele carriers, but the difference was not statistically significant ( $p=0.060$ ) between the two distinct genotypes. We wonder if this alteration may come from the ratio of ethnics and other subjects within our study population compared to other authors' study cohort. In obese subjects, soluble P-selectin levels were also elevated compared to all healthy controls, but without difference ( $p=0.777$ ) between AA and AC carriers (Figure 9). Miller *et al.* [46] found that in whites and South Asians the C allele was associated with lower levels of soluble P-selectin, but in blacks - in whom the C allele was very rare - it was not accounted for by the Thr715Pro polymorphism. We do not have any data whether gypsies would demonstrate distinct pattern of allele variation and thus soluble P-selectin levels.

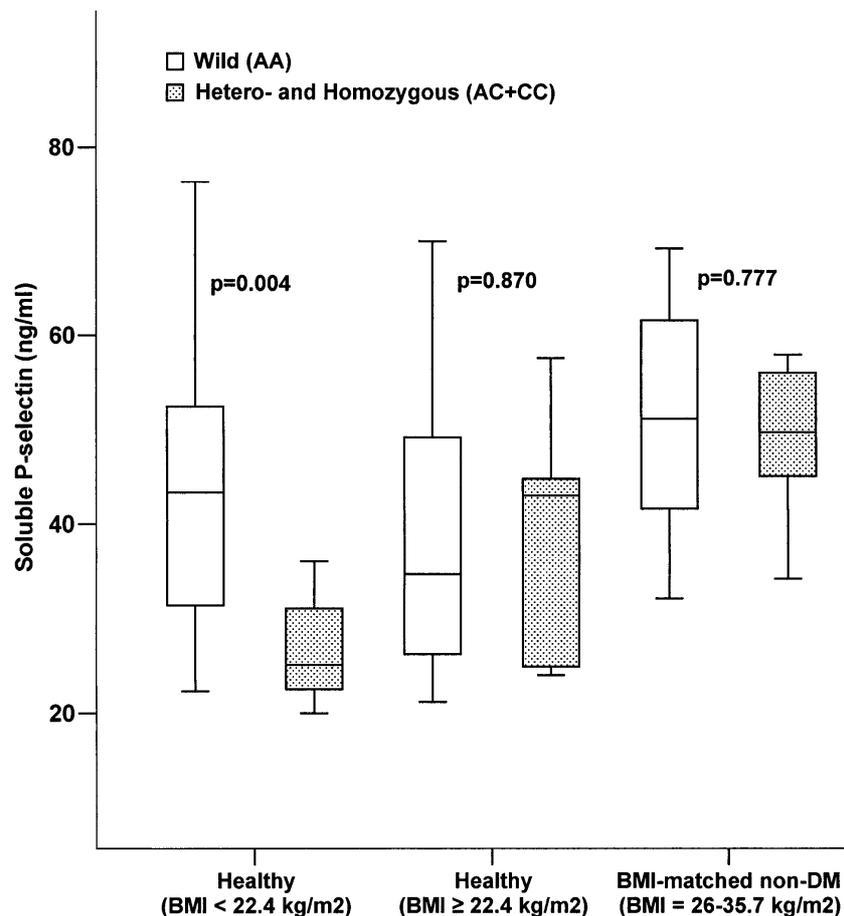


**Figure 8.** Comparison of soluble P-selectin levels in healthy and type 2 DM individuals according to their Thr715Pro genotype. Data were analyzed by Student's *t* test.

***Effect of BMI on soluble P-selectin levels in non-DM subjects***

The association between BMI and soluble P-selectin levels was analyzed in all non-diabetic subjects. The levels of soluble P-selectin in healthy carriers of the C allele with BMI < 22.4 kg/m<sup>2</sup> were significantly (p=0.004) lower compared to controls with the AA genotype. Interestingly, this difference was not detectable in healthy subjects with higher BMI (≥ 22.4 kg/m<sup>2</sup>) (Figure 9). Although we could confirm previous reports that lower soluble P-selectin levels could be measured in healthy Pro715 allele carriers, but this was only true in lean subjects. We can only speculate why we found this large difference in the levels of soluble P-selectin between healthy controls with higher and lower BMI. Barbaux *et al.* [44] also revealed a positive correlation between soluble P-selectin levels and BMI

only in their healthy controls, but no association was found in patients with cardiovascular disease (CVD). Thus, we think that the “very healthy” individuals with  $<22.4 \text{ kg/m}^2$  BMI values had no (or only few) risk factors for having increased platelet activation status, and thus higher soluble P-selectin levels. However, in case of “still healthy” people with normal BMI close to  $25 \text{ kg/m}^2$ , there might be several factors (diet, lack of exercises, etc.), which may slightly promote elevation of platelet activation marker levels, as we found the tendency in overweight and obese individuals with even higher BMI. In addition, there might be some differences in the demographical characteristics in our patients versus Western European or American subjects in other studies.



**Figure 9.** Soluble P-selectin levels in all non-diabetic subjects with different BMI values. Subgroups were compared to each other by using Student’s *t* test.

### ***Effect of different variables on soluble P-selectin levels in type 2 DM patients***

Previous studies have showed age- and sex-dependence of soluble P-selectin levels [44,45]. However, another report found no correlation between the levels of soluble P-selectin and age in type 2 DM [37]. In our DM group, age did affect the soluble P-selectin levels. There was a significant difference ( $p < 0.05$ ) in soluble P-selectin levels among older (median age of  $\geq 54$  years,  $111.4 \pm 61.7$  ng/ml) and younger (median age  $< 54$  years,  $64.7 \pm 47.1$  ng/ml) patients when all DM subjects were pooled, but genotype did not influence soluble P-selectin level even in these subgroups studied (Table 4). Since age significantly affected soluble P-selectin levels, we compiled 2 age-matched groups and created 57 pairs: one consisting of DM patients and the other of healthy controls. P-selectin levels were significantly ( $p < 0.001$ ) higher in the DM group (mean: 79.4 ng/ml, quartiles: 39.7-121 ng/ml) as compared to the age-matched healthy control group (mean: 46.6 ng/ml, quartiles: 33.2-57.6 ng/ml). The levels of soluble P-selectin did not vary with the genotype in this subgrouping either.

There was no statistically significant difference between male ( $91.5 \pm 65.5$  ng/ml) and female ( $86.4 \pm 44.6$  ng/ml) patients, and no variation could be demonstrated when the different sexes were analyzed according to genotype (Table 4). The soluble P-selectin levels did not differ with the smoking status, since no statistically significant difference was found between 'never smoker' DM patients ( $87.8 \pm 49.7$  ng/ml) and current DM smokers ( $90.9 \pm 80$  ng/ml). Here, too, no difference was found in terms of soluble P-selectin levels according to genotypes (Table 4). Some previous studies observed an association between Pro715 allele and lower soluble P-selectin levels, but this was true only in non-smokers with CVD [44,45]. However, others failed to find this dependence in a healthy cohort [46].

Groups	AA genotype	AC+CC genotype	<i>p</i> value
Total DM group (n=119)	89.9 (43.8-130) (n=89)	95.6 (42.6-122.6) (n=30)	0.642
Males (n=78)	91.5 (42.2-132.5) (n=60)	102.3 (40.6-163.6) (n=18)	0.726
Females (n=41)	86.4 (44.2-128.9) (n=29)	85.5 (64.1-120.3) (n=12)	0.746
Never smokers (n=86)	87.8 (43.9-127.7) (n=68)	80 (42.6-115.4) (n=18)	0.901
Current smokers (n=27)	90.9 (41.2-159.4) (n=16)	126 (63.3-171.2) (n=11)	0.293
Older (n=61)*	111.4 (63-162.7) (n=48)	117 (81.7-122.7) (n=13)	0.607
Younger (n=58)*	64.7 (36.4-86.1) (n=41)	79.2 (39.5-125.4) (n=17)	0.412
Higher BMI (n=60) **	82.5 (40-130.2) (n=44)	91.8 (39.9-122.9) (n=16)	0.523
Lower BMI (n=56) **	94.9 (47.2-129.5) (n=42)	99.9 (60.2-98.7) (n=14)	0.860
Longer DM duration (n=59) ***	92.5 (44.2-132.6) (n=44)	88.7 (53.5-113.9) (n=15)	0.902
Shorter DM duration (n=58) ***	88.5 (41.2-130.3) (n=44)	107.4 (39.5-163.6) (n=14)	0.499

**Table 4.** Soluble P-selectin levels in type 2 DM patients according to the Thr715Pro genotype. \*  $\geq$  or  $<$  median age of 54 years; \*\*  $\geq$  or  $<$  median BMI of 31 kg/m<sup>2</sup>; \*\*\*  $\geq$  or  $<$  median DM duration of 8 years. Results represent mean (1-3 quartile). Student's independent *t* test was used to compare the subgroups with different genotype after log transformation.

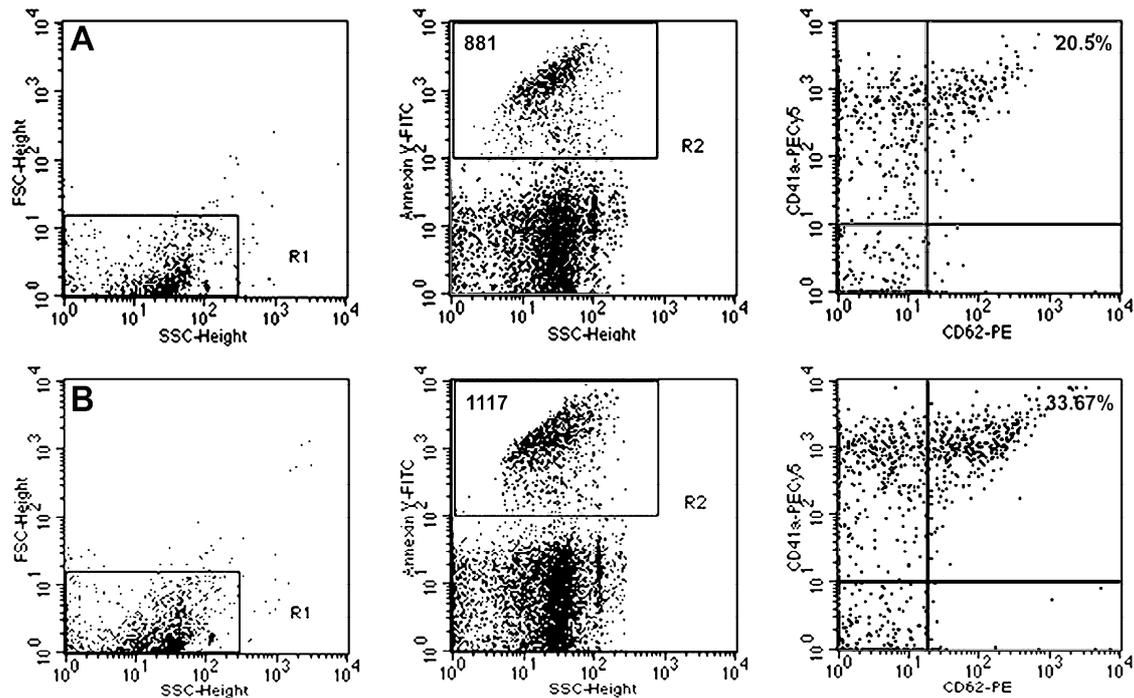
In the DM group, multiple regression analysis was used to test for significant association between soluble P-selectin level and all the continuous variables. Only age was found to be significant in this response. After adjustment for this parameter along with the categorical variables (gender, blood pressure, smoking habit), no significant ( $p=0.204$ ) difference was determined in the levels of soluble P-selectin by univariate analysis of variance among patients with AA and AC+CC genotypes. Future studies are required to investigate whether the chronic metabolic alterations might influence the shedding of surface P-selectin into soluble form resulting in no difference between obese or diabetic subjects with mutant and wild type genotype and healthy controls. The significance of this polymorphism and the haplotypes with additional P-selectin polymorphisms in type 2 DM and in obesity are also needed to be further studied.

#### ***Increased level of PMPs in patients after stenting***

Stents represent a thrombogenic surface resulting in activation and deposition of platelets. Earlier, Szűk *et al.* [87] investigated platelet P-selectin expression in patients with balloon angioplasty (PTCA) receiving two different clopidogrel regimens with

aspirin. They concluded that the measurement of platelet P-selectin was a sensitive tool to monitor the impact of angioplasty and the beneficial effect of antiplatelet drugs. Some previous clinical studies [55-60] have attempted to analyze the levels and characteristics of MPs especially those of platelet origin in patients who underwent PTCA [55,58,59,60], or PCI with stent implantation [56,57] by using flow cytometry [55,57,59], or solid phase capture assay [58], or ELISA [56,60] compared to healthy controls. Here, we applied blood sampling for PMP analysis along with alternative platelet activation markers at 15-minute time point after the intervention. Flow cytometry was assessed as the most widely used technique for the identification, quantitation and characterization of plasma MPs for its simplicity, and the wealth of information [55,57,59,64,67,82,83]. By now, this tool has been standardized and optimized for MP detection [67,88]. It is now generally accepted that only PS-exposing MPs i.e. Annexin V-positive events are considered as MPs, and PMPs are the most abundant form (80-95%), but the distribution of MPs with other cell origin is still a debate [58,62,64,66,82,83].

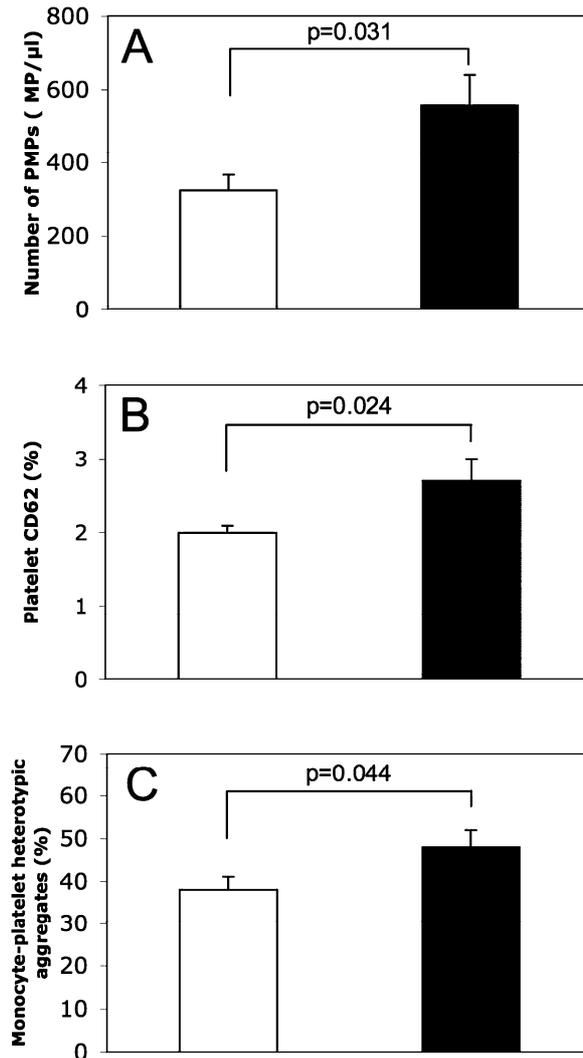
Thus, we determined the level of Annexin V-positive PMPs at the earliest time point that was technically feasible. Only one group [56] published data at 15 minutes as the first sampling time point to measure PMP levels, where no significant increase of PMP levels was found by using ELISA. In contrast, we showed that there was significantly ( $p=0.031$ ) higher level of PMPs ( $557 \pm 83/\mu\text{l}$  versus  $325 \pm 42/\mu\text{l}$ ) even by 15 minutes in patients with stent implantation compared to subjects with catheterization alone (Figures 10 and 11A).



**Figure 10.** Representative dot plots of PMP analysis. MPs were collected in R1 gate according to FSC and SSC parameters. These events were identified in R2 gate by their Annexin V positivity in patients with catheterization (A) and patients after stenting (B). During further analysis, PS-positive MPs gated in R2 were stained by anti-CD41a-PECy5 and anti-CD62-PE antibodies to measure the number of PMPs and their activation status.

#### *Elevated levels of other platelet activation markers in stented patients*

As expected, the procedure of stenting induced activation of platelets demonstrating significantly higher levels of surface P-selectin ( $2.7 \pm 0.3\%$  versus  $1.99 \pm 0.1\%$ ;  $p=0.024$ ; Figure 11B) as it was found earlier [87]. Interestingly, no soluble P-selectin level increase was measured in stented patients compared to controls ( $38 \pm 12$  ng/ml versus  $36 \pm 11$  ng/ml), which may be related to the early sampling time, and thus there was little time for surface P-selectin receptors to be shedded.



**Figure 11.** Flow cytometric analysis of the total number of PMPs, the platelet P-selectin percentage, and the ratio of platelet-monocyte aggregates. (A) The absolute number of PMPs was calculated by using fluorescent beads in patients (filled bar) and controls (open bar). (B) Platelets were stained for CD62 positivity to measure their activation status, and (C) platelet-monocyte interaction was studied based on the CD42a positivity of gated monocytes. Mean values  $\pm$  SEM are indicated, p values were calculated by using Student's *t* test.

Although there was a tendency towards higher level of CD62 positive PMP population (Figure 10A-B) with higher CD62 MFI values, the difference in these results were not statistically significant between stented patients and control groups. Furthermore, the ratio of leukocyte-platelet heterotypic aggregates was also elevated, but only monocyte-platelet aggregates displayed a statistically significant ( $p=0.044$ ) increase after

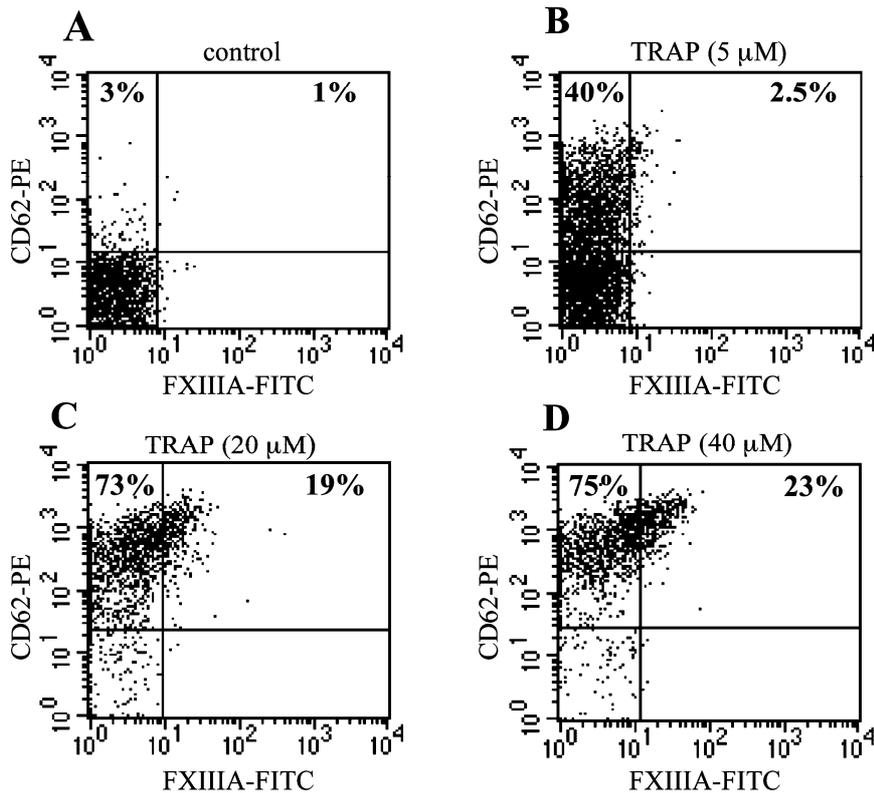
stenting ( $48 \pm 4\%$  versus  $38 \pm 3\%$ ; Figure 11C). These findings are in agreement with some previous reports [55-60], which detected increased levels of PMPs in stented patients measured in coronary or peripheral circulation. However, we do not exclude the significance of MPs shed from other source like endothelial cells. Although others found only mild endothelial injury after coronary angiography and PCI in stable angina patients when detecting the extent of vessel wall injury [89]. The platelet-activating effect of stent implantation in patients could be compared more relevantly to those ‘controls’, whose coronary arteries were also exposed to the similar degree of invasive procedures during catheterization except for the stenting [90]. This was evident from the data of these subjects who have elevated platelet P-selectin expression compared to former examined healthy controls in our polymorphism study (see Table 3). Although stents may induce platelet activation, a follow-up study up to 30 days described a beneficial effect of stenting by detecting decreasing levels of PMPs and other platelet activation markers [60].

Accordingly, the analysis of increased level of PMPs can be considered as an early and sensitive method to detect procedure-induced activation of platelets right after invasive cardiological interventions, and may supplement other tools like the measurement of surface CD63 expression [91,92], the plasma levels of soluble GPV [58], soluble CD40L and RANTES [60] measured by ELISA to evaluate platelet reactivity. We used non-eluting bare metal stents; so further studies are needed to study if these parameters are suitable to predict prothrombotic events. In addition, the cell-activating effect of other (e.g. drug-eluting) types of stents on platelet activation and PMP levels may be interesting for comparison with the impact of bare metal type.

### ***FXIII binds to TRAP-stimulated human platelets***

We investigated the binding of non-active FXIII to human platelets in whole blood stimulated with TRAP (Figure 12). Platelet activation was tested by measuring P-selectin (CD62) labeling and the co-expression of these markers was studied subsequently on flow cytometer. In the absence of agonist, unstimulated platelets failed to bind FXIII (Figure 12A). However, FXIII-A positivity significantly increased gradually upon platelet stimulation with TRAP in a concentration-dependent manner (Figure 12B-D) and reached the plateau level in response to  $40 \mu\text{M}$  TRAP when mean CD62 positivity was over 90% indicating maximum platelet activation. No further increase in positivity of either marker

was detected at higher TRAP concentrations (data not shown). Furthermore, FXIII-A and P-selectin displayed a co-expression when platelets were activated with TRAP as followed in dot plots. These data suggest that FXIII is expressed only on stimulated human whole blood platelets. Similarly, a saturable and specific binding of FXIIIa to thrombin-stimulated, but not to resting platelets was demonstrated earlier [75-77].

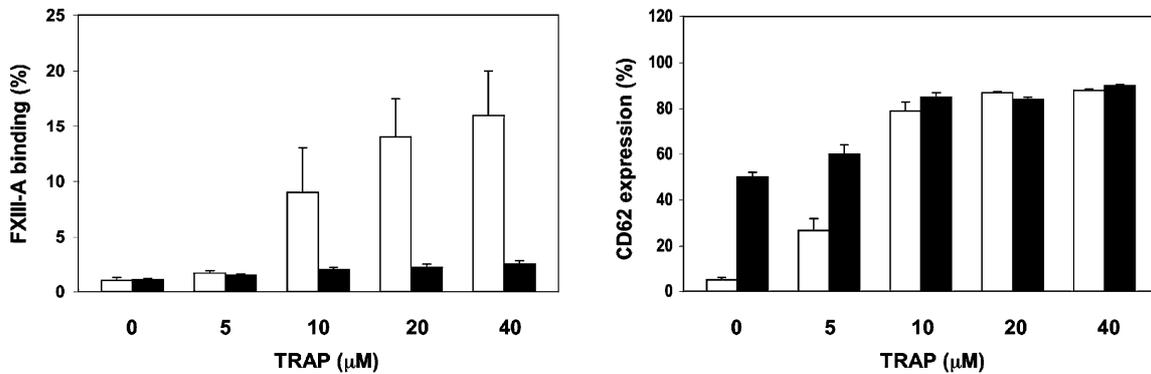


**Figure 12.** Representative dot plots of 4 experiments from different normal donors when unstimulated (A) and TRAP-activated platelet samples (B-D) in whole blood were analyzed by flow cytometry with anti-FXIII-A-FITC and CD62-PE and CD42a-PerCP antibodies. Values indicate the percentage positivity of gated events in each quadrant.

***Platelet-bound FXIII derives from plasma but not from platelet cytosol***

It was not clear before if intracellular FXIII becomes available on the platelet surface upon activation. Former studies suggested that FXIII was retained during platelet activation [93-95]. To determine whether platelet-bound FXIII derives from plasma or becomes released from intracellular localization and subsequently binds to the platelet surface, whole blood samples and washed human platelets were activated with increasing

concentrations of TRAP and compared for FXIII-A and CD62 positivity (Figure 13). In washed platelet samples, no significant elevation in FXIII-A positivity was detected in samples stimulated with various concentrations of TRAP. However, as seen earlier on Figure 12, human platelets in whole blood bound FXIII gradually in response to increasing agonist concentration. On the other hand, CD62 positivity significantly increased upon activation in both types of samples, which indicates that the proper platelet activation occurred. The basal P-selectin level was much higher in unstimulated washed samples compared to control whole blood sample, since platelets were artificially activated during isolation in the washing procedure (Figure 13).



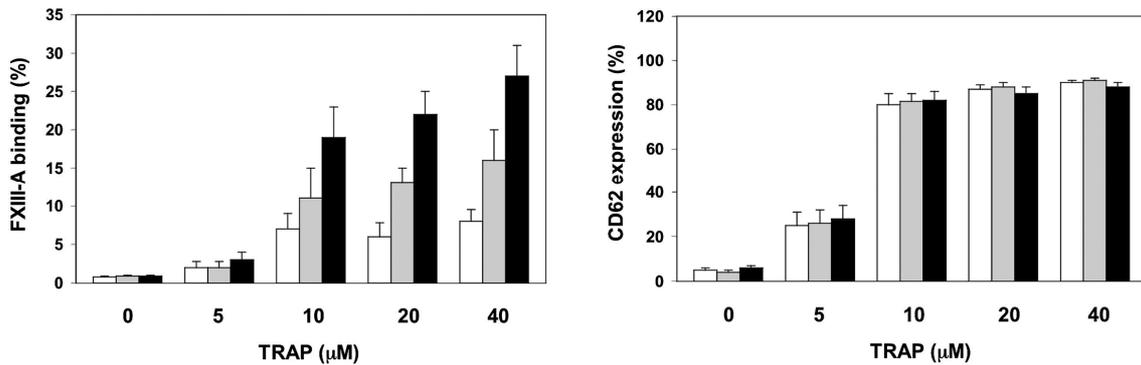
**Figure 13.** Analysis of FXIII-A and P-selectin (CD62) positivity measured on platelets from whole blood (open bars) and washed platelets (filled bars) stimulated by TRAP (0-40 μM). Data shown are representative of 4 experiments using different normal donors. Results are expressed as mean ± SEM.

According to these experiments, non-active FXIII from plasma binds to activated platelets in whole blood, and the intracellular form is not expressed on platelets in TRAP-activated samples. Surprisingly, in a former report, gel-filtered human platelets when co-stimulated with collagen and thrombin (called COAT-platelets) demonstrated enhanced surface expression of α-granule proteins as well as FXIII in the absence of exogenous FXIII [96]. One explanation might be the very high level of platelet activation induced by two strong platelet agonists causing maximum secretion of α-granule contents like fibrinogen with FXIII content, or probably even cell lysis with the release of intracellular FXIII. However, when we used very high TRAP or thrombin concentrations (without GPVI agonist) to

stimulate platelets without lysis, we could not observe FXIII expression on washed activated platelets without adding exogenous FXIII to the cells. Thus, we ruled out the possibility of FXIII release from platelet cytosol induced by PAR agonists, and we claim that FXIII-A positivity is due to the plasma FXIII binding to platelets.

***Platelets with different size showed distinct levels of FXIII-A positivity***

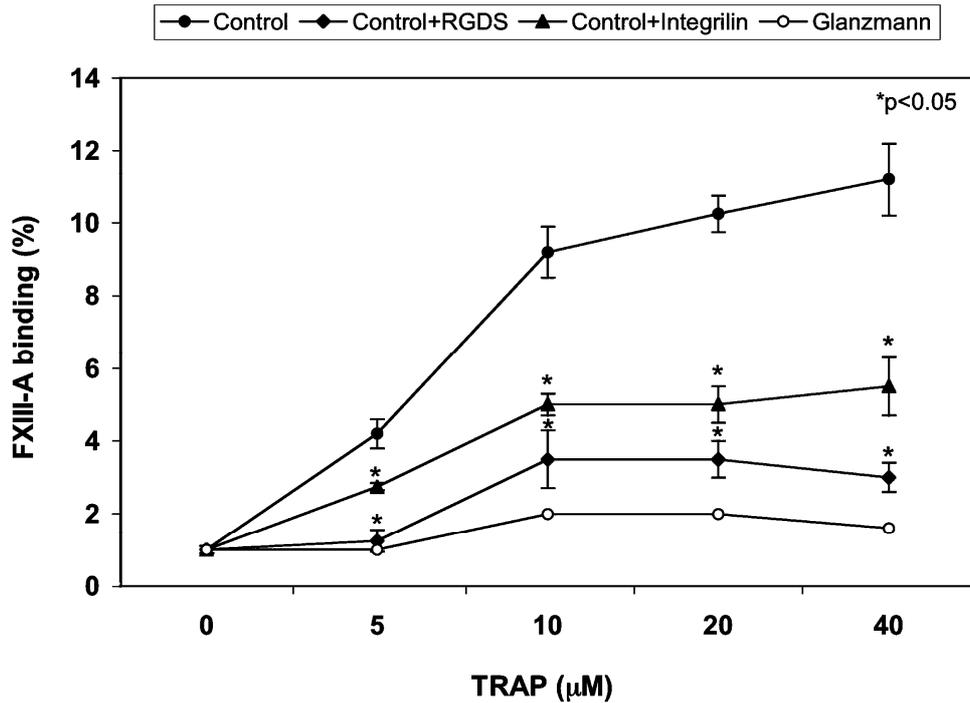
We further analyzed the FXIII-A and P-selectin positivity on large, medium and small platelets (Figure 14). Data were analyzed from experiments where whole blood platelets were activated by TRAP. We found that large platelets displayed the highest FXIII-A positivity, while small platelets showed the lowest level. However, activated platelets were equally positive for P-selectin expression regardless of platelet size (Figure 14). These data suggest that the binding site for FXIII may be a platelet receptor that correlates with platelet volume.



**Figure 14.** Analysis of platelet populations varying in size in terms of FXIII-A and P-selectin positivities. The whole platelet population was divided into three subgroups according to FSC signal to analyze the FXIII-A and P-selectin positivities on TRAP-stimulated large (black), medium (grey) and small (white) platelets. Data shown are representative of 4 experiments done from 4 different human donors. Results are expressed as mean  $\pm$  SEM.

***Eptifibatide and RGDS-tetrapeptide significantly inhibited FXIII-A positivity on stimulated platelets***

Activated platelets express approximately 80 000 copies of GPIIb/IIIa receptors on their surface and this activation-dependent receptor plays a significant role in fibrin(ogen) binding during platelet activation [97]. Moreover, the inhibition of these receptors by using F(ab')<sub>2</sub> fragments of the monoclonal antibody (7E3) reduced markedly the arterial eversion graft thrombosis and *ex vivo* platelet aggregation in dogs [98]. Here, we investigated the role of GPIIb/IIIa receptor in plasma FXIII binding to TRAP-stimulated platelets in whole blood using pharmacological agents against this receptor. Eptifibatide was used as a highly specific GPIIb/IIIa receptor antagonist at the typical concentration (2 µg/ml) as being administered during emergency cardiological intervention [84]. Platelets were stimulated with different concentrations of TRAP after 15 min of preincubation with eptifibatide. Fifty-five % of FXIII binding was prevented by eptifibatide treatment compared to the untreated activated sample (p<0.05) (Figure 15), but was not totally abolished. When platelets were pre-treated with RGDS tetrapeptide, the inhibition of fibrinogen binding to platelets resulted in even lower levels of non-active FXIII binding (Figure 15). FXIII-A positivity was inhibited by 73% in the presence of RGDS peptide versus control sample.



**Figure 15.** The role of GPIIb/IIIa receptor in FXIII binding to TRAP-activated human platelets in whole blood. Platelets were incubated with eptifibatide (2 μg/ml, ▲) and RGDS tetrapeptide (5 mM, ◆) (n=3). One patient with severe type I GT (○) was also analyzed. As a control, healthy platelets were examined without any inhibitor (●) (n=3). Data are expressed as mean ± SEM. \*p<0.05 compared to control by using Student's *t* test.

Platelets were properly activated by TRAP as measured by the P-selectin expression (data not shown). These results suggest that GPIIb/IIIa receptor and receptor-bound fibrinogen have an important role in FXIII binding. Earlier data on the binding site of FXIIIa were contradictory. In the first study, GPIIb/IIIa receptor and platelet-bound fibrin(ogen) were excluded as binding site for FXIIIa as RGD peptide did not block the interaction between FXIIIa and platelets [75]. The binding of FXIIIa to thrombin-activated platelets was inhibited by plasmin [76]. In a later study, the GPIIb/IIIa receptor was shown to mediate the binding of FXIIIa to thrombin-stimulated platelets [77].

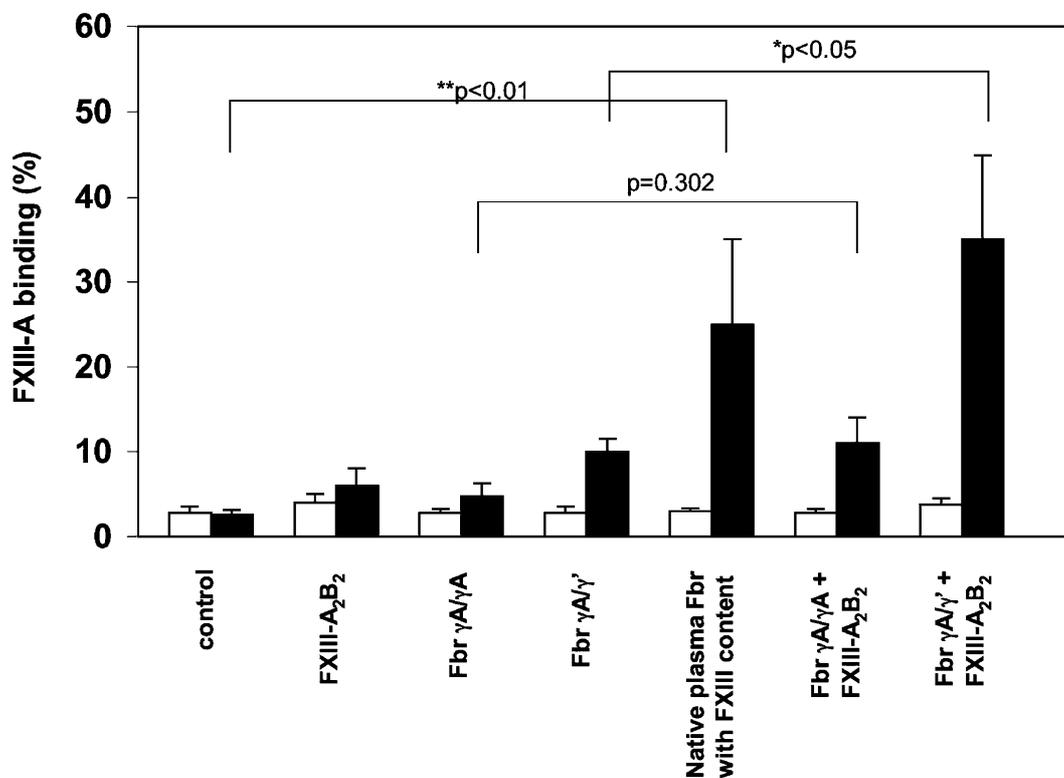
***Impaired binding of non-active FXIII to activated GT platelets***

In order to support the theory that GPIIb/IIIa receptor is involved in plasma FXIII binding, we analyzed the FXIII-A positivity on TRAP-activated platelets in whole blood from a patient with GT in the “absence” of this platelet receptor. Simultaneously, platelets were also examined from age-matched healthy controls. No difference in basal FXIII-A positivity was found between the unstimulated normal and GT samples. However, upon TRAP-induced platelet activation, insignificant FXIII-A positivity was measured on GT platelets compared to healthy platelets (Figure 15). Normal and GT platelets were both properly activated by TRAP as evidenced by the elevated P-selectin values (data not shown). These data confirm our previous findings that GPIIb/IIIa receptor has a pivotal role in FXIII binding during platelet activation. Surprisingly, FXIIIa binding to stimulated platelets from two patients with severe Glanzmann thrombasthenia was found to be normal [75].

#### ***FXIII-A<sub>2</sub>B<sub>2</sub> requires fibrinogen with $\gamma$ -chain to bind to activated platelets***

The next question was whether FXIII binds directly to GPIIb/IIIa receptor, or the presence of fibrinogen is essential for FXIII binding. To study the characteristics of FXIII binding process, we used two different variants of fibrinogen with distinct molecular structure. Fibrinogen molecules are comprised of two sets of three polypeptide chains termed A $\alpha$ , B $\beta$  and  $\gamma$  chain [99]. A splicing variant of the  $\gamma$ -chain, termed the  $\gamma'$ -chain, which is present only in approximately 15% of fibrinogen molecules [80,100] was found to bind strongly FXIII via its B subunits [101,102]. Sample with native plasma fibrinogen containing FXIII was used as a positive control showing significantly higher FXIII-A positivity compared to control sample ( $p < 0.01$ ) (Figure 16). TRAP-activated platelets displayed only slightly increased FXIII-A positivity when exogenous plasma FXIII-A<sub>2</sub>B<sub>2</sub>, or either variant of fibrinogen ( $\gamma$ A/ $\gamma$ A or  $\gamma$ A/ $\gamma'$ ) with minor FXIII content was added alone in samples. However,  $\gamma$ A/ $\gamma'$  fibrinogen in the presence of purified FXIII-A<sub>2</sub>B<sub>2</sub> significantly ( $p < 0.05$ ) augmented FXIII binding on TRAP-activated platelets compared to control sample and platelets treated with  $\gamma$ A/ $\gamma'$  fibrinogen only (Figure 16). On the other hand, washed platelets incubated with FXIII-A<sub>2</sub>B<sub>2</sub> in the presence of  $\gamma$ A/ $\gamma$ A fibrinogen showed only insignificant ( $p = 0.302$ ) increase in FXIII-A positivity as compared to platelet sample containing  $\gamma$ A/ $\gamma$ A fibrinogen alone. These data suggest that non-active FXIII-A<sub>2</sub>B<sub>2</sub> from plasma binds to thrombin-receptor activated human platelets via GPIIb/IIIa receptor-

bound fibrinogen containing  $\gamma$ -chain and it is not capable of direct platelet binding to the fibrinogen receptor.



**Figure 16.** Analysis of the mechanism of FXIII binding to TRAP-activated washed human platelets. Non-activated (open bars) and TRAP-activated human platelets (filled bars) were incubated with or without plasma FXIII-A<sub>2</sub>B<sub>2</sub> in the presence or absence of one of the following:  $\gamma$ A/ $\gamma$ ' fibrinogen,  $\gamma$ A/ $\gamma$ A fibrinogen, or purified plasma fibrinogen containing FXIII used as a positive control. Data shown are representative of 3 experiments done from 3 different human donors. Results represent mean  $\pm$  SEM. \* $p$ <0.05; \*\* $p$ <0.01 according to Student's  $t$  test analysis.

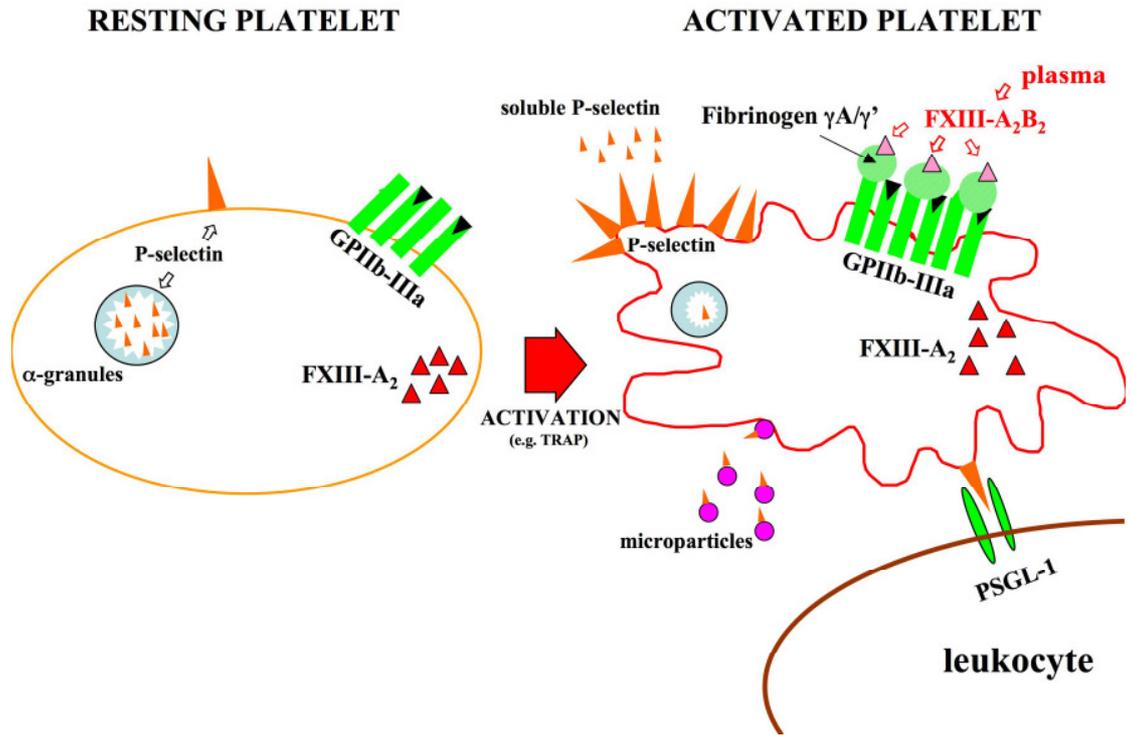
This is in agreement with a previous report [103] where it was shown that  $\gamma$ A/ $\gamma$ ' fibrinogen binds FXIII 20-fold more tightly than  $\gamma$ A/ $\gamma$ A fibrinogen. Cox and Devine also demonstrated the indirect binding of FXIIIa through fibrinogen associated with its receptor [77]. However, they did not provide more data about the mechanism of FXIII binding. This mechanism targets FXIII to the surface of stimulated platelets where thrombin and consequently fibrin are formed and FXIII becomes activated, and the cross-linking of fibrin by FXIIIa becomes promoted on the surface of activated platelets.

It was to be stressed that throughout our study, non-activated plasma FXIII-A<sub>2</sub>B<sub>2</sub>

was used. FXIIIa only consists of thrombin cleaved A subunits without the dissociated B subunits, while, as demonstrated above, plasma FXIII seems to bind to stimulated platelets through the B subunits. Therefore, the binding sites for FXIII and FXIIIa must be distinct and our results are not comparable with the earlier findings.

It is interesting that in a clinical study [104] in patients with PAD platelet-associated FXIII was found significantly higher than in healthy controls, and the detection of FXIII on platelets was proposed as a marker of platelet activation [104,105]. We also suggest that the detection of FXIII binding to activated platelets may be a useful alternative platelet activation marker during *ex vivo* sample analysis.

Here, we provide a model to summarize our findings about the different platelet activation markers that we measured *in vitro* and in *ex vivo* patient samples. Accordingly, resting platelets demonstrate a very low expression of P-selectin. GPIIb/IIIa receptors are in quiescent state without bound fibrinogen and thus plasma FXIII-A<sub>2</sub>B<sub>2</sub>. When platelets are activated in prothrombotic conditions *in vivo* or stimulated by TRAP *in vitro*, P-selectin receptors are expressed from  $\alpha$ -granules rapidly, and later partially shedded into the plasma becoming a soluble form. Exposed P-selectin binds to its counter-receptors like PSGL-1 receptors on leukocytes resulting in several cell-cell heterotypic interactions. GPIIb/IIIa receptors become activated during undergoing conformational change and then are capable to bind fibrinogen with FXIII-A<sub>2</sub>B<sub>2</sub>. Simultaneously, MPs are vesiculated by a budding process with high levels of platelet-specific receptors and PS on their surface to facilitate coagulation and thrombus formation. We think that the investigation of these sensitive markers is appropriate to detect the activation of platelets in prothrombotic conditions.



**Figure 17.** Overall schematic figure on the fate and role of different platelet activation markers examined in the dissertation.

## SUMMARY

Cardiovascular and cerebrovascular diseases are still the leading cause of death in the developed world. Activated platelets are fundamentally involved in the pathomechanism of thrombotic complications in these diseases. That is why the investigation of activated platelets has become more obvious in the daily routine. Our primary goal was to detect increased platelet activation in time that occurs often in acute or chronic vascular disorders or induced by invasive therapeutic intervention in such states.

In this present study, we found that in type 2 DM and obese patients, platelet and soluble P-selectin levels were significantly elevated compared to the findings in healthy controls, but the most studied P-selectin gene polymorphism (Thr715Pro) did not affect plasma soluble P-selectin values in the patient groups. In DM patients divided into different subgroups according to several demographical variables, the levels of soluble P-selectin still did not vary notably according to their genotype for this polymorphism.

In patients with stable angina, significantly increased levels of PMPs, platelet P-selectin and platelet-monocyte aggregates were measured compared to angina patients underwent catheterization alone. However, soluble P-selectin levels did not show marked difference between the two study groups. Thus, the measurement of PMP levels can be considered as an early sensitive activation marker to detect platelet activity right after invasive cardiological interventions.

*In vitro*, FXIII-A<sub>2</sub> was not expressed from its intracellular localization on washed platelets activated by TRAP. Thus, surface-bound FXIII on stimulated whole blood platelets is of plasma origin. The presence of  $\gamma$ A/ $\gamma$ ' fibrinogen significantly potentiated the binding of purified FXIII-A<sub>2</sub>B<sub>2</sub> on stimulated washed platelets, but no FXIII-A positivity was seen without this type of fibrinogen or with fibrinogen having  $\gamma$ A-chain only. Accordingly, plasma FXIII is unable to bind directly to the activated platelet surface, and significant binding of non-active FXIII occurs only when GPIIb/IIIa receptor-bound fibrinogen with  $\gamma$ '-chain is present. Analysis of FXIII binding to platelets may be an additional sensitive activation marker in the future.

## ÖSSZEFOGLALÁS

A kardio- és cerebrovaszkuláris betegségeket napjaikban is vezető halálökként tartjuk számon a fejlett országokban. Az aktíválódott vérlemezkék alapvető szerepet játszanak az ezekben a betegségekben bekövetkező trombotikus komplikációk pathomechanizmusában. Ezért van olyan nagy jelentősége az ilyen vérlemezkék kimutatásának a napi rutindiagnosztikában.

Vizsgálataink során a 2-es típusú cukorbetegségben szenvedő betegekben, illetve túlsúlyos egyéneknél a vérlemezkék felszíni P-szelektin, valamint annak szolubilis formájának mennyiségét szignifikánsan emelkedettnek találtuk szemben az egészséges kontroll személyekben mért értékekkel. A legvizsgáltabb P-szelektin gén polimorfizmus (Thr715Pro) egyik betegcsoportban sem befolyásolta szignifikánsan a szolubilis P-szelektin plazma szintjét. A cukorbeteg esetén a különböző betegparaméterek alapján kialakított alcsoportokban a szolubilis P-szelektin szintje - a genotípus alapján - továbbra sem különbözött jelentős mértékben.

Stabil anginában szenvedő betegekben szignifikánsan megemelkedett volt a vérlemezke-eredetű mikropartikulák mennyisége, a thrombocytá felszíni P-szelektin és a vérlemezke-monocytá aggregátumok aránya ellentétben olyan, szintén anginás betegekkel, akik "csak" diagnosztikai katéterezéssel estek át. Ugyanakkor a szolubilis P-szelektin mérése jelentős különbséget nem mutatott a két betegcsoport között. Ezek alapján a thrombocytá-eredetű mikropartikulák vizsgálata egy érzékeny korai vérlemezke aktivációs markernek tekinthető az invazív kardiológiai beavatkozásokkor aktíválódott vérlemezkék vizsgálatára.

*In vitro* kísérleteinkben, a thrombocyták intracelluláris részében lévő FXIII-A<sub>2</sub> nem expresszálódott a TRAP agonistával aktivált mosott thrombocytákon. A teljes vérben vizsgált aktivált vérlemezkékhez kötődő FXIII ezek alapján plazma eredetű. A  $\gamma$ A/ $\gamma$ ' láncú fibrinogén jelenléte szignifikánsan növelte a tisztított FXIII-A<sub>2</sub>B<sub>2</sub> kötődését az aktivált mosott vérlemezkék feksznén, de a FXIII-A pozitívitas nem volt mérhető ennek a fibrinogénnek a hiányában, vagy olyan fibrinogénnel, aminek csak  $\gamma$ A lánc van. Ez alapján úgy tűnik, hogy a plazma eredetű nem aktivált FXIII nem képes közvetlenül kötődni az aktív vérlemezkékhez, és jelentős FXIII kötődés csak a GPIIb/IIIa receptorhoz

kötődő fibrinogén  $\gamma$ -láncán keresztül lehetséges. A FXIII kötődésének vizsgálata egy újabb thrombocytá aktivációs marker lehetőségét vetheti fel a későbbiekre.

## REFERENCES

1. Ross R. Atherosclerosis – an inflammatory disease. *N Eng J Med* 1999; 340: 115-126.
2. Packard RRS, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin Chem* 2008; 54: 24-38.
3. Watanabe J, Wohltmann HJ, Klein RL, Colwell JA, Lopes-Virella MF. Enhancement of platelet aggregation by low-density lipoproteins from IDDM patients. *Diabetes* 1988; 37: 1652–1657.
4. Tschoepe D, Driesch E, Schwippert B, Nieuwenhuis HK, Gries FA. Exposure of adhesion molecules on activated platelets in patients with newly diagnosed IDDM is not normalized by near-normoglycemia. *Diabetes* 1995; 44: 890-894.
5. Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost* 2004; 2: 1282-1291.
6. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. *Cell* 1993; 74: 541-544.
7. Subramaniam M, Frenette PS, Saffaripour S, Johnson RC, Hynes RO, Wagner DD. Defects in hemostasis in P-selectin-deficient mice. *Blood* 1996; 4: 1238–1242.
8. Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. *Circulation* 2000; 102: 1931–1936.
9. McEver RP. Properties of GMP-140, an inducible granule membrane protein of platelets and endothelium. *Blood Cells* 1990; 16: 73-80.
10. Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP. Identification of a specific glycoprotein ligand-1 for P-selectin (CD62) on myeloid cells. *J Biol Chem* 1992; 118: 445-456.
11. Frenette PS, Denis CV, Weiss L, Jurk K, Subbarao S, Kehrel B, Hartwig JH, Vestweber D, Wagner DD. P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet-endothelial interactions in vivo. *J Exp Med* 2000; 191: 1413-1422.

12. Watson ML, Kingsmore SF, Johnston GI, Siegelman MH, Le Beau MM, Lemons RS, Bora NS, Howard TA, Weissman IL, McEver RP. Genomic organization of the selectin family of leukocyte adhesion molecules on human and mouse chromosome 1. *J Exp Med* 1990; 172: 263-272.
13. McEver RP. Selectins. *Curr Opin Immunol* 1994; 6: 75-84.
14. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B. PADGEM protein: a receptor that mediates the interaction of activated platelets with neutrophils and monocytes. *Cell* 1989; 59: 305-312.
15. Geng JG, Bevilacqua MP, Moore KL, McIntyre TM, Prescott SM, Kim JM, Bliss GA, Zimmerman GA, McEver RP. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* 1990; 343: 757-760.
16. Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol* 1985; 101: 880-886.
17. Johnston GI, Pickett EB, McEver RP, George JN. Heterogeneity of platelet secretion in response to thrombin demonstrated by flow cytometry. *Blood* 1987; 69: 1401-1403.
18. Ault KA, Rinder HM, Mitchell JG, Rinder CS, Lambrew CT, Hillman RS. Correlated measurement of platelet release and aggregation in whole blood. *Cytometry* 1989; 10: 448-455.
19. Weikert U, Kühl U, Schultheiss HP, Rauch U. Platelet activation is increased in patients with cardiomyopathy: myocardial inflammation and platelet reactivity. *Platelets* 2002; 13: 487-491.
20. Furman MI, Beniot SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, Hechtman HB, Michelson AD. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. *J Am Coll Cardiol* 1998; 31: 352-358.
21. Marquardt L, Ruf A, Mansmann U, Winter R, Schuler M, Bugge F, Mayer H, Grau AJ. Course of platelet activation markers after ischemic stroke. *Stroke* 2002; 33: 2570-2574.
22. Tschoepe D, Driesch E, Schwippert B, Lampeter EF. Activated platelets in subjects at increased risk of IDDM. DENIS Study Group. Deutsche Nikotinamid Interventionsstudie. *Diabetologia* 1997; 40: 573-577.

23. Yamazaki M, Uchiyama S, Iwata M. Measurement of platelet fibrinogen binding and P-selectin expression by flow cytometry in patients with cerebral infarction. *Thromb Res* 2001; 104: 197-205.
24. Rinder HM, Murphy M, Mitchell JG, Stocks J, Ault KA, Hillman RS. Progressive platelet activation with storage: Evidence for shortened survival of activated platelets after transfusion. *Transfusion* 1991; 31: 409-414.
25. Berger G, Hartwell DW, Wagner DD. P-selectin and platelet clearance. *Blood* 1998; 92: 4446-4452.
26. Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, Valeri CR. In vivo tracking of platelets: Circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci USA* 1996; 93: 11877-11882.
27. Ishiwata N, Takio K, Katayama M, Watanabe K, Titani K, Ikeda Y, Handa M. Alternatively spliced isoform of P-selectin is present in vivo as a soluble molecule. *J Biol Chem* 1994; 269: 23708-23715.
28. Dunlop LC, Skinner MP, Bendall LJ, Favalaro EJ, Castaldi PA, Gorman JJ, Gamble JR, Vadas MA, Berndt MC. Characterization of GMP-140 (P-selectin) as a circulating plasma protein. *J Exp Med* 1992; 175: 1147-1150.
29. Conway DS, Pearce LA, Chin BS, Hart RG, Lip GY. Plasma von Willebrand factor and sPsel as indices of endothelial damage and platelet activation in 1321 patients with nonvalvular atrial fibrillation: relationship to stroke risk factors. *Circulation* 2002; 106: 1962-1967.
30. Fijnheer R, Frijns CJ, Korteweg J, Rommes H, Peters JH, Sixma JJ, Nieuwenhuis HK. The origin of P-selectin as a circulating plasma protein. *Thromb Haemost* 1997; 77: 1081-1085.
31. Verhaar MC, Beutler JJ, Giallard CA, Koomans HA, Fijnheer R, Rabelink TJ. Progressive vascular damage in hypertension is associated with increased levels of circulating P-selectin. *J Hypertens* 1998; 16: 45-50.
32. Walcheck B, Kahn J, Fisher JM, Wang BB, Fisk RS, Payan DG, Feehan C, Betageri R, Darlak K, Spatola AF, Kishimoto TK. Neutrophils rolling altered by inhibition of L-selectin shedding in vitro. *Nature* 1996; 380: 720-723.
33. Fox JE. Shedding of adhesion receptors from the surface of the activated platelets. *Blood Coagul Fibrinol* 1994; 5: 291-304.

34. Blann AD, Seigneur M, Boisseau MR, Taberner DA, McCollum CN. Soluble P-selectin in peripheral vascular disease: relationship to the location and extent atherosclerosis disease and its risk factors. *Blood Coagul Fibrinol* 1996; 7: 789-793.
35. Gurbel PA, O'Connor CM, Dalesandro MR, Serebruany VL. Relation of soluble and platelet P-selectin to early outcome in patients with acute myocardial infarction after thrombolytic therapy. *Am J Cardiol* 2001; 87: 774-777.
36. Ikeda H, Takajo Y, Ichiki K, Ueno T, Maki S, Noda T, Sugi K, Imaizumi T. Increased soluble form of P-selectin in patients with unstable angina. *Circulation* 1995; 92: 1693-1696.
37. Jilma B, Fasching P, Ruthner C, Rumplmayr A, Ruzicka S, Kapiotis S, Wagner OF, Eichler HG. Elevated circulating P-selectin in insulin dependent diabetes mellitus. *Thromb Haemost* 1996; 76: 328-332.
38. Kopp HP, Hopmeier P, Schernthaner G. Concentration of circulating P-selectin are increased in patients with newly diagnosed insulin-dependent diabetes mellitus. *Exp Clin Endocrinol Diabet* 1998; 106: 41-44.
39. Minamino T, Kitakeze M, Sanada S, Asanuama H, Kurotobi T, Koretsune Y, Fukunami M, Kuzuya T, Hoki N, Hori M. Increased expression of P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation: role of nitric oxide. *Circulation* 1998; 98:1721-1727.
40. Zeiger F, Stephan S, Hoheisel G, Pfeiffer D, Ruehlmann C, Kokschi M. P-selectin expression, platelet aggregates, and platelet-derived microparticle formation are increased in peripheral arterial disease. *Blood Coagul Fibrinol* 2000; 11: 723-728.
41. Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. *Circulation* 2001; 103: 491-495.
42. Herrmann SM, Ricard S, Nicaud V, Mallet C, Evans A, Ruidavets JB, Arveiler D, Luc G, Cambien F. The P-selectin gene is highly polymorphic: reduced frequency of the Pro715 allele carriers in patients with myocardial infarction. *Hum Mol Genet* 1998; 7: 1277-1284.
43. Kee F, Morrison C, Evans AE, McCrum E, McMaster D, Dallongeville J, Nicaud V, Poirier O, Cambien F. Polymorphisms of the P-selectin gene and the risk of myocardial infarction in men and women in the ECTIM extension study. *Heart* 2000; 84: 548-552.

44. Barboux SC, Blankenberg S, Rupprecht HJ, Francomme C, Bickel C, Hafner G, Nicaud V, Meyer J, Cambien F, Tiret L. Associations between P-selectin gene polymorphisms and soluble P-selectin levels and their relation to coronary artery disease. *Atheroscler Thromb Vasc Biol* 2001; 21: 1668-1673.
45. Carter AM, Anagnostopoulou K, Mansfield MW, Grant PJ. Soluble P-selectin levels, P-selectin polymorphisms and cardiovascular disease. *J Thromb Haemost* 2003; 1: 1718-1723.
46. Miller MA, Kerry SM, Dong Y, Strazzullo P, Cappuccio FP. Associations between the Thr715Pro P-selectin gene polymorphism and soluble P-selectin in a multiethnic population in South London. *Thromb Haemost* 2004; 92: 1060-1065.
47. Liu Y, Burdon KP, Langefeld CD, Beck SR, Wagenknecht LE, Rich SS, Bowden DW, Freedman BI. P-selectin gene haplotype associations with albuminuria in the Diabetes Heart Study. *Kidney Int* 2005; 68: 741-746.
48. Volcik KA, Ballantyne CM, Coresh J, Folsom AR, Wu KK, Boerwinkle E. P-selectin Thr715Pro polymorphism predicts P-selectin levels but not risk of incident coronary heart disease or ischemic stroke in a cohort of 14595 participants: The Atherosclerosis Risk In Communities Study. *Atherosclerosis* 2006; 186: 74-79.
49. Jilma B. The Thr715Pro single nucleotide polymorphism of P-selectin: does it really matter in cardiovascular disorders? *Thromb Haemost* 2004; 92: 896-897.
50. Zalewski G, Ciccarone E, Di Castelnuovo A, Zito F, Capani F, de Gaetano G, Donati MB, Iacoviello L. P-selectin gene genotypes or haplotypes and cardiovascular complications in type 2 diabetes mellitus. *Nutr Metab Cardiovasc Dis* 2006; 16: 418-425.
51. Bugert P, Vosberg M, Entelmann M, Jahn J, Katus HA, Klüter H. Polymorphisms in the P-selectin (CD62) and P-selectin glycoprotein ligand-1 (PSGL-1) genes and coronary heart disease. *Clin Chem Lab Med* 2004; 42: 997-1004.
52. Tregouet DA, Barboux S, Escolano S, Tahri N, Golmard JL, Tiret L, Cambien F. Specific haplotypes of the P-selectin gene are associated with myocardial infarction. *Hum Mol Genet* 2002; 11: 2015-2023.
53. Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin. *Circulation* 2001; 104: 1533-1537.

54. Káplár M, Kappelmayer J, Kiss A, Szabó K, Udvardy M. Increased leukocyte-platelet adhesion in chronic myeloproliferative disorders with high platelet counts. *Platelets* 2000; 11: 183-184.
55. Craft JA, Marsch NA. Increased generation of platelet-derived microparticles following percutaneous transluminal coronary angioplasty. *Blood Coagul Fibrinol* 2003; 14: 719-728.
56. Inoue T, Hikichi Y, Morooka T, Yoshida K, Fujimatsu D, Komoda H, Kameda M, Nonaka M, Sohma R, Hashimoto S, Node K. Comparison of changes in circulating platelet-derived microparticles and platelet surface P-selectin expression after coronary stent implantation. *Platelets* 2006; 17: 416-420.
57. Vidal C, Spaulding C, Picard F, Schaison F, Melle J, Weber S, Fontenay-Roupie M. Flow cytometry detection of platelet procoagulation activity and microparticles in patients with unstable angina treated by percutaneous coronary angioplasty and stent implantation. *Thromb Haemost* 2001; 86: 784-790.
58. Morel O, Hugel B, Jesel L, Mallat Z, Lanza F, Douchet MP, Zupan M, Chauvin M, Cazenave JP, Tedgui A, Freyssinet JM, Toti F. Circulating procoagulant microparticles and soluble GPV in myocardial infarction treated by primary percutaneous transluminal coronary angioplasty. A possible role for GPIIb-IIIa antagonists. *J Thromb Haemost* 2004; 2: 1118-1126.
59. Craft JA, Masci PP, Roberts MS, Brighton TA, Garrahy P, Cox S, Marsh NA. Increased platelet-derived microparticles in the coronary circulation of percutaneous transluminal coronary angioplasty patients. *Blood Coagul Fibrinol* 2004; 15: 475-482.
60. Nomura S, Uehata S, Saito S, Osumi K, Ozeki Y, Kimura Y. Enzyme immunoassay detection of platelet-derived microparticles and RANTES in acute coronary syndrome. *Thromb Haemost* 2003; 89: 506-512.
61. Freyssinet JM. Cellular microparticles: what are they bad or good for? *J Thromb Haemost* 2003; 1: 1655-1662.
62. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. *Cardiovasc Res* 2003; 59: 277-287.
63. Flaumenhaft R, Dilks JR, Richardson J, Alden E, Patel-Hett SR, Battinelli E, Klement GL, Sola-Visner M, Italiano JE Jr. Megakaryocyte-derived

- microparticles: direct visualization and distinction from platelet-derived microparticles. *Blood* 2009; 113: 1112-1121.
64. Biró E, Sturk-Maquelin KN, Vogel GM, Meuleman DG, Smit MJ, Hack CE, Sturk A, Nieuwland R. Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. *J Thromb Haemost* 2003; 1: 2561-2568.
  65. Polgár J, Matuskova J, Wagner DD. The P-selectin, tissue factor, coagulation triad. *J Thromb Haemost* 2005; 3: 1590-1596.
  66. George FD. Microparticles in vascular diseases. *Thromb Res* 2008; 122: S55-59.
  67. Shah MD, Bergeron AL, Dong JF, López JA. Flow cytometric measurement of microparticles: pitfalls and protocol modifications. *Platelets* 2008; 19: 365-372.
  68. Ndrepepa G, Mehilli J, Schulz S, Iijima R, Keta D, Byrne RA, Pache J, Seyfarth M, Schömig A, Kastrati A. Patterns of presentation and outcomes of patients with acute coronary syndromes. *Cardiology* 2009; 113: 198-206.
  69. Hermann A, Rauch BH, Braun M, Schrör K, Weber AA. Platelet CD40 ligand (CD40L) – subcellular localization, regulation of expression, and inhibition by clopidogrel. *Platelets* 2001; 12: 74-82.
  70. Muszbek L, Bagoly Z, Bereczky Z, Katona E. The involvement of blood coagulation factor XIII in fibrinolysis and thrombosis. *Cardiovasc Hematol Agents Med Chem* 2008; 6: 190-205.
  71. Muszbek L, Yee VC, Hevessy Z. Blood Coagulation factor FXIII: Structure and function. *Thromb Res* 1999; 94: 271-305.
  72. Ádány R, Bardos H. Factor XIII subunit A as an intracellular transglutaminase. *Cell Mol Life Sci* 2003; 60: 1049-1060.
  73. Reed GL, Matsueda GR, Haber E. Platelet factor XIII increases the fibrinolytic resistance of platelet-rich clots by accelerating the crosslinking of alpha<sub>2</sub>-antiplasmin to fibrin. *Thromb Haemost* 1992; 68: 315-320.
  74. Hevessy Z, Haramura G, Boda Z, Udvardy M, Muszbek L. The role of platelet factor XIII in fibrin-alpha-2-antiplasmin crosslinking. *Thromb Haemost* 1996; 75: 161-167.
  75. Greenberg CS, Shuman MA. Specific binding of blood coagulation factor XIIIa to thrombin-stimulated platelets. *J Biol Chem* 1984; 259: 14721-14727.

76. Kreager JA, Devine DV, Greenberg CS. Cytofluorometric identification of plasmin-sensitive factor XIIIa binding to platelets. *Thromb Haemost* 1988; 60: 88-93.
77. Cox AD, Devine DV. Factor XIIIa binding to activated platelets is mediated through activation of glycoprotein IIb/IIIa. *Blood* 1994; 83: 1006-1016.
78. Losonczy G, Rosenberg N, Kiss C, Kappelmayer J, Vereb G, Kerényi A, Balogh I, Muszbek L. A novel homozygous mutation (1619delC) in GPIIb gene associated with Glazmann thrombasthenia, a decay of GPIIb-mRNA and the synthesis of a truncated GPIIb unable to form complex with GPIIIa. *Thromb Haemost* 2005; 93: 904-909.
79. Lorand L, Credo RB, Janus TJ. Factor XIII (fibrin-stabilizing factor). *Methods Enzymol* 1981; 80: 333-341.
80. Wolfenstein-Todel C, Mosesson MW. Human plasma fibrinogen heterogeneity: evidence for an extended carboxyl-terminal sequence in a normal gamma chain variant (gamma'). *Proc Natl Acad Sci U S A* 1980; 77: 5069-5073.
81. Katona E, Haramura G, Karpati L, Fachel J, Muszbek L. A simple, quick one-step ELISA assay for the determination of complex plasma factor XIII (A2B2). *Thromb Haemost* 2000; 83: 268-273.
82. Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romijn FP, Hack CE, Sturk A. Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. *Thromb Haemost* 2001; 85: 810-820.
83. Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut JG, Arnoux D, Charpiot P, Freyssinet JM, Oliver C, Sampol J, Dignat-George F. Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. *Diabetes* 2002; 51: 2840-2845.
84. Leclerc JR. Platelet glycoprotein IIb/IIIa antagonists: lessons learned from clinical trials and future directions. *Crit Care Med* 2002; 30: 332-340.
85. Plow E, Marguerie G. Inhibition of fibrinogen binding to human platelets by the tetrapeptide glycyl-l-prolyl-l-arginyl-l-proline. *Proc Natl Acad Sci USA* 1982; 79: 3711-3715.

86. Rák K, Beck P, Udvardy M, Pfliegler G, Misz M, Boda Z. Plasma levels of beta-thromboglobulin and factor VIII-related antigen in diabetic children and adults. *Thromb Res* 1983; 29: 155-162.
87. Szűk T, Nagy B Jr, Bereczky Z, Kőszegi Z, Édes I, Kappelmayer J. Effects of ad hoc clopidogrel loading versus pre-treatment on P-selectin expression after coronary stent implantation. *Platelets* 2006; 17: 344-346.
88. Robert S, Poncelet P, Lacroix R, Arnaud L, Giraudo L, Hauchard A, Sampol J, Dignat-George F. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *J Thromb Haemost* 2009; 7: 190-197.
89. Vargová K, Toth-Zsamboki E, Beres BJ, Bencze J, Kerecsen G, Gulacsi-Bardos P, Kiss RG, Preda I. Circulating endothelial cell count, plasma vWF and soluble ICAM-1 levels following primary or elective percutaneous coronary intervention. *Atherosclerosis* 2008; 198: 366-372.
90. Jung F, Spitzer SG, Pindur G. Effect of an ionic compared to a non-ionic X-ray contrast on platelets and coagulation during diagnostic cardiac catheterization. *Pathophysiol Haemost Thromb* 2002; 32: 121-126.
91. Tan KT, Tayebjee MH, Macfadyen RJ, Lip GY, Blann AD. Elevated platelet microparticles in stable coronary artery disease are unrelated to disease severity or to indices of inflammation. *Platelets* 2005; 16: 368-371.
92. van der Zee PM, Biró E, Ko Y, de Winter RJ, Hack CE, Sturk A, Nieuwland R. P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction. *Clin Chem* 2006; 52: 657-664.
93. Lopaciuk S, Lovette KM, McDonagh J, Adany R, Muszbek L, Chuang HYK, McDonagh RP. Subcellular distribution of fibrinogen and factor XIII in human blood platelets. *Thromb Res* 1974; 8: 453-456.
94. Broekman MJ, Handin RJ, Cohen P. Distribution of fibrinogen and platelet factor 4 and factor XIII in subcellular fractions of human platelets. *Br J Haematol* 1975; 31: 51-55.
95. Sixma JJ, Van den Berg A, Schiphorst M, Geuze HJ, McDonagh J. Immunocytochemical localization of albumin and factor FXIII in thin cryo sections of human blood platelets. *Thromb Haemost* 1984; 51: 388-391.

96. Dale GL, Friese P, Batar P, Hamilton SF, Reed GL, Jackson KW, Clemetson KJ, Alberio L. Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature* 2002; 415: 175-179.
97. Collier BS, Shattil SJ. The GPIIb/IIIa (integrin  $\alpha$ IIB $\beta$ 3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood* 2008; 112: 3011-3025.
98. Kiss RG, Lu HR, Roskams T, Jang IK, Plow EF, Gold HK, Collen D. Time course of the effect of a single bolus injection of F(ab')<sub>2</sub> fragments of the antiplatelet GPIIb/IIIa antibody 7E3 on arterial eversion graft occlusion, platelet aggregation, and bleeding time in dogs. *Arterioscler Thromb* 1994; 14: 367-374.
99. Henschen A, Lottspeich F, Kehl M, Southan C. Covalent structure of fibrinogen. *Ann NY Acad Sci* 1983; 408: 28-43.
100. Mosesson MW, Finlayson JS, Umfleet RA. Human fibrinogen heterogeneities: III. Identifications of  $\gamma$  chain variants. *J Biol Chem* 1972; 247: 5223-5227.
101. Siebenlist KR, Meh DA, Mosesson MW. Plasma factor XIII binds specifically to fibrinogen molecules containing  $\gamma$  chains. *Biochemistry* 1996; 35: 10448-10453.
102. Siebenlist KR, Meh DA, Mosesson MW. Protransglutaminase (Factor XIII) mediated crosslinking of fibrinogen and fibrin. *Thromb Haemost* 2001; 86: 1221-1228.
103. Moaddel M, Farrell DH, Daugherty MA, Fried MG. Interaction of human fibrinogens with factor XIII: roles of calcium and the  $\gamma$  peptide. *Biochemistry* 2000; 39: 6698-6705.
104. Devine DV, Andestad G, Nugent D, Carter CJ. Platelet-associated factor XIII as a marker of platelet activation in patients with peripheral vascular disease. *Arterioscler Thromb* 1993; 13: 857-862.
105. Devine DV, Bishop PD. Platelet-associated factor XIII in platelet activation, adhesion, and clot stabilization. *Semin Thromb Haemost* 1996; 22: 409-413.

## LIST OF ORIGINAL PUBLICATIONS RELATED TO THE DISSERTATION

**Nagy B Jr**, Csongrádi E, Bhattoa HP, Balogh I, Blaskó G, Paragh G, Kappelmayer J, Káplár M. The lack of association between Thr715Pro P-selectin gene polymorphism and soluble P-selectin levels in type 2 diabetes mellitus. *Thromb Haemost* 2007; 98: 186-191.

**Impact factor: 3.501**

**Nagy B Jr**, Simon Z, Bagoly Z, Muszbek L, Kappelmayer J. Binding of plasma factor XIII to thrombin-receptor activated human platelets. *Thromb Haemost* 2009; 102: 83-89.

**Impact factor: 3.803**

**Nagy B Jr**, Szúk T, Debreceni IB, Kappelmayer J. Platelet-derived microparticle levels are significantly elevated in patients treated by elective stenting compared to subjects with diagnostic catheterization alone. *Platelets* 2009; (in press)

**Impact factor: 2.271**

Kappelmayer J, **Nagy B Jr**, Miszti-Blasius K, Hevessy Z, Setiadi H. The emerging value of P-selectin as a disease marker. *Clin Chem Lab Med* 2004; 42: 475-486.

**Impact factor: 1.685**

**Nagy B Jr**, Veszprémi A, Kiss F, Miszti-Blasius K, Kappelmayer J. Thrombocytá aktivációs markerek összehasonlító elemzése áramlási citometriával in vitro aktiváció során. *Klin Kísérl Lab Med* 2003; 30: 46-54.

**Total impact factors of original publications related to the dissertation: 11.260**

## LIST OF ORIGINAL PUBLICATIONS NOT CLOSELY RELATED TO THE DISSERTATION

Hevessy Z, **Nagy B Jr**, Kiss F, Kiss Sziráki V, Kiss A, Reményi G, Kappelmayer J. Paroxysmalis nocturnalis haemoglobinuria laboratóriumi diagnosztikája. *Klin Kísérl Lab Med* 2004; 31: 66-76.

Kappelmayer J, **Nagy B Jr**, Losonczy G. Thrombocyták rendellenességeinek vizsgálata áramlási citométerrel. *Modern sejtanalitikai módszerek, ISBN 963 472 810 3* 2004; 45-48.

Hevessy Z, **Nagy B Jr**, Kiss F, Kiss A, Kappelmayer J. Mean fluorescence intensity rate is a useful marker in the detection of paroxysmal nocturnal hemoglobinuria clones. *Clin Chem Lab Med* 2005; 43: 919-923.

**Impact factor: 1.918**

Szűk T, **Nagy B Jr**, Bereczky Z, Kőszegi Z, Édes I, Kappelmayer J. Effects of "ad hoc" clopidogrel loading versus pre-treatment on P-selectin after coronary stent implantation. *Platelets* 2006; 17: 344-346.

**Impact factor: 1.679**

Schlamadinger Á, Tóth J, **Nagy B Jr**, Fazakas F, Hársfalvi J, Kappelmayer J, Muszbek L, Radványi G, Boda Z. Bernard-Soulier-szindróma: a herediter thrombocytopeniák ritka oka. *Hemat Transzf* 2007; 40: 40-46.

**Nagy B Jr**, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C-theta isoform. *Blood* 2009; 113: 2557-2567.

**Impact factor: 10.432**

Chari R, Getz T, **Nagy B Jr**, Bhavaraju K, Mao Y, Bynagari YS, Murugappan S, Nakayama K, Kunapuli SP. Protein kinase C delta differentially regulates platelet functional responses. *Arterioscler Thromb Vasc Biol* 2009; 29: 699-705.

**Impact factor: 6.858**

Bynagari YS, **Nagy B Jr**, Tuluc F, Bhavaraju K, Kim S, Vijayan KV, Kunapuli SP. Mechanism of activation and functional role of protein kinase C eta in human platelets. *J Biol Chem* 2009; 284: 13413-13421.

**Impact factor: 5.520**

**Total impact factor of in extenso publications: 37.667**

**Total international citations: 36**

#### **LIST OF ORAL PRESENTATIONS AT INTERNATIONAL CONGRESSES**

**Nagy B Jr**. Functional role of protein kinase C theta isoform in agonist-induced functional responses in platelets. *28<sup>th</sup> Annual Dr. Erwin Marguiles Award in Thrombosis Research Conference*, 2008. 06. 13., Temple University, Philadelphia, PA, USA

**Nagy B Jr**, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C-theta isoform. *The XXII<sup>nd</sup> Congress of International Society on Thrombosis and Haemostasis*, 2009. 07.10-17. Boston, MA, USA

Bynagari YS, **Nagy B Jr**, Tuluc F, Bhavaraju K, Kim S, Vijayan KV, Kunapuli SP. Mechanism of activation, regulation and functional role of protein kinase C eta in human platelets: pharmacological and gene knockout studies. *38<sup>th</sup> Annual Philadelphia Workshop on Hemostasis, Thrombosis and Atherosclerosis*, 2009. 02. 06., University of Pennsylvania, Philadelphia, PA, USA

## LIST OF POSTER PRESENTATIONS AT INTERNATIONAL CONGRESSES

**Nagy B Jr**, Hevessy Z, Katona E, Polgár J, Kappelmayer J. Comparative evaluation of FXIIIa and P-selectin expression on the surface of thrombin-activated platelets and microparticles. *The XXth Congress of International Society on Thrombosis and Haemostasis*, 2005. 08. 06-12. Sydney, Australia

**Nagy B Jr**, Csongrádi E, Balogh I, Káplár M, Paragh G, Kappelmayer J. Evaluation of increased platelet activation and the effect of P-selectin gene (T715P) polymorphism in type 2 diabetes mellitus and obesity. *European Platelet Group Meeting*, 2006. 06. 25-28. Lodz, Poland

**Nagy B Jr**, Simon Z, Bagoly Z, Muszbek L, Kappelmayer J. Binding of plasma factor XIII to thrombin-receptor activated human platelets. *The XXIIInd Congress of International Society on Thrombosis and Haemostasis*, 2009. 07.10-17. Boston, MA, USA

**Nagy B Jr**, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Functional role of protein kinase C theta isoform in agonist-induced functional responses in platelets. *Hemostasis Gordon Research Meeting*, 2008. 06. 29-07. 04., Waterville Valley, NH, USA

**Nagy B Jr**, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C-theta isoform. *ASH meeting*, 2008. 12. 06-09., San Francisco, CA, USA

**Nagy B Jr**, Bhavaraju K, Getz T, Bynagari YS, Kim S, Kunapuli SP. Impaired activation of platelets lacking protein kinase C-theta isoform. *38<sup>th</sup> Annual Philadelphia Workshop on Hemostasis, Thrombosis and Atherosclerosis*, 2009. 02. 06., University of Pennsylvania, Philadelphia, PA, USA

## LIST OF ORAL AND POSTER PRESENTATIONS AT NATIONAL CONGRESSES

**Nagy B.** XIII-as véralvadási faktor „A” alegységének (FXIII-A) és a P-szelektin receptor (CD62) expressziójának összehasonlító vizsgálata thrombin aktiválta thrombocyták és mikropartikulák felszínén. (előadás) *PhD és TDK tudományos diáktalálkozója*, 2005. 02. 14-22., Debrecen

**Nagy B Jr**, Polgár J, Katona É, Hevessy Z, Kappelmayer J. XIII-as véralvadási faktor „A” alegységének (FXIII-A) és a P-szelektin receptor (CD62) expressziójának összehasonlító vizsgálata thrombin aktiválta thrombocyták és mikropartikulák felszínén. (előadás) *MTHT kongresszus*, 2005. 10. 06-08., Alsópáhok

**Nagy B.** Eltérő clopidogrel kezelések hatása a thrombocyta aktivációra koronária sztent beültetést követően. (előadás) *PhD és TDK tudományos diáktalálkozója*, 2006. 04. 10-14., Debrecen

**Nagy B Jr**, Csongrádi É, Balogh I, Káplár M, Paragh G, Kappelmayer J. Thrombocyta aktiváció vizsgálata 2-es típusú diabetes mellitusban és túlsúlyos egyéneknél. (poszter) *MLDT konferencia*, 2006. 08.30-09.02., Szeged

**Nagy B Jr**, Szűk T, Debreceni BI, Kappelmayer J. Mikropartikula képződés vizsgálata perkután koronária angioplasztika (PTCA) alkalmazása esetén. (előadás) *Tudományos szimpózium Laki Kálmán emlékére*, 2009. 09. 26., Abádszalók

## **KEYWORDS**

Platelet activation

P-selectin

Flow cytometry

Thr715Pro P-selectin polymorphism

Type 2 diabetes mellitus

Factor XIII

Fibrinogen

TRAP

Stenting

Microparticle

## **TÁRGYSZAVAK**

Vérlemezke aktiváció

P-szelektin

Áramlási citometria

Thr715Pro P-szelektin polimorfizmus

2-es típusú diabetes mellitus

XIII-as faktor

Fibrinogén

TRAP

Sztentelés

Mikropartikula

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Prof. János Kappelmayer, who has been my advisor since 2001 when I started working as a medical student researcher and later as a PhD student at his Department. It has been an honor to be his PhD student. He provided me with many valuable suggestions and constant encouragement during the course of this work. I appreciate all his contributions of time, ideas, and funding to make my PhD work stimulating and productive. His dedication and enthusiasm for research were contagious and motivational for me. I am also happy to thank him for those personal conversations we had together simultaneously. Special thanks for making me possible to get a successful fellowship at Temple University in Philadelphia, PA.

I would like to acknowledge my dept to my PhD program supervisor, former head of the Department, Prof. László Muszbek for his useful advices and personal support.

I am also thankful to István Balogh, Zsuzsa Simon, Zsuzsa Bagoly, Éva Katona, Zsuzsa Hevessy, Tibor Szűk, Miklós Káplár, Harjit Bhattoa, and Éva Csongrádi for their help and practical comments to my work in addition to the fruitful collaborations. Special thanks to Prof. Csongor Kiss for providing us the samples from the GT patient.

My keen appreciation goes to Anikó Veszprémi Gyórfi, Erika Dzsudzsák, Ildikó Tóth Trefán, Ildikó Debreceni Beke, Gizella Haramura, Zsolt Kanyári, Valéria Sziráki Kiss, Ildikó Kópis and Csaba Antal for their valuable technical assistance.

Finally, I would like to express my special thanks to my Family and Friends for their patience and attention. They helped me to concentrate on completing this dissertation and supported me mentally. Without their love and encouragement, this study would not have been completed.

These studies were supported by grants from the Hungarian National Foundation T049392/2005, K75199/2008 [to JK]; OTKA-NKTH NI69238 and by a grant from the Hungarian Academy of Sciences (MTA 2006TKI227) [to LM].

## **APPENDIX**

### **Author contribution statement**

Béla Nagy Jr designed and performed most experiments, and analyzed data. Zsuzsa Simon performed some FXIII experiments; Zsuzsa Bagoly provided the two variants of fibrinogen; Prof. László Muszbek designed some FXIII experiments; István Balogh designed the molecular biology experiments, and Harjit Bhattoa helped me in the analysis of data. The monoclonal mouse anti-human-FXIII-A antibody was kindly provided by Éva Katona, and the purified plasma FXIII was prepared by Gizella Haramura. Ildikó Debreceni Beke performed some MP experiments. Miklós Káplár, Tibor Szúk, Prof. Csongor Kiss and Éva Csongrádi provided the patients and healthy controls. Prof. János Kappelmayer designed experiments and gave overall direction.

### **Original publications related to the dissertation**