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PLATELET FUNCTION IN DIABETES MELLITUS

Relationships to hyperglycaemia, antidiabetic treatment and microangiopathy

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ABSTRACT

Diabetes mellitus is associated with accelerated atherosclerosis and increased morbidity and mortality in micro-and macrovascular complications. The metabolic derangements that accompany diabetes can adversely influence platelets and vascular endothelial function, which may contribute to the pathogenesis of diabetic angiopathy. This thesis aimed to examine the effect of acute and postprandial hyperglycaemia, as well as the impact of treatment with insulinotropic drugs and improved metabolic control on platelet function in patients with diabetes mellitus. The relationship between platelet function and microvascular complications was also investigated. In addition, platelet function was related to endothelial and inflammatory markers.

Acute hyperglycaemia, elicited by an oral glucose tolerance test, induced platelet activation as indicated by elevated plasma levels of soluble P-selectin in patients with diet-treated type 2 diabetes. In a cross-over study, evaluating the effects of two oral antidiabetic treatments, platelet hyperreactivity (increased ADP-induced P-selectin expression) was observed after a carbohydrate-rich meal in type 2 diabetes patients. Premeal treatment with repaglinide or glibenclamide reduced postmeal hyperglycaemia, but not the meal-induced platelet activation. Repaglinide treatment was associated with attenuated platelet and endothelial activity in the fasting state, but this effect was not related to glycaemic control or reduced postmeal hyperglycaemia. Platelet function in the fasting state was similar in well-controlled patients with type 2 diabetes, without macrovascular complications and healthy controls, but the plasma levels of inflammatory markers (e.g. ICAM-1, TNF-α) were significantly elevated in the patients.

Type 1 diabetes was associated with platelet and leukocyte hyperreactivity to *in vitro* stimulation, and this was more marked in patients with microangiopathy. Agonist-induced leukocyte-platelet cross-talk was enhanced in type 1 diabetes and was correlated to platelet hyperreactivity in patients with microangiopathy. Furthermore, patients with type 1 diabetes and microangiopathy had elevations of sCD40L, C-reactive protein and soluble E-selectin in serum, compared to healthy controls, indicating low-grade inflammation and vascular endothelial perturbation.

In well-controlled patients with type 2 diabetes undergoing coronary angioplasty, platelet reactivity (ADP-induced P-selectin expression) was reduced in patients with tight glycaemic control compared to patients with deteriorated glycaemic control at 3 months after coronary angioplasty.

In conclusion, acute and postprandial hyperglycaemia in type 2 diabetes as well as microangiopathy in type 1 diabetes are associated with certain aspects of platelet activation. The insulinotropic drug repaglinide, but not glibenclamide, attenuates fasting, but not postmeal platelet reactivity. Improved glycaemic control reduces platelet reactivity in type 2 diabetes patients undergoing coronary angioplasty. This thesis also supports the existence of an inflammatory component early on in type 2 diabetic disease, and in type 1 diabetic microangiopathy.

Keywords: Diabetes mellitus, platelet function, P-selectin, leukocyte-platelet cross-talk, endothelium, inflammation, coagulation, hyperglycaemia, metabolic control, microvascular complications, percutaneous coronary intervention

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers:

I Yngen M, Östenson C-G, Li N, Hjemdahl P, Wallén NH. Acute hyperglycemia increases soluble P-selectin in male patients with mild diabetes mellitus. Blood Coagulation and Fibrinolysis 2001; 12: 109-116

II Yngen M, Östenson C-G, Hjemdahl P, Wallén NH. Meal-induced platelet activation in type 2 diabetes mellitus: effects of treatment with repaglinide and glibenclamide. Manuscript.

III Yngen M, Östenson C-G, Hu H, Li N, Hjemdahl P, Wallén NH. Enhanced P-selectin expression and increased soluble CD40 ligand in patients with type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. Diabetologia 2004; 47: 537-540

IV Hu H, Li N, Yngen M, Östenson C-G, Wallén NH, Hjemdahl P. Enhanced leukocyte-platelet cross-talk in type 1 diabetes mellitus: relationship to microangiopathy. Journal of Thrombosis and Haemostasis 2004; 2: 58-64

V Yngen M, Norhammar A, Hjemdahl P, Wallén NH. Effects of improved metabolic control on platelet reactivity in patients with type 2 diabetes mellitus following coronary angioplasty. Manuscript.

CONTENTS

ABBREVIATIONS

ADP	Adenosine diphosphate
Ang-II	Angiotensin-II
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
CD40L	CD40 ligand
CVD	Cardiovascular disease
DM	Diabetes mellitus
EIA	Enzyme immuno assay
ET-1	Endothelin-1
F1+2	Prothrombin fragment 1 +2
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GP	Glycoprotein
$HbA1_c$	Glycated haemoglobin
HDL	High-density lipoprotein
Hepes	N-2-hydroxy-ethyl-piperazine-N-2-ethanesulfonic acid
hsCRP	High sensitivity measurement of C-reactive protein
ICAM-1	Intercellular adhesion molecule-1
IGT	Impaired glucose tolerance
LDL	Low-density lipoprotein
MAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MFI	Mean fluorescence intensity
MPV	Median platelet volume
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PAD	Peripheral arterial disease
PCI	Percutaneous coronary intervention
PKC	Protein kinase C
PLA	Platelet-leukocyte aggregate
P-Lym	Platelet-lymphocyte aggregate
P-Mon	Platelet-monocyte aggregate
PMN	Polymorphonuclear leukocytes
P-Neu	Platelet-neutrophil aggregate
PSGL-1	P-selectin glycoprotein ligand-1
sE-selectin	Soluble E-selectin
sP-selectin	Soluble P-selectin
TAT	Thrombin-antithrombin
TNF-α	Tumor necrosis factor-alpha
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
vWf	Von Willebrand factor

INTRODUCTION

General background

Diabetes mellitus (DM), characterized by chronic hyperglycaemia, is a rapidly growing worldwide health problem. The prevalence of DM has been estimated to 3-5% of Western populations. The incidence of DM is increasing, and will be more than doubled within 15 years mainly due to adverse life style changes with excess in caloric intake and reduced physical activity, which will in turn lead to obesity, insulin resistance and consequently impaired glucose tolerance and type 2 DM [1, 2]. Worldwide incidence data indicate an increase also in type 1 DM (juvenile diabetes) [3].

Type 2 DM accounts for over 80% of cases of DM and is a slow-onset, heterogenous disorder, resulting from interactions between environmental factors and polygenetic inheritance [4]. Impaired insulin secretion and reduced insulin sensitivity (insulin resistance in the liver, skeletal muscle and adipose tissue) are the main pathophysiological features responsible for development of the disease [5]. Along with worsened glucose metabolism, impaired glucose tolerance (IGT) appears and results in an increase in postprandial hyperglycaemia, which can be detected by an oral glucose tolerance test (OGTT).

A majority of patients with type 2 DM, as well as subjects with IGT, have signs of the metabolic syndrome (also called dysmetabolic syndrome, insulin resistance syndrome or syndrome X)[6], which is a cluster of phenotypes associated with a substantially increased risk for cardiovascular disease (CVD)[7]. Insulin resistance plays a central role in this syndrome. Other components are centripetal obesity, hypertension, dyslipidemia (low HDL, high TG, small dense oxidized LDL) and endothelial dysfunction (microalbuminuria)[8]. Recently, a prothrombotic state, characterized by abnormalities in platelet function and elevated circulating levels of C-reactive protein (CRP), PAI-1 and fibrinogen, has been recognized as a component of the metabolic syndrome[9].

Microvascular complications (retinopathy, nephropathy and neuropathy) contribute importantly to the increased morbidity in DM as retinopathy and nephropathy are major causes of blindness and end-stage renal disease, respectively. However, the major cause of morbidity and mortality in DM is macrovascular complications. More than 75% of all diabetic patients die of CVD[10]. Insulin resistance, IGT and overt type 2 DM are associated with an increased risk for CVD[11] and patients with type 2 DM have a 2-4 fold increased risk for coronary artery disease and peripheral arterial disease, and a 3-fold increased risk for stroke compared to non-diabetic subjects [12]. Diabetes also worsens early and late outcomes in acute coronary syndromes and after coronary interventions[13-16]. There is now consensus that patients with DM without previous myocardial infarction should be treated with multifactorial interventions against modifiable risk factors as aggressively as in non-diabetic individuals with a previous myocardial infarction [10, 17]. Although type 2 DM is associated with a cluster of cardiovascular

risk factors, the exact causes of the substancially increased risk of suffering CVD are not fully understood. Premature, accelerated macrovascular disease occurs both in type 1 and type 2 DM. Recent epidemiological studies indicate that type 1 DM is as great a risk factor for cardiovascular mortality and stroke as type 2 DM, and that these complications also can occur at a young age [18, 19]. Thus, early detection and treatment of risk factors for cardiovascular disease are important achievements also in type 1 DM.

Platelet function is of pathophysiological importance in atherothrombotic disease [20] and there is strong support for platelet dysfunction with platelet hyperreactivity in both type 1 and type 2 DM [21-24]. It may be hypothesized that platelets, acting in concert with the vascular endothelium, leukocytes and coagulation, play a key role in the development of diabetic angiopathy. While platelet dysfunction is clearly involved in the pathogenesis of macroangiopathy [20-22, 25-27], the role of platelets in microangiopathy is less clear [28, 29]. The metabolic state that accompanies DM may alter platelet and endothelial function already in early stages of diabetic disease. However, it is debatable whether antidiabetic treatment and improved metabolic control can restore the observed platelet hyperactivity in DM. In addition, studies of the effect of acute hyperglycaemia on platelet function in patients with DM are sparse [30-32].

The present work concerns platelet function in DM and the possible influence of acute hyperglycaemia, antidiabetic treatment and improved metabolic control, as well as the relationship to microvascular complications. In addition, platelet function was related to endothelial, inflammatory and coagulation markers.

Platelet physiology

Platelet morphology

Platelets are anuclear cell fragments shed from megacaryocytes in the bone marrow. The resting, discoid platelet which is 2-4 μm diameter and has a median platelet volume (MPV) of 7-9 fl, is the smallest corpuscular component in the circulation. The normal platelet count in peripheral blood is 150-400 x 10^9/L. Approximately 2/3 of the platelets circulate in the blood and 1/3 are stored in the spleen. The lifespan of a platelet is 7-10 days, with a daily renewal rate of about 20% of the platelet count. The platelet cytoplasmic membrane is composed of a bilayer of polarized phospholipids containing arachidonic acid. The organization of the phospholipids changes during platelet activation. This favors an interaction with coagulation factors (FV, FX) and formation of a catalylic prothrombinase complex (FXa, FVa, Ca^{2+}, prothrombin), as well as the release of arachidonic acid and formation of thromboxane A_2. The external layer of the platelet membrane contains numerous glycoproteins (e.g. GPIIb/IIIa, GPIb/IX/V, GPIV) which

act as receptors for various ligands and are essential for platelet adhesion and aggregation. The most abundant of these platelet membrane glycoproteins is GPIIb/IIIa, often called the fibrinogen receptor, with about 80.000 surface copies per platelet. GPIIb/IIIa also binds other ligands such as von Willebrand factor (vWf) and fibronectin. The cytoplasmatic membrane invaginates into the platelet to form the open canalicular system. This system enlarges the platelet surface area and provides routes for the release of platelet granular contents and is a storage site for membrane receptors. The dense tubular system is a part of the platelet internal membrane system. It is derived from the endoplasmatic reticulum of the megacaryocytes and is the main site for Ca^{2+} storage and prostaglandin synthesis. There are three different platelet specific secretory granules: α-granules, dense bodies and lysosomes. The α-granules are the numerically most abundant granules and contain a variety of high-molecular weight proteins with various biological functions. Examples of proteins stored in α-granules are P-selectin, vWf, fibrinogen, GPIIb/IIIa, FV, FX, PAI-1, growth factors, cytokine-like proteins like β-thromboglobulin, platelet factor 4 and probably also CD40 ligand and interleukin-1. The dense granules contain low-molecular compounds which promote platelet activation (e.g. ADP, serotonin and Ca^{2+}). Lysosomes contain hydrolytic enzymes, e.g. elastase, which affect elastic tissue of the vessel wall and may promote atherogenesis. Elastase is also released from activated neutrophils and can induce platelet activation [33].

Platelet activation

Hemostasis involves interactions between the vessel wall, platelets, coagulation and fibrinolysis. Under normal conditions, these systems are in balance and prevent thrombus formation and bleeding. The main physiological function of blood platelets is to maintain hemostasis by the initiation and formation of a hemostatic plug and by the secretion of various biologically active factors leading to repair of vascular injuries. Pathophysiologically platelets may cause vascular injury and tissue damage by three principal mechanisms: triggering of acute arterial thrombosis, microembolisation of the capillaries and enhancing the local progession of vascular lesions [34].

Platelets are dynamic cell fragments, influenced by several activating and inhibiting pathways which can be regulated by various endogenous and exogenous stimuli. Adhesion of resting platelets to the damaged vessel wall is the first step of primary hemostasis, and is mediated mainly through the interactions between platelet GPIb-V-IX and GPIa-IIa receptors, and subendothelial compunds like vWf and collagen. Binding of these ligands to the GP receptors leads to shape change, spreading or rolling, and activation of the adhering platelets with subsequent secretion of granule components and formation of aggregates (platelet plug; white thrombus) (Figure 1b and 3).

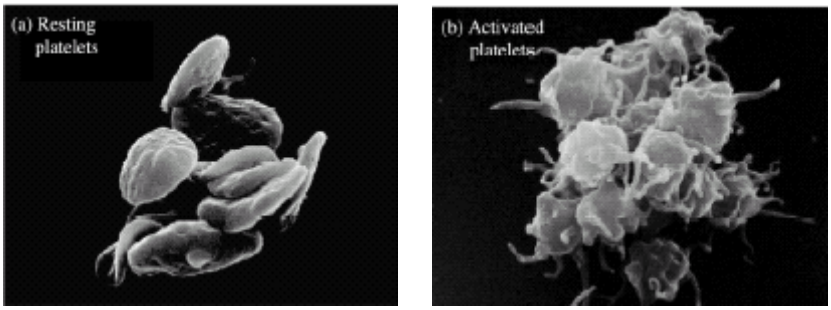

Figure 1. Resting and activated platelets*

Shape change with formation of pseudopods occurs when the intracellular Ca2+ concentration exceeds a specific threshold. During shape change, platelet fibrinogen receptors (GPIIb/IIIa) are exposed and activated, and platelet-platelet aggregation is initiated (reversible, primary aggregation). Notably, resting platelets are not able to bind fibrinogen.

An important amplifying mechanism in platelet activation is the arachidonic acid-thromboxane pathway. During platelet activation, arachidonic acid is cleaved from the platelet membrane by the enzyme phopholipase A_2. The arachidonic acid is then oxygenated by cyclooxygenase (COX) and the platelet stimulating endoperoxides prostaglandin G_2 (PGG_2) and PGH_2 are formed. PGH_2 is then converted, via the action of thromboxane synthase, to TxA_2, a potent platelet activating and vasoconstricting agent. TxA_2 is extremely unstable with a very short half-life in plasma (t1/2 around 30 sec). It is rapidly converted to TxB_2, which is further metabolised to the stable metabolites 11-dehydro-TxB_2 and 2, 3-dinor-TxB_2 (figure 2).

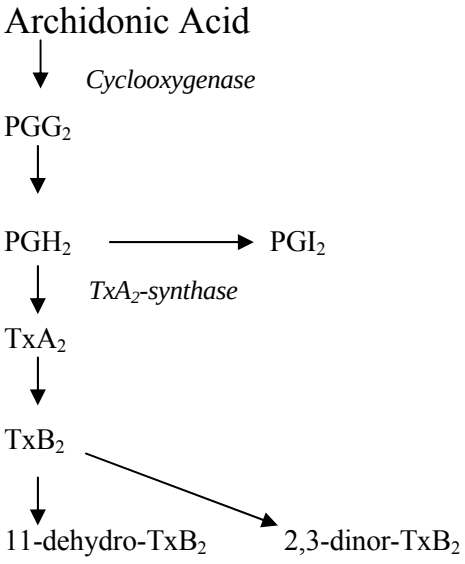

Figure 2. Biosynthesis of thromboxane

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Aspirin inhibits platelet aggregation through irreversible acetylation and inactivation of COX, resulting in blockade of TxA_2 production.

ADP is another important platelet activator. Several ADP-specific purinoreceptors have been charachterized. The P2Y12 receptor is present on the platelet membrane and is coupled to inhibitory G-proteins and mediates ADP-induced release of Ca^{2+}, inhibits adenylate cyclase and activates the GPIIb/IIIa receptor leading to platelet aggregation. The thienopyridines ticlopidine and clopidogrel inhibit platelet activation via blockade of the P2Y12 receptor.

Thromboxane A_2 , ADP and other substances such as serotonin, released from the activated platelet, provide important positive feedback and strengthen the platelet-rich clot (irreversible, secondary aggregation) (Figure 3). The substances released or externalized from the platelet granules serve to recruit more platelets and other blood cells, thereby amplifying the platelet response. The platelet plug initially formed in primary hemostasis is relatively unstable. Secondary hemostasis begins with the activation of coagulation cascade and formation of thrombin and fibrin. Platelet membrane phospholipids become negatively charged during platelet activation, which favors coagulation activation (e.g. FV, FVIIIa, FIXa, FX) and binding of the prothrombinase complex (FXa, FVa, Ca^{2+}, prothrombin) to the platelet membrane. The thrombin formed causes further platelet activation. The procoagulant activity in the vicinity of a platelet aggregate can also be reinforced by so called microparticles. Activated platelets extrude small membrane vesicles that exhibit a high binding affinity for FV and FVIIIa and possess a high procoagulant activity. Finally, deposition of fibrin on the platelet aggregate leads to consolidation via cross-linking and the platelet-fibrin conglomerate contracts (clot retraction) and thus further strengthens the blood clot (red thrombus) [33].

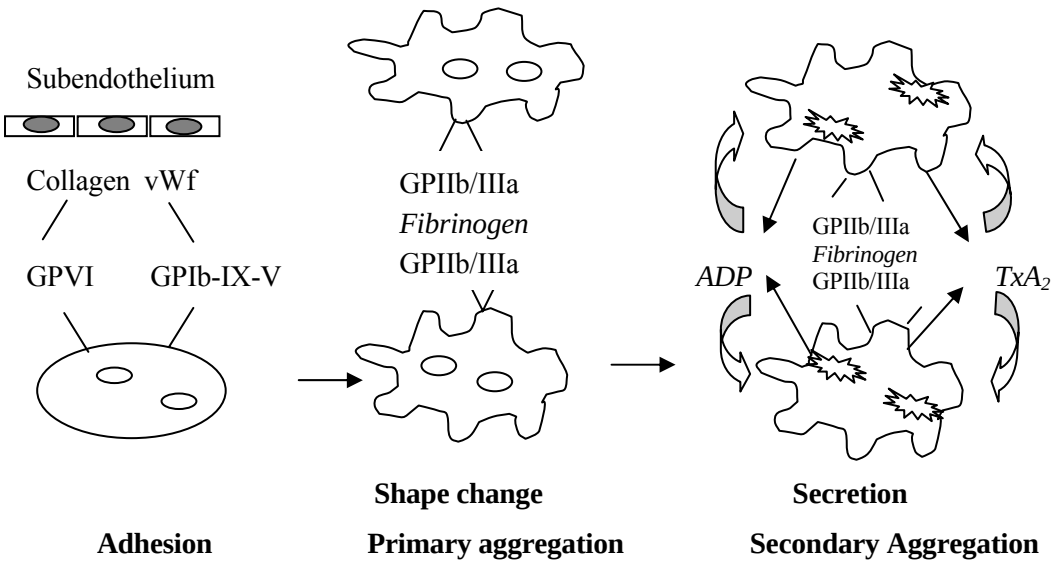


Figure 3. Platelet activation

Platelet function and inflammation

P-selectin, CD40 ligand and cell-cell interactions

Hemostasis and inflammation are closely linked and are often concomitantly activated [35, 36]. In response to inflammatory stimuli, circulating leukocytes roll and then arrest on endothelial cells and finally migrate into the surrounding tissues. In response to vascular damage, circulating platelets adhere to subendothelial tissues and then recruit more platelets into aggregates that function as procoagulant surfaces [37]. These processes are progressively linked and mediated by cell adhesion molecules. Platelet adhesion receptors are classified into four groups according to their molecular structure: selectins (P-selectin), integrins (e.g. GPIa-IIa, GPIIb/IIIa), leucine-rich glycoproteins (GPIb-V-IX and GPIV) and receptors of the immunoglobulin type (ICAM-1 and PECAM-1). Selectins mediate the early tethering and rolling of leukocytes on the vascular endothelium, leading to weak attachment of leukocytes to the vessel wall. Integrins enable firm adhesion of leukocytes to the vascular endothelium and immunoglobulins mediate migration of leukocytes between endothelial cells into the surrounding tissues [37]. Migrating poly-morphonuclear leukocytes (PMNs) can also carry adhering platelets to inflammatory extravascular tissue [38].

P-selectin expression

P-selectin is an integral membrane protein located in the α-granules of the platelets [39] and is expressed on the cell surface upon cell activation [40]. This membrane protein mediates interactions between platelets, leukocytes and endothelial cells and seems to be the most important receptor for the binding to leukocytes [41]. P-selectin stabilizes inital platelet aggregation[42] and synergizes with platelet activating factor (PAF) and/or RANTES to induce synthesis of cytokines like interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1), providing localized signals for monocyte adhesion [43]. Moreover, P-selectin has been shown to be required for monocyte and macrophage accumulation and intimal hyperplasia, and may thus be involved in neointima formation after arterial injury [44].

Soluble P-selectin

P-selectin is expressed on platelets and vascular endothelial cells [39]. The expression is transient, as it is endocytosed [45] or proteolytically shed from the platelet surface into plasma in a biologically active soluble form, while the platelet continues to circulate and function [46]. Soluble P-selectin has been found to be dependent on the time of sample collection and related to platelet count [46, 47] and has been proposed to be a reliable marker for *in vivo* platelet activation [48, 49]. However, P-selectin in plasma may also be partly derived from the endothelium since P-selectin is a component of the membrane of the Weibel Palade bodies in these cells [39].

CD40 ligand (CD40L)

CD40L, a member of the TNF-α family and originally identified on activated T-cells, is also expressed by platelets [50]. Upon stimulation of platelets with ADP or thrombin, CD40L is rapidly mobilized from intracellular granules to the platelet surface and trigger an inflammatory response of endothelial cells [50]. CD40L is cleaved into a soluble form, sCD40L, and shed from the platelet surface over a period of minutes to hours. Studies indicate that >95% of the CD40L in whole blood is found in platelets [51]. sCD40L has been shown to induce the production and release of proinflammatory cytokines from vascular cells in the atheroma, to stabilize platelet-rich thrombi, and to inhibit the reendothelization of injured vessel. It has been postulated that CD40L may play an important role in plaque instability and restenosis after PCI [51]. Interestingly, GPIIb/IIIa antagonists block the hydrolysis and subsequent release of sCD40L from platelets and the biological activites of sCD40L can be retained by virtue of sCD40L to bind to GPIIb/IIIa [52]

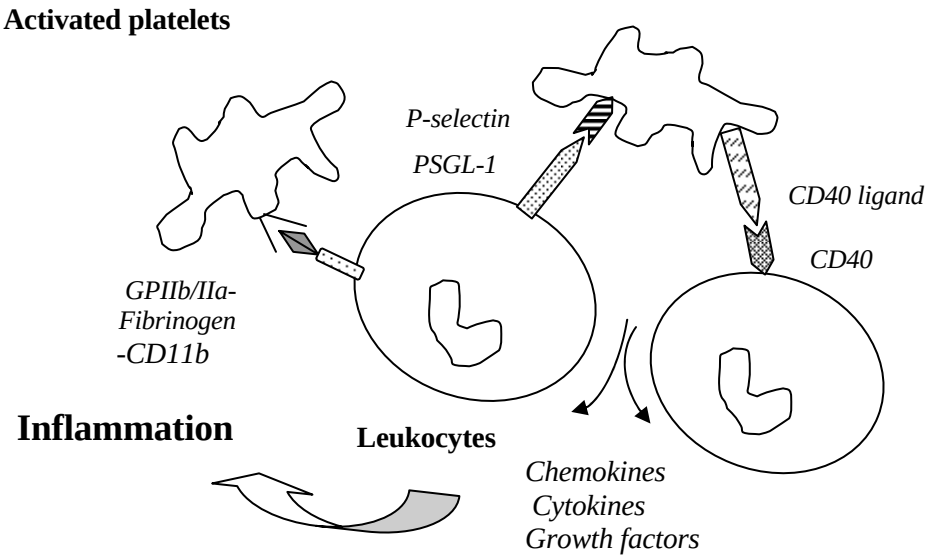


Figure 4. Platelet involvement in inflammation and some of the ligand-receptor systems resposible for leukocyte-platelet interactions.

Platelet-leukocyte aggregates

Platelet-leukocyte aggregates represent an interface between inflammatory, atherogenic and thrombogenic responses. The propensity to form heterotypic aggregates differs between leukocyte subpopulations, with monocytes showing the greatest and lymphocytes the least propensity [53]. Also, experimental studies suggest that the heterotypic aggregation is more dependent on platelet activation than leukocyte activation [54]. PMN and platelet-monocyte interactions mediate targeting of leukocytes to the injury and may enhance the synthesis of

chemokines and cytokines and adhesion molecules, thus further increasing platelet and leukocyte reactivity [38]. P-selectin promotes platelet adhesion to the stimulated vessel wall and evokes a process that induces leukocytes tethering and rolling via the ligand P-selectin glycoprotein-1 (PSGL-1), constitutively expressed on the surface of PMNs and monocytes. Interactions between P-selectin and PSGL-1, in concert with other ligand-receptor systems and platelet derived mediators, activate leukocytes[55]. Increased leukocyte integrin expression (e.g. CD11/CD18, expressed on monocytes, neutrophils and natural killer cells) results in leukocyte adhesion and migration on/into the vessel wall [56]. P-selectin/PSGL-1 binding also induces the generation of procoagulant microparticles from leukocytes, containing active tissue factor [57], and may enhance coagulation and fibrin deposition at the site of injury [57, 58]. Thus, interactions between platelets and leukocytes involving P-selectin constitute a link between coagulation and inflammation. Platelet-leukocyte interactions mediated by P-selectin and PSGL-1 may be an important effector mechanism in the pathogenesis of diseases that involve dysregulated inflammation (e.g. coronary artery disease, restenosis after PCI and diabetic angiopathy) [59, 60]. Interaction of CD40L on T-cells and CD40 on B-cells are of importance in the immune system. However, CD40 is not only constitutively expressed by B-cells, but is also found in monocytes, macrophages and endothelial cells [50]. Thus, CD40 on monocytes can bind to CD40L on platelets, as depicted in figure 4, and trigger inflammatory reactions.

Platelet-leukocyte cross-talk

Platelets and leukocytes may regulate each others functions, i.e. platelet-leukocyte cross-talk, via direct cell-cell contact and soluble mediators. Platelet-leukocyte conjugation enhances the proinflammatory and prothrombotic activities of platelets and leukocytes. Thus, monocytes are actively intergrated in the platelet-rich thrombus, and promote its growth[61]. Leukocyte-bound platelets increase leukocyte tissue factor [62] and adhesion molecule expression [63], as well as cytokine [64] and superoxide anion production [65]. Direct cell to cell contact also enhances transcellular ararachidonic acid metabolism [66]. Platelet-leukocyte cross-talk can be regulated by soluble mediators. Thus, leukocyte function can be influenced by platelet-released soluble mediators that stimulate (e.g. ADP, serotonin, PF4, β–TG) or inhibit (e.g. NO, TGF-β) leukocytes. Similarly, platelet function can be influenced by leukocyte-released mediators that enhance (e.g. PAF, superoxide anion) or inhibit (e.g. proteases, NO and adenosine) platelet function. Taken together, platelet-leukocyte cross-talk clearly influences platelet and leukocyte function. However, the importance of this cross-talk in the pathogenesis of thrombosis and inflammation is not fully known.

Platelet alterations in diabetes mellitus

Effects of hyperglycaemia and relation to vascular complications

Diabetes mellitus is considered to be a prothrombotic state with chronic platelet activation, activation of the coagulation system and decreased fibrinolytic potential [67]. A number of studies performed during the past decades have documented several different platelet function alterations in patients with DM. Three main explanations for abnormal platelet function in DM have been proposed: (1) Immature, larger and more reactive platelets are synthesized in the bone marrow. (2) Platelets are activated when exposed to the metabolic milieu in DM. (3) Platelets are activated due vascular damage. Figure 5 shows some of the reported platelet abnormalities associated with diabetic disease.

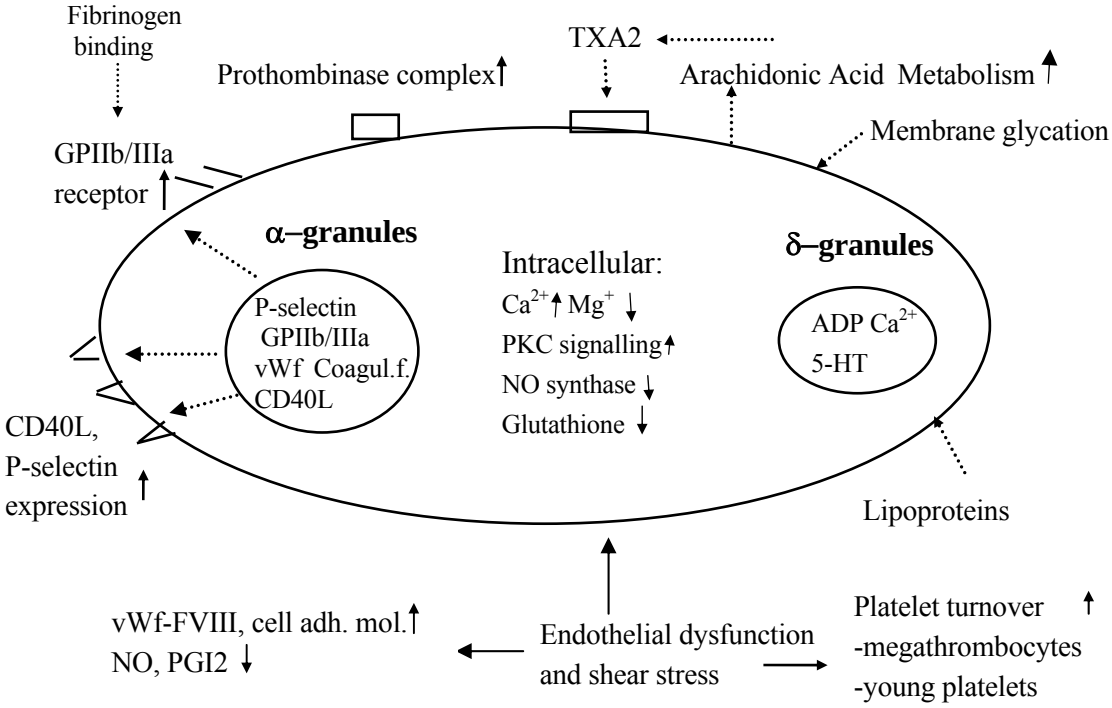


Figure 5. Platelet abnormalities in diabetes

Platelet aggregability

Platelet hyperaggregability in response to glucose was recognized already in 1965 [68]. Enhanced platelet aggregation in response to various agonists (ADP, thrombin, collagen, arachidonic acid, epinephrine), using older platelet function techniques (e.g. Born aggregometry), has been shown in patients with both type 1 and type 2 DM compared to non-diabetic individuals [69, 70]. The alteration may occur early in diabetic disease [71] and may precede the development of angiopathy [72].

Thromboxane production

An early observation was that diabetic platelets exhibit enhanced thromboxane production *in vitro*, as evidenced by increased Tx synthesis in platelet rich plasma or washed platelets following addition of platelet agonists [73, 74] as well as *in vivo,* as evidenced by increased urinary excretion of 11-dehydro-TxB_2 [75, 76]. TxA_2 production has been positively correlated with fasting plasma glucose and HbA1c, and improved glycaemic control has been shown to reduce TxA_2 production in several, [73, 75, 77] but not all studies [78, 79]. A lipid and arachidonate peroxidation marker (8-*iso*-$PGF_{2\alpha}$), has been correlated with TxA_2 biosynthesis and suggested to be a link between glycaemic control, oxidative stress and platelet activation [80]. Increased Tx production has been related to both diabetic micro- and macroangiopathy [75, 76]. However, increased excretion of the metabolite TxB_2 has also been found in type 1 and type 2 diabetic patients without vascular complications, and associated with decreased oxidant status especially in type 2 DM [81]. In contrast, others have not found any difference in urinary TxB2 excretion between type 1 diabetic patients with or without retinopathy and nondiabetic controls [79].

Membrane glycation

Impaired platelet membrane fluidity due to glycation of proteins and to changes in lipid composition have been reported in DM [82]. In experimental models, glycosylated LDL has been shown to increase platelet aggregability. Small oxidized LDL inhibits platelet membrane Ca^{2+}-ATPase which may lead to increased intracellular Ca^{2+} concentration and decreased nitric oxide synthase [83]. Lipoproteins also enhance thromboxane generation during platelet activation [84]. Oxidized lipids provide a surface for activation of the prothrombinase complex in DM [85].

Alterations in platelet size and platelet survival

It has been reported that predominantly large platelets circulate in patients with DM and this has been considered to reflect an activated megacaryocyte-platelet system [86-88]. Larger and younger platelets are considered to be more reactive. The platelet size distribution (mostly assessed as median platelet volume; MPV) correlates positively with the number of platelet glycoproteins (GPIb and GPIIb/IIIa) on the platelet membrane, the thromboxane synthesizing capacity, and the platelet granule contents of various platelet specific proteins [87, 89, 90]. An increased MPV is an independent risk factor for myocardial infarction [91] and increased MPV has also been associated with proliferative diabetic retinopathy [87]. However, MPV does not seem to be influenced by glycaemic control [86]. Studies on platelet survival in DM have produced conflicting results. Decreased platelet survival in patients with DM has been associated with overt vascular complications [92]. However, one study showed no difference in platelet survival in DM patients compared to healthy controls, and yet another study failed to show a relationship between platelet survival and vascular complications [28, 93].

Platelet glycoprotein receptors

Platelets from diabetic subjects show increased adhesiveness and increased numbers and activity of several platelet specific surface glycoprotein receptors (GPs). Thus, increases in GPIIb/IIIa ("fibrinogen receptor") [90, 94], GPIb-IX ("von Willebrand factor receptor")[90], GPIa/IIa ("collagen receptor"), CD62 (P-selectin)[94-98] and CD63 [98] have been observed in DM. Platelet receptor activation has been correlated to glycaemia in clinical [98] and experimental studies [99] and to vascular complications [95, 97]. Other studies show no correlation between GP expression in DM and glycaemic control [94]. Also, upregulation of the CD40-CD40 ligand system [100] have been observed in patients with DM and CD40L on platelets has been correlated to HbA1c concentrations [100].

Platelet secretion products

Cytokine-like proteins are released from the α-granules upon platelet activation. Elevated plasma levels of β-thromboglobulin (β-TG) and platelet factor 4 (PF4) have been observed in DM patients [78, 101] . Elevated levels of these proteins have been associated with diabetic angiopathy, e.g. proliferative retinopathy, in several studies. Other studies show normal levels in well-controlled patients without microangiopathy [102 103]. As described above, P-selectin and CD40L are shed from the platelet surface into plasma and into biologically active soluble forms[46, 104]. Recent observations show elevated levels of soluble P-selectin [96, 105] and CD40L [97, 106-108] in DM and in cardiovascular disease [109-112]. Multifactorial risk management have shown to reduce high levels of sCD40L in DM with and without CVD [113]. Elevated levels of these compounds may reflect accelerated atherosclerosis and a prothrombotic state [48, 114-116].

Intracellular mechanisms

Functional platelet abnormalities in DM have been associated with several intracellular alterations. Thus, diabetic platelets have reduced Na+/K+ ATPase activity and increased Ca2+ ATPase activity resulting in elevated intracellular Ca2+ concentrations and platelet hyperactivity [117]. Also, decreased intracellular Mg^{+} in diabetic platelets may enhance platelet activity [118]. Moreover, hyperglycaemia is associated with increased PKC activity [31] and superoxide anion production, as well as reduced antioxidant (glutathione) levels and nitric oxide (NO) synthase activity [119]. These alterations may induce oxidative stress and platelet activation.

Coagulation and fibrinolytic alterations in diabetes mellitus

Most of the factors of both the intrinsic (contact activation; increased levels of kallikrein, von Willebrand factor and FVIII, XI and XII) and the extrinsic (increased tissue factor and factor VII) pathways of coagulation have been reported to be altered in DM [67]. Coagulation activation markers reflecting the final steps of the coagulation cascade *in vivo*, such as prothrombin fragment 1+2 (F1+2; released when thrombin is formed from prothrombin) and thrombin-antithrombin complexes (TAT; inactivated free thombin), have also been reported to be increased in DM [67]. Elevated plasma levels of F1+2 have been found during acute hyperglycaemia in type 2 DM [120] and have been associated with progession of retinopathy in both type 1[121] and type 2 DM [122]. Furthermore, patients with DM may have lower activities of anticoagulants such as antithrombin III (leading to increased thombin activity) and protein C (leading to increased factor V and VIII activity) [67]. Lastly, elevated plasma levels of fibrinogen have consistently been reported in DM [67]. Increased fibrinogen levels and/or impaired metabolic control may result in a tight, more thrombogenic fibrin gel structure [123, 124]. The most important inhibitor of the fibrinolytic system is plasminogen activator inhibitor (PAI). PAI-1 levels are typically elevated in obese insulin resistant patients and in type 2 diabetic patients, but are commonly in the normal range in patients with type 1 DM [9].

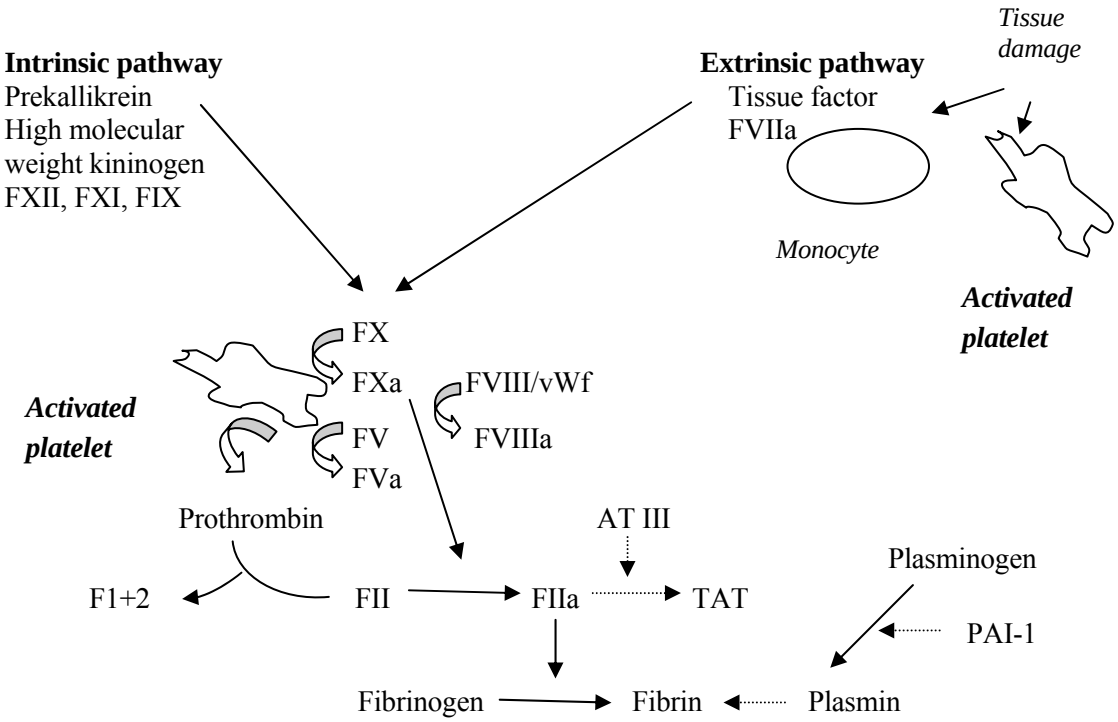


Figure 6. Platelet involvement in the coagulation cascade
(activating ; inhibiting)

Endothelial dysfunction in diabetes mellitus

The endothelial cells synthesize both platelet inhibiting and dilating substances, such as nitric oxide (NO) and prostaglandin I_2 (PGI_2) and platelet activating and constricting substances like endothelin (ET-1) and angiotensin II (Ang-II). In DM, there seems to be an imbalance between these two groups of compounds with an increased production of platelet activating and vasoconstricting substances. Platelet dysfunction in DM may not only derive from hyperfunction, but also to loss of antiaggregatory mechanisms. There are reports of decreased vascular synthesis[125] of, and decreased vascular sensisitivity [126] to, prostacyclin (PGI2) and of decreased synthesis and release of NO [127-129] (see figure 5). Impaired NO-mediated vasodilation seems to precede atherosclerosis in DM [130]. Hyperglycaemia inhibits NO production by blocking eNOS activation, by activating protein kinase C and by increasing the production of reactive oxygen specimens [21, 130]. Subsequently, transcription factors, such as nuclear factor κB (NF-κB), activated by hyperglycaemia and/or dyslipidaemia induce inflammatory gene expression, resulting in increased production of leukocyte-attracting chemokines, increased production of inflammatory cytokines and augmented expression of cell adhesion molecules [130]. These changes of endothelial cells and monocytes may lead to increased production of tissue factor (TF), the major procoagulant found in atherosclerotic plaques, along with platelet activation and aggregation, as well as alterations in coagulation and fibrinolytic factors. Interestingly, proatherogenic effects of C-reactive protein (CRP) have been shown in endothelial cells. Thus, CRP decreases platelet inhibiting prostacyclin (PGF1α) and NO, and increases cell adhesion molecules, MCP-1, ET-1, IL-8 and PAI-1 [131].

Von Willebrand factor

An established plasma marker for generalized endothelial cell damage or dysfunction is vWF [132-134], a multimeric glycoprotein which is mainly stored in the Weibel-Palade bodies within the endothelial cell and also in the α-granules of platelets. vWf carries factor VIII in the circulation, mediates platelet adhesion and may also participate in aggregation of platelets. vWf levels correlate with the age of the patients and the duration of DM which is one of the major risk factors for the development of microvascular complications [135]. Thus, positive relationships with nephropathy and retinopathy in DM have been reported and correlations with microalbuminuria are commonly described [136]. Also, high levels of vWf may predict cardiovascular disease progression [137]. Glycosylation of vWf may not alter its function since vWf antigen levels do not seem to be influenced by acute changes in blood glucose or the degree of glycaemic control [135].

Soluble E-selectin

Soluble E-selectin (sE-selectin), another endothelial marker, is exclusively derived from cytokine-activated endothelial cells and modulates granulocyte adhesion to the vascular endothelium. Elevated levels of sE-selectin have been reported in type 1 and type 2 DM and positive correlations with hyperglycaemia [138] and micro-[97, 139] and macroangiopathy[136, 140] have been shown. Interestingly, sE-selectin and the cell adhesion molecules ICAM-1 and VCAM-1 were in a recent prospective study found to predict the development of type 2 DM in initially non-diabetic women, independently of other known risk factors, including obesity and subclinical inflammation [141]. Thus, endothelial dysfunction may precede the development of DM.

Antidiabetic treatment and platelet function

Four main classes of oral antidiabetic agents are currently available for the treatment of type 2 DM. Insulinotropic drugs (long-acting sulphonylureas, short-acting meglitinides) increase pancreatic inulin secretion. Biguanides (metformin) mainly decrease hepatic glucose. α-Glucosidase inhibitors (Acarbose) decrease gut carbohydrate absorption and thiazolidinediones (glitazones) are insulin sensitizers which increase peripheral glucose disposal [142]. While there are numerous studies confirming platelet hyperactivity in DM, studies concerning effects of oral antidiabetic treatment on platelet function are sparse. In this thesis, the effects of insulinotropic drugs (glibenclamide and repaglinide) on platelet function were investigated.

Sulphonylureas

Different sulphonylurea derivatives have been shown to exhibit antiplatelet effects by inhibiting arachidonic acid metabolism [143] and by reducing agonist-induced platelet aggregation in some (glibenclamide, glimepiride) [144, 145], but not all studies (glibenclamide) [146]. However, whether the antiplatelet effect is drug specific or an effect of improved metabolic control has been debated [145]. Gliclazide, a sulphonylurea not available in Sweden, has been reported to have a free radical scavenging ability resulting in reduced platelet reactivity and increased prostacyclin synthesis and thus beneficial effects beyond those related to glycaemic control [147]. Gliclazid has also been found to reduce platelet aggregation, enhance fibrinolysis [148] and inhibit neutrophil-endothelial cell adhesion, and to inhibit the surface expression of endothelial adhesion molecules [149].

Meglitinides

To our knowledge, there are no published data on the effect of the novel short-acting insulin secretagogues repaglinide and nateglinide on platelet function. However, reduced postprandial glycaemia and improved glycaemic control during repaglinide treatment has been associated

with decreased concentrations of interleukin-6 and CRP [150] and a beneficial effect on oxidative stress [151].

Biguanides

Metformin treatment caused a 40% reduction of cardiovascular complications in overweight patients with type 2 DM in the UKPDS study [152]. Metformin is thus first choice treatment in these patients. A systematic evaluation of the effect of metformin treatment on platelet function is lacking and existing results are inconsistent. Decreased sensitivity to platelet-aggregating agents during metformin treatment has been demonstrated in type 2 DM (metformin vs gliclazide)[153] and in insulin-dependent diabetic patients (metformin added to insulin treatment)[154]. Metformin may have platelet stabilizing effects as shown by reduced platelet density, β-TG [153, 155] and platelet superoxide anion production [156]. However, others observed no effect on metformin on spontaneous or ADP-induced platelet aggregation in a placebo-controlled study and there were no effects of the drug on markers of platelet secretion *in vivo* (βTG, platelet factor 4) [157]. Metformin is a pleiotropic drug with beneficial treatment effects on fibrinolysis (reduced PAI-1 in plasma), coagulation (reductions of FVII, FXIII), lipids (reduced hypertriglyceridaemia) and blood flow (increased vasodilator responses to L-arginine and post ischemic blood flow)[158].

α-glucosidase inhibitors

The α-glucosidase inhibitor Acarbose is a mild antidiabetic drug which lower postprandial glucose levels. Acarbose treatment in patients with IGT has recently been associated with a reduction of cardiovascular disease and hypertension, and a reduced incidence of type 2 DM [159]. Information on platelet function during acarbose treatment is sparse. Acarbose has been shown to decrease platelet-monocyte aggregate formation by reducing postprandial hyperglycaemia in patients with DM [60].

Thiazolidinediones

The thiazolidinediones (glitazones) are a new class of compunds activating the nuclear peroxisome proliferator-activated receptor-γ (PPARγ) which is expressed in several tissues, including vascular tissue. The receptor has a regulatory role in the differentiation of cells, particularly in the adipose tissue. These drugs exhibit several non-hypoglycaemic effects (reviewed in [160]). Recent experimental studies show that human bone marrow megacaryocytes and platelets express PPARγ, and PPARγ agonists blunt platelet release of CD40L and thromboxane [161]. Rosiglitazone treatment has been associated with decreased serum sCD40L concentrations [106, 107] in patients with type 2 DM and coronary artery disease, and reduced platelet P-selectin expression in non-diabetic patients with coronary artery disease [162].

Insulin

Insulin therapy improves clinical outcomes in patients with type 2 DM and acute myocardial infarction [163] and in critically ill patients with only mildly elevated glucose levels [164]. The biochemical mechanisms underlying these beneficial effects of insulin are not fully known. Hyperinsulinaemia (endogenous or exogenous) has been implicated in a variety of mechanisms that may predispose diabetic patients to increased rates of restenosis after PCI[165, 166]. However, data are inconclusive [166]. The higher risk of restenosis found in insulin-treated patients compared to non-insulin treated patients may be due to confounding by indication as insulin treatment may merely be a marker for more advanced disease. Insulin has been suggested to exert direct as well as indirect effects on platelet function [23]. *In vitro* studies in platelets from healthy subjects have shown direct platelet inhibiting effects of insulin at low concentrations [167], wheras high supraphysiological concentrations enhance platelet activity [168-170]. However, data are inconsistent since other *in vitro* studies have not revealed any effects of insulin on platelets from healthy subjects[171, 172] or dose-dependent platelet activating effects of insulin on platelets in healthy subjects [170] and patients with type 1 DM [173]. Exogenous insulin can restore platelet abnormalities as well as inflammatory markers by improving glycaemic control in patients with DM [73, 75, 77, 124, 138, 174]. Thus, beneficial effects of insulin on platelet function *in vivo* may be related to improved metabolic control, rather than to direct platelet stabilizing effects in patients with DM.

Antiplatelet therapy in diabetes mellitus

Platelet inhibition with aspirin is a cornerstone in the prevention and treatment of occlusive macrovascular arterial disease [175]. A recent meta-analysis of aspirin treatment for the primary prevention of cardiovascular events shows that the net benefit of aspirin increases with increasing cardiovasular risk [176]. Unfortunately, the proportion of patients with DM was small (2-17%) in these trials. In a recent study primary prevention of CVD with low-dose of aspirin in diabetic patients was less efficient compared to subjects with other cardiovascular risk factors [177]. Thus, despite the high cardiovascular risk in DM, clear evidence for efficient primary prevention of antiplatelet therapy in DM is lacking.

Pooled data from subgroup analyses of patients with DM in secondary prevention trials [178] plus data from the only trial in diabetic patients (the ETDRS study; mixed primary and secondary prevention with aspirin 650 mg/day) [179] suggest that patients with DM benefit as much, or more from aspirin treatment, as non-diabetic patients. However, a recent meta-analysis failed to show a clear reduction of macrovascular events by antiplatelet therapy in diabetic patients (only 7% risk reduction in DM patients compared to 25% in other high risk patients) [175].

The findings mentioned above may reflect that platelets from patients with DM are less sensitive to the antiaggregating effect of aspirin. The mechanism behind this so called ”aspirin resistance” is not known. Possible explanations for reduced aspirin efficacy in diabetic disease include increased platelet turnover (increased production of platelets with non-acetylated COX), upregulated inflammatory activity (inflammation-induced COX-2 expression in monocytes-macrophages as an additional source of endoperoxides and subsequently TxA_2 formation) [180, 181] and hyperglycaemia (leading to glycosylated platelet membranes and/or increased production of thromboxane bypassing the COX step) [182, 183]. Also, stress[184], hypercholesterolaemia [185] and hypertension [186] may contribute to “aspirin resistance” in patients with DM.

Another group of antiplatelet agents are the thienopyridines ticlopidine and clopidogrel. There are limited data on the effectiveness of clopidogrel treatment in preventing macrovascular complications in DM (20% of the patients in the CAPRIE study had DM)[187]. Retrospective subgroup analysis of patients with DM and a history of atherothrombosis in the CAPRIE study found that clopidogrel (75 mg) was more effective than aspirin (325 mg) in reducing recurrent ischemic event [188].

Data from studies in acute coronary syndromes support the concept of platelet hyperreactivity in diabetic macroangiopathy. GPIIb/IIIa inhibitors are more effective in reducing cardiovascular complications in patients with DM than in non-diabetic patients [189]. This may be explained by decreased fibrinogen binding due to glycation of platelet membrane proteins in DM [190].

There are a few antiplatelet treatment studies supporting the involvement of platelets also in the pathogenesis of diabetic microangiopathy. The DAMAD study showed that aspirin treatment slowed the progression of early diabetic retinopathy [191]. Moreover, ticlopidine treatment reduced the progression of microaneurysm in patients with nonproliferative retinopathy[192] . However, a large study with aspirin treatment in diabetic patients (the ETDRS study) showed no effect on progression of retinopathy, despite reductions of cardiovascular mortality and morbidity [179].

Pathogenesis of diabetic vascular complications

The pathogenesis of diabetic angiopathy is complex and involves hemostatic alterations, endothelial dysfunction and inflammation (figure 7). Some of the major contributing metabolic factors to diabetic angiopathy are listed below. In addition pathogenetic differences between type 1 and type 2 DM are briefly described.

Hyperglycaemia

Microvascular complications (retinopathy, nephropathy, neuropathy) have clearly been related to the degree of hyperglycaemia. Improving glycaemic control has been shown to reduce microvascular complications in type 1 [193] and type 2 diabetic patients [194]. The association between reducing hyperglycaemia and preventing macrovascular complications (CVD, stroke, PAD) has been less consistently demonstrated [193, 194]. However, a recent meta-analysis has found a progressive relationship between glucose levels and cardiovascular risk extending below the diabetic threshold [195, 196]. Studies indicating a benefit of improving glycaemic control regarding macrovascular complications in DM are emerging [197]. Postprandial hyperglycaemia is an early defect in type 2 DM and the possible importance of postprandial hyperglycaemia versus fasting hyperglycaemia in the pathogenesis of angiopathy in DM has recently been debated [198-200]. Based on epidemiological studies involving oral glucose tolerance tests, it has been suggested that the postmeal glucose value is a better predictor of HbA1c [201-203], CRP levels [204] and cardiovascular complications [202, 203] than fasting hyperglycaemia.

Biochemical mechanisms of hyperglycaemia-induced vascular damage involve non-enzymatic glycation (irreversible formation of advanced glycation end products, AGE, during chronic hyperglycaemia), increased sorbitol pathway, protein kinase C (PKC) activation, decreased production of NO, oxidative stress (reviewed in[205]) and haemodynamic changes that may cause platelet activation [23, 31, 129], endothelial dysfunction [205-209] and inflammation [210, 211].

Dyslipidaemia

An established risk factor for accelerated atherosclerosis in DM is dyslipidaemia. Impaired lipid metabolism is considered to be a primary event in type 2 DM [212]. Excessive efflux of free fatty acids (FFA) from adipose tissue and impaired insulin-mediated skeletal uptake increase hepatic FFA concentrations and cause the dyslipidaemia typically seen in type 2 DM. Lipoprotein abnormalitites in DM include increased plasma triglycerides (TG), low high density lipoprotein (HDL), and increased highly atherogenic, small, dense low-density lipoproteins (LDL) particles that have undergone glycooxidation [213]. All these abnormalities may affect platelet function by interfering with membrane fluidity or with intracellular events reviewed above. Also, oxidized LDL stimulates the biosynthesis of cell adhesion molecules (ICAM-1, E-selectin) in diabetic endothelial cells, which promote interactions with leukocytes and macrophages. LDL particles accumulate in macrophages, leading to the formation of foam cells, an early event in the atherosclerotic process [214].

Insulin resistance

Insulin resistance (i.e. resistance to insulin-stimulated glucose uptake) leads to elevated levels of circulating insulin. Evidence is accumulating showing that insulin resistance is associated with activated haemostasis, impaired fibrinolysis, endotheliopathy and inflammation, and that

these disturbances are central to the development of cardiovascular complications in type 2 DM [215, 216 9]. In addition, markers of insulin resistance have shown to be strong risk factors for retinopathy in type 1 DM [217].

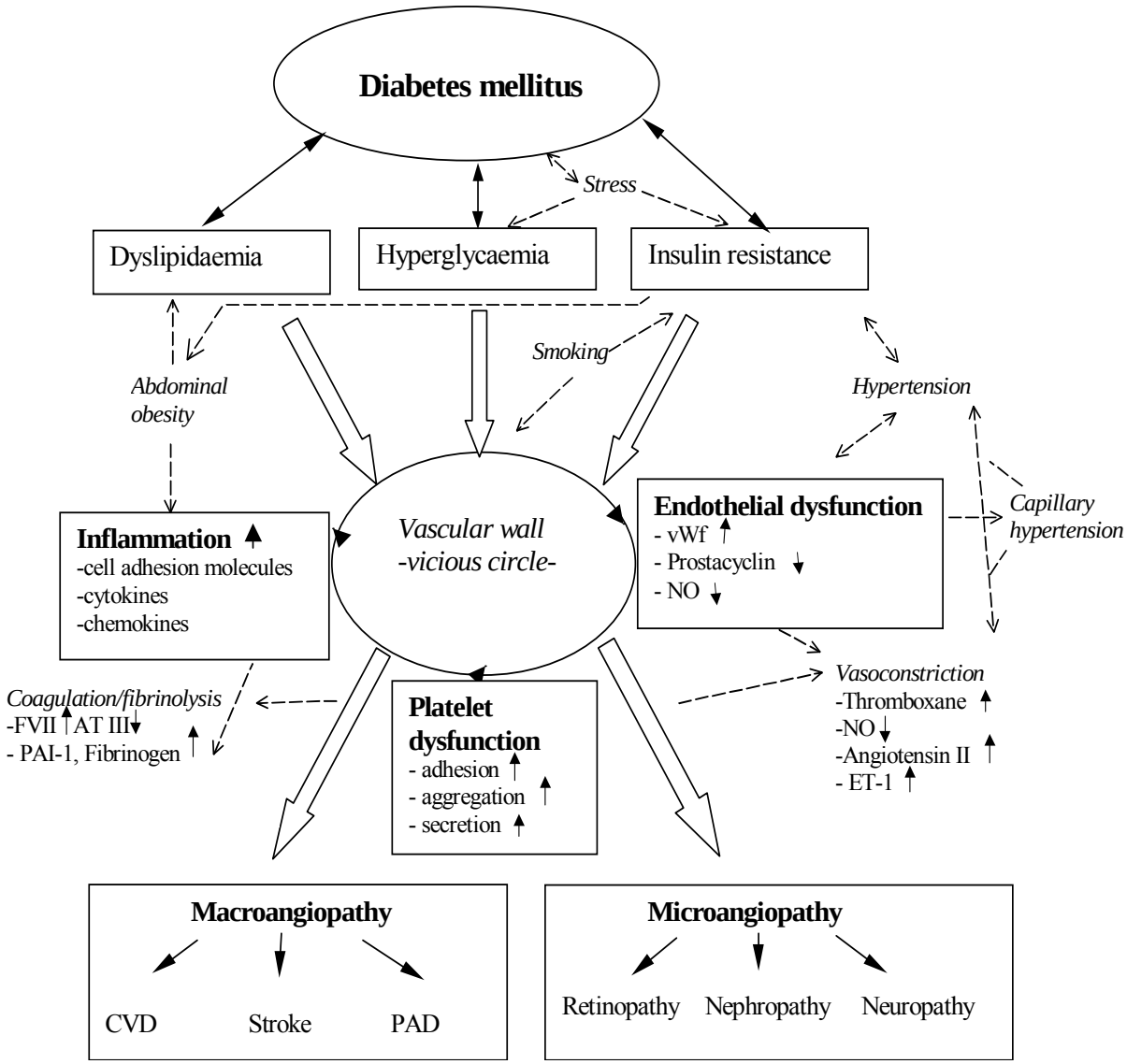


Figure 7. Pathogenesis of diabetic angiopathy

Platelets possess insulin receptors [172] and it has be proposed that platelets also are insulin resistant [218, 219 21]. However, glucose entry into the platelet does not depend on insulin [23] and the physiological relevance of these receptors are not clear [220]. The inhibiting effects of insulin on NO-mediated increases in intraplatelet nucleotides (cyclic guanosine 3´, 5´-monophoasphate; cGMP) are blunted in obese subjects and patients with type 2 DM [219, 221], and there is a decreased number of platelet insulin receptors and lower affinity in type 2 DM [222]. Also, *in vivo* euglycaemic hyperinsulinaemia raises intra-platelet Ca^{2+} in insulin resistant subjects, but not in

insulin sensitive subjects [223]. Moreover, hyperinsulinemia has been associated with elevated levels of fibrinogen, PAI-1 and vWf [224]. Thus, insulin resistance with attendant hyperinsulinaemia may create a pro-aggregatory state.

NO-mediated endothelium-dependent vasodilatation is disturbed in the insulin resistant state, and endothelial dysfunction has been suggested to be a common antecedent to the many features of insulin resistance [141, 225].

Insulin resistance is frequently associated with central obesity. Pro-inflammatory cytokines generated in adipose tissue, such as TNF-α and interleukin-6, result in insulin resistance in several cell types and contribute to a persistent systemic low-grade inflammation and endothelial oxidative stress, thus leading to a prothrombotic condition [226 216 227].

Insulin resistance (i.e. hyperinsulinemia) is associated with hypertension due to sympathetic nervous system activation, renal sodium retention and arterial wall thickening [228, 229]. More than 50% of subjects with essential hypertension are insulin resistant [27] and hypertension is a common feature in IGT [230] and type 2 DM [231]. The risk of developing diabetic micro- and macrovascular complications is related to elevations of systolic blood pressure [232]. Hypertension reduces endothelium-dependent vasodilatation [233] and enhances the interaction of platelets and monocytes with vascular endothelial cells [234].

Differences in the pathogenesis of angiopathy in Type 1 vs Type 2 DM

Although hyperglycaemia is a major determinant in both types of DM, the patho-physiological mechanisms behind diabetic angiopathy seem to differ between type 1 and type 2 DM [235]. The interaction of insulin resistance and inflammation typically seen in IGT and type 2 DM is central for the development of macrovascular complications [27]. Hypertension (without/with microalbuminuria) and dyslipidaemia are more common in type 2 than in type 1 DM and may antedate the emergence of overt type 2 DM [235]. In type 1 DM, the basis of hypertension is often microalbuminuria and nephropathy [213]. Endothelial dysfunction is closely associated with diabetic retinopathy, nephropathy and atherosclerosis in both type 1 and type 2 DM [228]. The development of microalbuminuria is related to atherosclerosis and microalbuminuria is an independent predictor for cardiovascular mortality, progressive renal disease and retinopathy in both type 1 and type 2 DM [133, 213, 236].

AIMS OF THE STUDY

The aims of the present study were:

- To investigate the effect of short-term hyperglycaemia on platelet function in patients with diabetes mellitus

- To evaluate the impact of various antidiabetic treatments on platelet function in patients with diabetes mellitus

- To study relationships between diabetic microangiopathy and platelet function

- To relate platelet function in diabetes mellitus to inflammatory and endothelial function markers and to activity of the coagulation system

PATIENTS AND METHODS

Study population

A total of 87 patients with DM and 53 healthy controls were investigated in papers I-V. The study subjects were investigated with platelet function tests on 270 separate occasions. The patient groups were well defined and were overall well metabolically controlled.

Fourty-eight patients had DM type 2. Twenty-six of them had no history of macrovascular disease; eleven had mild diabetes and were treated with diet (paper I); fifteen had oral antidiabetic treatment (paper II). Twenty-two of the type 2 diabetic patients had severe coronary artery disease and were treated with diet, tablets or insulin (paper V). In addition, six individuals with IGT participated in study I, but the results were not included in the published paper due to the small number of subjects.

Thirthy-nine patients had type 1 DM, with 10-20 years duration of disease; 19 patients did not have and 20 patients had microangiopathy (paper III and IV).

The patients with type 2 DM in paper I and subjects with IGT were recruited from the Stockholm Diabetes Prevention Program, identified by random screening of the population. None of these patients had any symtoms of diabetes when the diagnosis was made. Most of the typ 2 diabetic patients in paper II were recruited from the outpatient clinic of the Department of Endocrinology and Diabetology, Karolinska University Hospital, Solna. The patients in paper V were enrolled from the Department of Cardiology, Karolinska University Hospital, Solna. The patients with type 1 DM in paper III and IV were selected from a register at the Department of Endocrinology and Diabetology, Karolinska University Hospital, Solna.

The healthy subjects were recruited from a register at Metabolic Unit, Karolinska University Hospital, Solna in study I. In paper II-IV the healthy subject were mainly recruited among the staff of the hospital.

Diagnosis of diabetes

The subjects in paper I were classified as having normal glucose tolerance, impaired glucose fasting/impaired glucose tolerance (IGF/IGT) or type 2 DM according to the criteria based on oral glucose tolerance testing, OGTT.

Table 1. Diagnosis of diabetes and IGF/IGT

Group	Blood glucose, mmol/L		
	Fasting		2h*
normal	≤ 5,5	and	< 6,7
IGF/IGT	5,6-6,0	and/or	6,7-9,9
diabetes	≥ 6,1	and/or	≥ 10,0

*2h after intake of 75 gram glucose in 200 mL water.

The patients in paper II were previously diagnosed as type 2 DM and were all on oral antidiabetic treatment before study entry.

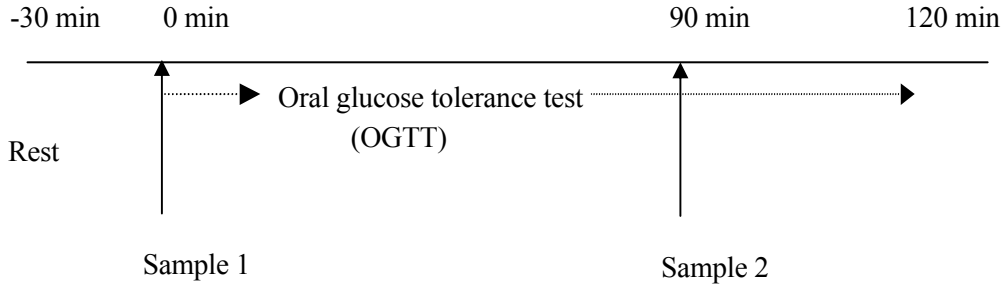
In paper III and IV, the diagnosis of type 1 DM was documented in the charts and the patients had C-peptide levels in plasma <0.3 pmol/mL.

In paper V, type 2 DM was defined on the basis of a fasting blood glucose >6.0 mmol/L, and a fasting plasma C-peptide value >0.30 pmol/mL or >0.60 pmol/L 6 min after a glucagon test. Previously known DM was considered as type 2 DM if the patient was >35 years of age at diagnosis, non-insulin-requiring for at least two years after the onset and not prone to ketoacidosis.

Study design

Study I

This study was a pilot study, designed to elucidate the influence of acute hyperglycaemia on platelet function and endothelial and coagulation markers in subjects with mild type 2 DM compared to non-diabetic subject (n=28 in total).



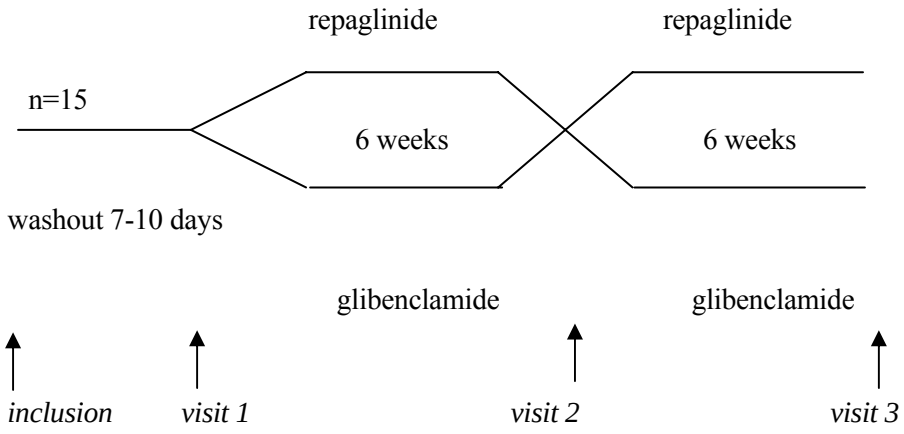
The patients and controls were all men and were matched for age and BMI. The patients with DM (n=11) were diet treated and had a known duration of disease of 3.4 ± 0.6 years. None of the patients had any symptom of DM when the diagnosis was made and none had any history or clinical sign of macro- or microvascular disease.

In addition, six subjects that were initially included as healthy (n=4) or type 2 DM (n=2) fulfilled the criteria of having impaired glucose tolerance (IGT), since they had fasting blood glucose levels between 5.6-6.0 mmol/L and/or blood glucose levels between 6.7-9.9 mmol/L after the glucose load. Four of these subjects had IGF and IGT and two had IGT only. The subjects with IGT were not included in the published paper I, but data from these subjects are shown in the results section of this thesis (tables 3 and 4; figures 9 and 10; page 40-42) .

Between group comparisons of patients with type 2 DM, subjects with IGT and subjects with normal glucose tolerance were made (see table 5; page 43).

Study II

The study was an open, randomized, cross-over study comparing the effects of treatment with repaglinide and glibenclamide on platelet and inflammatory parameters in patients with type 2 DM with previous oral antidiabetic treatment, and a known duration of disease of 4.6 ± 2.6 years. Measurements were performed before and after a standardized carbohydrate–rich meal (consisting of 54% carbohydrates, 30% fat, 16% protein and containing 621 kcal). The patients were investigated on three occasions; after a one week washout period without antidiabetic drug treatment, and after six weeks of treatment with repaglinide or glibenclamide, respectively.



Fifteen of 19 eligible patients completed the study. The drop outs (n=4) were due to infectious disease, need for surgery of a hernia, non-compliance and sampling difficulties, respectively. The patients measured their blood glucose levels at home and the study drugs were titrated every week according to the fasting plasma glucose level.

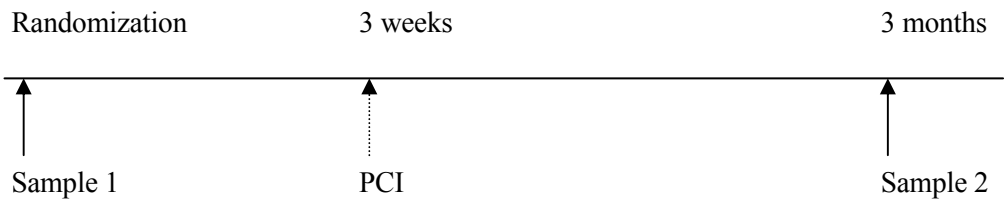
Samples for measurements of platelet, endothelial and coagulation parameters were drawn in the morning with the patient fasting, and 1.5 h after the meal. Repaglinide and glibenclamide were administered directly before the meal. Blood glucose and insulin were measured premeal and every 10 min until 1.5 h after the meal. The premeal results in the patients were compared to healthy controls, matched for sex, age, smoking and BMI.

Study for paper III and IV

This study was cross-sectional and compared type 1 diabetic patients with mainly early stages of retinopathy to patients free from microangiopathy, and healthy control subjects. The aims were to investigate alterations of platelet function, inflammatory parameters and platelet-leukocyte cross-talk in diabetic microangiopathy. The two patient groups were matched for sex, age and BMI, as well as for $HbA1_c$ and duration of disease, the two strongest predictors for developing retinopathy. Patients with severely impaired metabolic control (HbA1c >9%) and other concomitant diseases including macrovascular disease were excluded. The healthy control subjects were matched for sex and age. Sampling was performed in the morning with all subjects fasting.

Study V

This study was a substudy to a Swedish multicenter trial of antidiabetic treatment in DM patients undergoing PCI. The study was a prospective, randomised and open with blinded evaluation (PROBE-design). The aim was to test the hypothesis that instensified metabolic control, by the use of multidose insulin, would reduce the rate of restenosis during the first 6 months among type 2 diabetic patients subjected to coronary angioplasty. Our substudy evaluated platelet function at randomisation and at 3 months after elective percutaneous coronary intervention (PCI). The aim was to optimize the metabolic control 3 weeks prior to angioplasty in the intensively insulin treated group and to maintain optimal metabolic control during the study. These patients measured the blood glucose at home and were followed up weekly before angioplasty and thereafter every second week or at least once every month. The control group continued to receive usual care, but were followed in similar manner. Two of the 24 patients were excluded from the study because their post PCI treatment with clopidogrel was still ongoing at the 3 month sampling.



Blood sampling

Platelets are extremely easily activated. Thus, in order to avoid artefactual results it is important to standardize the sampling procedure and to perform clean venipunctures. In the present project subjects had antecubital veins that allowed technically good sampling for

platelet function testing and the blood was, with few exceptions, collected by a single laboratory technician/nurse in each study. Venipunctures were always performed without stasis with the subjects in a semi-recumbent position. Blood sampling was performed after an overnight fast and after 30 min of rest in all studies. In studies I and II samples were also taken 1.5 h after OGTT or meal intake, respectively. Samples for blood glucose and insulin were drawn from an indwelling catheter inserted into an antecubital vein (paper I and II). All subjects abstained from tobacco and caffeine-containing beverages on the day of sampling and the subjects in studies I-IV were instructed not to take any platelet inhibiting drugs during 14 days preceding the sampling. In study V all patients received chronic aspirin treatment (75-160 mg/day).

Platelet function tests

There is no "gold standard" method for platelet function testing. Platelets are dynamic cells and interact with each other, as well as with vascular endothelial cells and leukocytes. There are several different techniques which assess different aspects of platelet function. Examples of platelet function tests are listed in the table:

Table 2. Platelet function tests

Flow cytometry (*in vivo*)
- Expression of GPs, e.g. GPIIb/IIIa, GPIb-IX and P-selectin
- Platelet-platelet aggregates
- Platelet-leukocyte aggregates
Aggregation (*in vitro*)
- Impedance aggregometry (whole blood)
- Born aggregometry (platelet rich plasma)
Platelet counting techniques in whole blood
Soluble markers (plasma/urine)
- Thromboxane metabolites
- β-Thromboglobulin
- Platelet factor 4
- Serotonin
- P-selectin
- CD40L

Flow cytometric analysis

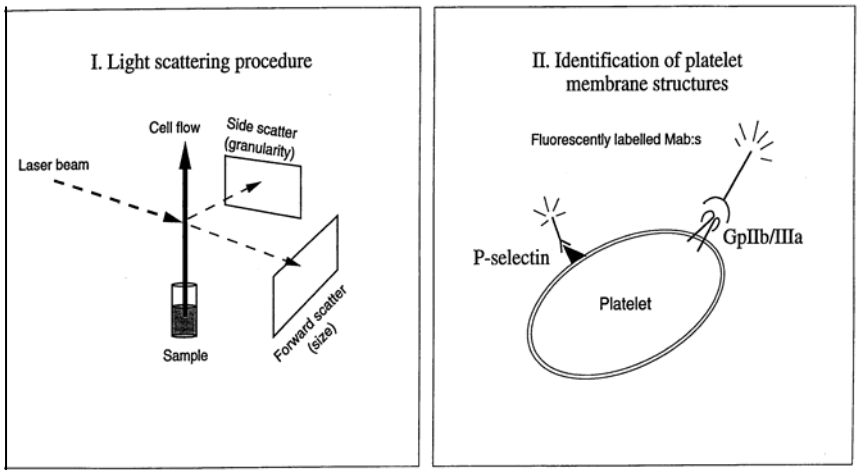
Flow cytometry with fluorochrome-labeled monoclonal antibodies makes it possible to detect specific alterations on the surface of activated platelets. Flow cytometry analyses individual cells/particles or cellular-conjugates aggregates that are detected by a laser beam during a

continuous flow of cells. The scatter of the light and the intensity of the fluorescence are recorded by specific photodiodes. The size (forward scatter), granularity (side scatter), and surface expression of antigens following a binding of an antibody (mean fluorescence intensity, MFI or % positive cells), can be determined (figure 8) [33]. The flow cytometric analysis of platelets, leukocytes and platelet-leukocyte cross-talk is based on previuosly described methods [237, 238].

Within 3 min of blood sampling, 5 µL citrated whole blood was added to 45 µL of Hepes-buffered saline, in order to minimize *in vitro* aggregate formation, containing appropriately diluted antibodies and agonists for the detection of platelet P-selectin expression, leukocyte CD11b expression or platelet-leukocyte conjugation and incubated at room temperature for 20 min. Samples were then further diluted and mildly fixed with 0.5% formaldehyde saline before analysis in a Coulter EPICS XL-MCL flow cytometer (Coulter Corp., Hialeah, FL).

Single platelet analysis: Platelets were identified with a fluorescein isothiocyanate (FITC) conjugated anti-CD42a (glycoprotein IX) monoclonal antibody Beb1 (Becton Dickinson, San Jose, CA, USA). Single platelets were discriminated from other blood cells by their light scattering characteristics and live gated into an electronic bit map, in which the gated particles were more than 98% positive for the platelet marker, FITC-CD42a. Platelet P-selectin expression was determined by a R-phycoerythrin (RPE)-conjugated anti-P-selectin monoclonal antibody, AC1.2 (Becton Dickinson). The gated cells were subsequently subjected to single colour analysis of RPE-CD62P fluorescence to obtain the percentage of P-selectin positive cells in the platelet population (figure 8) (paper I-V).

Figure 8. Flow Cytometry in Whole Blood



Agonists used to elucidate platelet reactivity *in vitro* were ADP and human α-thrombin in rising concentrations (except for in paper IV where ADP was not used and in study III where only one concentration of ADP was used). In addition, in study IV, the Tx A_2 analogue, U446619 was used to induce platelet activation. When thrombin was used, fibrin crosslinking was inhibited by the peptide GPRP (gly-pro-arg-pro). Platelet P-selectin expression data are reported as % P-selectin positive cells in the platelet population (paper I-V).

Platelet-leukocyte aggregates (PLA) were analysed by whole blood flow cytometry using RPE-CD45 fluorescence triggering. All RPE-CD45 positive particles, i.e. total leukocytes were gated, and differentiated as lymphocytes, monocytes and neutrophiles, according to CD45 expression and light scattering characteristics. Total leukocytes and the different subgroups were then subjected to two colour analysis (RPE-CD45 vs FITC-CD42a) to discriminate platelet-coupled and platelet-free leukocytes. Heterotypic aggregates are presented as precentages of platelet-conjugated leukocytes in the total leukocyte population (PLA), as well as in the subpopulations of lymphocytes (P-Lym), monocytes (P-Mon), and neutrophils (P-Neu) (paper IV).

Leukocyte CD11b expression was analysed by the above described gating and separation of leukocytes and subsequent single colour (FITC-CD11b) analysis. Leukocyte CD11b expression is reported as mean fluorescence intensity (MFI) (study IV).

Platelet-leukocyte cross-talk: Hirudinized blood (200 μL) was incubated at 37^0C in a platelet aggregometer, without stirring for 5 min. Then fMLP (1 μM), the TxA_2-analog U46619 (1 μM) or vehicle (Hepes buffered saline) were added and further incubated for 5 min with stirring (900 rpm). Thereafter, blood was labeled for flow cytometric analysis of single platelet P-selectin expression, leukocyte CD11b expression and PLAs (paper IV).

Intra- and interassay CV for the flow cytometry analyses are <5% and <10%, respectively.

Platelet aggregation

Platelet aggregation was studied in paper II, using a four channel whole blood impedance aggregometer (Chrono-Log model 570-VS Four Sample; Chrono-log Corp, Havertown PA) after dilution of citrated blood 1:1 with physiological saline. Samples were preincubated at 37^0C for 5 min after which collagen (0.5; 1.0; 2.0 μg/mL, final concentrations; Horm, Nycomed Arznemittel, München, Germany). The slope and amplitude of aggregation were measured after eight minutes; the means of duplicate measurements were registrered. Measurements were performed within 45 minutes after sampling.

Urinary-11-dehydro-thromboxane-B_2

The determination of urinary 11-dehydro-Thromboxane B_2 (11-dehydro-TxB_2) was based on a commercially available enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor, USA), and a sample work-up procedure developed and validated in our laboratory [239]. After thawing

and centrifugation (5 min at 1400 x *g)*, the urine was diluted with ammonium bicarbonate buffer and left overnight at room temperature to convert 11-dehydro-TxB_2 to its open ring form. The samples were then purified by modified solid-phase extraction (Bond-Elut Certify II, Analytichem International), and eluted with 2% formic acid in methanol. The samples were taken to dryness overnight in a vacuum centrifuge and resuspended in an EIA buffer. After an overnight incubation, the samples were further diluted with the EIA buffer and applied to a Maxisorb plate (Nunc immunoplate) precoated with mouse monoclonal antibodies. The analysis was then performed according to instructions from the manufacturer and the results were adjusted to urinary creatinine (analysed by colorimetry; the Jaffe reaction, DRI® Creatinine-Detect® Test; Microgenics GmbH, Passau, Germany). Intra- and interassay CV are <3% and <14%, respectively [239]

Plasma and serum variables

Median platelet volume (MPV) was determined in whole blood anticoagulated with EDTA by a semi-automated cell analyzer (Medonic, CA 460, Solna, Sweden). Fibrinogen levels in plasma were determined by the clotting method of Clauss, in the presence of an excess of thrombin (Diagnostica Stago, Asnieres-sur-Seine, France) and thrombin generation was measured as prothrombin fragment 1+2 (F1+2), (Enzygnost F1+2; Behring Diagnostics, Marbourg, Germany) and thrombin-antithrombin complexes (TAT) (Enzygnost TAT micro, Behring Diagnostics, Marburg, Germany). Blood samples for analyses of sP-selectin, vWf and elastase in plasma were anticoagulated with 3.8% citrate and centrifuged for 25 min at 2000 x *g* (sP-selectin in paper II, III and V) and 10 min at 1400 x *g* respectively at 4^0 C. Enzyme immunoassays (EIA) were used to determine sP-selectin (R & D Systems, Abingdon, Oxfordshire, UK), vWf (Asserachrom, Diagnostica Stago, France) and elastase (DPC Biermann GmbH, Bad Nauheim, Germany). Concentrations of sCD40L, sE-selectin and soluble intercellular adhesion molecule-1 (ICAM-1) were determined in serum, after centrifugation for 10 min at 1400 x *g* and 4^0C using EIA kits from R&D systems (Abingdon, U.K.). CRP was determined in serum by means of a high sensitivity assay with particle enhanced immunonephelometry (BN™ Systems, Dade Behring, Marburg GmbH, Germany). All samples were stored at $–80^0$ before analysis.

Metabolic variables

Blood glucose concentrations were assayed using a glucose oxidase method in a Yellow Springs Glucose Analyzer II (Yellow Springs Inc., Yellow Spring, Ohio, USA) in paper I. Fasting blood glucose was determined by the coronary care nurse using a bedside HemoCue photometer (HemoCue AB, Ängelholm, Sweden) in paper V. Plasma glucose was measured using a glucose oxidase method with a Vitros GLU Slide analyzer (Johnson & Johnson Clinical Diagnostics, Inc) in paper II-IV. Glycosylated hemoglobin was determined by a turbidimetric

inhibition immunoassay (TINIA) for hemolyzed whole blood (Roche diagnostics GmbH, Mannheim, Germany) in papers II-IV, and by high-performance liquid chromatography (Department of Laboratory Medicine, Malmö Hospital, Sweden) in study V (Boeringer Mannheim Scandinavia AB, Bromma, Sweden). Insulin in plasma was measured by radioimmunoassay, using rabbit antiporcine insulin antibodies, human standard insulin and charcoal addition to separate antibody-bound and free insulin. Human plasma C-peptide was determined by a commercial radioimmunoassay kit (Eurodiagnostica, Malmö, Sweden). The glucose and insulin responses to the meal were calculated as the glucose and insulin incremental areas under the curve (AUC), respectively, substracting the fasting value, using the trapezoid rule. Microalbuminuria and creatinine were analyzed using Beckmann IMMAGE® Immunochemistry Systems (Beckman Instruments, Inc, Richmond, CA, USA) and colorimetry (the Jaffe reaction) (DRI® Creatinine-Detect® Test; Microgenics GmbH, Passau, Germany), respectively. The cholesterol and triglyceride contents of the various lipoprotein fractions were assessed by standard enzymatic techniques (Vitros Chemistry Products, Ortho-Clinical Diagnostics, Inc, Rochester, NY, USA). LDL cholesterol was calculated by the Friedewald formula..

Statistical analysis

Descriptive statistics are presented as mean values ± SEM (papers I, III and IV) or ± SD (papers II and V) for parameters that were normally distributed, and as medians (25^{th}-75^{th} percentiles) for skewed data. Between group comparisons were evaluated by Student´s unpaired *t*-test or the Mann-Whitney *U* test (two groups) and analysis of variance (ANOVA) (three groups). Dose-response curves for agonist-induced platelet P-selectin expression were analyzed by two-way repeated measures ANOVA. Skewed data were logarithmically transformed before the statistical evaluation. Hyperglycaemia-induced changes (paper I) and effects of improved metabolic control (paper V) were analyzed by Student´s *t*-test for paired data or Wilcoxon´s signed rank test. Antidiabetic treatment effects and effects of the meal on agonist-induced platelet P-selectin expression were analyzed by three-way ANOVA. There was no sequence effect of randomized treatment (paper II). Postprandial glucose and insulin excursions and premeal hemostatic parameters (paper II) were compared using one-way ANOVA; post hoc tests were performed using Fisher´s LSD test. Correlations were assessed by the Spearman test. The software used was Statistica; Statsoft, Tulsa, Oklahoma, USA.

Ethical considerations

The studies were approved by the local Ethics Committee of the Karolinska Hospital (paper I-IV), the Ethics Committee of the Karolinska Institute (paper V) and by the Swedish Medical Products Agency (paper II). All subjects gave their informed consent before participating.

RESULTS AND DISCUSSION

Acute hyperglycaemia and platelet activation in type 2 diabetes mellitus (I)

The oral glucose tolerance test (OGTT) elicited significant increases in blood glucose in all the groups, i.e. in healthy controls, in subjects with IGT and in type 2 DM (table 3.). Acute hyperglycaemia elevated sP-selectin in plasma in patients with mild type 2 DM and IGT, but not in the healthy subjects (figure 9). Soluble P-selectin levels were correlated to blood glucose levels during OGTT (figure 10), and AUC for glucose ($r=0.57$; $p<0.001$ for both; all subjects included) and the sP-selectin increments correlated with the AUCs for glucose ($r=0.40$; $p<0.05$).

Platelet P-selectin expression in unstimulated samples was not significantly altered by the OGTT, but ADP- and thrombin-induced platelet P-selectin expression was decreased in samples collected 1.5 h after the OGTT (table 4.). Endothelial vWf and coagulation markers (F1+2 and TAT) in plasma were not altered by the glucose load.

Table 3. Patient characteristics and metabolic parameters

Variable	**Controls** (n=11)	**IGT** (n=6)	**Type 2 DM** (n=11)	**p**[a]
Age (years)	49.1 ± 1.8	52.5 ± 0.8	49.8 ± 0.8	ns
BMI (kg/m^2)	26.2 ± 0.5	25.5 ± 1.0	27.4 ± 0.7	ns
Glucose (mmol/L)				
Basal	4.6 ± 0.1	5.2 ± 0.3	6.8 ± 0.2	<0.001[b]
90 min	6.2 ± 0.1[e]	10.0 ± 0.8[d]	13.7 ± 0.5[e]	<0.001
120 min	5.4 ± 0.3[c]	8.6 ± 0.4[e]	12.9 ± 0.6[e]	<0.001
AUC (mmol/L x h)	9.3 ± 1.1	12.4 ± 2.0	15.1 ± 2.0	<0.001
Insulin (μU/mL)				
Basal	18.4 ± 0.9	20.8 ± 1.6	23.8 ± 3.1	ns
90 min	81.4 ± 43.6[e]	132.5 ± 35.5[e]	69.1 ± 16.1[d]	ns
120 min	63.5 ± 8.0[e]	125.7 ± 47.9[e]	78.2 ± 21.0[e]	ns
AUC (μU/mL x h)	90.1 ± 45.9	104.5 ± 57.4	69.4 ± 44.9	ns
C-peptide (pmol/mL)				
Basal	0.93 ± 0.08	1.24 ± 0.13	1.30 ± 0.12	<0.05
120 min	2.94 ± 0.38[e]	4.06 ± 0.84[c]	3.27 ± 0.35[e]	ns

Values are presented as mean ± SEM. p[a] Unpaired two-tailed t-test (Type 2 DM vs controls; IGT vs controls) [b] $p< 0.05$; IGT vs controls [c, d, e] $p<0.05$; $p<0.01$; $p<0.001$ vs basal levels

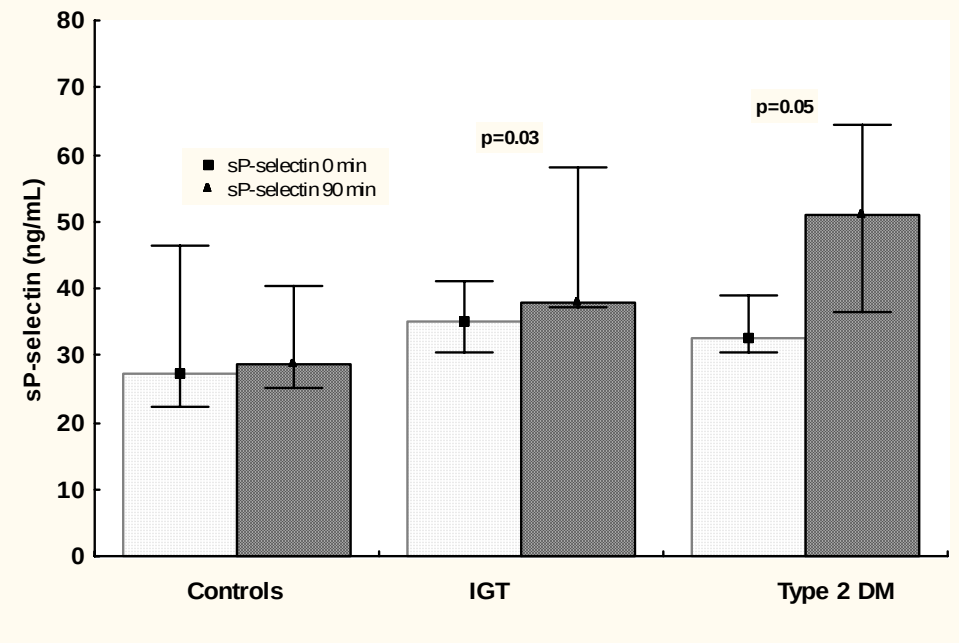


Figure 9. Soluble P-selectin before (0 min) and after (90 min) OGTT.
Values are median with 25th and 75th percentiles; Wilcoxon signed rank test.

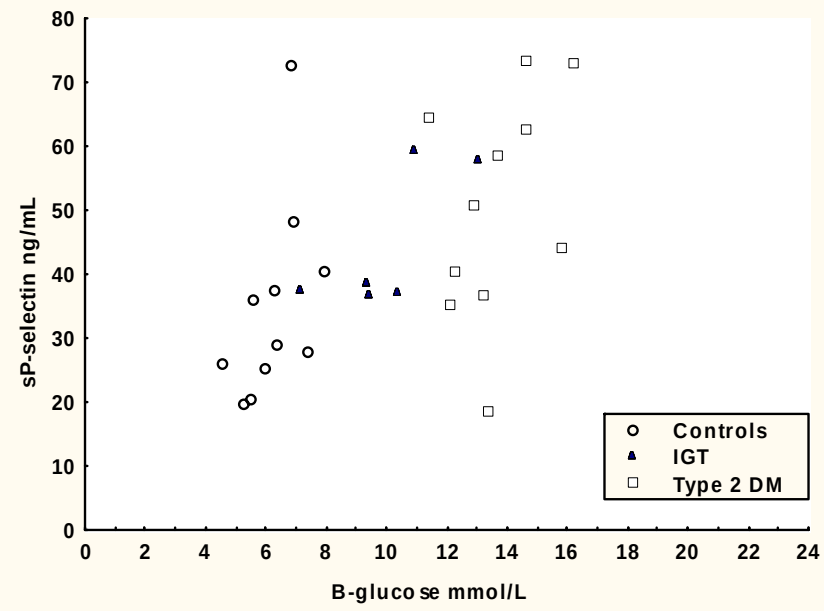


Figure 10. Relationships between blood glucose and soluble P-selectin 90 min after OGTT.

Table 4. Effects of OGTT on platelet P-selectin expression (% P-selectin positive cells)

Agonist	**Controls** (n=11)		**IGT** (n=6)		**Type 2 DM** (n=11)	
ADP (mol/L):	**Basal**	**90 min**	**Basal**	**90 min**	**Basal**	**90 min**
Rest	0.7 ± 0.2	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.7 ± 0.1
10^{-7}	1.6 ± 0.3	1.6 ± 0.3	2.1 ± 0.3	1.8 ± 0.3	2.0 ± 0.3	1.6 ± 0.3
3×10^{-7}	8.0 ± 1.4	8.0 ± 1.4	9.3 ± 1.1	7.8 ± 0.8	8.2 ± 1.0	6.5 ± 0.9
10^{-6}	26.2 ± 3.5	25.6 ± 3.9	32.7 ± 3.5	31.0 ± 2.6	29.0 ± 2.3	24.8 ± 2.1
10^{-5}	52.5 ± 4.4	51.6 ± 4.6	59.6± 11.3	59.0 ± 4.1	54.4 ± 2.6	50.2 ± 2.8[b]
Thrombin (U/mL):						
Rest	0.7 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.7 ± 0.1
0.02	47.9 ± 8.9	45.4 ± 8.8	40.3 ± 12.8	37.3 ± 15.2	34.1 ± 7.2	27.5 ± 7.6
0.04	82.3 ± 4.8	81.2 ± 5.2	87.9 ± 2.3	86.0 ± 2.7	84.0 ± 3.2	78.3 ± 4.2
0.08	92.9 ± 1.4	92.6 ± 1.6[a]	94.2 ± 0.8	93.4 ± 0.6	93.4 ± 0.7	93.1 ± 0.6[b]

Values are presented as mean ± SEM. Statistical analyses is based on two way repeated measures ANOVAs comparing concentration-effect curves for basal vs 90 min measurements. [a] $p<0.05$; [b] $p<0.01$

The samples in this study were collected 1.5 h after the glucose load. One interpretation of the present findings is that activated platelets by that time point have lost the expressed P-selectin due to shedding into plasma[46, 47]. The slight decrease in platelet P-selectin expression after OGTT could be explained by loss of activated platelets *in vivo* resulting in more stable platelets for the agonist stimulation *in vitro.* Our findings of unaltered plasma levels of vWf suggest that the sP-selectin appearing in plasma after hyperglycaemia originated from platelets, rather than endothelial cells. In conclusion, the OGTT induced mild platelet activation in IGT and type 2 DM, as measured by sP-selectin in plasma.

Between group comparisons in the fasting state showed that platelet, endothelial and coagulation markers did not differ between subjects with type 2 DM, IGT and normal glucose tolerance. However, the levels of TNF-α, ICAM-1 and leukocyte counts were elevated in patients with type 2 DM compared to controls and monocyte chemoattractant protein-1 (MCP-1) tended to be elevated in patients with type 2 DM compared to controls and to IGT ($p<0.10$) (table 5). TNF-α levels were correlated to sICAM-1 ($R=0.52$; $p<0.01$; all subjects) and fasting plasma insulin concentrations ($R=0.66$; $p<0.05$; type 2 diabetes), but not to BMI. These results are in line with other recent studies demonstrating elevations of TNF-α and IL-6 among insulin resistant subjects and in patients with overt type 2 diabetes [240, 241].

Table 5. Baseline values of platelet, endothelial and inflammatory markers

Variable	Controls (n=10)	IGT (n=6)	Type 2 diabetes (n=10)
TNF-α (pg/mL)	2.5 (2.2; 2.8)	2.7 (2.1; 3.2)	3.0 (2.6; 3.3)*
ICAM-1 (ng/mL)	183 (179; 197)	218 (190; 241)	234 (206; 265)**
sP-selectin (ng/mL)	27.3 (22.4; 46.5)	35.0 (30.6; 41.1)	32.5 (30.5; 39.1)
vWf (%)	114 (103; 154)	120 (92; 167)	124 (104; 162)
MCP-1 (pg/mL)	167 (155; 184)	153 (135; 186)	202 (165; 261)
Monocytes (10^9/L)	0.30 (0.30; 0.40)	0.25 (0.20; 0.40)	0.40 (0.30; 0.40)
WBC (10^9/L)	4.6 (3.1; 5.1)	4.5 (3.5; 4.6)	4.9 (4.5; 6.5)*

Values are medians (interquartiles). *p<0.05 **p<0.01; Type 2 diabetes vs controls; Mann Whitney *U*-test. WBC; white blood count

Meal-induced platelet activation in type 2 diabetes mellitus: effects of insulinotropic drugs (II)

Based on the findings in paper I the effects of postmeal hyperglycaemia were examined in 15 type 2 diabetic patients (age 53 ± 6 years; BMI 28 ± 4 kg/m^2) treated with oral antidiabetic drugs. In addition, comparisons were made with healthy controls in the fasting state.

The carbohydrate rich meal induced significant increases in glucose and insulin at baseline (without antidiabetic treatment), as well as after treatment with repaglinide or glibenclamide. ANOVA analysis showed decreased postmeal glucose and AUC glucose values after treatment with the insulinotropic drugs, and this decrease was more pronounced after glibenclamide treatment (table 6). Triglycerides, total cholesterol, LDL and HDL were not altered by the meal.

ADP-induced platelet P-selectin expression increased significantly following the meal and this effect was seen at baseline (without antidiabetic treatment), as well as after treatment with repaglinide and glibenclamide (table 7). Premeal treatment with repaglinide or glibenclamide did not attenuate the meal-induced platelet activation. Treatment with repaglinide, but not glibenclamide, reduced the overall (pre-and postmeal values) ADP-induced P-selectin compared to baseline (p=0.09), but this effect was not related to postmeal hyperglycaemia (p=0.32).

In this experimental set-up sP-selectin was unaffected by the meal. These results are in contrast to the increases in sP-selectin seen after OGTT (study I). The discrepancy may be related to more pronounced incremental rises in glucose and insulin after OGTT. In accordance with paper I, we found no postmeal increases of vWf or the trombin generation marker F1+2. The finding that hyperglycaemia is associated with platelet hyperreactivity is in line with experimental *in vitro* studies showing increased P-selectin expression and fibrinogen binding when blood from individuals with and without diabetes is incubated with glucose [99].

Table 6. HbA1c and plasma glucose and insulin values pre-and postmeal in type 2 diabetes patients at baseline and after treatment with repaglinide and glibenclamide

Variable	Baseline	Repaglinide	Glibenclamide	p
HbA1c (%)	6.8 ± 1.7	6.8 ± 1.5	6.7 ± 1.5	ns
Premeal glucose (mmol/L)	10.5 ± 2.7	9.7 ± 2.5	9.2 ± 2.0[b]	<0.01
Postmeal glucose (mmol/L)	13.6 ± 3.6	12.3 ± 3.2	11.2 ± 2.9[b]	<0.05
AUC glucose (mmol/L x h)	10.6 ± 2.3	10.1 ± 2.8	9.2 ± 2.2[a]	0.13
Δ peak glucose (mmol/L)	4.2 ± 1.1	3.8 ± 1.5	3.3 ± 1.2[a]	0.07
Time to peak (min)	63 ± 22	53 ± 16	53 ± 14[a]	0.07
Premeal insulin (μU/mL)	19.9 ± 5.5	21.5 ± 6.8	21.4 ± 5.4	ns
Postmeal insulin (μU/mL)	43.9 ± 16.0	56.9 ± 24.4[b]	58.2 ± 29.4[b]	<0.01
AUC insulin (μU/mL x h)	51.2 ± 24.6	70.8 ± 36.6[a]	71.7 ± 34.2[a]	<0.01

Data are mean ± SD; p-values are treatment effect Type 2 DM patients; one-way ANOVA.
[a]p<0.05; [b]p<0.01 post hoc test of antidiabetic treatment vs baseline

Table 7. Effects of a standardized carbohydrate-rich meal on ADP-induced P-selectin expresssion (% P-selectin positive cells) in type 2 DM at baseline and after treatment with repaglinide and glibenclamide

ADP (μmol/L)	Baseline		Repaglinide		Glibenclamide	
	Premeal	Postmeal	Premeal	Postmeal	Premeal	Postmeal
0.0	1.2 ± 0.4	1.3 ± 0.5	1.4 ± 0.5	1.3 ± 0.6	1.3 ± 0.4	1.4 ± 0.9
0.3	20.5 ± 6.3	21.7 ± 5.6	19.2 ± 4.8	21.7 ± 8.5	20.4 ± 6.9	22.3 ± 8.7
1.0	42.3 ± 10.1	46.4 ± 9.0	40.1 ± 7.6	45.0 ± 13.0	42.3 ± 9.2	45.8 ± 9.7
10.0	60.3 ± 9.9	64.1 ± 8.6[a]	56.4 ± 8.2[b]	61.9 ± 12.5[a]	59.4 ± 9.0	63.4 ± 9.1[a]

Data are mean ± SD. [a]p<0.01; premeal vs postmeal values. [b]p=0.01; baseline vs repaglinide premeal values; two-way repeated measures ANOVA of the dose-response curves

The premeal results were compared to results from 15 healthy controls, matched for sex, age, smoking and BMI. Platelet reactivity, thrombin markers or fibrinogen did not differ between patient and controls, but sICAM-1 and vWf levels were significantly higher in type 2 DM patients compared to controls. This is in accordance with a low-grade chronic inflammatory activation of the vessel wall present in type 2 diabetic disease [227]. Treatment with the insulinotropic drugs significantly decreased both sICAM-1 and vWf, and this effect was more pronounced after the repaglinide treatment (table 8).

In conclusion, the postmeal state was associated with platelet activation in patients with type 2 DM, but premeal administration of repaglinide or glibenclamide did not attenuate the meal-induced platelet

activation. Repaglinide *per se* seem to have platelet inhibiting effects as well as beneficial effects on the vascular endothelium. However, these effects were not related to the control of postmeal hyperglycaemia.

Table 8. Hemostatic, endothelial and inflammatory markers and plasma lipids in control subjects compared to Type 2 DM patients at baseline and after treatment with repaglinide and glibenclamide

Variable	Control subjects	Type 2 DM Baseline	Type 2 DM Repaglinide	Type 2 DM Glibenclamide	p
Mean platelet volume (fL)	8.6 ± 1.0	9.1 ± 0.5	9.1 ± 0.7	9.0 ± 0.5	ns
sP-selectin (ng/mL)	34.3 ± 11.2	33.4 ± 8.1	36.4 ± 14.3	34.8 ± 13.1	ns
11-dehydro-thromboxaneB_2 (ng/mmol)	69 (48; 84)	82 (56; 94)	72 (65; 101)	78 (72; 82)	ns
Fibrinogen (g/L)	3.2 ± 0.8	3.3 ± 0.8	3.1 ± 0.7	3.2 ± 0.8	ns
F1+2 (nmol/L)	0.78 ± 0.28	0.77 ± 0.16	0.78 ± 0.19	0.82 ± 0.14	ns
vWf antigen (kIU/L)	0.94 ± 0.24	1.20 ± 0.33[a]	1.09 ± 0.40[b]	1.13 ± 0.34	0.02
sE-selectin (ng/mL)	43.5 ± 18.9	54.8 ± 18.5	53.0 ± 18.7	51.6 ± 16.8	ns
sICAM-1 (ng/mL)	248 ± 53	286 ± 48[a]	266 ± 46[b]	273 ± 38	0.03
CRP (mg/L)	0.9 (0.5; 1.2)	0.8 (0.6; 2.2)	0.7 (0.5; 1.5)	0.6 (0.5; 1.5)	ns
Cholesterol (mmol/L)	4.9 ± 0.6	5.1 ± 0.7	5.2 ± 0.6	5.0 ± 0.6	ns
HDL (mmol/L)	1.2 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	ns
LDL (mmol/L)	3.2 ± 0.6	3.3 ± 0.8	3.3 ± 0.8	3.1 ± 0.7	ns
Triglycerides (mmol/L)	1.3 ± 0.8	2.0 ± 1.6	2.0 ± 2.0	1.7 ± 0.8	ns

Data are mean ± SD and medians and interquartiles. [a]$p<0.05$; Type 2 DM patients vs control subjects. Student´s t-test for in dependent samples. p-values are treatment effect in Type 2 DM patients; ANOVA. [b]$p<0.01$; post hoc test of treatment effect.of repaglinide vs baseline

Microangiopathy in type 1 diabetes mellitus: evidence for platelet activation and chronic inflammation (III-IV)

In paper III we found that platelet reactivity, as measured by thrombin-induced platelet P-selectin expression (figure 11), and sP-selectin were significantly enhanced in type 1 diabetes patients with microangiopathy compared to patients without microangiopathy and healthy controls, even though the patients were metabolically well controlled (see table 9). Furthermore, sE-selectin, sCD40L and CRP levels in serum were elevated in patients with microangiopathy compared to controls (table 9). There was also a correlation between thrombin-induced P-selectin expression and sCD40L ($r=0.30$; $p<0.05$; all subjects and $r=0.46$; $p<0.05$; patients with microangiopathy), indicating a relationship between platelet reactivity and inflammation. sCD40L also correlated to F1+2 ($R=0.37$; <0.05; all subjects). We found no significant

correlations between P-selectin expression or sCD40L and endothelial or inflammatory markers, but the parallell increase in circulating sE-selectin and CRP supports the existence of endothelial dysfunction and chronic inflammation in patients with type 1 diabetic microangiopathy.

Table 9. Characteristics and biochemical analyses in controls and patients

Variable	**Controls** (n=27)	**Type 1 DM w/o microangiopathy** (n=19)	**Type 1 DM with microangiopathy** (n=20)
Age (years)	43.5 ± 2.2	42.3 ± 2.8	42.4 ± 2.8
Sex (female/male)	14/13	9/10	10/10
Smoking (yes/occasionally/no)	6/1/20	5/3/11	3/0/17
Duration of disease (years)		14.7 ± 0.7	15.9 ± 0.7
BMI (kg/m^2)	23.1 ± 0.4	25.4 ± 0.7**	25.0 ± 0.7*
Blood pressure (mmHg)			
Systolic	116 ± 2	121 ± 3	128 ± 3**
Diastolic	74 ± 1	73 ± 1	76 ± 2
HbA1c (%)[a]	4.6 ± 0.05	6.5 ± 0.2***	6.9 ± 0.2***
U-albumin (μg/mg of creatinine)	3.8 (2.9; 5.4)	4.2 (3.8; 7.0)	5.9 (4.4; 14.4)*
Triglycerides (mmol/L)	1.1 ± 0.1	0.9 ± 0.05	0.9 ± 0.1
HDL–cholesterol (mmol/L)	1.4 ± 0.1	1.6 ± 0.1	1.7 ± 0.1*
LDL-cholesterol (mmol/L)	3.0 ± 0.1	2.8 ± 0.2	2.5 ± 0.2
sP-selectin (ng/mL)	40.8 ± 1.7	43.0 ± 2.5	50.5 ± 2.3**
sCD40L (ng/mL)	3.95 ± 0.32	4.09 ± 0.40	5.36 ± 0.64*
CRP (mg/L)	1.1 ± 0.4	1.6 ± 0.3	3.1 ± 0.8**
sE-selectin (ng/mL)	37.1 ± 2.2	38.7 ± 3.4	46.3 ± 3.1*
von Willebrand factor (KIU/L)	1.00 ± 0.07	1.12 ± 0.08	1.19 ± 0.10
Fibrinogen (g/L)	2.8 ± 0.1	2.9 ± 0.1	3.0 ± 0.1
F1+2 (nmol/L)	0.63 (0.52; 0.81)	0.79 (0.52; 0.95)	0.60 (0.51; 0.82)
Elastase (ng/mL)	23.0 ± 1.8	29.1 ± 2.7*	28.0 ± 1.8*
11-dehydro-Thromboxane B_2 (pg/mmol creatinine)	45.6 ± 3.1	58.0 ± 6.5	48.0 ± 4.0

Values are presented as mean ± SEM, except for U-albumin and F1+2 where median with 25^{th} and 75^{th} percentiles are displayed. *p<0.05, **p<0.01, ***p<0.001; patients vs controls

[a] Reference <5.2% for non-diabetic subjects

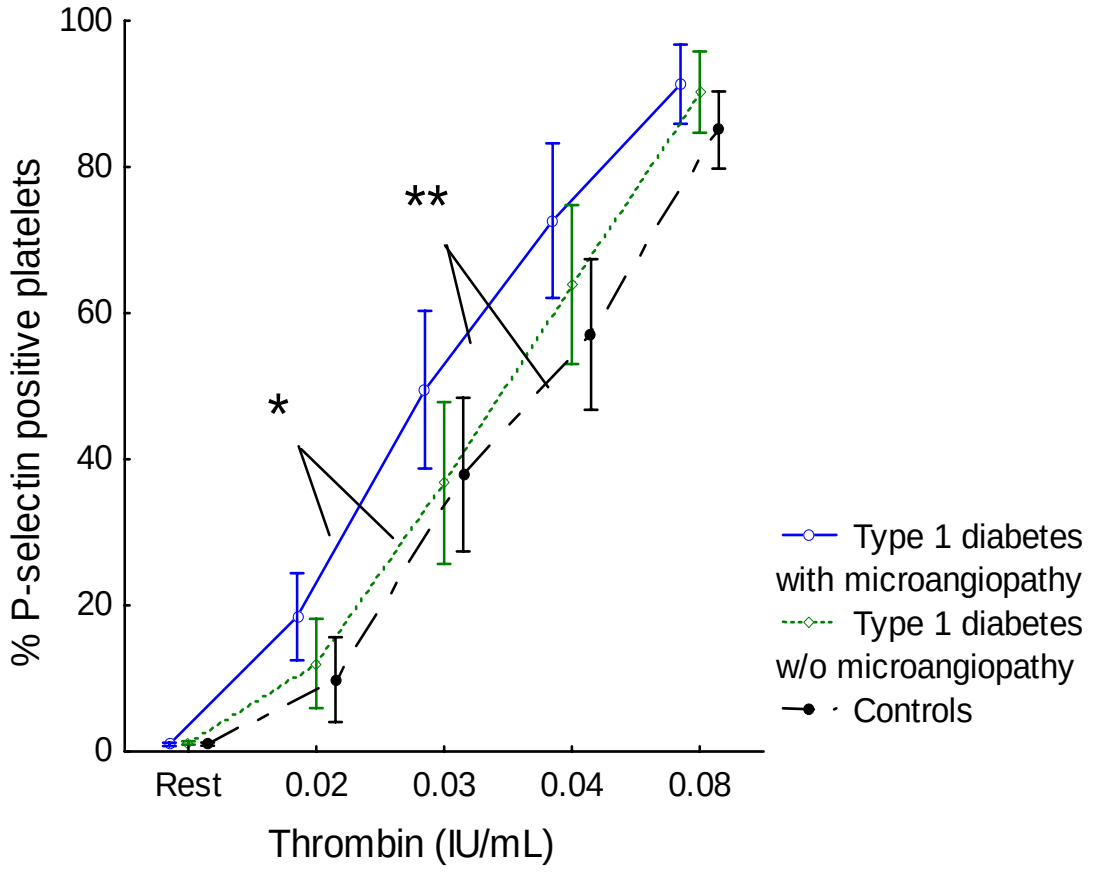


Figure 11. Platelet responsiveness to thrombin stimulation.
Data are means ± SEM.Two-way repeated measures ANOVA.
*p<0.05; **p<0.01

Plasma concentrations of F1+2, fibrinogen and urinary 11-dehydro-thromboxane B_2 excretion did not differ between the groups (table 9).

The finding in paper III that diabetic microangiopathy was associated with platelet activation and low-grade inflammation was confirmed in paper IV. Thus, circulating platelet-monocyte and platelet-neutrophil aggregates were elevated in the type 1 DM patients with microangiopathy. Leukocyte hyperactivity was more prominent among patients with microangiopathy, as evidenced by enhanced fMLP-induced leukocyte CD11b expression. Also, the patients with microangiopathy had increased PLA formation in response to stimulation with the TxA_2 analogue U46619. In fact, platelets had enhanced responses to *in vitro* stimulation with U46619 in both patient groups, but this effect was more pronounced in the patients with microangiopathy (figure 12).

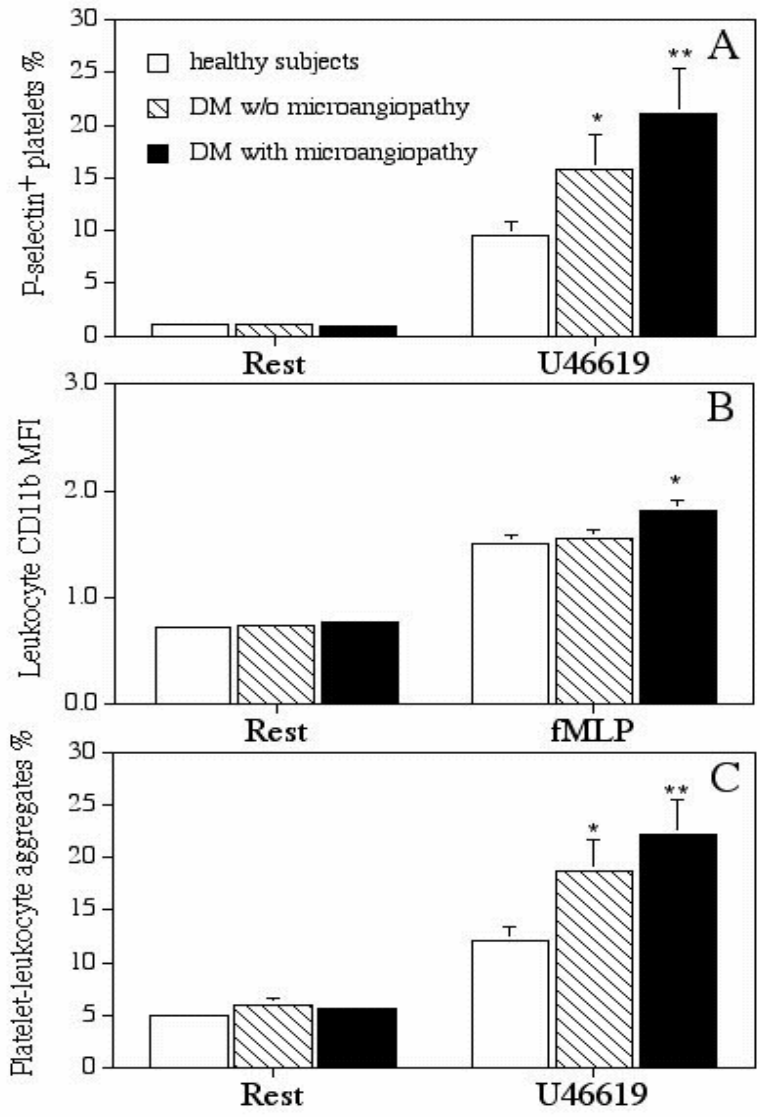


Figure 12. Platelet and leukocyte reactivity to *in vitro* stimulation.Whole blood from patients with type 1 diabetes without (hatched bars) and with (filled bars) microangiopathy, and controls (open bars) was incubated without stirring in the absence or presence of 0.3 mM U44619 and 0.1 mM fMLP, respectively. Platelet activation is shown as % P-selectin positive platelets (panel A). Leukocyte CD11b expression was measured as mean fluorescence intensity (MFI) (panel B). Platelet-leukocyte aggregation was measured as the percentage of the platelet-bounded leukocytes (panel C). *$p<0.05$ and **$p<0.01$ as compared to corresponding stimulating samples from controls.

Leukocyte-platelet cross-talk was more marked among type 1 DM patients compared to controls (figure 13) and the extent of leukocyte-platelet cross-talk was positively correlated to platelet hyperreactivity in the patients with microangiopathy. These findings suggests that enhanced leukocyte-platelet cross-talk may contribute to platelet hyperreactivity in type 1 DM patients with microangiopathy. The results in paper IV are in line with the findings in paper III, in which a relationship between platelet reactivity and an inflammatory marker (sCD40L) was shown. Taken together, data from paper III and IV show that type 1 DM with microangiopathy is a low-grade inflammatory state with elevated plasma levels of inflammatory markers, enhanced leukocyte and platelet reactivity. Clearly, type 1 DM with microangiopathy is a condition with enhanced interactions between inflammation (leukocytes) and hemostasis (platelets).

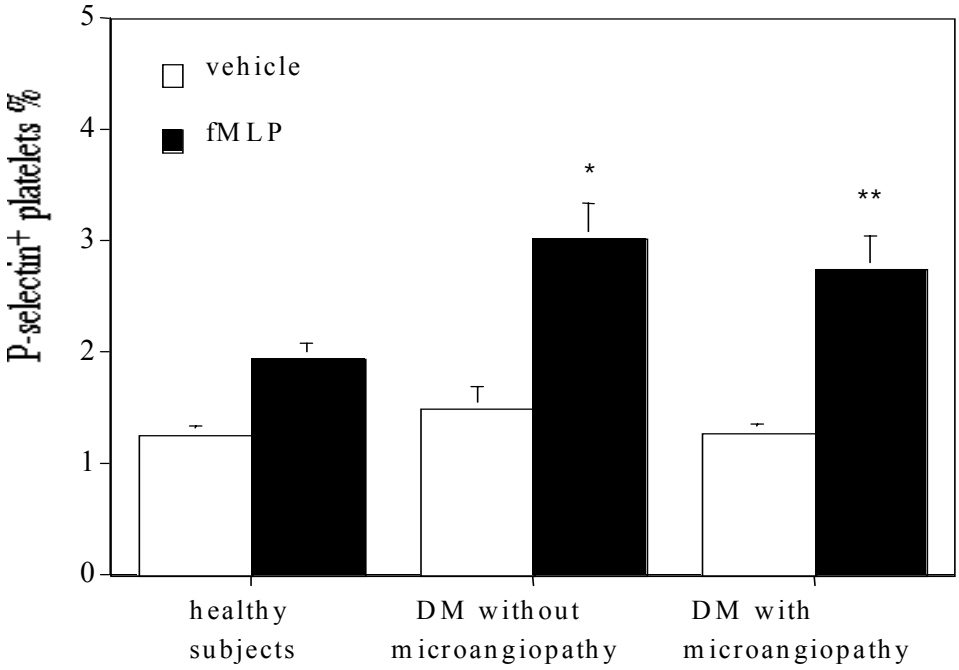


Figure 13. Leukocyte-platelet cross-talk. Hirudized whole blood was pre-incubated at 37^0C without stirring for 5 min, then further incubated with stirring for 5 min in the absence (open bars) or presence (filled bars) of 1 mM fMLP. Platelet activation is shown as the percentage of P-selectin positive platelets.*$p<0.05$; **$p<0.01$ as compared to stimulated samples from healthy controls.

Effects of improved metabolic control on platelet reactivity in type 2 diabetes mellitus following coronary angioplasty (V)

In this study 22 patients with type 2 DM undergoing elective PCI were randomized to intensive insulin treatment or to conventional antidiabetic treatment. The aim was to optimize metabolic control 3 weeks prior to percutaneous coronary intervention (PCI), and maintaining this during the study in the intensively treated group. Platelet function samples were taken at randomization and at 3 months after PCI. Metabolic control (HbA1c and plasma lipids) as well as platelet P-selectin expression and sP-selectin were similar between groups at 3 months after PCI (Hba1c was 6.0% ± 0.7 at baseline and 6.0% ± 1.0 at 3 months in the intensive insulin treatment group; n=12, and 5.9% ± 1.1 at baseline and 6.2% ± 1.4 at 3 months in the conventional antidiabetic treatment group; n=10). However, 6 of the 12 patients in the intensive group showed impaired metabolic control after 3 months and 3 weeks, and 3 of 10 patients in the conventionally treated group showed improved control.

An evaluation based on metabolic control (i.e. HbA1c levels) showed that ADP-induced P-selectin expression was significantly lower ($p=0.02$) among patients with improved metabolic control, regardless of the type of antidiabetic treatment, compared to patients with deteriorated metabolic control 3 months after PCI (see table 10 and figure 14). HbA1c and fasting glucose values were correlated to ADP-induced P-selectin expression ($r=0.34$; $p<0.05$ and $r=0.31$; $p<0.05$ respectively; all subjects). Platelet responses to thrombin did not differ between the two groups. Thus, it seems that thrombin-induced platelet reactivity – in contrast to ADP-induced platelet reactivity - is not attenuated by improved metabolic control.

As expected, the patients with improved glycemic control also had significantly lower TG levels after 3 months. However, no significant correlations between BMI or plasma lipid values and platelet reactivity were found, indicating that glycaemic control rather than plasma TG is of importance for platelet reactivity in this study population.

The serum levels of sCD40L in study V were in the same range as previously found in male patients with acute coronary syndromes and at high risk for new cardiac events [112]. This suggests that our patients had a prothombotic and proinflammatory milieu in their vasculature. However, in contrast to another recent study [100] we could not detect any correlation between glycaemic control and the levels of sCD40L.

Table 10. Patient charachteristics, metabolic control, sP-selectin and sCD40L

Variable	Improved metabolic control (n=9)	Impaired metabolic control (n=13)	P
Sex (male/female)	9/0	8/5	
Age (years)	61 ± 4	66± 8	ns
Smoking (yes/no/former)	1/5/3	1/6/6	
BMI (kg/m^2)	26 ± 3	32 ± 4	<0.01
Duration of diabetes (years)	9 ± 6	12 ± 8	ns
U-Albumin (mg/L)	6 (4; 10)	7 (4; 26)	ns
S-Creatinine	92 ± 22	83 ± 15	ns
HbA1c (%)			
Baseline	6.1 ± 0.7	5.9 ± 1.0	ns
3 months	5.7 ± 0.5[b]	6.5 ± 1.4[b]	0.10
fB-glucose (mmol/L)			
Baseline	7.7 ±1.9	7.8 ± 2.2	ns
3 months	6.8 ± 1.2	8.9 ± 1.4	<0.01
HDL (mmol/L)			
Baseline	1.1 ± 0.4	1.1 ± 0.3	ns
3 months	1.1 ± 0.3	1.2 ± 0.4	ns
LDL (mmol/L)			
Baseline	2.4 ± 0.6	2.3 ± 0.9	ns
3 months	2.6 ± 0.4	2.2 ± 0.6	ns
Triglycerides (mmol/L)			
Baseline	1.8 ± 0.6	2.3 ± 1.3	ns
3 months	1.5 ± 0.8[a]	2.3 ± 1.7	ns
Blood pressure (mmHg)			
Baseline systolic/diastolic	150 ± 14 / 84 ± 5	146 ± 28 / 82 ± 11	ns
3 months systolic/diastolic	146 ± 16 / 84 ± 5	153 ± 27 / 82 ± 10	ns
sP-selectin (ng/mL)			
Baseline	43.8 ± 17.6	46.8 ± 16.2	ns
3 months	41.0 ± 19.4	41.5 ± 13.0	ns
sCD40L (ng/mL)			
Baseline	4.8 ± 2.1	4.3 ± 1.8	ns
3 months	4.9 ± 2.0	4.1 ± 1.6	ns

Data are means ± SD, except for U-albumin were data are presented as medians and interquartiles. p-values are derived from between group comparisons; Student´s t-test for unpaired data.. [a]p<0.05; [b]p<0.01; Student´s t-test for paired data, baseline vs 3 months

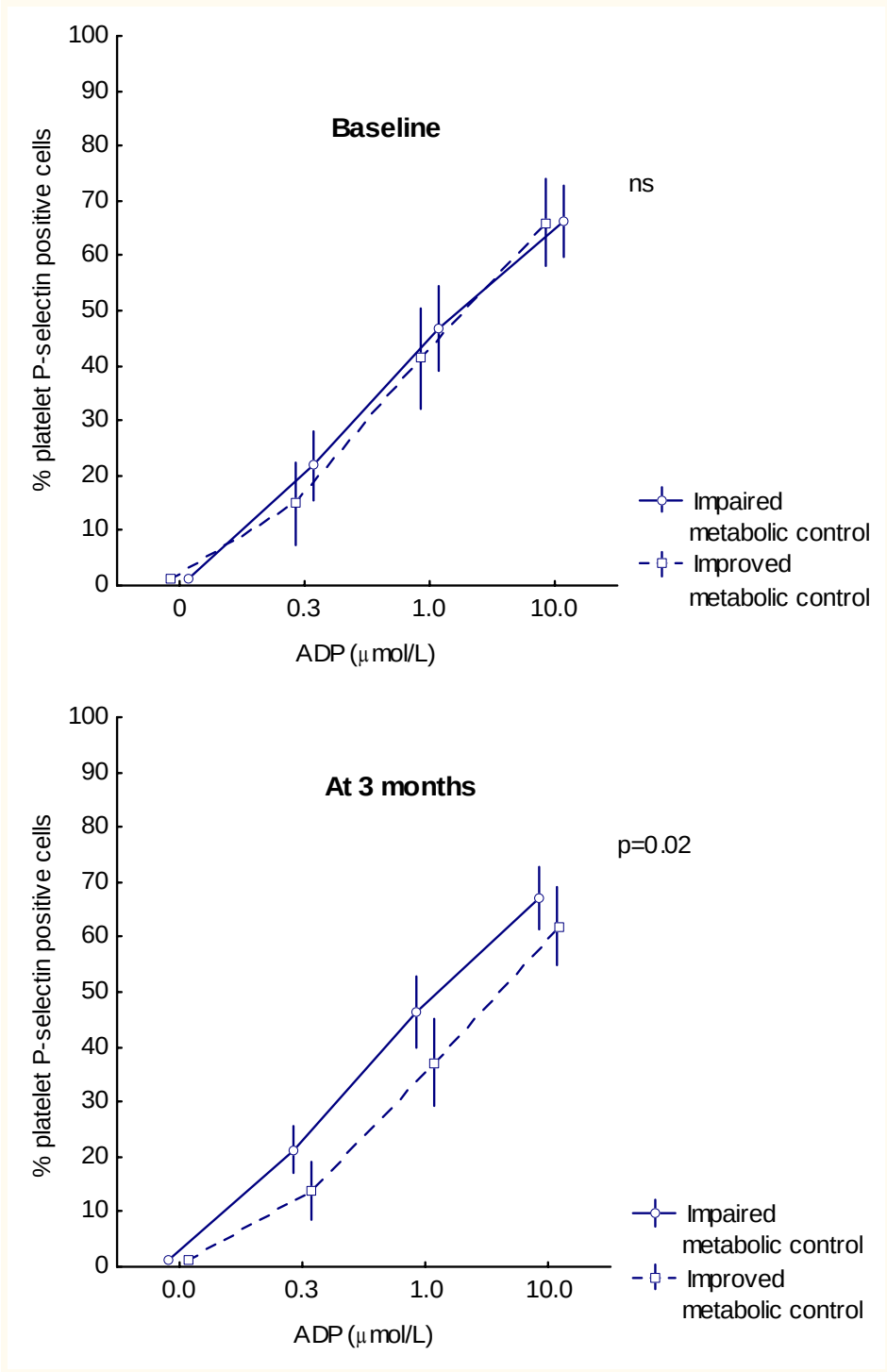


Figure 14. Platelet P-selectin expression at baseline and at 3 months after PCI in patients with impaired metabolic control (n=13) compared to patients with improved metabolic control (n=9). Data are means ± SD; Two-way repeated measures ANOVA for the dose-response curves.

GENERAL DISCUSSION

Diabetes mellitus is characterized by chronic hyperglycaemia and associated with accelerated atherosclerosis and atherothrombotic disease. The pathogenesis of diabetic complications, including microangiopathy (retinopathy, nephropathy and neuropathy) and macroangiopathy (CVD, stroke and PAD) is complex and cannot only be explained by the co-existence of other risk factors like hypertension or dyslipidaemia. The role of platelets in atherothrombotic complications has clearly been established during the past decades. Thus, it might be hypothesized that hyperreactive platelets are implicated in the pathogenesis and progress of vascular complications in DM. In summary, platelet abnormalities in DM can be explained by both increased intrinsic platelet activation and decreased influences of endogenous inhibitors of platelet function. These abnormalities, in close interaction with inflammation, endothelial dysfunction and coagulation, seem to contribute to the enhanced thrombotic potential in diabetes. Platelet hyperreactivity has been shown in diabetic patients with already established vascular angiopathy in the present and previous studies. However, since platelet alterations in diabetes in general are similar to those seen in non-diabetic patients with atherothrombotic disease, it is not clear whether these platelet abnormalities are due to diabetes *per se* (metabolic disturbances) or a consequence of the vascular disease. Thus, the metabolic derangements that accompany diabetes may adversely influence platelet function. However, limited attention has been paid to acute hyperglycaemia as a possible contributor to platelet activation and the mechanisms underlying this effect.

The present thesis supports the idea that hyperglycaemia *per se* contributes to the platelet hyperreactivity in diabetes mellitus. Thus, we found signs of platelet activation in individuals with IGT and in diet treated type 2 DM following an oral glucose tolerance test (OGTT). However, the OGTT did not affect endothelial or coagulation markers. A more physiological approach taken to confirm these findings was to evaluate platelet function following a standardized carbohydrate-rich meal. The meal induced platelet hyperreactivity, but again, no effects on endothelial or coagulations markers in plasma could be found. Our data do not support the idea that postmeal hyperglycaemia causes dysfunction of the vascular endothelium. However, functional methods such as flow mediated vasodilatation were not used in the present project. Thus, the effects of hyperglycaemia on the function of vascular endothelium have not been fully investigated in this thesis.

We hypothesized that diabetes-associated platelet hyperreactivity could be reduced by lowering the hyperglycaemic spikes by premeal treatment with insulinotropic drugs. However, this hypothesis could not be confirmed with treatment with either repaglinide or glibenclamide. Both drugs reduced the postprandial hyperglycaemia. In our experimental set-up glibenclamide was even more effective in lowering both pre- and postmeal glucose levels than repaglinide. Surprisingly, repaglinide – but not glibenclamide - had a slight platelet inhibiting effect in the fasting state. Repaglinide also reduced the plasma levels of some vascular endothelial markers. However, these effects were not related to the reduction of postmeal hyperglycaemia. Previous studies report reduced plasma levels of interleukin-6 and CRP [150] , and reduced

oxidative stress [151]-following treatment with repaglinide. However, these results were explained by reduced postprandial glycaemia and improved glycaemic control during repaglinide treatment. Thus, further studies are needed to elucidate the mechanisms behind the present findings regarding platelet function. In conclusion, the postprandial state – during which patients with mild DM have enhanced blood-glucose elevations - platelets may be "temporarily" hyperreactive. Thus, important information can be obtained by investigating platelet function not only in the basal state, but also during a physiological intervention like after a carbohydrate-rich meal.

Platelet function and coagulation were similar in the patients with type 2 DM and matched healthy controls when investigated in the fasting state. All patients in the present study had mild DM with short known duration of disease (around 3-5 years). They were overall well controlled (HbA1c<6.5%), and had no history of macrovascular disease or nephropathy. Nonetheless, we observed enhanced inflammatory activity and signs of vascular endothelial cell perturbation as some circulating inflammatory and endothelial markers were elevated in the patients compared to the controls (ICAM-1, TNF-α in study I; ICAM-1 and vWf in study II). Recent evidence points towards subclinical chronic inflammation as an important pathogenetic factor in the development of insulin resistance and type 2 DM [141, 242] . Subclinical inflammation is also a precursor of cardiovascular disease [36]. Thus, our findings support the existence of an inflammatory component early on in type 2 DM. This may contribute to the premature development of atherosclerosis in diabetes. We would also suggest that inflammation and endothelial dysfunction precede platelet activation and hypercoagulation in type 2 diabetic disease. However, this needs to be proven in a longitudinal study.

While platelets definitively play a key role in macrovascular complications, the role of platelets in microvascular disease has been questioned. Our findings in paper III-IV reveal an association between platelet hyperactivity and type 1 diabetic microangiopathy. Recent observations that type 1 DM is at least as great a risk factor for cardiovascular mortality as type 2 DM [18] have emphasized the need for early detection and treatment of risk factors for cardiovascular disease also in type 1 DM. Interestingly, the patients with microangiopathy in study III had a mean value of CRP >3 mmol/L. CRP is an established risk marker for cardiovascular disease and CRP levels have been shown to correlate with endothelial vasoreactivity [131]. According to Framingham Risk Score, CRP levels of 1.0-3.0 mg/L indicate an average risk for cardiovascular events, whereas CRP levels >3.0 indicate a high risk [131]. Thus, our findings suggest that platelet hyperreactivity is involved in type 1 diabetic microangiopathy and that the increased platelet reactivity and inflammatory parameters may reflect an increased risk of developing overt cardiovascular disease.
The results in studies III and IV may be of pathophysiological importance since one of the early detectable signs of microvascular damage in type 1 DM is retinopathy. Our patients with microvascular complications had mainly early stages of retinopathy. Diabetic retinopathy progresses from mild non-proliferative abnormalities (increased vascular permeability due to e.g. hyperglycaemia, reduced ET-1 production and pericyte loss), to moderate and severe nonproliferative diabetic retinopathy (vascular occlusion induced by

increased coagulation and abnormal leukocyte function), and finally to proliferative diabetic retinopathy (growth of new blood vessels induced by growth factors and hypoxic activation of PKC). Experimental evidence supports the concept that leukocytes may be involved in the pathogenesis of retinopathy [243]. Leukocytes can occlude retinal capillaries and leukocytes are deformed and activated as they pass through the capillary network. Thus, the increases in leukocyte CD11/CD18 expression and platelet-leukocyte aggregates observed in paper IV may represent an enhancing factor in the development of microangiopathy [56]. In addition, leukocyte-platelet cross talk and sCD40L levels were positively correlated to platelet hyperactivity**,** and sE-selectin levels were elevated in the patients with microangiopathy. Thus, the findings in study III and IV suggest that platelets are involved not only in hemostasis, but may participate in a persistent inflammatory response of the vessel wall, inducing a vicious circle of endothelial perturbation, and leukocyte and platelet activaton. Taken together, the results propose that patients with type 1 DM and microvascular complications would benefit from anti-inflammatory and antiplatelet drug treatment.

Diabetes is a well-recognized risk factor for adverse outcomes after percutaneous coronary intervention (PCI) including a higher restenosis rate[13-16]. A difference of 0.8% in HbA1c in the patients with type 2 DM in study V was associated with a significant reduction in platelet reactivity (platelet P-selectin expression) when comparing groups with deteroriated and improved metabolic control at 3 months after PCI. Thus, improved metabolic control seems to exhibit a beneficial effect on one of the potential pathophysiological factors involved in complications after PCI, i.e. platelet activation. The complex pathophysiological processes that determine outcomes after PCI include endothelial denudation with platelet deposition and mural thrombosis. Also, the risk of restenosis following PCI may be enhanced by activated platelets that secrete growth factors at the site of injury and thus contribute to vascular smooth muscle cell proliferation and intimal hyperplasia [165]. Intererstingly, platelet P-selectin (the expression of which we presently found to be reduced in the patients with improved metabolic control) is required for monocyte and macrophage accumulation and intimal hyperplasia[44].
It is noteworthy that our findings were obtained in patients who were well controlled with HbA1c levels around 6% already at baseline. Cross-sectional studies show that pre-procedural optimal glycaemic control in type 2 DM is associated with improved clinical outcome after PCI [166]. Even mild elevations in pre-procedural fasting glucose are associated with a >3 fold increased risk regarding mortality after PCI [244]. Moreover, a recent epidemiological study shows that small shifts (0.1-0.2%) in the level of HbA1c >5% can affect the risk of suffering cardiovascular events in the general population [196]. Thus, further improvement of the glycaemic control may be an important treatment goal in type 2 diabetic patients with cardiovascular disease, even in patients with good metabolic control by conventional standards.

CONCLUSIONS

The following conclusions were drawn from the studies:

- Platelets in individuals with mild type 2 diabetes mellitus are activated when exposed to acute hyperglycaemia elicited by an oral glucose tolerance test or a carbohydrate rich meal.

- Treatment with the insulinotropic drug repaglinide, but not glibenclamide, attenuates platelet reactivity and reduces circulating endothelial markers. These effects are not related to reduced postmeal hyperglycaemia.

- Tight glycaemic control is associated with reduced platelet reactivity at 3 months following coronary angioplasty in type 2 diabetic patients. This may influence outcomes after intervention.

- Microangiopathy in well-controlled type 1 diabetes mellitus is associated with platelet hyperreactivity and chronic inflammation indicating an increased risk for developing macrovascular complications. Also, enhanced leukocyte-platelet cross-talk and leukocyte reactivity in these patients indicate a pathogenetic role for platelets and leukocytes in type 1 diabetic microangiopathy.

- No major alterations in platelet function or coagulation activity are present in patients with mild type 2 diabetes mellitus without macrovascular complications or in well-controlled type 1 diabetic patients without vascular complications compared to healthy controls.

- The study support the existence of an inflammatory component early in the course of diabetic disease, which may contribute to the premature development of atherothrombotic complications in diabetes mellitus.

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