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Karolinska Institutet

Stockholm 2014

Published by Karolinska Institutet.
Printed by Åtta.45 Tryckeri AB

ISBN 978-91-7549-636-8

PLATELET FUNCTION IN DIABETES MELLITUS

STUDIES ON POSTPRANDIAL PLATELET ACTIVATION AND ASPIRIN TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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This picture, overlooking on the hills of Jerusalem was taken from my home.
On the right side- Hadassah University hospital in Ein kerem, where I graduated medical school, and hematology and where I am currently working as a hematologist. On the left is the beautiful Church of the Holy Trinity (with the golden dome).

To Omri, Tamar and Amitai

ABSTRACT

Diabetes mellitus (DM) is a prothrombotic disease which is associated with a high risk for cardiovascular events, especially in type 2 DM (T2DM). The aim of this work is to contribute to the improvement of cardiovascular risk in DM. Patients with DM have reduced responses to antiplatelet treatment with aspirin, perhaps due to an increased platelet turnover, and this is associated with a poor outcome. Postprandial hyperglycemia is an independent risk factor for cardiovascular complications.

We compared the laboratory responses to twice daily dosing of aspirin 75 mg, the regular once daily dose of 75 mg or a higher once daily dose of 320 mg in an open cross-over study in 25 patients with T2DM and micro/macrovascular complications, We found an improved response to 75 mg twice daily as compared to both once daily doses using two whole blood methods; impedance platelet aggregometry and the IMPACT-R cone and platelet analyzer. Both patients with a high and low platelet turnover could benefit from twice daily dose of aspirin.

The role of postprandial hyperglycemia in postprandial platelet activation was examined in a randomized cross-over study comparing premeal insulin (0.1 and 0.2 U/kg insulin aspart) and placebo in 18 patients with T2DM. Platelet activation was studied in the fasting state, before and after normalizing glucose levels, and 90 min after a carbohydrate rich meal. Although postprandial glucose levels decreased after insulin injection, platelet activation increased. Glucose normalization with IV insulin aspart before the meal also resulted in platelet activation mainly via the thromboxane pathway as studied with the thromboxane analogue U46619 using flow cytometry. The postprandial platelet activation was correlated directly to insulin levels and inversely to glucose levels. We therefore concluded that postprandial platelet activation in T2DM is related to insulin rather than to glucose levels.

The role of insulin in postprandial platelet activation was further investigated by comparing T1DM (n=11) and T2DM (n=9), without premeal insulin before and after a carbohydrate rich meal. T1DM patients, who had very high postprandial glucose levels due to inability to secrete insulin, had no platelet activation after the meal. These findings further support the role of insulin in postprandial platelet activation.

Microparticles derived from platelets, monocytes and endothelial cells were formed after a carbohydrate meal in both patients with T1DM and T2DM in the above study. The microparticles had a prothrombotic potential (thrombin generation assay) which was related to phosphatidylserine and not to tissue factor expression. As opposed to the importance of insulin in postprandial platelet activation seen in T2DM, the procoagulant microparticles increased similarly or even more in T1DM. Therefore microparticle release may be related to hyperglycemia rather than hyperinsulinemia.

This work suggests that twice daily dosing can improve the response to low-dose aspirin treatment in T2DM which might improve cardiovascular prognosis, and that liberal usage of premeal insulin may not benefit patients with T2DM as it contributes to postprandial platelet activation, and insulin treatment has been related to increased cardiovascular risk in DM. Postprandial platelet activation does not occur without premeal insulin in T1DM but should also be studied after insulin. Prothrombotic microparticle release however occurs in both T1DM and T2DM after the meal.

LIST OF SCIENTIFIC PAPERS

Spectre G, Arnetz L, Östenson CG, Brismar K, Li N, Hjemdahl P.
Twice daily dosing of aspirin improves platelet inhibition in whole blood in patients with type 2 diabetes mellitus and micro- or macrovascular complications.
Thromb Haemost. 2011 Sep;106(3):491-9.

Spectre G, Östenson CG, Li N, Hjemdahl P.
Postprandial platelet activation is related to postprandial plasma insulin rather than glucose in patients with type 2 diabetes.
Diabetes. 2012 Sep;61(9):2380-4.

Spectre G, Stålesen R, Östenson CG, Hjemdahl P.
Meal-induced platelet activation differs between patients with type 1 and type 2 diabetes.
Submitted for publication

Spectre G, Mobarrez F, Stålesen R, Varon D, Wallén H, Östenson CG, Hjemdahl P.
Meal intake increases procoagulant microparticle formation in diabetes.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACS	Acute coronary syndrome
BID	Twice daily
CAD	Coronary artery disease
CPA	Cone and plate(let) analyser
COX	Cyclooxygenase
DM	Diabetes mellitus
EMPs	Endothelial microparticles
IGF-R	Insulin like growth factor receptor
IR	Insulin receptor
LTA	Light transmission aggregometry
MP	Microparticle
NO	Nitric oxide
OD	Once daily
MMPs	Monocyte microparticles
PGI_2	Prostacycline
PMPs	Platelet microparticles
PPH	Postprandial hyperglycemia
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein 1 ligand
SC	Surface coverage
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TF	Tissue factor
Tx	Thromboxane
WBA	Whole blood aggregometry

1 INTRODUCTION

1.1 General background

Diabetes mellitus (DM) is a prothrombotic disease associated with inflammation and accelerated atherosclerosis. The prevalence of DM is increasing worldwide; 360 million people were estimated to have DM in 2011, 95% of them having DM Type 2 (T2DM), and the incidence of DM is rapidly increasing so that 552 million people are expected to have DM by 2030 (1). Type 1 DM (T1DM) is an autoimmune disease leading to destruction of pancreatic beta cells and an absolute insulin deficiency, and usually occurs in young and slim people. T2DM, on the other hand, usually occurs in obese subjects in the context of a westernized lifestyle, with high fat diets and little exercise. T2DM patients are often overweight with an abdominal fat distribution, and they have insulin resistance with elevated plasma insulin levels. In early stages of the disease, impaired first phase insulin secretion leads to postprandial hyperglycemia (PPH), which is followed by a deteriorating second phase insulin response and persistent hyperglycemia in the fasting state in later stages (1).

Macrovascular complications such as acute myocardial infarction and ischemic stroke are the leading causes of death in DM. Patients with T2DM have a 2-4 fold increased risk of suffering coronary artery disease, and their risk of suffering a myocardial infarction is similar to that of patients without diabetes who already had myocardial infarction (2) (3). DM further worsens the prognosis of patients with coronary artery disease, such as after a major ischemic event and after revascularization (4).

1.2 Prothrombotic alterations in Diabetes Mellitus

DM is associated with a hypercoagulable state which is multifactorial. There is enhanced thrombin generation by platelets, impaired fibrinolysis secondary to elevated levels of plasminogen activator inhibitor (PAI-1), and low grade inflammation leading to elevated levels of IL-6 and fibrinogen, and increased tissue factor expression in the endothelium (5).

Platelets play an important role in the initiation and progression of atherothrombosis (6). Platelets in DM have an increased tendency toward adhesion and (7) and a high reactivity, meaning that they can easily be activated even with lower concentrations of agonists. The platelets in DM may have a higher turnover, they are "bigger" and they have more binding sites for agonists. Arachidonic acid (AA) metabolism is increased in DM and patients produce more of

the platelet agonist thromboxane A_2 (TxA_2) which is a positive feed-back mechanism in platelet-dependent thrombosis. Platelets from diabetic patients seem to have "insulin resistance", and absence of an inhibitory effect of insulin as described further below. There is decreased production of nitric oxide (NO) and prostacyclin (PGI_2) by endothelial cells and there are also impaired responses to the antiplatelet and vasodilatory effects of NO and PGI_2 in DM (8).

1.3 Aspirin treatment in diabetes

AA is metabolized in platelets to TxA_2 which is a platelet aggregant and a vasoconstrictor, and in endothelial cells to PGI_2 which is antiaggregatory and a vasodilator. Low-dose aspirin (acetylsalicylic acid) irreversibly inhibits the platelet cyclooxygenase, COX-1, and blocks the platelet-dependent production of TxA_2. Low-dose aspirin is considered to act presystemically on platelets in the portal system with little systemic bioavailability and minimal effects on the endothelial production of PGI_2 via COX-2. The balance between TxA_2 and PGI_2 production is important for the efficacy of aspirin as an antiplatelet drug. With high doses aspirin is able to inhibit PGI_2 and shift the balance toward a more prothrombotic one, even though endothelial cells are able to synthetize new COX-2 (9). Greater than 95% inhibition of platelet-dependent TxA_2 synthesis appears to be required for effective inhibition of Tx-dependent platelet aggregation (10).

Low-dose aspirin treatment reduces morbidity and mortality in patients with acute coronary syndromes (ACS) and its use is clearly indicated in all such patients for secondary prevention, including patients with DM (1). Dual antiplatelet treatment with aspirin and the ADP blocker clopidogrel has further improved outcome in patients with ACS (11). However, DM patients have a higher risk for cardiovascular complications after ACS under dual antiplatelet treatment with aspirin and clopidogrel (12) and diabetic patients were shown to have reduced responses to both aspirin and clopidogrel (13-15), indicating that new approaches to antiplatelet treatment in DM are needed, and that diabetic patients might need more efficient platelet inhibition after ACS.

Aspirin was previously recommended for primary prevention among all patients with DM above the age of 40, based on their increased cardiovascular risk (16). However, trials on primary prevention followed by several metaanalyses failed to show benefit from the use of aspirin in patients with DM, and the use of aspirin for primary prevention in DM has therefore been questioned (17-21).

Different mechanisms have been proposed to explain the reduced response to aspirin in patients with DM including increased TxA_2 synthesis, glycation of platelet proteins which might compete with acetylation by aspirin, and a high platelet turnover (22).

Platelets normally persist in the blood stream approximately 7-10 days. They appear in several sizes. When platelet turnover is high, the proportion of large/reticulated platelets is increased. Those platelets are more active. They contain mRNA and are capable of producing membrane and secretory proteins with potentially prothrombotic effects such as GPIIb/IIIa and COX-2 (23). In addition, aspirin, has a short half-life of only 20 min in blood and newly released platelets are likely to be unaffected during a large part of the normal 24 h dosing interval. Since ≈10% of the circulating platelets are newly formed each day in healthy subjects, some recovery of TxA_2 synthesis might occur within a 24-hour dosing interval. We previously found that inhibition of Tx-dependent platelet aggregation in whole blood by aspirin – even at high doses – was not fully sustained during 24 hours in normal subjects (24).

Several recent studies have indicated that both healthy subjects and patients with coronary artery disease with increased platelet turnover may have reduced responses to antiplatelet drugs (23, 25-26). Increased platelet turnover might result from either abnormal megakaryopoiesis or increased peripheral consumption by injured blood vessels (27). Patients with DM are of particular interest due to their high risk for cardiovascular events. The evidence for increased platelet turnover in DM is derived mainly from old studies, where accelerated entry of newly formed platelets (measured by platelet aggregation, thromboxane formation and ATP secretion) was observed in patients with DM and angiopathy compared to healthy subjects (28). The proportion of unblocked platelets at the end of a 24 hour dosing interval could thus be expected to be higher in such DM patients. We therefore suggested that aspirin given twice daily might be beneficial in patients with DM and angiopathy.

1.4 Postprandial blood glucose

Glucose begins to rise about 10 minutes after a meal due to the absorption of dietary carbohydrates. The postprandial blood glucose profile is determined by carbohydrate absorption, insulin and glucagon secretion, and their effects on glucose metabolism in the liver and peripheral tissues (29). The magnitude and time to peak of postprandial hyperglycemia (PPH) depend both on the composition of the meal and the subject. In non–diabetics, plasma glucose

levels peak 60 min after the meal, rarely exceed 7,8 mmol/L (140 mg/dl), and return to preprandial levels within 3 hours, even though absorption of the ingested carbohydrates continues for at least 5-6 hours after a meal. In patients with T1DM who are unable to secrete insulin, the postprandial glucose levels depend on the type, dose and route of administration of exogenous insulin. In T2DM patients the peak insulin levels are delayed and insufficient to control PPH. Abnormalities in glucagon secretion, hepatic glucose uptake, suppression of glucose production and peripheral glucose uptake all contribute to the delayed and higher postprandial glucose levels in both T1DM and T2DM patients (29). Postprandial glucose levels are usually measured 90-120 minutes after the meal and should be below 10 mmol/L (180 mg/dL) to achieve adequate metabolic control with HbA1c levels in the target range of below 7% (30-31). PPH develops early in the course of the disease in T2DM patients, and is thought to be an independent risk factor for cardiovascular complications in DM (32-33). PPH is associated with increased production of free radicals (34), endothelial dysfunction (35-36), and platelet activation (37-39).

Rapidly acting "meal insulin", as well as several other new drugs targeting PPH have been developed in recent years (33). Ceriello et al have shown that insulin aspart reduces PPH as compared to soluble insulin, and that this improves endothelial dysfunction in DM patients (36). However, it is still controversial whether achieving established PPH targets add benefit to CVD outcome. (32, 40-42).

Hyperglycemia has been considered to be a major cause of the platelet hyperactivity in DM (8,38,43). Several studies, including work from our laboratory, have shown that high blood glucose can increase platelet reactivity via an elevation of osmolality (44-45). We also found that a carbohydrate rich meal increases platelet activation and platelet–leukocyte conjugation in response to stimulation with ADP (39) and, in particular, with the thromboxane A_2 analogue U46619 (37) in T2DM patients. Since healthy controls did not develop PPH and did not show increased platelet reactivity after the same meal (37), it was hypothesised that the postprandial platelet activation was related to the hyperglycemia. Santilli et al (38) showed that acarbose treatment, which attenuates PPH, reduced the urinary Tx metabolite excretion in patients with T2DM and that this effect was correlated to changes in PPH. However, treatment with the oral hypoglycemic agents repaglinide or glibenclamide reduced PPH only mildly, and did not counteract the postprandial platelet activation in our previous studies (37,39). Thus we speculated

that a more aggressive approach is needed to control the PPH and, possibly, the accompanying platelet activation after a meal intake.

1.5 Microparticles

Microparticles (MPs) found in the blood stream are bioactive sub-micron (0.1–1 μm) membrane vesicles shed from activated and apoptotic cells in culture and in vivo. They are detectable in healthy subjects but their numbers are greatly increased in a variety of diseases such as cancer, venous thromboembolism, coronary artery or cerebrovascular disease, hypertension and diabetes (46, 47). MPs are derived from a variety of cell types such as platelets, monocytes, endothelial cells and red blood cells. They bear surface antigens from their parent cells and express anionic phospholipids such as phosphatidylserine (PS). Their origins can be identified by their surface antigens using flow cytometry. For example, in study number IV we identified platelet derived MPs by the integrin alpha IIb (CD41) of the GPIIb/IIIa complex, endothelial derived MPs by VE-cadherin (CD144), activated endothelial derived MPs by E-selectin (CD62E), and monocyte derived MPs by CD14.

Platelet derived MPs are the most abundant and can be identified in both healthy and sick patients, while monocyte derived MPs are low or even undetectable in healthy subjects (46). MPs are believed to support coagulation and promote thrombin generation, and it was recently shown that MPs from platelets and monocytes differentially modulate clot formation, structure and stability, suggesting that the various MPs may provide unique contributions to thrombosis (46).

The number of MPs in plasma is elevated in patients with DM compared to healthy subjects (48-49) and among diabetic patients they are increased in patients with vascular complications (50) and they might contribute to the increased thrombotic risk in those patients. There is very limited data on MPs in the postprandial phase. Endothelial derived microparticles are increased after a fat rich meal in patients with T2DM, but there is no information regarding other types of MPs or their procoagulant potential in this setting (51).

2 AIMS OF THE PROJECT

The overall aim of this work is to contribute to the improvement of prevention and treatment of cardiovascular risk in DM. Specific aims in this project are:

Study 1. To evaluate whether twice daily dosing of aspirin improves its antiplatelet effect as compared to the usual once daily dosing in high risk patients with T2DM and micro- and/or macrovascular complications.

Study 2. To evaluate if the platelet activation seen after a carbohydrate rich meal in T2DM patients is related to their postprandial hyperglycemia, and thus can be attenuated by premeal insulin treatment.

Study 3. To compare postprandial platelet activation in patients with T1DM and T2DM, when no insulin is given prior to the meal, in order to further investigate the role of insulin in posprandial platelet activation.

Study 4. To investigate whether intake of a carbohydrate rich meal has an effect on microparticle (MP) production and procoagulant activity in diabetes, and whether those parameters differ between patients with T1DM and T2DM.

3 STUDY DESIGN

3.1 Study population

Patients were recruited from the Diabetology and Cardiology outpatient clinics of the Karolinska University Hospital.

For study 1 we included 25 patients with T2DM, 51-75 years old with microvascular complications (retinopathy, neuropathy or nephropathy) and/or patients with stable macrovascular complications (ischemic heart disease, cerebrovascular disease or peripheral vascular disease) who had not had any clinical event in the preceding 6 months. They were either high risk patients who already took aspirin for cardiovascular protection (n= 21) or had multiple risk factors and an indication for aspirin treatment according to the AHA/ADA guidelines at the time (16).

For study 2 we included 18 patients with T2DM, 51-69 years old, who did not have macrovascular complications and did not use antiplatelet drugs that may interfere with meal effects on platelets.

For studies 3-4, nine T2DM patients with the same characteristics as in study 2 were compared to 11 patients with T1DM who were 40-72 years old, did not take antiplatelet drugs, did not have insulin secretion, and had a BMI below 26 kg/m^2.

For all studies we included patients who had HbA1c levels below 90 mmol/mol (IFCC method), or 10.4 % (NGSP method) to avoid very poorly controlled and unstable patients. The patients abstained from food, tobacco or nicotine on the days of experiments. For the "meal studies" (studies 2-4), patients who took basal insulin were instructed to take half of the evening dose of insulin to avoid problems with hypoglycemia without eating in the morning. The patients took no glucose lowering medications in the morning of the experiments, to avoid hypoglycemia or effects of oral medications on postprandial glucose. All patients had good antecubital veins which is important for the minimization of sampling artifacts in platelet function testing and they all rested for 30 minutes before the first blood draw.

3.2 Study design

Study 1 was a randomized, open, cross-over study with three visits. Patients took three different doses of aspirin for at least two weeks each: the regular dose of 75 mg once daily (OD), an intermediate dose of 320 mg OD (previously often used in the USA) or 75 mg twice daily (BID) according to a randomization list.

Study 2 was a randomized, double-blind, cross-over study with three visits. At each visit the premeal glucose levels were standardized with IV insulin aspart to achieve ≈6 mmol/L. Fifteen minutes after achieving this target, one of 3 subcutaneous injections were given according to a randomization list (saline as placebo, insulin aspart 0.1U/kg or insulin aspart 0.2U/kg). Only the nurse giving the injection had the randomization list, and both the researchers and the patients were unaware of the content of the injection. Immediately after the injection patients ingested the same carbohydrate rich meal prepared by the hospital kitchen during 15 min. The meal contained 620Kcal, 54% carbohydrates, 30% fat 16% protein, and 6.5 grams of fiber. (chicken, rice, boiled vegetables, a carrot salad, bread, margarine and an apple). Three sets of blood samples for platelet function tests were taken: before and 15 minutes after glucose standardization, and 90 minutes after the meal (Figure 1). Samples for glucose, insulin and c-peptide measurements were taken together with platelet functions tests and also immediately, 15, 30, 45 and 60 minutes after the meal.

Study 3+4 was a single visit, comparing T1DM and T2DM patients without premeal insulin. Platelet function (study 3) and MPs (study 4) were measured at baseline (after 30 min rest) and 90 minutes after the same meal as in study 2.

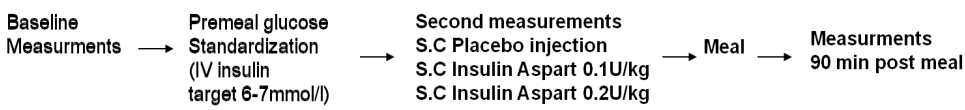

Figure 1: Study 2 design

4 METHODS

4.1 Responses to aspirin treatment

Several different methods have been used to test laboratory responses to aspirin treatment. There is no "gold standard", although aggregometry in platelet rich plasma (PRP) is the one most widely accepted as such. Each method has its advantages and disadvantages, and they are more or less directly related to platelet COX-1 function. The following methods were used in study 1:

Turbidimetric aggregometry in platelet rich plasma

Turbidimetric aggregometry in PRP according to Born (52), which is often called light transmission aggregometry (LTA), measures the increase of light transmission through platelet rich plasma when platelets aggregate in response to an agonist. This is the historical gold standard and has been widely used to measure responses to aspirin. It has been correlated with clinical events (53). When stimulating platelet aggregation in PRP with AA it specifically reflects platelet COX-1 inhibition by aspirin. Blood was anticoagulated with citrate (final concentration of 0.38%) and centrifuged at 190 x g for 10 minutes to prepare PRP and 1400 x g for 10 min to obtain platelet poor plasma (PPP). The following agonists were used: AA 0.5 and 1 mmol/L (Sigma Chemical Co, St. Louis, MO , USA) (24), collagen 1 µg/mL (type 1; Horm, Nycomed Arzneimittel, Munich, Germany), and ADP 5 µmol/L (Sigma).

Impedance aggregometry

Impedance aggregometry is also called whole blood aggregometry and measures the change in electrical impedance between two electrodes when platelets are aggregated by an agonist (54). The advantage of this method is that it is performed in whole blood, where interactions between platelets and red and white blood cells take place and might influence aggregation. For study 1 we used the Chrono-log model 570-VS Four Sample. The blood was anticoagulated with hirudin to maintain normal extracellular calcium levels and diluted 1:1 with physiological saline. The following agonists were used: AA (Sigma) at final concentrations of 0.5 and 1 mmol/L, and collagen (type 1; Horm, Nycomed) at a final concentration of 1 µg/mL. The amplitude of aggregation was measured after 6 minutes and is expressed in Ohms. For study 3 we used multiple electrode aggregometry with the ***Multiplate***® technology and reagents (Roche Diagnostics International, Rotkreuz, Switzerland). Agonists used were: ADP (6.5 µmol/L; ADPtest®), AA (0.5 mmol/L; ASPItest®), collagen (3.2 µg/mL; COLtest®), as well as U46619 (0.3 µmol/L; no commercial test). Results are expressed as area under the curve (AU/min).

The IMPACT-R Cone and Plate(let) Analyser (CPA)

The IMPACT-R Cone and Plate(let) Analyser (CPA) (Diamed, Cresier, Switzerland) is a point of care method for the monitoring of platelet adhesion and aggregation in whole blood under shear stress (55). It has been modified to test responses to anti-platelet drugs and showed a good correlation with turbidimetric aggregometry (56). 130 μl of citrated whole blood is placed on a polystyrene well and subjected to an arterial shear rate of 1800 s^{-1} for 2 minutes. Adhesion of single platelets and platelet aggregates to the surface is measured as % surface coverage (%SC) of adhered platelets. To evaluate the response to aspirin, samples were preincubated with AA 0.32 mM for 1 minute under gentle mixing at 10 RPM. In patients with a poor response to aspirin, platelet microaggregates are formed during preincubation resulting in adhesion refractoriness and decreased platelet adhesion to the well (55), while in patients with good responses to aspirin, microaggregates are not formed and platelet adhesion to the well is normal. Therefore, increased SC% in this method represents a good response to aspirin as opposed to PRP or whole blood aggregometry in which reduced platelet aggregation reflects a good response to aspirin. Patients with acute coronary syndromes had a high rate of unresponsiveness to aspirin by both tubidimetric aggregometry and the IMPACT-R, and this was related to a poor clinical outcome (57).

Analysis of 11-dehydro-thromboxane B_2 in urine:

TxA_2 is the major product of AA in platelets (58). TxA_2 is unstable and is rapidly converted to TxB_2 which is further metabolized to stable metabolites including the major metabolite 11-dehydro-TxB_2. The urinary excretion of 11-dehydro-TxB_2 thus reflects thromboxane generation and platelet activation (59-60) and has been correlated with clinical events (61). 11-dehydro-TxB_2 excretion in urine depends directly on the function of aspirin's therapeutic target, COX-1, but is not platelet specific, as other cells like monocytes and polymorphonuclear cells can produce thromboxane. Approximately 80% of 11-dehydro-TxB2 excreted in the urine is platelet derived and 20% is of non-platelet origin (24, 62) Urinary 11-dehydro-TxB_2 was measured by enzyme immunoassay (Cayman Chemicals, Ann Arbor, USA) using a sample work–up procedure previously described and validated by us (24,63). Results are expressed in relation to creatinine excretion. Measurements were performed in morning urine to obtain an index of Tx production toward the end of the dosing interval.

4.2 Platelet turnover

Reticulated platelets

Reticulated platelets are young and large platelets that contain RNA, and a high percentage of reticulated platelets indicate a high platelet turnover. Poor responses to aspirin treatment are associated with higher percentages of reticulated, less mature platelets (23, 64). Measurements in study 1 were made by flow cytometry (Beckman-Coulter EPICS XL-MCL flow cytometer) after incubation of blood with thiazole orange (Retic-COUNT ™; Becton Dickenson, San Jose, CA, USA), a fluorescent dye that permeates the plasma membrane and binds to RNA (65).

Mean platelet volume

Mean platelet volume (MPV) is positively correlated with platelet turnover (66), and was measured in EDTA anticoagulated blood using a MICROS 60 cell counter (ABX Diagnostics, Montpellier, France).

4.3 Assessment of platelet activation (studies 2-3) and microparticles (study 4) by flow cytometry

P-selectin (CD62) is a protein stored in α granules of platelets. Upon activation of platelet secretion P-selectin translocates to the plasma membrane and can serve as a marker for platelet activation. Platelet activation through different pathways also results in a conformational change in the GPIIb/IIIa receptor, which enables it to bind fibrinogen and form platelet aggregates. In addition, leukocytes – predominantly monocytes and polymorphonuclear neutrophils – can bind activated platelets via P-selectin glycoprotein 1 ligand (PSGL-1) on leukocytes and P-selectin on platelets. Platelet–leukocyte aggregates are also considered as markers for platelet activation (67).

Flow cytometric procedure: Aliquots of 5μl of citrated whole blood were added to Hepes-buffered saline containing appropriately diluted fluorescent monoclonal antibodies and incubated at room temperature for 20 min. After incubation, the samples were fixed with 0.5% (v/v) formaldehyde saline before flow cytometric analysis. Analyses were performed with a Beckman-Coulter EPICS XL-MCL flow cytometer. Platelets were identified by their light scattering signal and staining with fluorescein isothiocyanate (FITC) conjugated anti-CD42a (GPIX) MAb (Becton Dickenson), P-selectin positive platelets by phycoerythrin(PE)-conjugated anti CD62 (Becton Dickenson), and fibrinogen binding by FITC-conjugated polyclonal rabbit anti-human fibrinogen antibodies (DAKO, Glostrup, Danmark). Leukocytes were identified by anti CD45-PE (Beckman Coulter), and then further discriminated by their side scatter characteristics into lymphocytes, monocytes and neutrophils (68). Monocytes were also identified by anti CD14-

PC5 (Beckman Coulter). For platelet-leukocyte aggregate analysis, leukocytes were subjected to two-color analysis (RPE-CD45 versus FITC-CD42a).

Results are expressed as percentages of platelets expressing P-selectin, percentages of platelets binding fibrinogen, and percentages of leukocytes binding platelets in the total or subtype leukocyte population.

4.4 Microparticles

Platelet free plasma was obtained by high speed centrifugation of PPP that had been stored at -80^{o}C (69). MPs were measured by flow cytometry using a Beckman Gallios instrument (Beckman Coulter, Brea, CA, USA). The MP gate in flow cytometry was determined using Megamix beads**.** MPs were defined as particles less than 1.0 µm in size and negative to phalloidin (to exclude cell membrane fragments**).** MPs from all cell origins binding lactadherin were defined as posphatidylserine positive (PS^{+}). Platelet-MPs (PMPs), were defined as $PS^{+}CD41^{+}$, endothelial-MPs (EMPs) as $PS^{+}CD144^{+}$ or $PS^{+}CD62E^{+}$ (E-selectin positive), and monocyte-MPs (MMPs) as $PS^{+}CD14^{+}$. Further phenotyping included measurements of tissue factor (TF) (CD142) on PMPs, EMPs and MMPs, and P-selectin (CD62P) expression on PMPs. The absolute numbers of MPs were calculated by the following formula: (MPs counted x standard beads/L)/standard beads counted. Data are expressed as 10^{6} MPs/L.

4.5 Thrombin generation

The procoagulant potential of MPs in vitro was measured by a calibrated automated thrombogram (CAT) technique (70). High speed centrifugation of PPP was used to obtain MP-enriched pellets (20 800 g for 45 min at RT). The pellet was then added to previously centrifuged normal pool plasma. Thrombin generation was measured during 60 minutes at 37°C according to instructions from the manufacturer of the equipment (Thrombinoscope BV, Maastricht, the Netherlands). The calculated area under the curve represents the total amount of thrombin generated over time, and is called *endogenous thrombin potential* (ETP). Time to the start of thrombin generation is the *lag time*, the maximal concentration of thrombin generated is called *peak thrombin* and the time to maximal thrombin generation is called *time to peak.*

In order to further investigate the mechanisms of action involved in MP-induced thrombin generation, experiments were also performed after the addition of an antibody against TF (40

ng/mL, monoclonal anti-human tissue factor IgG, Sekisui Diagnostics, LLC, MA, USA) or incubation with lactadherin 30 μmol/L (purified lactadherin, Haematologic Technologies, Vermont, USA) which blocks the negatively charged surface by binding to PS.

4.6 Urinary 8-isoprostane

Urine samples for measurements of 8-isoprostane (8-iso prostaglandin F2α) excretion, a measure of reactive oxygen species (ROS) and lipid peroxidation analysed by an enzyme immunoassay kit (Cayman Chemical , Ann Arbor, MI, USA), were obtained before and 90 min after the meal intake. Butyrated ydroxyl toluene (0.005% final concentration) was added to prevent oxidative degradation of the analyte, and samples were stored at -80^{o}C before analysis. The 8-isoprostane excretion rate is expressed as ng/nmol creatinine.

4.7 Statistical analysis

Continuous data were compared by repeated measures ANOVA, with Fisher´s post hoc testing to control for multiplicity. Differences between two groups of patients were analysed by Student´s unpaired t-test for continuous variables for uncorrected means (after validation for normal distribution by the Shapiro-Wilk test), or the chi square test for non-continuous variables. Trend analysis was performed by means of regression analysis. Mean values and standard deviation are given for studies 1 and 2, and errors of the mean for studies 3-4 are given unless otherwise stated. Analyses were performed using STATISTICA software (StatSoft, Tulsa, OK) and a p-value <0.05 was considered to indicate a statistically significant difference.

5 RESULTS

5.1 Study 1:

We found better platelet inhibition with BID compared to OD dosing of aspirin in patients with T2DM and micro/macrovascular complications. Improved inhibition of Tx dependent platelet activation in whole blood was observed by two methods.

Using impedance aggregometry aspirin 75 mg BID reduced AA included platelet aggregation in whole blood by a mean of 22% compared to the regular dose of 75 mg OD (from 12.6±3.5 to 9.7±4.6 Ohms; p=0.003) and by 9% compared to aspirin 320 mg OD (from 12.6±3.5 to 9.7±4.6 Ohms; p=0.003). Aspirin 75 mg BID also reduced collagen induced whole blood platelet aggregation by a mean of 16% (from 14.6±5.0 to 12.2±5.4 Ohms; p=0.02). The higher aspirin dose of 320 mg OD also reduced platelet aggregation in whole blood as compared to 75 mg OD, although to a lesser extent than did BID dosing. AA induced aggregation decreased by a mean of 13% (from 14.6±5 to 12.7±5.3; p=0.02) and collagen induced aggregation by a mean of 9% (from 12.6±3.5 to 11.5±4.2 Ohms; p=0.05) with 320 mg OD (Figure 2).

With shear induced platelet activation, i.e., when using the IMPACR-R CPA method after incubation with AA, aspirin 75 mg BID increased the %SC by a mean of 20% as compared to 75 mg OD (from 3.4±1.6 % to 4.1±2.2%; p=0.05) indicating a better response to the BID dose. The %SC response to 320 mg OD was intermediate (and not significantly different from either 75 mg dose regimen) (Figure 3).

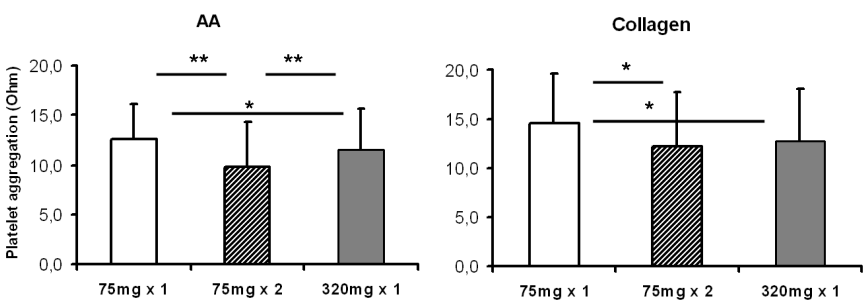

Figure 2: Responses to agonist stimulation using impedance aggregometry in whole blood (WBA). AA; arachidonic acid 0.5 mM, Coll; collagen 1 µg/ml.

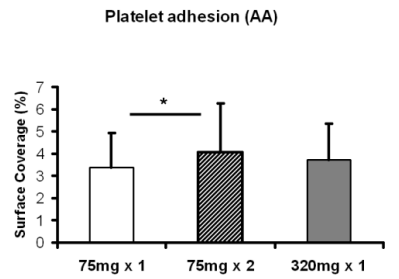

Figure 3: Surface coverage (%) after preincubation with arachidonic acid (AA) 0.32 mM in the IMPACT–R test

All patients had good responses to aspirin treatment with turbidimetric aggregometry in PRP. Platelet aggregation with AA 0.5mM was markedly reduced on all occasions with no differences between the doses: 2.9±1.4% with 75 mg OD, 3.1±1.4% with 75 mg BID and 3.4±3.4% with 320 mg OD. There was high platelet reactivity to both ADP and collagen with no differences on the three occasions; mean ADP aggregation was 80±14%, 81±13% and 78±13%, and mean collagen induced aggregation 58±22%, 57±19% and 52±18% when treating with 75 mg OD, 75 mg BID and 320 mg OD, respectively.

Urinary 11-dehydro–TxB_2 decreased with 320 mg OD as compared to 75 mg OD; (from 30.8±11.3 to 25.9±7.5 ng/mmol creatinine, p=0.002), but was not significantly reduced by 75 mg BID (from 30.8±11.3 to 27.8±10.9; p=0.07).

There was a very good correlation between the two measures of platelet turnover; percent reticulated platelets and mean platelet volume; R^2=0.74 (Figure 4). However, improvements in platelet responses with BID treatment were not correlated with platelet turnover, as both patients with low and high turnover could benefit from BID aspirin treatment (see Figure 2 in paper I).

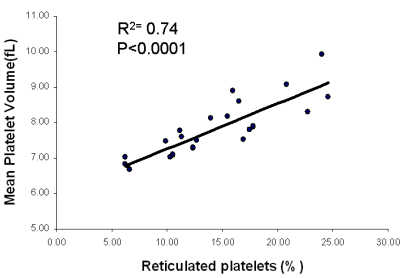

Figure 4: Correlation between % reticulated platelets and mean platelet volume.

5.2 Study 2:

Patients arrived at the laboratory three times in the fasting state. Their fasting glucose levels were high and comparable at the three visits (mean levels between 8.9-9.4 mmol/L). Premeal glucose normalization and standardization with IV insulin infusion successfully reduced the glucose levels to mean values of 5.3-5.6 mmol/L which did not differ between the visits. 90 min after the meal glucose levels increased on all three occasions, with the highest levels after placebo (11.7±0.5 mmol/L). Compared to placebo the postprandial glucose levels were reduced to 9.9±0.4 mmol/L ($p=0.002$) with 0.1 U/kg and to 8.7±0.5 mmol/L ($p<0.001$) with 0.2 U/kg insulin aspart (Figure 5).

Insulin levels in plasma were also comparable on the three occasions in the fasting state (mean levels between 34.3-36.8 µU/mL), and they increased similarly on all occasions after IV insulin infusion (to mean levels between 47.3-47.9 µU/mL). Postprandial insulin levels increased dose-dependently after the administration of meal insulin - highest with insulin aspart 0.2U/kg (109.7±74.2 µU/mL), followed by 0.1U/kg (94.8±72.4 µU/mL) and placebo (61.9±40 µU/mL) (Figure 5).

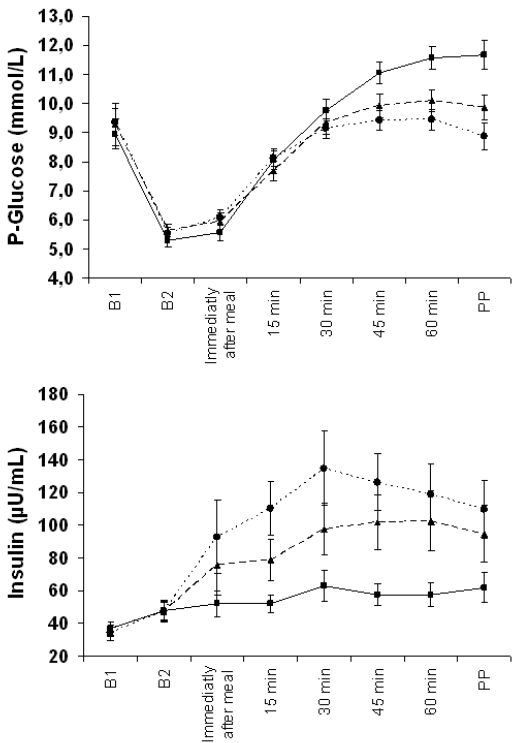

Figure 5: Mean concentrations (± SEM) for plasma (P) glucose and insulin. Measurements were performed in 18 patients before (B1) and after (B2) glucose standardization by insulin infusion (if needed) and 90 min after the meal (PP). Premeal treatments were placebo (solid lines), insulin aspart 0.1 units/kg (dashed lines) and 0.2 units/kg (dotted lines).

The platelet activation markers P-selectin and fibrinogen binding, and responses to agonist stimulation did not differ either before or after the glucose normalization procedure that preceded the meal intake on the three occasions.

Platelets were activated after the premeal IV infusion of insulin. P- selectin positive platelets increased after insulin infusion both without agonist stimulation (+10%), and with ADP (+7.5%) or U46619 stimulation (+67%; from 19.1±13.7 to 31.7±21% P-selectin positive platelets).

Platelets were activated after the meal on all three occasions. Platelet activation was markedly increased postprandially with the thromboxane A_2 analogue U46619, and mildly so with ADP. There was no meal effect with Collagen Related Peptide (a GPVI agonist). The meal itself activated platelets (after placebo injection): U46619 induced P-selectin expression increased by 23% ($p=0.02$) (Figure 6), and ADP induced P-selectin expression increased by 5% ($p=0.005$). However, after insulin injections, when glucose levels reduced, platelets were more markedly activated, with no difference between the two insulin doses. P-selectin expression increased without agonist stimulation by 10-15% with the two insulin doses, by 54-57% after stimulation with U46619 and by 5-9% after stimulation with ADP. U46619 induced fibrinogen binding increased by 46-49% with premeal insulin (Figure 6). However, fibrinogen binding was decreased by 5-8% after stimulation with ADP.

Postprandial platelet activation was inversely correlated with glucose levels and positively correlated with insulin levels (Figure 7).

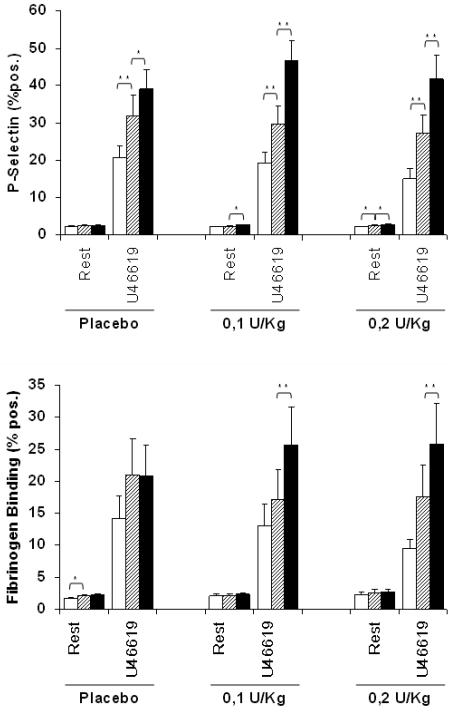

Figure 6: Platelet P-selectin expression and fibrinogen binding expressed as percentage of positive platelets without (rest) and with stimulation with the TxA_2 agonist U46619 (0.3 µmol/L). Measurements were performed before (white bars) and after (gray bars) glucose standardization with IV insulin aspart, and 90 min after the meal (black bars).

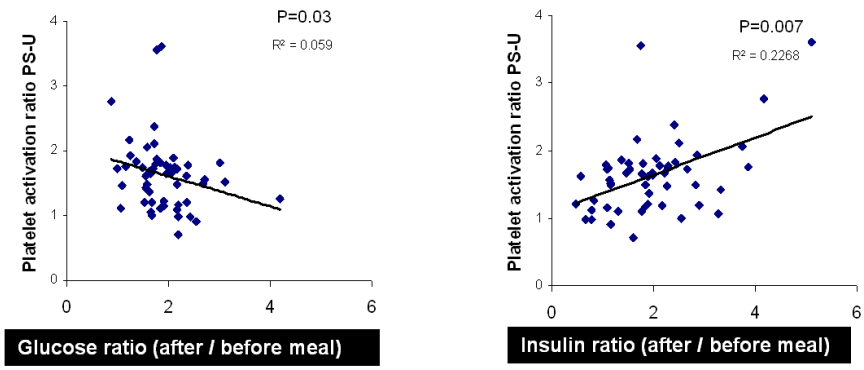

Figure 7: Correlations between postprandial glucose (left) and insulin responses (right) and platelet P-selectin responses to U46619.

5.3 Study 3:

Patients with T1DM or T2DM arrived at the laboratory in the fasting state for a single visit and had the same meal as in study 2 without premeal insulin. Before the meal patients with T1DM and T2DM had similar fasting glucose and insulin levels and the platelet activation markers P-selectin and fibrinogen binding did not differ between T1DM and T2DM. Platelet-leukocyte aggregates were elevated in T1DM compared to T2DM. Meal intake increased glucose levels in both groups, but more markedly so in T1DM (from 7.9±0.7 before meal to 11.6±0.6 mmol/L 90 min after meal in T2DM and from 9.2±0.85 to 21.3±1.6 in T1DM; $p<0.001$ for group difference). Postprandial insulin levels increased as expected in T2DM and did not change in T1DM (figure 8).

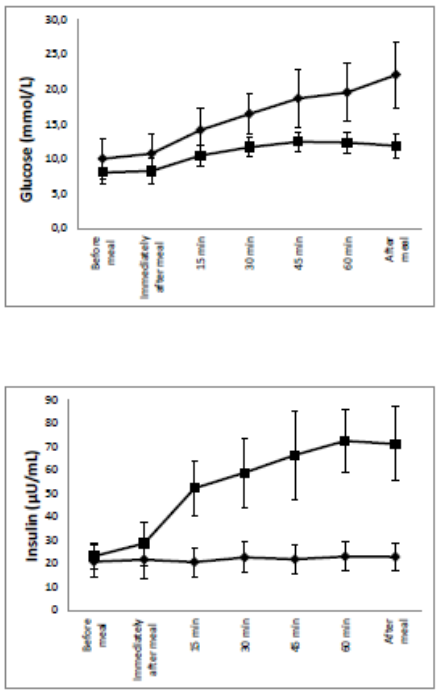

Figure 8: Mean concentrations (± SEM) for plasma (P) glucose and insulin. Measurements were performed in patients with T1DM (n=11, rhomboid line) or T2DM (n=9, square line) before and up to 90 min after the meal. No premeal insulin was given.

Postprandial platelet activation occurred as expected in T2DM but not in T1DM patients. Platelet P-selectin expression stimulated by U46619 was approximately doubled in T2DM but unchanged in T1DM ($p=0.003$ for group difference) (figure 9). ADP-induced P-selectin expression was also

increased after the meal in T2DM only, although to a lesser degree than with U46619 (from 52.7±15.9 to 57.8±16.6; p=0.006 for group difference).

Fibrinogen binding in resting platelets did not increase after the meal in either patient group but the response to stimulation by U46619 was mildly enhanced (p=0.03; no significant group difference) and the response to ADP stimulation was mildly decreased, more so in T2DM (from 60.5±20 to 52.5±21%) than in T1DM (from 62.3±11 to 59.7±12%; p=0.01 for group difference).

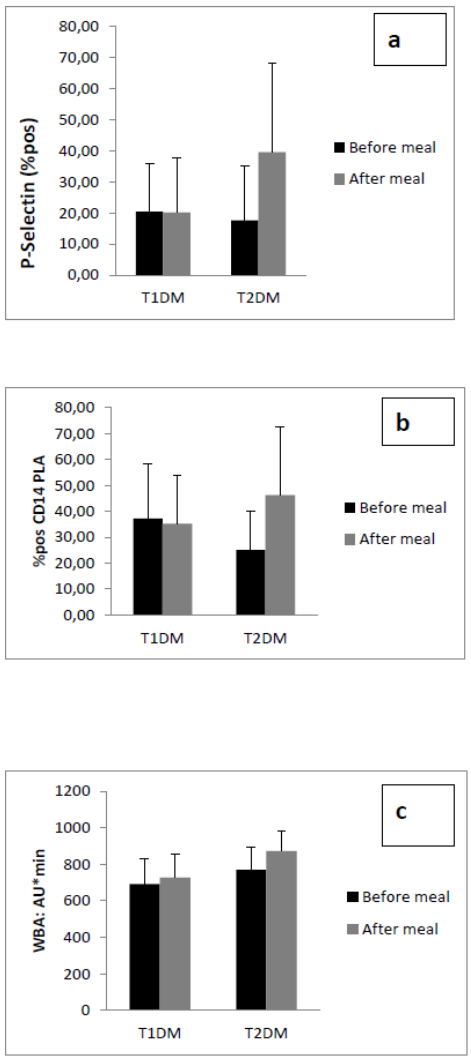

Figure 9: Platelet activation stimulated by the thromboxane analog U46619 before (black columns) and after the meal (hatched columns) in T1DM and T2DM patients. P-selectin expression (% positive platelets, panel a) and platelet-leukocyte aggregate formation (CD14 PLA; % positive, panel b) measured by flow cytometry, and whole blood aggregometry (WBA) using the Multiplate® technique (AU x min; panel c). Mean values ±SEM.

Hemoglobin , WBC and platelet counts did not differ between T1DM and T2DM either before or after the meal, therefore the two groups were analyzed together. The meal intake induced an increment in total WBC, from 5.1±0,1 to 5.5±0.3 ($p=0.009$). This resulted from an increment of neutrophils (from 2.9±0.2 to 3.4±0.2; $p<0.001$) whereas lymphocytes and monocytes decreased slightly (lymphocytes from 1.61±0.09 to 1.51±0.1; $p=0.03$, and monocytes from 0.64±0.04 to 0.59±0.05; $p=0.03$) after the meal. There was also a slight but significant decrease in platelet counts (from 226±11 to 217±9; $p=0.009$). Hemoglobin levels did not change after the meal.

All subtypes of PLAs except platelet-lymphocyte aggregates were elevated in T1DM compared to T2DM before the meal. Thus, platelet-monocyte and platelet-neutrophil aggregates were 7.0±2.5% and 4.1±1.5% in T1DM versus 4.3±1.9% and 2.9±0.8% in T2DM ($p<0.05$ for both group differences). However, increased responses to U46619 stimulation after the meal were observed in the T2DM group only. For example, platelet-monocyte aggregates stimulated by U46619 were unchanged at 37.2±6.3% in T1DM but increased from 25.1±5.0% to 46.1±8.8% with U46619 after the meal in T2DM ($p<0.001$ for group difference) (Figure 9). After the meal U46619 also increased platelet-lymphocyte aggregates (from 2.0±0.3 to 3.1±0.5%; $p=0.02$) and platelet-neutrophil aggregates (from 13.56±3.0 to 28.4±6.8%; $p=0.01$) in the T2DM group, but there were no meal effects on any PLA subtype in the T1DM group. ($p<0.01$ for group differences).

Platelet aggregation in whole blood stimulated by U46619 increased by 14% after the meal in T2DM (from 768±41 to 873±27.6 AUC; within group $p=0.02$) but not in T1DM (from 691±41 to 727±38 AUC) (figure 9). No postprandial enhancements were observed with ADP, arachidonic acid or collagen in either T1DM or T2DM.

In vitro incubation of blood samples with 100 µU/Ml insulin increased platelet P-selectin expression by ≈10% both in resting and U46619 stimulated platelets ($p<0.005$ for both; no group differences) before the meal. After the meal the responses to insulin in vitro were retained in resting samples ($p<0.01$) but blunted with U46619 stimulation, especially in the T2DM group which had high endogenous insulin levels after the meal (resulting in no significant overall effect) (Figure 10).

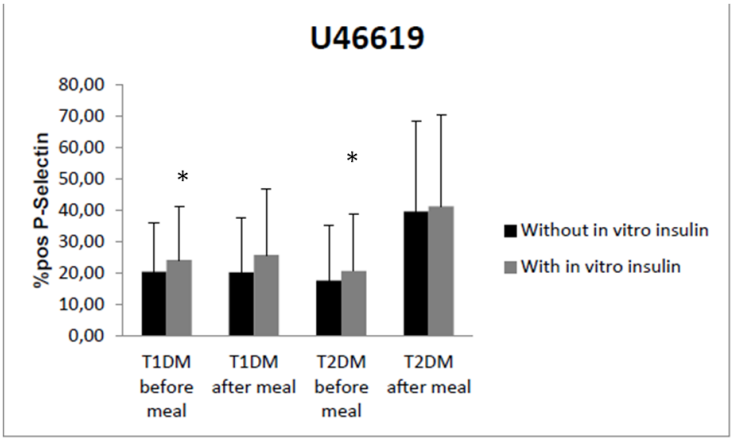

Figure 10: U46619 induced platelet P-selectin expressed as percentage of positive platelets, before and 90 min after the meal, without and with in vitro incubation with insulin (100 µU/Ml, 3 min) in patients with T1DM and T2DM.

The urinary excretion of 8-isoprostane did not differ between T1DM and T2DM patients before the meal (96±13 *vs* 96±36 ng/mmol creatinine), and did not change 90 min after the meal (103±25 *vs* 94±28 ng/mmol creatinine).

5.4 Study 4:

Microparticle counts (total PS^+ MPs, PMPs, EMPs and MMPs) did not differ significantly between T1DM and T2DM before the meal. After the meal MPs derived from all cell origins increased in both groups with some differences in platelet derived MPs. The total number of PS^+ MPs increased by 39-40% in T2DM and T1DM, monocyte derived MPs carrying TF increased by 164% and 135% in T1DM and T2DM patients, respectively. Endothelial derived MPs carrying E-selectin increased in both groups (by 96% in T1DM and 59% in T2DM) while PS^+ and TF^+ EMPs did not change in either group. All types of platelet derived MPs, i.e., PS^+, P-selectin$^+$, and TF^+ PMPs, increased in T1DM (by 83%, 115% and 157%, respectively) while only TF+ PMPs increased in T2DM (by 78%) (Table 1).

	All PS^+ MPs	PS^+ PMPs	PS^+ & $CD62P^+$ PMPs	TF^+ PMPs	PS^+ & $CD144^+$ EMPs	$CD62E^+$ EMPs	TF^+ EMPs	TF^+ & $CD62E^+$ EMPs	TF^+ MMPs
T1DM (n=11)	Lac.	Lac. + CD41	Lac. + CD41 + CD62P	CD41 + CD142	Lac.+ CD144	Lac. + CD62E	CD144 + CD142	CD62E + CD142	CD14 + CD142
Before meal	23159	9944	4841	159	900	2209	541	644	775
After meal	32432	18184	10416	406	1038	4328	622	1150	2044
Ratio after/before	1.4***	1.83*	2.15***	2.57***	1.15	1.96**	1.15	1.79**	2.64**
T2DM (n=9)									
Before meal	17834	12169	5669	228	909	1922	547	756	1153
After meal	24764	15538	8800	406	938	3056	563	906	2709
Ratio after/before	1.39**	1.28	1.55	1.78**	1.03	1.59*	1.03	1.2	2.35**
Overall group diff. by ANOVA (P value)	0.17	0.96	0.85	0.48	0.87	0.3	0.87	0.67	0.23
Overall meal effect by ANOVA (P value)	<0.001	0.02	<0.001	<0.001	0.22	<0.001	0.21	0.007	<0.001

Table 1: Mean concentrations (x 10^6/L) of microparticles (MPs) derived from platelets (PMPs), endothelial cells (EMPs) and monocytes (MMPs). Phosphatidylserine (PS) positive MPs were identified with lactadherin (Lac). CD62P = P-selectin; CD62E = E-selectin; CD142 = tissue factor (TF); CD142 = VE-cadherin (correct?). The ratio after/before indicates the size of the meal effect for each variable within the T1DM and T2DM groups. Two factor repeated measures ANOVAs were used to analyze overall group differences and overall meal effects; there were no significant interaction terms for group x meal effect. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ for meal effects within groups

Meal intake increased MP-induced thrombin generation in normal platelet poor plasma in the two groups and they were therefore analyzed together: the endogenous thrombin potential increased by 26% (from 756±50.6 to 950±68 nmol thrombin x min), the time to peak decreased by 8% (from 19.7±0.4 to 18.2±0.5 min; p= 0.02, and the peak increased by 61% (from 41.2±3.3 to 66.4±9.4 nM thrombin ; p=0.03) (Figure 11). Thrombin generation was completely abolished by PS blockade with lactadherine, but was uninfluenced by TF blockade both before and after the meal. (Figure 12).

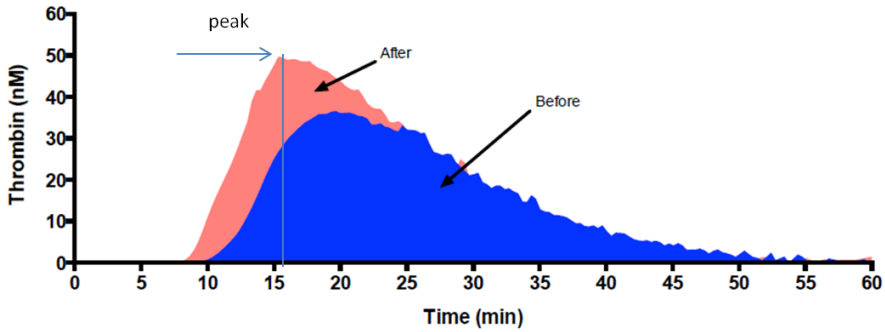

Figure 11: Thrombin generation: Endogenous thrombin potential before (blue) and after (red) the meal.

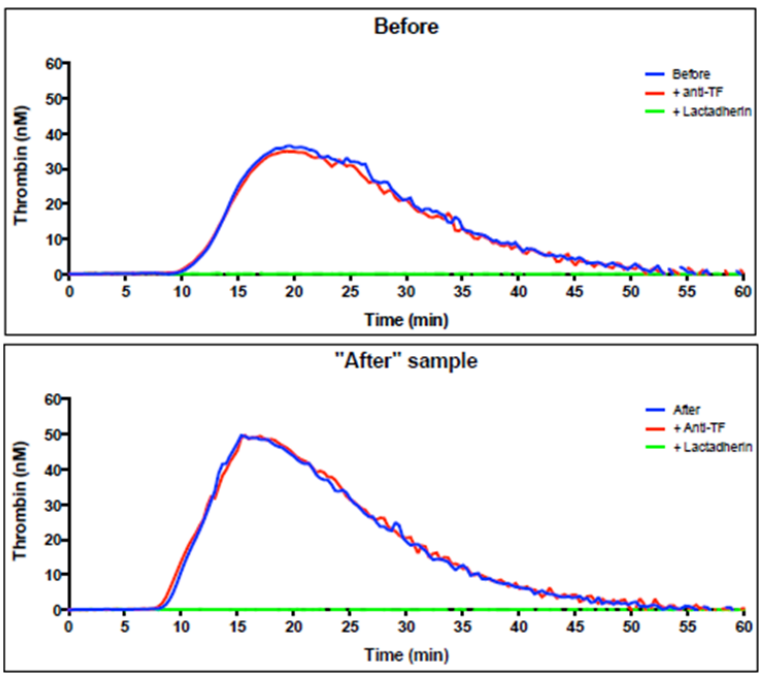

Figure 12: Figure 3: MP-induced thrombin generation without (blue curves) and with PS inhibition by lactadherin (green curves) or an anti-TF antibody (red curves) before and after the meal.

DISCUSSION

This thesis focuses on two issues of interest in relation to the cardiovascular risk in diabetes, i.e., improving the response to aspirin treatment in high risk patients with T2DM (study 1), and what determines the presence and extent of postprandial platelet activation (studies 2-3) as well as microparticle formation (study 4), by evaluating the effects of insulin treatment and by comparing T2DM and T1DM patients.

In study 1 we found an improved laboratory response to aspirin 75 mg twice daily (BID) compared to once daily (OD) by two whole blood methods. Until now, three other studies using different methods have compared OD and BID aspirin dosing in patients with T2DM and they have all shown that BID dosing improves the response to aspirin (Table 2).

Rocca et al analyzed TxB_2 recovery in serum 12-24 hours after aspirin intake in 100 patients with T2DM who took aspirin for either primary or secondary prevention and selected patients in the upper tertile of Tx recovery (i.e., patients with rapid platelet turnover) for a dose comparison (71). Aspirin OD 100 mg BID abolished the increase in TxB_2 recovery seen in this subgroup with 100 mg OD , while 200 mg OD had an intermediate effect. Aspirin BID had no effect on the VerifyNow aspirin assay or on urinary 11-dehydro-TxB_2 excretion. Higher platelet recovery after aspirin, i.e., a faster platelet turnover, was correlated with MPV, BMI and young age (71).

The studies by Capodanno et al (72) and Dillinger et al (73) included T2DM patients with stable coronary artery disease (CAD). Capodanno found that 81 mg BID or 162 mg BID, improved the response to aspirin (VerifyNow Aspirin test, and collagen induced platelet aggregation in PRP) as compared to 81mg OD (72). Dillinger found that 75mg BID gave better platelet inhibition than 150mg OD (light transmission aggregometry with AA and the PFA-100 method in whole blood) (73).

Both Rocca et al and we included patients who received aspirin for either primary or secondary prevention. When we planned our study during 2006, aspirin was recommended for cardiovascular protection for patients with diabetes, regardless of prior vascular events (16). However, we decided to include high risk diabetic patients with micro- and/or macrovascular complications who according to DiMinno et al were supposed to have a high platelet turnover, and 11/25 patients did not have macrovascular complications (28). The recommendations for aspirin use at that time were based on subgroup analysis of large cohorts of high risk patients receiving aspirin for either primary or secondary prevention, and the number of diabetic patients was relatively low (only 4000 diabetics among 95000 patients) (74). Later on, a controversy

regarding aspirin for primary prevention in diabetes rose when some studies dedicated to diabetic patients failed to demonstrate efficacy in primary prevention (17-19,75). The current american guidelines still recommend aspirin for primary prevention in diabetes for patients whose 10 year risk for cardiovascular events is above 10% (21) and the european guidelines recommend aspirin for secondary prevention for all DM patients and for primary prevention in selected high risk patients (1). If we had performed the study today we would probably have selected secondary prevention patients only. However, perhaps BID dosing of aspirin might be able to show greater efficacy and thereby increase the rationale for aspirin treatment also in primary prevention.

We studied a population of patients with T2DM and micro/macrovascular complications, similar to the population studied by DiMinno et al (28). However, there was considerable variability in the reticulated platelet percentages among our patients, between 6% and 26%, despite this selection of patients. We found a strong correlation between the percent reticulated platelets and mean platelet volume, as did Rocca et al (71). Our study had a crossover design in which each patient served as his/her own control and we did not include a control group of healthy subjects, therefore we cannot conclude that diabetic patients indeed have a higher platelet turnover as compared to other high risk patients or healthy subjects. Interestingly, the response to BID aspirin did not correlate with either MPV or reticulated platelets, and patients could benefit from BID dosing of aspirin whether they had low or high reticulated platelets or MPV. In agreement with our findings, Dillinger et al found that MPV was not related to an improved response to BID aspirin (73).

Reference	Patients	Aspirin dose	Platelet studies	Results
Spectre (study 1)	T2DM (n=25) with complications	75mg OD 75mg BID 320 mg OD	WBA ; AA, Collagen LTA- AA Impact R with AA 11-dehydro-TXB_2 in urine	75 mg BID improved WBA, Impact R. Higher doses reduced urinary 11-dehydro TXB_2 LTA was low and not affected
Capodanno (72)	T2DM (n=20) with stable CAD	81 mg OD 81 mg BID 162mg OD 162 BID 325 OD	LTA AA, ADP, Collagen Verify now aspirin Serum TXB2	BID regimes- improved reactivity (Verify now + LTA collagen, no effect on LTA ADP) Higher doses- reduced serum TXB2
Rocca (71)	T2DM (n=33) on ASA. Primary and secondary prevention and rapid turnover. Control-patients on ASA	100 mg OD 100 mg BID 200 mg OD	Serum TxB_2 Verify now Aspirin 11-dehydro-TXB_2 in urine	BID abolished high serum TxB2 recovery 200 mg- intermediate effect No effect on Verify now + urinary 11-dehydro-TXB_2
Dillinger (73)	T2DM (n=92) , stable CAD+ smoker/elevated CRP, fibrinogen, or platelets	150 mg OD 75 mg BID	LTA-AA 0.5 PFA-100 (coll –epi)	Improved "biological resistance" (<20% agg PRP)

Table 2: Studies of twice daily dosing of aspirin in patients with T2DM. CAD;coronary artery disease, OD; once daily, BID; twice daily, WBA; whole blood aggregometry, AA; arachidonic acid, LTA; Light transmission aggregometry

Grove et al studied patients with stable CAD and found a positive correlation between platelet turnover (by flow cytometry) and platelet aggregation in whole blood one hour after low-dose (75 mg) aspirin ingestion in 85 patients with T2DM and 92 non-diabetics (23). In that study, the level of reticulated immature platelets was correlated with thrombopoetin levels, smoking and diabetes in multivariate analysis. However, neither reticulated platelets nor MPV differed between patients with stable CAD with or without diabetes (personal communication with Grove (23), Therefore, the concept of high platelet turnover in DM should be reevaluated.

We observed differences in the effectiveness of aspirin treatment between platelet aggregation in PRP and in whole blood. The nearly complete inhibition of AA induced platelet aggregation in PRP suggests that aspirin had reached its target in platelets and that our patients had good compliance. At the same time there was clearly residual AA induced platelet aggregation in whole blood. These differences in platelet aggregation between PRP and whole blood are interesting and have also been observed in another study (76). Whole blood is the natural platelet environment, where other blood cells as erythrocytes and leukocytes are also present, and may be the most relevant matrix for platelet function studies.

It is known that platelets interact with leukocytes. Tx and AA can also be produced by other blood cells such as monocytes (77) and polymorphonulear cells (78), and can be delivered to platelets by transcellular metabolism and bypass platelet COX-1 inhibition. Platelets inhibited by aspirin can thus be activated in the presence of monocytes (77), and platelets produced more TxB_2 in the presence of activated PMN cells (78). Tx can also be formed via COX-2 in young/reticulated platelets. Aspirin has a lower affinity to COX-2 which therefore is not inhibited by low dose aspirin (64). The newly released large/reactive platelets may be lost during centrifugation for the preparation of PRP which could contribute to the lack of platelet activation by AA in PRP despite residual AA responses in whole blood. Finally, RBCs which are present in whole blood contain ADP which can activate platelets (79). Aggregation in PRP used to be the “gold standard” for studying responses to antiplatelet treatment. However, this should probably be reconsidered, and future studies should more often concentrate on assays performed in whole blood.

A higher dose of aspirin has also been suggested to provide better platelet inhibition in DM (76). In our study 320 mg OD improved the response to aspirin as compared to 75 mg OD when using whole blood aggregometry, but 75 mg BID was more effective than 320 mg OD.

Higher doses of aspirin are less attractive since they might increase the systemic bioavailability with more COX-2 inhibition leading to reduced PGI_2 formation, and higher doses of aspirin are also associated with a higher gastrointestinal bleeding risk. Thus, a low BID dose of aspirin may be preferable to a higher OD dose both from the efficacy and safety points of view.

Urinary 11-dehydro TxB_2 excretion was reduced by the higher aspirin dose of 320 mg OD, but there was only a trend toward a reduction with 75 mg BID (p=0.07) in our study. Rocca et al also measured urinary 11-dehydro-TxB_2 and found that it was uninfluenced by platelet turnover or by dosing of aspirin (up to 200 mg per day) (71) Tx is also produced by other blood cells and endothelial cells, and approximately 20% of the stable Tx metabolite excretion in urine is of non-platelet origin (23,61). Therefore, the reduced TxB_2 excretion with the higher dose of aspirin could be related to increased systemic bioavailability of aspirin and inhibition of endothelial cells, rather than to better platelet inhibition.

Until now the twice daily dose of aspirin has only been studied in small-scale studies of laboratory responses to the drug. Based on these studies which have all shown an improvement in the laboratory response to aspirin there should be a place for a clinical trial of BID aspirin for secondary prevention in patients with T2DM. Very large studies of thousands of diabetic patients will, however, be needed to prove improved clinical outcomes with BID compared to the conventional OD dosing of aspirin. In such a study, patients should not be selected according to their platelet turnover, since this is very impractical in an ordinary clinical setting, and since also patients with a low % reticulated platelets or a low MPV could benefit from BID dosing according to our study and that of Dillinger et al (73).

Possible drawbacks of twice daily use of aspirin are the problems of compliance and safety, mainly with regard to gastrointestinal toxicity. Twice daily dosing may, however, also be "compliance forgiving" since one missed dose results in a 24 h rather than a 48 h interval until the next dose. Twice daily dosing of aspirin has also been studied in patients with essential thrombocythemia who have an abnormal megakaryopoesis and a high platelet turnover. In these patients aspirin 100 mg BID provided better platelet inhibition as compared once daily dosing (80). In addition, the excretion of a stable PGI_2 metabolite in urine was not changed supporting cardiovascular safety of 100 mg aspirin BID treatment (81). Thus, an outcome study of BID aspirin in high risk patients would be of considerable value. Unfortunately, however, such large studies require large resources and aspirin is an old and cheap drug with no or very limited commercial interest. The likelihood that a large outcome study with BID dosing of aspirin will be undertaken is therefore small.

Postprandial platelet activation

The second part of this thesis is related to postprandial platelet activation and the mechanisms involved. Yngen et al from our group had previously shown that a carbohydrate rich meal activates platelets in patients with T2DM (39). Patients were tested, in a randomized cross-over study ,without any glucose lowering treatment and after taking repaglinide or glibenclamide, two medications that are known to increase insulin secretion and reduce postprandial glucose levels. In that study, both medicines reduced glucose levels mildly and neither attenuated the meal-induced platelet activation. Healthy individuals ingesting the same meal had neither postprandial hyperglycemia nor platelet activation (37). We therefore hypothesized that a stronger postprandial glucose lowering agent is needed to reduce glucose levels sufficiently in T2DM to also prevent postprandial platelet aggregation.

To our great surprise, the opposite results were obtained. Insulin aspart administered immediately before the same carbohydrate rich meal reduced, but did not abolish postprandial hyperglycemia and the postprandial platelet activation increased further with both insulin doses. In addition, we observed platelet activation following the glucose normalization procedure with intravenous insulin that preceded the meal. We concluded that postprandial platelet activation is not related to glucose levels but to insulin levels. We then compared patients with T1DM who are unable to secrete insulin to patients with T2DM, having the same meal but without premeal insulin. Indeed we found that patients with T1DM had very high levels of posprandial glucose but no platelet activation, proving that postprandial hyperglycemia is not the reason for postprandial platelet activation in diabetic patients.

Table 3 summarizes our results from four carbohydrate rich meal studies in patients with DM and healthy individuals.

Subjects (ref)	**Postprandial hyperglycemia**	**Postprandial insulin elevated**	**Postprandial platelet activation**
Healthy individuals (37)	No	Yes (not measured in the study)	No
T1DM (study 3)	Yes (marked)	No	No
T2DM (37, 39, studies 2-3)	Yes	Yes	Yes

Table 3: Postprandial glucose, insulin and platelet activation in patients with DM and healthy individuals

Platelets carry receptors for insulin (IR) and insulin like growth factor (IGF-R) which share a lot of homology (82). A human platelet contains about 570 IR (83) and many more IGF-R Both receptors are transmembrane glycoproteins composed of 2 extracellular α subunits and 2 transmembrane β subunits (82). The α subunit binds ligands and the β subunit has tyrosine kinase activity. When insulin binds to the α subunit, it leads to autophosphorylation of the β subunit, a conformational change and phosphorylation of other proteins. The literature is inconsistent with regard to the effects of insulin on platelet activation, as some report platelet activation by insulin while others report platelet inhibition or no effect (82-87). It has been suggested that physiological or high physiological concentrations of insulin (0.25-2 nmol/L = 26-288 μU/mL) inhibit platelet activity, whereas supraphysiological concentrations (25-200 nmol/L = 2600-29000 μU/mL) enhance platelet activity (84).

Different mechanisms of platelet inhibition by insulin have been suggested. Based on studies in healthy individuals, Ferriera et al suggested that insulin attenuates platelet activation by ADP and thrombin by interfering with cAMP suppression through IRS-1 and G_i (83). Hunter et al showed that platelets express (IR) homodimers or IR/IGF-R hybrids (82). However, they found no direct effect of insulin on platelet activation at physiological concentrations and speculated that this is due to the fact that IGF-R is much more abundant and most of the IR is in hybrids which are not sensitive to insulin. Anffosi et al (84) suggested that insulin is able to increase both cGMP synthesis through guanylate cyclase activation, and cGMP catabolism through phosphodiesterase activation. At physiological or slightly supraphysiological concentrations the first phenomenon

dominates so that intraplatelet cGMP levels increase and insulin shows antiaggregating properties, whereas insulin reduces cGMP levels through phosphodiesterase activation at supraphysiological concentrations, leading to platelet activation (84).

We have consistently found in vitro platelet activating effects of physiological or slightly above physiological concentrations of insulin (10-300 μU/ml) in vitro in healthy individuals (87-88) and in patients with T1DM (86), and in vivo postprandial activation with in T2DM (37,39). These findings are in agreement with the findings in studies 2 and 3. Even groups that have found platelet inhibitory effects of insulin in healthy volunteers did not find that in patients with T2DM or obesity. Those patients seem to have "platelet insulin resistance" with a loss of the platelet inhibitory responsiveness to insulin (89-91). Our results with markedly enhanced platelet activation in the presence of elevated insulin levels in vivo (after the meal, or after insulin infusion) in response to the Tx analogue U46119, but only mild enhancement of responses to ADP suggest a role of the Tx pathway in the platelet activating effect of insulin. This is a novel mechanism for the effect of insulin on platelets and it would be interesting to explore whether aspirin treatment could reduce postprandial platelet activation inT2DM.

The inconsistencies in the literature with regard to the effects of insulin on platelets are not understandable at the moment and may result from, e.g., different methods of testing platelet activation (flow cytometry in our laboratory and mostly platelet aggregation in PRP in other laboratories), different doses of insulin, and/or the complexity of platelet activation with different stimuli and response variables. For example fibrinogen binding was decreased when stimulated by ADP but increased when stimulated by U46619 in studies 2 and 3 which was opposite to all other results and we cannot presently explain this discrepancy.

The findings of a role of insulin in postprandial platelet activation may be of clinical importance considering the findings that insulin treated T2DM patients have a higher ADP induced platelet aggregation than T2DM patients not treated with insulin (92) and the growing evidence for worsened clinical outcome in patients who receive insulin. In the DIGAMI 2 trial the risk of suffering non-fatal myocardial infarction or stroke increased significantly with insulin treatment while metformin was protective (93). In a retrospective Swedish study based on two registries that included 12500 diabetic patients after coronary angiography, T2DM patients treated with insulin who underwent coronary angiography had a higher long-term mortality risk (94), and among patients with DM who were treated with metformin, the addition of insulin vs a sulfonylurea was associated with an increased risk of

suffering a composite endpoint of nonfatal cardiovascular outcomes and all-cause mortality (95).

Other possible mechanisms that might contribute to the differences in postprandial platelet activation between T1DM and T2DM besides the different insulin levels could be related to parameters that were not studied such as triglyceride levels which could influence platelet activation (96), age differences (patients with T1DM were younger) and insulin resistance. Patients with T1DM in our study had lower BMI´s and were less likely to have insulin resistance. Insulin may therefore have platelet inhibiting effects in lean T1DM as in healthy subjects but there was no postprandial insulin response in the T1DM patients. A study with premeal insulin in obese versus non-obese T1DM patients would clarify the question of the role of platelet insulin resistance in postprandial platelet activation.

After obtaining the different results between T1DM and T2DM we tried to recall patients with T1DM for another visit with meal intake after taking their regular premeal insulin. Six of the 11 patients agreed to come, but this group is too small to allow any firm conclusions. Our impression is that there was platelet activation also in T1DM patients after receiving insulin. For example, stimulation with U46619 increased platelet P-selectin expression from a mean of 16.7+2.2 to 29.6+8.0% after the meal with premeal insulin (p=0.19), as compared to a slight decrease/no change for the same six patients in the previous visit without premeal insulin. Five patients showed platelet activation after the meal and one patient who was well trained (a marathon runner) and was treated with an insulin pump had results that differed from the others with lower insulin levels in plasma and no platelet activation after the meal even with meal insulin. A proper meal insulin study comparing lean and obese T1DM patients is of considerable interest.

In vitro incubation of samples from T1DM patients with insulin at similar plasma concentrations as the postprandial insulin levels (100 μU/ml) in T2DM patients resulted in mild platelet activation both before and after the meal (figure 10). Reasons behind the smaller effect of insulin in vitro compared to in vivo could be other effects of insulin occurring in vivo, or the time of platelet exposure to insulin – only 20 min in vitro versus 90 min in vivo. However, in vitro systems are often less responsive than in vivo systems also from a more general point of view.

The impression from the above-mentioned results is thus that platelets from patients with T1DM can be activated postprandially by insulin, and it would be interesting to further characterize the platelet responses to insulin in T1DM.

Boden et al performed clamp studies in healthy volunteers and in patients with T2DM (97-98). They observed that selective hyperinsulinemia increased tissue factor (TF) procoagulant activity

by 30%, and that hyperglycemia and hyperinsulinemia had additive effects as TF procoagulant activity increased by 80% when they were combined. If the same is true for platelet activation, it is comprehensible why patients with T2DM in our study had marked platelet activation with both hyperinsulinemia and postprandial hyperglycemia. We have shown in patients with T1DM that postprandial hyperglycemia alone does not cause postprandial platelet activation, but hyperglycemia might still contribute to platelet activation seen in the presence of hyperinsulinemia. Clamp studies focused on platelet activation would be able to solve this question.

Postprandial microparticle formation

The meal induced increased MP formation in both patients with T1DM and T2DM. The MPs were derived from all cell types, platelets, monocytes and endothelial cells. Endothelial cells released E selectin positive MPs which is a marker for endothelial activation (99), but not VE-cadherin (CD 144) positive EMPs. VE-cadherin is a cell adhesion glycoprotein located in the tight junction between endothelial cells and perhaps greater endothelial damage may be required for its release. Alternatively, the release of VE-cadherin^{+} MPs may be related to the content of the meal, as it was elevated in T2DM patients after two consecutive fat rich meals in another study (51). Monocytes carrying TF increased in both patients with T1DM and T2DM. Monocytes are the main cells that carry tissue factor in blood (100) and activated platelets are able to bind TF from MMPs through P-selectin on platelets and PSGL-1 on MMPs (100-101).

All types of platelet derived MPs increased in T1DM (PS^{+}, P-selecin^{+} and TF^{+}), while only TF^{+} PMPs were increased significantly in T2DM. These results were unexpected as we observed postprandial platelet activation in T2DM and not T1DM in this study (study 3). One explanation could be that increased platelet activation results in higher MP formation but also higher MP consumption, or adherence of MPs to other cells (102). Of note, both platelet and monocyte counts decreased after the meal which could possibly reflect their consumption. Decreases in monocyte and lymphocyte counts have also been observed in healthy volunteers after a light meal (103). Another explanation for the increased postprandial PMP levels in T1DM is the complexity of biological responses of the coagulation system; it could be that MP release is related to high postprandial glucose levels and not to insulin levels (contrary to the platelet activation response) and therefore their levels tended to increase more markedly in T1DM in this study.

The increment in MPs after the meal also resulted in increased MP-dependent thrombin generation. The CAT assay was modified and was performed without adding phospholipids or tissue factor to assure that the prothrombotic potential is driven by the MPs. Blocking PS expressed on the MPs abolished the thrombin generation, reflecting the role of the negatively charged surface of MPs in promoting thrombin generation, while an antibody towards TF did not influence the thrombin generation. The number of MPs carrying TF was relatively small as compared to the number of PS^+ MPs (table 1), and perhaps the role of MPs carrying TF is to deliver TF to the site of injury where it can be activated while it may present in a non-functional form on circulating MPs (104). Nonetheless, the postprandial increases in MPs with procoagulant activity due to PS exposure are a novel prothrombotic mechanism which may contribute to the increased cardiovascular risk of DM patients.

CONCLUSIONS

The following conclusions can be drawn from this work:

- Twice daily dosing of aspirin 75 mg improves the laboratory response to aspirin in whole blood, as compared to the regular once daily dose of 75 mg or to a higher dose of 320 mg once daily, in high risk patients with T2DM and micro/macrovascular complications.
- The improved laboratory response to twice daily dosing of aspirin is not related to the turnover of platelets, as both patients with low and high turnover could benefit from twice daily use of aspirin.
- Reducing postprandial glucose levels by rapid "meal insulin" did not reduce postprandial platelet activation in patients with T2DM. On the contrary, use of premeal insulin increased the postprandial platelet activation in T2DM.
- When no insulin is given prior to the meal, patients with T1DM and no insulin secretion do not have postprandial platelet activation, despite very high glucose levels.
- Postprandial platelet activation is seen mainly when the Tx pathway of platelets is stimulated and is related to high insulin levels rather than to hyperglycemia in patients with DM.
- Prothrombotic microparticles derived from platelets, monocytes and endothelial cells are released after the meal in patients with both T1DM and T2DM.
- Postprandial microparticle formation may be related to postprandial hyperglycemia and not to hyperinsulinemia, since patients with T1DM had low insulin and high postprandial glucose levels and similar or even increased microparticle release as compared to T2DM patients.
- Postprandial platelet activation and release of procoagulant microparticles may contribute to the increased cardiovascular risk of T2DM patients. Further studies are needed to clarify the role of insulin in postprandial platelet activation in T1DM patients.

ACKNOWLEDGEMENTS

It has been a while since I first came to Sweden on a snowy day on January 2006, and also a while since I returned back home three years later. It is indeed an exciting moment for me to finally complete my studies, but at the same it is not easy for me to say goodbye.

I feel privileged to have this experience at your lab and to get to know many wonderful people. I have learned to appreciate good, well organized and precise clinical research and I cannot thank you enough for helping me finalizing the lab work while I am being away.

I would first like to express my deepest gratitude and appreciation to my main supervisor and mentor Paul Hjemdahl. I thank you for the support, patience and open mind, for many hours of fruitful discussions, hundreds of emails and for being such a wonderful research and writing model. I will sure miss your clear and precise thinking and your red "track changes" corrections in my future papers.

To Maud Daleskog, Ragnhild Stålesen and Charlotte Ander, I wouldn't be able to complete this without you!! I thank you for your excellent laboratory expertise and statistical assistance and also for your friendship and many enjoyable moments together.

To ClaesGoran Östensson my co-supervisor, thank you for being so positive and cooperative, for sharing your vast knowledge in the field of diabetes and for many interesting and pleasant meetings.

To Nailin Li my co-supervisor, thank you for always having your door open for questions and discussions, for your care and friendship, and for our collaborative work on platelet- lymphocyte interactions.

To David Varon my co-supervisor and mentor in Israel. Thank you for introducing me to the field of platelets, for making the contact with the lab in Sweden and providing us with the IMPACT-R machine, for being my tutor in both research and clinic, and for making the effort to come to my defense .

To my co-authors Lisa Arnetz, Kerstin Brismar, Fari Mobarrez and Håkan Wallén. Many thanks for your contribution to my studies and for the fruitful discussions we've had.

To Lillemor Mellander, Sandra Chesley and Annika Jouper, many thanks for your excellent secretarial help and your kindness.

To Kajsa Sundqvist and Maria Wärn, thank you for being cooperative, for helping us finding patients for the studies as well for your excellent contribution to the meal study.

I also would also like to thank all the other friends and colleagues at the lab in Stockholm and my colleagues in Israel, especially Yossi Kalish who has worked very hard to enable me to finalize my thesis and Dina Ben -Yehuda for your support and for enabling me to have time for research.

And finally, to my parents Sivana and Shmulick, my three children Omri, Tamar and Amitai, to Uri and my friends in Israel, I thank you for your immense love and support.

The study was supported by grants from the Swedish Research Council/Medicine (5930), the Swedish Heart Lung Foundation, the Erling-Persson Foundation, Karolinska Institutet, the Stockholm County Council, and a research grant from Novo Nordisk.

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