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PLATELET ACTIVATION AND ASSOCIATED TRANSCRIPTIONAL SIGNATURE IN TYPE 2 DIABETIC PATIENTS WITH STABLE CORONARY ARTERY DISEASE: INSIGHTS INTO THEIR THROMBOTIC PROPENSITY.

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INTRODUCTION

Type-2 diabetes mellitus

Type-2 Diabetes Mellitus (T2DM) is a metabolic disorder of fuel homeostasis.

According to the American Diabetes Association (ADA), T2DM should be considered a metabolic disorder characterized by a hyperglycaemic state, as a result of chronic insulin resistance, which leads to pancreatic β cell dysfunction and subsequently inadequate insulin secretion in response to varying degrees of overnutrition, inactivity and consequential overweight or obesity.

DM chronic hyperglycaemia has been associated with long-term target organ damage, dysfunction, and collapse especially among ophthalmologic, renal, neurologic, and cardiovascular systems [1].

It should be noted that T2DM is an independent risk factor for coronary artery disease, stroke, peripheral vascular disease, and heart failure, which are the main causes of death in these patients [2].

Although the T2DM pathogenesis is still not fully elucidated, there are many contributing factors such as advanced age, familial history, and behavioural and environmental factors that play a relevant role in disease prognosis and evolution.

Epidemiology

Prevalence of diabetes mellitus is increasing worldwide.The estimated worldwide prevalence of diabetes among adults was 285 million (6.4%) in 2010 and this value is predicted to rise to around 439 million (7.7%) by 2030. T2DM is the predominant form and accounts for at least 90% cases. The rise in prevalence is predicted to be much greater in developing than in developed countries (69% *vs*. 20%); this increase is due to a change towards a western lifestyle (high-energy diet with reduced physical activity) [3].

T2DM occurs most commonly in adults aged 40 years or older and the prevalence of the disease increases with advancing age. Indeed, the aging of the population is one reason that T2DM is becoming increasingly common.

Mortality and complications

The excess global mortality in 2000 attributable to diabetes overall, most of which was attributable to T2DM, was 2.9 million (5.2%) deaths. In 2004 heart disease and stroke were reported on 68% and 16% respectively of diabetes-related death certificate in the USA. Furthermore, diabetes is the leading cause of blindness among adults and leads to around 44% of end-stage renal failure and 60% of non-traumatic lower-limb amputation in the USA [4].

Diagnosis

The American Diabetes Association (ADA) criteria for the diagnosis of diabetes are any of the following [5]:

> • an HbA1c level of 6.5% or higher; the test should be performed in a laboratory using a method that is certified by the National Glycohaemoglobin Standardization Program (NGSP) and standardized or traceable to the Diabetes Control and Complications Trial (DCCT) reference assay, **or**

> • a fasting plasma glucose (FPG) level of 126 mg/dL (7.0 mmol/L) or higher; fasting is defined as no caloric intake for at least 8 hours, **or**

> • a 2-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or higher during a 75-g oral glucose tolerance test (OGTT), **or**

> • a random plasma glucose of 200 mg/dL (11.1 mmol/L) or higher in a patient with classic symptoms of hyperglycaemia (ie, polyuria, polydipsia, polyphagia, weight loss) or hyperglycaemic crisis [1, 5].

If a patient has had 2 different tests and the results are discordant, the test that has a result above the diagnostic threshold should be repeated. A second abnormal result on this test will confirm the diagnosis.

In asymptomatic patients whose random serum glucose level suggests diabetes (>140 mg/dL), an FPG or HbA1c level should be measured. An FPG level of 100-125 mg/dL is considered an impaired fasting glucose (IFG), and an FPG level of less than 100 mg/dL is considered a normal fasting glucose. However, an FPG of 91-99 mg/dL is a strong independent predictor of future type 2 diabetes [6].

An HbA1c below 6% is considered normal glucose tolerance (using an assay that has been standardized to the DCCT normal range of 4-6%). An HbA1C of 6-6.4% is neither normal glucose tolerance nor diabetes. Binding of glucose to haemoglobin A is a non-enzymatic process

that occurs over the lifespan of a red blood cell, which is 120 days in average. Measurement of glycated haemoglobin thus reflect plasma glucose levels over the preceding 2-3 months. HbA1c measurements are the criterion standard for monitoring long-term glycaemic control.

Measuring concentrations of insulin or C-peptide, a fragment of proinsulin that serves as a marker for insulin secretion is rarely necessary to diagnose T2DM, but it is useful to understand β-cells functionality/failure. Insulin levels generally are high early in the course of T2DM and gradually wane over time. Fasting C-peptide level more than 1 ng/dL in a patient who has had T2DM for more than 1-2 years is suggestive of residual beta-cell function; stimulated C-peptide concentrations are preserved until late in the course of T2DM; absence of a C-peptide response to carbohydrate ingestion may indicate total beta-cell failure.

Signs and symptoms

Typical clinical manifestations include:
• classic symptoms: polyuria, polydipsia and polyphagia;
•blurred vision
•lower extremity paresthesias
•yeast infections.
The diagnosis of T2DM is readily entertained when a patient presents with classic symptoms. However, many patients with T2DM are asymptomatic or have symptoms so mild that they go annoticed and their disease remains undiagnosed for many years.

Etiology and pathophysiology

The etiology of T2DM appears to involve complex interactions between environmental and genetic factors. The disease develops when a diabetogenic lifestyle (ie, excessive caloric intake, inadequate caloric expenditure, obesity) is superimposed on a susceptible genotype.

Chronic fuel surfeit is the primary pathogenic event that drives the development of T2DM in genetically susceptible people. Many chronically overnourished and overweight or obese individuals, however, do not develop diabetes at all or develop it very late in life. They remain resistant to T2DM and safely deposite excess calories to subcutaneous adipose tissue (SAT) rather than to the heart, skeletal muscle, liver, and islet β cells, owing to the following mechanisms: successful islet β-cell compensation; maintenance of near-normal blood nutrient concentrations; development of minimal insulin resistance; increased expansion of SAT relative

to visceral adipose tissue (VAT); and limited increase in liver fat. In this way, key organs of the body avoid nutrient-induced damage (Figure 1) [7].

Susceptible overnourished individuals develop T2DM owing to the failure of these adaptive responses to safely dispose of the fuel surfeit. The following metabolic defects are crucial to the development of T2DM: inability of islet β cells to compensate for the fuel surfeit; increased glucagon secretion and reduced incretin response; impaired expansion of SAT and deposition of fuel surfeit in less healthy VAT and organs, causing tissue damage, hypoadiponectinaemia, and inflammation of adipose-tissue; increased endogenous glucose production and development of insulin resistance (Figure 1) [8].
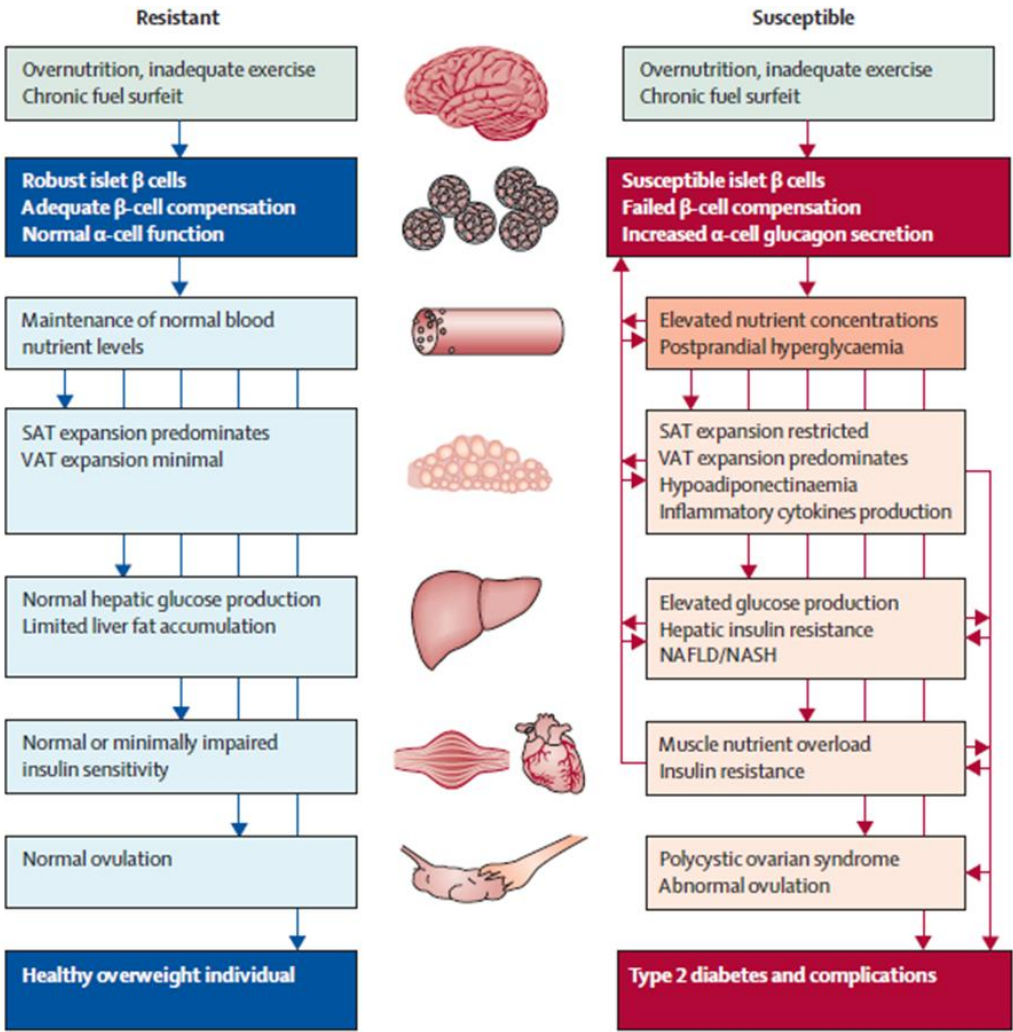

Figure 1. Pathway to T2DM and related complications. From Nolan, J.C et al, *Lancet* 2011.

Genetic factors

Many different susceptibility genes have been identified that interact with environmental factors during gestation, early childhood, and later in life.

The heritability of T2DM is high (estimated to be >50%), as indicated by the high concordance rates in monozygotic twins and the notably raised risk in individuals with affected first-degree relatives [9, 10]. Genome-wide association studies have identified more than 40

diabetes-associated loci. A greater number of these loci are associated with impaired β-cell function (KCNJ11, TCF7L2, WFS1, HNF1B, SLC30A8, CDKAL1, IGF2BP2, CDKN2A, CDKN2B, NOTCH2, CAMK1D, THADA, KCNQ1, MTNR1B, GCKR, GCK, PROX1, SLC2A2, G6PC2, GLIS3, ADRA2A, and GIPR) than impaired insulin sensitivity (PPARG, IRS1, IGF1, FTO, and KLF14) or obesity (FTO). Of these, TCF7L2 is the strongest susceptibility locus for T2DM, being associated with β-cell dysfunction [11]. Nevertheless, only around 10% of the heritability of T2DM can be explained by susceptibility loci identified so far, with each locus having a low effect size [12]. The remaining heritability might be related to a large number of less common variants (allele frequency <5%).

Environmental factors

Westernised lifestyle, which involves a high-energy diet and reduced physical activity, is indisputably linked to the pandemics of obesity and T2DM.

Micronutrient imbalances, including deficiency in concentrations of vitamin D, vitamin B12 in individuals replete with folic acid, and increased body iron stores have been implicated in the pathogenesis of T2DM [13-15].

Evidence also suggests that exposure to synthetic organic pollutants (eg, pesticides and plasticisers) affects endocrine cells and increases the risk of developing T2DM [16].

Increased use of technologies to reduce energy expenditure, including cars, and raised television viewingtimes, contribute to sedentary lifestyles, which are strongly associated with overweight, obesity, and T2DM. Low socioeconomic status and depression also affect risk [17, 18].

Major risk factors

The major risk factors for T2DM are the following:
- Age greater than 45 years
- Weight greater than 120% of desirable body weight
- Family history of T2DM in a first-degree relative (eg, parent or sibling)

- Hispanic, Native American, African American, Asian American, or Pacific Islander descent
- History of previous impaired glucose tolerance (IGT) or impaired fasting glucose (IFG)
- Hypertension (>140/90 mm Hg) or dyslipidemia (HDL cholesterol level < 40 mg/dL or triglyceride level >150 mg/dL)
- History of gestational diabetes mellitus
- Polycystic ovarian syndrome (which results in insulin resistance)

Pathobiology and molecular mechanisms

T2DM is characterized by a combination of peripheral insulin resistance and inadequate insulin secretion by pancreatic β-cells. Insulin resistance leads to decreased glucose transport into muscle cells, elevated hepatic glucose production, and increased breakdown of fat.

A role for excess glucagon cannot be underestimated; indeed, T2DM is an islet paracrinopathy in which the reciprocal relationship between the glucagon-secreting alpha cell and the insulin-secreting beta cell is lost, leading to hyperglucagonemia and hence the consequent hyperglycemia [19].

B-cells dysfunction

In human beings islet β cells are vulnerable to nutrient induced damage and, therefore, contribute notably to the development of T2DM. β-cells have to: maintain synthesis of proinsulin with correct posttranslational modification; ensure secretory granules be ready for secretion; sense nutrient concentrations in blood, mostly via intracellular metabolism with the production of nutrient-secretion coupling factors; sense other neurohormonal signals; and appropriately execute insulin granule release via activation of a complex exocytosis machinery. The mechanisms underlying β-cell failure, therefore, can be many, varied, and complex [20]. Islet β-cell dysfunction generally occurs when compensation is required for fuel excess. Nolan et al proposes that is the mix of β-cells susceptibility factors that determines the initial mechanism of damage, but that once substantial hyperglycaemia has developed, glucotoxic and glucolipotoxic mechanisms ensue in most patients, resulting in acceleration of the rate of failure [7, 21]. One factor peculiar to islet β-cells in human T2DM is their propensity to develop islet amyloid polypeptide deposits, but these probably play a part in disease progression rather than initiation [22]. Loss of 40−60% of β-cell mass has been seen in pancreas samples from people with impaired fasting glucose and T2DM, but less than 24% at 5 years after disease onset has also been reported [23]. Whether a subset of people with T2DM have predominant functional deficiencies in β-cells without loss of mass is unknown.

Glucagon secretion and incretin effect

Glucagon secretion and the incretin effect, which involves glucagon like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), are disturbed in T2DM [24]. Glucagon secretion is increased during fasting and fails to suppress after meals. This could be caused by impaired GLP-1 production (although the overall evidence for this is not strong) and reduced sensitivity of β-cells to gastric inhibitory polypeptide [25]. All these disturbances aggravate hyperglycemia, but are unlikely to be primary defects in the pathogenesis of T2DM. Gut hormones, including GLP-1, also have role in central nervous system (CNS) regulation of energy balance and appetite [26].

Neurohormonal weight control networks

Dysfunction of the mechanisms that control the body's energy balance and weight and cause overweight and obesity is of major importance in T2DM pathogenesis. This dysfunction occurs within the complex neurohormonal weight control network of the body in which central signals (from the brainstem and higher cortical centres, eg, cognitive, visual) and peripheral signals of energy stores (from adipose tissue eg, leptin) or related to hunger (from the gut, eg, ghrelin) and to satiety (from gut and pancreas, eg, vagal afferent neural signals, cholecystokinin, glucagon-like peptide 1 [GLP-1], insulin, and nutrient levels) feed into the hypothalamus and other key areas in the CNS to control appetite, physical activity, and bodyweight [27].

Adipose tissue and inflammation

Healthy white adipose tissue prevents nutrient spillover to other tissues and protects against metabolic disease. White adipose tissue in metabolic syndrome or T2DM is abnormal in multiple ways: distribution favours VAT; reduced adipocyte differentiation and adiponectin expression and secretion; suppression of lipolysis by insulin is impaired; increased expression and secretion of inflammatory cytokines (eg tumour necrosis factor α, interleukin-1β, and monocyte chemoattractant protein-1) and increased tissue inflammation (eg, macrophage infiltrates) [28].

The liver

Increases in endogenous glucose production, predominantly of hepatic origin, are a major determinant of fasting hyperglycaemia in T2DM. Mechanisms involved are increased supply of gluconeogenic substrate from peripheral tissues, an effect of raised concentrations of non-esterified fatty acid to activate hepatic gluconeogenesis, and the hepatic response to raised concentration of glucagon [29].

<u>Skeletal and cardiac muscle</u>

T2DM is a disease of relative inactivity and overnutrition with failure of the body to safely contain fuel excess. As discussed above, this failure can be explained by islet β-cell and adipose tissue deficiencies, with secondary contributions from the liver. Skeletal muscle inactivity (lack of exercise) certainly contributes to fuel surfeit, but this is not a consequence of insulin resistance [21]. Rather, insulin resistance is downstream from failure to contain the fuel surfeit. Skeletal muscle in individuals with T2DM is nutrient replete, or even nutrient overloaded, such that it responds with insulin resistance as protection against steatosis or metabolic stress of the tissue. Skeletal and cardiac muscles develop insulin resistance to divert excess nutrients to safe storage in adipose tissue [30].

<u>Hyperglycaemia and advanced glycation end products (AGEs)</u>

Chronic hyperglycaemia is a common feature of diabetes and is an important initiator of vascular complications, many of which have been related to vascular and inflammatory cell interactions with advanced glycation end products (AGEs) [31]. These heterogeneous, long-lived protein adducts are produced from a nonenzymatic chemical reaction between sugars and the amino groups of proteins. Circulating levels of AGEs, detected most commonly as hemoglobin A1C, are clinically important biomarkers used for monitoring diabetes therapy. On a pathophysiologic level, cellular interactions with AGEs induce biologic responses that have been linked directly to the development of diabetic vascular complications and that are mediated by specific cell-surface receptors. The best-studied AGE receptor is the Ig superfamily member receptor for AGE (RAGE), although limited studies suggest that scavenger receptors such as SR-A, SR-BI, and CD36 may also serve this function [32].

Diabetes complications

T2DM is associated with both microvascular, macrovascular and neuropathic complications. The duration of diabetes is significant because the chronic complications of diabetes are related to the length of time the patients has had the disease and to the severity.

Microvascular complications include retinal, renal and, possibly, neuropathic disease; diabetes neuropathy affects autonomic and peripheral nerves.

Macrovascular complications include coronary, cerebrovascular and peripheral vascular disease. Hyperglycaemia appears to be the determinant of microvascular complication, whereas macrovascular disease is less related to glycaemia, in fact improved metabolic control is associated with a significant reduction in the risk of developing microvascular complications, but with only a modest and barely significant reduction in cardiovascular disease (CVD) risk [33].

Macrovascular risk reduction is accomplished through controls of lipids and hypertension, smoking cessation and aspirin therapy.

As a matter of fact, several other factors, in addition to hyperglycaemia per se concur in determining accelerated atherosclerosis in diabetic patients [34].

Diabetes and cardiovascular disease

T2DM is a major cardiovascular risk factor. It is associated with accelerated atherosclerosis, clinically resulting in premature coronary artery disease (CAD), increased risk of cerebrovascular and severe peripheral vascular disease [35]. Patients with T2DM have a two-to four- fold increase in the risk of CAD and patients with T2DM but without previous myocardial infarction (MI) carry the same level of risk for acute coronary event as non-diabetic patients with previous history of MI [36]. DM patients also have a higher risk of cardiovascular complications and recurrent atherothrombotic events as well as worse cardiovascular outcomes following CVD complications [37]. CVD is the leading cause of premature mortality in patients with T2DM [38] The effect of diabetes on atherosclerosis is so pronounced that the benefit of female gender is eliminated in women with diabetes, who have an event rate similar to that of men with diabetes [39].

The increased CVD risk is independent of and additive to other CVD risk factors, such as hypertension, albuminuria, obesity, cigarette smoking and dyslipidemia, relative to nondiabetic patients with these comorbidities. Microalbuminuria is a common findings in T2DM and it is a risk factor for macrovascular (especially coronary heart) disease. A strong association has been reported between microalbuminuria and cardiovascular outcomes in patients with type 2 diabetes. An analysis of 3,498 patients with diabetes and 5,545 patients without diabetes in the Heart Outcomes Prevention Evaluation (HOPE) study found that microalbuminuria increased the adjusted relative risk (RR) of major cardiovascular events (RR 1.83, 95% CI 1.64–2.05) [40]. Participants with diabetes had a RR of 1.97 (95% CI 1.68–2.31) and those without diabetes had an RR of 1.61 (95% CI 1.36–1.90).

Aspirin selectively acetylates the hydroxyl group of a serine residue at position 529 (Ser529) of the cyclooxygenase-1 (COX-1) enzyme, thereby blocking platelet formation of thromboxane A_2 (TXA$_2$) and thus diminishing platelet aggregation mediated by thromboxane and prostaglandin endoperoxide (TP) receptors pathway. This effect is irreversible because platelets are anucleate and therefore unable to resynthesize COX-1. TXA$_2$ binds to TP receptors, which results in changes in platelet shape and enhancement of recruitment and aggregation of platelets.

Even with glucose levels at below the threshold used to define diabetes, impaired glucose metabolism and insulin resistance are linearly associated with increased CVD risk. Despite the increasing clinical appreciation of the incremental CVD risk associated with diabetes and the improved application of risk-modifying therapies, there remains a residual degree of CVD risk associated with T2DM [41]. An effective strategy for modifying CVD risk in patients with diabetes is the control of conventional risk factors, which seems to be more important than glycaemic control since the risk of developing macrovascular complication appears to be related to conventional risk factor rather than to glycaemic values. Treatment with statin and blood pressure control, with therapeutic targets more intensive for diabetic than for nondiabetic patients, are important in reducing CVD risk.

The ADA recommends that patients with diabetes who are at high risk for cardiovascular events receive primary preventive therapy with low-dose aspirin [42]. High risk for CV events is defined on the basis of the following risk factors: family history of CAD, cigarette smoking, hypertension, weight >120% of ideal body weight, microalbuminuria and total cholesterol >200 mg/dL. This evidence is further supported by the results of the Primary Prevention Project in which low-dose aspirin (100 mg/day) was evaluated for the prevention of CV events in individuals with one or more of the following: hypertension, hypercholesterolemia, diabetes, obesity, family history of premature CAD (n=4495). After a mean follow up of 3.6 years, aspirin was found to significantly lower the frequency of CV death (from 1.4 to 0.8%, relative risk [RR] 0.56 [CI 0.31-0.99]) and total CV events (from 8.2 to 6.3%, RR 0,77 [0.62-0.95]) [43].

However, aspirin has a paradoxical effect because it also inhibits prostacyclin synthesis by endothelial cells and platelets, which might be expected to favour thrombus formation. For this reason, low-dose aspirin is favoured for cardioprotection and this may be important in patients with diabetes, whose levels of prostacyclin are already reduced.

The abnormal metabolic state that accompanies diabetes is responsible for abnormalities in endothelial and platelet function, coagulation and fibrinolysis, and lead to oxidative stress and inflammation; these alterations may contribute to the cellular events that cause accelerated atherosclerosis and subsequently increase the risk of adverse cardiovascular events. Hyperactivated platelets at injured endothelial surfaces act together with an increased availability of thrombotic precursors, reduced coagulation inhibitors and diminished fibrinolysis [44]. The balance in normal haemostasis is shifted to favour thrombosis, leading to a prothrombotic phenotype and, as a consequence, increasing CV risk. Thus, T2DM is also associated with a hypercoagulable state (Figure 2).
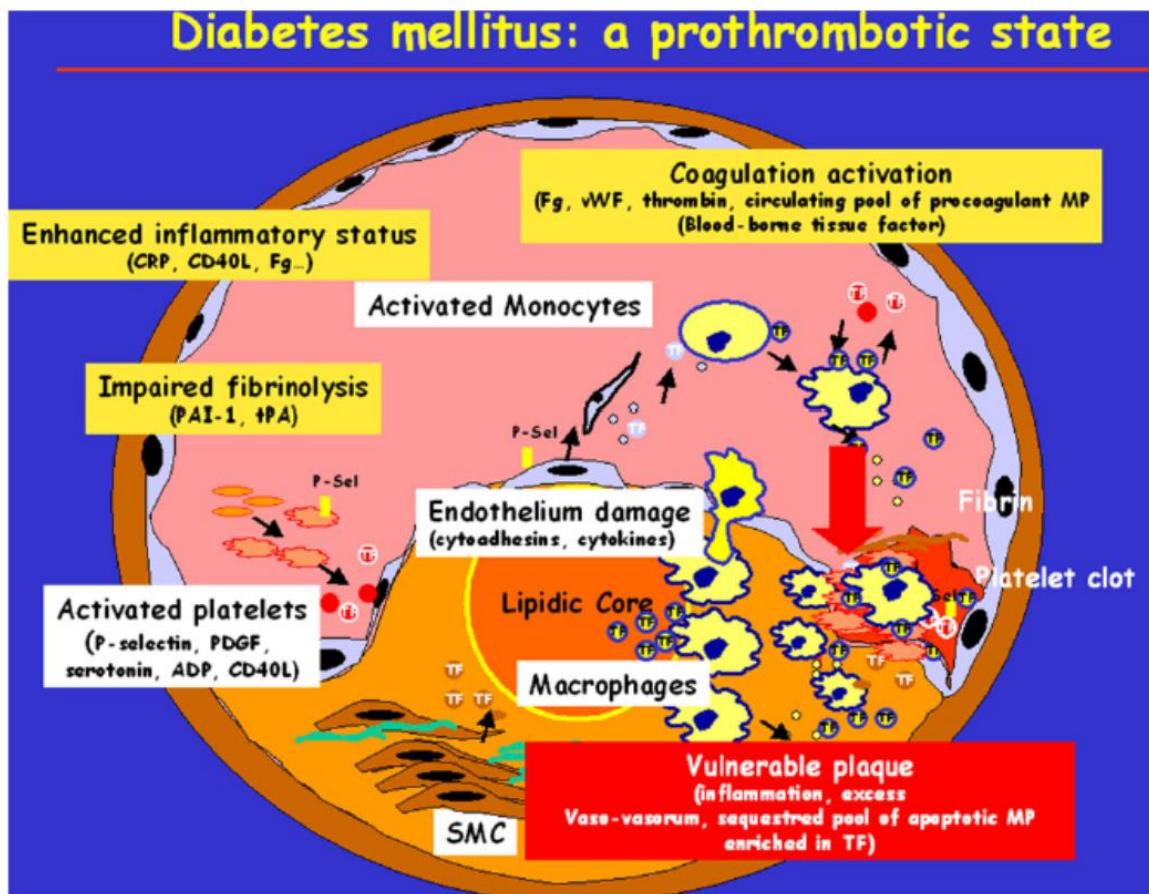
Figure 2. Possible pathways involved in the thrombotic state observed in T2DM. Numerous factors may contribute to the occurrence of a vulnerable blood in patients with diabetes. These include, but are not limited to, endothelial damage, monocyte activation, plaque neovascularization and inflammation and the shedding of procoagulant microparticles by stimulated or apoptotic cells. From Morel, O. et al., *Atherosclerosis* 2010.

Diabetic patients show increased plasma thrombin generation and a higher number of total microparticles, platelet-derived microparticles and Tissue Factor positive-microparticles, which are procoagulant microparticles, compared to controls [45, 46].

Patients with T2DM are characterized by increased levels of procoagulant factors such as thrombin-antithrombin III complex (TAT) and thrombin activation fragment 1+2 (F1+2), both thrombin generation and coagulation activation markers, plasminogen activator inhibitor-1 (PAI-1) and fibrinogen compared to healthy subjects [47, 48]. In diabetic patients plasma fibrinogen levels positively correlate with HbA1c and body mass index [49].

Moreover higher levels of fibrinogen, TAT, F1+2, and PAI-1 have been described in CAD patients with T2DM compared to CAD patients without T2DM [50]. Plasma fibrinogen, a readily measurable systematic inflammatory marker, appear to be an independent predictor for the severity of CAD in diabetic patients [51].

There is evidence for higher plasma levels of factor VII, factor VIII, factor XI, factor XII, kallikrein and von Willebrand factor (vWF) antigen in diabetes [52-55].

An increased clot firmness at the thomboelastometric assay has been reported in diabetic patients compared to healthy subjects, indicating an increased global haemostatic function in T2DM [56]. Fibrin clot formation is the final step in the atherothrombotic process and the structure of the fibrin network may predict a predisposition to cardiovascular events. Both CAD and T2DM are associated with altered fibrin clot properties and increased resistance to fibrinolysis [50]. T2DM is associated with prothrombotic changes in fibrin clot properties in patients with CAD; these patients have more compact, denser and less porous clot structure with prolonged lysis time compared with CAD patients without T2DM, despite treatment with aspirin. The change in fibrin clot properties are related to quantitative rather than qualitative changes in fibrinogen, with an additional possible influence of inflammatory protein since levels of fibrinogen, C-reactive protein and complement C3 are significantly increased in patients with T2DM compared with patients without T2DM [57]. Complement C3, a key inflammatory protein, has been shown to modulate fibrinolysis, particularly in patients with diabetes [58].

By sharing classic risk factors with atherosclerosis, T2DM may also share a common pathway, which is the inflammatory pathway. Hyperglycaemia itself, a characteristic of glucose intolerance, is related to the synthesis of inflammatory markers such as interleukin 6 (IL-6) and interleukin 18 (IL-18), with serum level variations positively correlated and with more significant increases in hyperglycaemic spikes, a situation that is common in diabetic patients [59]. Yudkin et al. demonstrated that CRP, IL-6 and TNF-α levels are positively correlated with measurements of insulin resistance [60]. Experimental studies show that IL-6 interferes with insulin synthesis in pancreatic β cells and reduces insulin-stimulated glycogen synthesis in hepatocytes [61]. Atheroma plaques of diabetic patients with coronary syndrome exhibit a larger content of lipid, thrombosis and macrophage infiltration than the coronary tissue from patients without diabetes [62]. This high proportion of infiltrated inflammatory cells suggests not only that inflammation plays a key role in atherosclerosis, but also that the proinflammatory state is more active in patients with diabetes. Souza et al. found that CRP is 2.6 fold higher in diabetic patients with acute coronary syndrome (ACS) compared to patients with ACS without diabetes. There is also a trend of increased CRP levels in diabetic patients with chronic coronary disease when compared with non-diabetic patients with chronic coronary disease, thus signaling a high inflammatory activity in diabetic patients even in the stable phase of coronary artery disease [63]. Initially described as a marker of the inflammatory response, an active role of CRP as a mediator of atherogenesis has been demonstrated in recent studies. CRP induces apoptosis of endothelial cells, inhibits angiogenesis and stimulates transcription of several genes of

proinflammatory cytokines. On the other hand, it stimulates endothelin-1 and IL-16 release, leading to an increased expression of adhesion molecules and chemokines involved in the recruitment of inflammatory cells such as monocyte chemoattrant protein 1 (MCP-1), that has a function in the development of atherosclerosis by recruiting monocytes into the subendothelial cell layer.

There is considerable evidence that hyperglycaemia results in increased generation of reactive oxygen species (ROS), leading to increased oxidative stress in several tissues. This in turn, activates stress-sensitive intracellular signalling pathways leading to the expression of gene products that cause cellular damage and are ultimately responsible for late complications of diabetes [64]. Indeed, circulating level of ROS could play a pivotal role in diabetes-related CVD. Excessive formation of ROS can affect endothelial NO production, leading to endothelial dysfunction, a common feature in atherosclerosis; in diabetic vessels, the bioavailability of NO is markedly reduced with a concomitant increase of superoxide (mainly derived from NAD(P)H oxidase) [65, 66]. So patients with diabetes have early development of abnormal endothelial function, resulting in an aggressive form of atherosclerosis and adverse arterial remodelling [67].

Furthermore, hyperglycaemia-induced oxidative stress is responsible for enhanced peroxidation of arachidonic acid to form biologically active F2-isoprostanes [68]; among them, an increased formation of 8-iso-prostaglandin (PG) $F_{2\alpha}$, a nonenzymatic oxidation product of lipid peroxidation on cell membranes and circulating low-density lipoprotein (LDL) which induces vasoconstriction and modulates platelet function [69], has been demonstrated in diabetes [70]. This suggests that enhanced peroxidation of arachidonic acid to form biologically active isoprostanes may represent an important link between impaired glycaemic control and persistent platelet activation in this setting. Moreover Davì et al. demonstrated enhanced thromboxane (TX) biosynthesis in T2DM patients and provided evidence for its platelet origin [71]. Thus, diabetes is associated with increased oxidative stress leading to enhanced lipid peroxidation and platelet activation, both likely contributing to accelerated atherosclerosis.

Management

Diabetes mellitus is a chronic disease that requires long-term medical attention to limit the development of its devastating complications and to manage them when they do occur.
The clinical management of established T2DM involves optimum control of factors that cause complications, such as blood glucose and lipid concentrations, blood pressure, bodyweight, and smoking, as well as regular screening for and appropriate management of microvascular (eye,

renal, and neural) and macrovascular (coronary, cerebral, and peripheral) complications. Local practice guidelines, such as the ADA Clinical Practice Recommendations 2011, should be used:

1. Individualized glycemic targets and glucose lowering therapies

2. Diet, exercise and education as the foundation of the treatment program

3. Use of metformin as the optimal first-line drug unless contraindicated

4. After metformin, the use of 1 or 2 additional oral or injectable agents, with a goal of minimizing adverse effects if possible

5. Ultimately, insulin therapy alone or with other agents if needed to maintain blood glucose control

6. Where possible, all treatment decisions should involve the patient, with a focus on patient preferences, needs, and values

7. A major focus on comprehensive cardiovascular risk reduction (preventive therapy woth low dose aspirin).

Approaches to prevention of diabetic complications include the following:
- HbA1c every 3-6 months
- Yearly dilated eye examinations
- Annual microalbuminuria checks
- Foot examinations at each visit
- Blood pressure < 130/80 mm Hg, lower in diabetic nephropathy
- Statin therapy to reduce low-density lipoprotein cholesterol.

Since platelet activation and aggregation, which result enhanced in T2DM, play a key role in thrombus formation, treatment strategies have focused on antiplatelet agents to prevent or treat CAD in diabetic patients.

All the abnormalities described in T2DM patients lead to a high trombogenicity and a consequent low responsiveness to antithrombotic treatment. Current antiplatelet therapies appear less effective in T2DM patients as compared to non-diabetics with a similar risk profile, both in primary and secondary CV prevention [72, 73]. This suggests a disease-associated change in platelet responsiveness to current antiplatelet strategies, including aspirin (ASA) and thienopyridines, or the presence of additional, T2DM-specific mechanisms not targeted by current antiplatelet drugs, contributing to thrombus formation (e.g., haemostatic adhesive molecules such as von Willebrand factor, TF-related coagulation pathways, inflammatory or oxidative mechanisms).

Moreover, despite their dysregulated glucose metabolism, intensive glycemic control has proven insufficient to reduce thrombotic complications.

Platelet biology

Platelets, also defined thrombocytes, are discoid cell fragments without nucleus originated from the cytoplasmic fragmentation of their precursors cells, megakaryocytes, in the bone marrow. Platelets are very small, their thickness is 1 μm and their maximum diameter is 4 μm. Megakaryocytes are rare myeloid cells that reside within the bone marrow and represent less than 0,1% of the myeloid cells [74]; they might be found, in adults, also in the lungs and the peripheric bloodstream. Thrombopoietin (TPO) controls thrombocytopoiesis by binding to the c-Mpl receptor; interleukins (IL) such as IL-3, IL-6 and IL-11 modulate thrombopoiesis but are not fundamental as TPO for megakaryocyte maturation and for maintenance of platelet mass.

Megakaryocytes tailor their cytoplasm and membrane systems for platelet biogenesis. Before having the capacity to release platelets, megakaryocyte enlarges considerably to an approximate diameter of 100 μm and fills with high concentrations of ribosomes which allow the production of platelet-specific proteins. Cellular enlargements is mediated by repeated cycles of endomitosis, a process that amplifies the DNA by as much as 64-fold. Platelet release by megakaryocytes involves the development of cytoplasmic ramifications, the so called "pro-platelets", of 100-500 μm of length. This process starts from a single site on megakaryocyte with the emission of pseudopodal extensions that stretch into pro-platelets. In the final portion of the pro-platelet the bundles of microtubules form loops that come out and fall in pro-platelet causing the formation of bulbous tips of 3-5 μm in diameter [75]. The individual platelets are then released from the ends of pro-platelets into the bloodstream (Figure 3).

In human physiological condition, 10^{11} platelets are produced every day and their average lifespan is about 8-10 days; platelet count in peripheral blood is approximately 250.000/μl. Senescent platelets are removed from the bloodstream by spleen and liver. The rate of platelet production may change in pathologic condition: a platelet count less than 100.000 platelets/μl is defined as thrombocytopenia and can cause increased risk of bleeding; an increased platelet count, higher than 600.000 platelets/μl, is defined as thrombocytosis and leads to increased risk of thrombotic events.
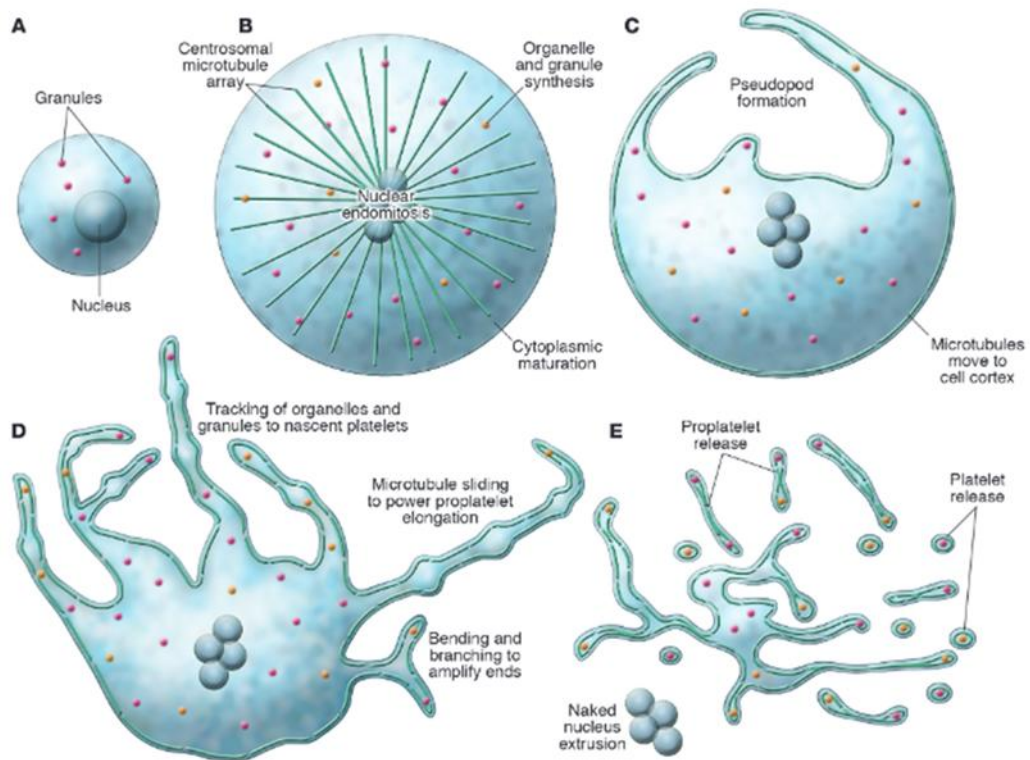
Figure 3. Schematic overview of platelets release by megakaryocytes. (B) Megakaryocytes maturation is characterized by repeated cycles of endomitosis and by a significant maturation of the cytoplasm with the formation of internal membranes, granules and organelles while the microtubules extend (C). Shortly before the formation of pro-platelets, microtubules are consolidated into a mass beneath the plasma membrane and blend in linear beams thicker branches that fill pro-platelets determining the elongation (D). In the terminal portion of the pro-platelets, the bundles of microtubules form loops that come out and fall in pro-platelets causing the formation of bulbous tips. (E) The entire cytoplasm of megakaryocytes is converted into pro-platelets that are released from the cell. The individual platelets are then released from the ends of pro-platelet.

Newly formed platelets are larger and contain an increased amount of megakaryocyte-derived m-RNAs and proteins, express more cyclooxygenase-2 (COX-2) and higher levels of adhesive protein on their surface. There are evidences suggesting that these young platelets, called immature or reticulated platelets, are more reactive [76].

Structurally, platelets consists of:

- A plasma membrane rich in phospholipids including phosphatidylserine and phosphatidylinositol, negatively charged and abundant in the cytoplasmic side of the membrane where they act as a substrate for the phospholipase. The glycocalix is rich in glycoprotein, some of which perform receptor functions in response to stimuli and agonists, giving to the platelets the ability to convey information and to give rise to a cellular response.

- Internal membranes formed by the open canalicular system and by the tubular dense on which the enzymes involved in the synthesis of prostanoids are anchored.

- A cytoskeleton composed of microtubules and microfilaments, which take part in the process of platelet activation and secretion.

- Mitochondria, lysosomes and peroxisomes.

- Secretory granules: lysosomes, dense granules (also referred to as delta granules) and alpha granules. The first contain hydrolytic enzymes such as elastase, which may affect the vessel wall and thus promote atherosclerosis. The second contain ADP, calcium, serotonine, the latter contain P-selectin, glycoprotein IIb/IIIa, CD40L, von Willebrand factor, factor V, factor X, growth factors, fibrinogen, thrombospondin, platelet factor-4 (PF-4), interleukin 1, beta thrombomodulin and PAI-1.

The content of these granules is secreted during platelet activation.

Platelet functions

Blood platelets are key players in vascular homeostasis and coagulation [77].

For long time it was believed that the contribution of platelets in atherothrombotic disease was confined only to thrombus formation. In recent years, the knowledge about platelet functions and activities has changed. We were used to think that platelets are short-acting cells when organized into a clot, but today we know that platelets are long-lived and can mediate cell-cell interactions for many hours after initial activation. Platelets are no more considered key player only in thrombus formation occurring upon plaque rupture, but they also play an important role in the inflammatory process, contributing in the early stage of plaque development by releasing numerous cytokines (IL-1, CD40L, beta-thromboglobulin, etc.) chemokines (RANTES, PF4, etc.), growth factors (PDGF, TGF, EGF, etc.) and adhesion proteins (fibrinogen, fibronectin, von Willebrand factor, etc.).

Platelets have an important physiological role in primary haemostasis and they are involved in endothelial damage repair to arrest bleeding after a tissue or vascular damage. Glycoprotein complexes (GP) Ib/V/IX on platelet membrane, by binding specific factors such as von Willebrand, mediate adhesion at the site of injury and platelet activation. Several mediators (ADP, thrombin and thromboxane A_2) amplify platelet response by activating specific receptors. The effect of these agonists leads to reduced cAMP and increased intracellular Ca^{++}, activation of GPIIb/IIIa which binds to fibrinogen, activation of phospholipase A2 and consequent increase of acid arachidonic to form thromboxane A2, degranulation and aggregation. Although platelet adhesion and activation represents a physiological response to repair also a fissured plaque, this process, by mechanisms of amplification, may lead to thrombus formation and vessel occlusion causing clinical events as ischemia or myocardial infarction.

Platelet transcriptome

Platelets, although they do not have a nucleus, contain between 2000 and 7000 transcripts derived from megakaryocytes. Besides this, they have the translational capacity necessary for protein synthesis [78], thus they have the ability of de novo protein synthesis through translation of megakaryocyte-derived mRNA. In 2011 Cecchetti et al. provided the evidence that the transcriptome is derived from megakaryocytes through a very well controlled mechanism since not all mRNAs are transferred to platelets, depending on physiological and pathological conditions [79].

Analysis of platelet transcriptome from healthy individuals identified mRNAs that encode for surface receptors and glycoproteins, as well as for proteins involved in metabolism, signalling, inflammation, and immunity [80, 81]. Functional genomic studies have allowed identification of gene previously unknown and attribution of a function such as gene that encodes for platelet-endothelial aggregation receptor-1 (PEAR-1), associated with platelet aggregation response to multiple agonists [82]; Goodall et al. found that COMD7 and LRRFIP1 genes show evidence of genetic association with myocardial infarction and in follow up functional studies LRRFIP1 demonstrated evidence for effects on thrombus formation and interaction with platelet cytoskeleton [83].

In response to activation platelets can use their transcriptome to perform new protein synthesis, thus modifying their proteome and, as a consequence, their functions [84]. Specific mRNAs may vary in clinical conditions such as sickle cell disease [85], ST-elevation myocardial infarction [86] and NSTE-ACS [87]. In addition, it has been recently shown that platelets harbour an abundant array of microRNAs (31), and some of them have been found to coordinate with reactivity to specific agonists and to pathological states [88]. Thus, anucleate platelets may make use of post-transcriptional gene regulation for their morphology and physiologic functions, and microRNAs may play a significant role by binding to their target mRNAs.

Platelet and Tissue Factor

Tissue factor structure

Tissue Factor (TF), or thromboplastin or CD142, is a 47 KDa trasmembrane glycoprotein belonging to the cytokine-receptor superfamily and consisting of 263 aminoacids organized into a 219 aminoacids extracellular domain, a transmembrane region of 23 aminoacids and a short 21 aminoacids intracellular tail (Figure 4) [89]. The extracellular domain contains a catalytic site responsible for the binding with factor VII/VIIa (FVII/FVIIA) binding domain; the intracellular region could undergo serine phosphorylation, which could modify its function, for example the

cytoplasmic region negatively regulates TF expression, which is mediated by suppressed Erk1/2 phosphorylation.
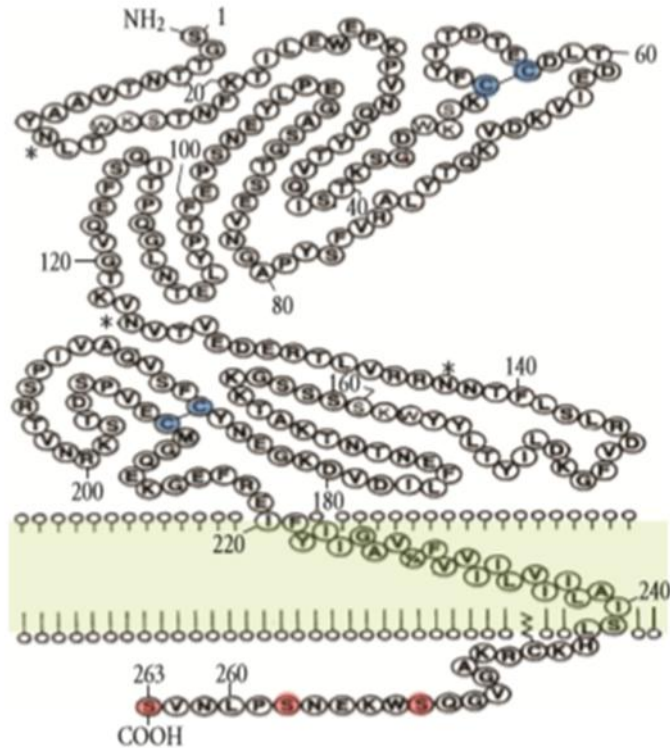
Figure 4. Tissue factor structure. The 47-kDa membrane bound single polypeptide chain consists of extracellular, transmembrane and cytoplasmic domains. There are two intrachain disulfide bridges, shown in blue, in the extracellular region which also contains factor VII/factor VIIa binding domain. There are three serine residues, shown in red, in the cytoplasmic domain for undergoing phosphorylation.

Tissue Factor role

TF is currently considered a major player in blood coagulation and thrombotic complications of atherosclerosis [90]. TF triggers the extrinsic blood coagulation pathway, which proceeds as calcium-dependent extracellular signalling to sequentially activate zymogens factor VII, factor X (FX) and prothrombin for the generation of FVIIa, FXa and thrombin respectively. As a result, thrombin cleaves off fibrinogen into fibrin monomers that cross-link to produce insoluble blood clot (Figure 5). Thus, TF is involved in thrombus formation and stabilization. The complex TF-FVII can be activated by circulating FVIIa (only 1% of FVII present in the bloodstream circulates in the activated form) or by the TF-FVIIa using a self-triggering mechanism or through a positive feedback activation by other proteases (Xa, IXa, thrombin).
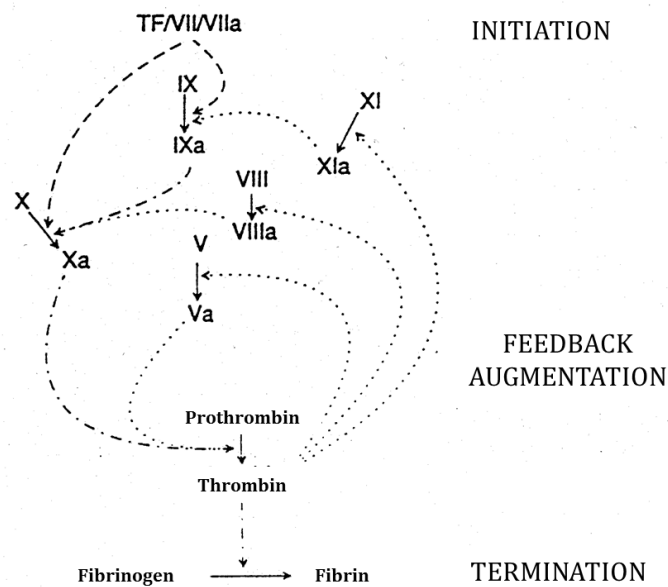
Figure 5. TF-depending pathway of coagulation cascade.

The physiological substrates of this complex are FIX and FX, which bind reversibly to anionic phospholipids of the membrane. FX can be activated by TF-FVIIa complex or by the complex IXa-VIIIa-phospholipid-Calcium. The factor IXa is assembled on membrane phospholipids and, in the presence of its cofactor, factor VIIIa, catalyzes the activation of FXa that induces platelet activation and fibrin clot formation [91]. The anionic membrane phospholipids, such as phosphatidylserine, play an important role in the beginning of TF-dependent coagulation, promoting both TF-FVIIa complex and the binding of FIX and FX.

The only physiologic inhibitor of the TF-FVIIa complex is the tissue factor pathway inhibitor (TFPI), a serine protease synthesized mainly by the vascular endothelium. A small percentage of TFPI is found also in platelets or in plasma, free or associated with lipoproteins.

Although the main physiologic function of TF is the regulation of the processes of haemostasis and thrombosis, emerging evidence showed a broad spectrum of biological function for TF. Indeed it is involved in cardiovascular complications [92], angiogenesis [93], tumor metastasis [94], wound repair, embryonic development, cell adhesion/migration and in autoimmune disorders. In condition of hyperglycaemia, such as T2DM, the increased circulating advanced glycation end products (AGEs) lead to an increased expression of Tissue factor [52] and it can assume a pathogenic role in diabetic progression related to inflammatory process being responsible for insulin resistance [95].

While the binding of TF with FVII on the extracellular surface initiates the extrinsic pathway of coagulation, some signalling pathways are also triggered intracellularly. The initial stages of signal transduction are still little known. TF does not behave like a receptor but it causes a conformational change in FVIIa that activates a membrane protein, probably the receptor protease activated receptor-2 (PAR-2) [96]. PAR-2 is a G-protein coupled receptor; its activation leads to phosphorylation of kinases giving rise to a phosphorylation cascade that lead to the activation of specific metabolic pathway. The TF-FVIIa complex formation can also induce mobilization of intracellular calcium after the activation of Gprotein/phospholipase C, activation of small G proteins such as Rac, phosphorylation of tyrosine kinases of the Src family and activation of phosphatydilinositol 3 kinase (PI3K) [97]. PI3K phosphorylates Akt, responsible for the cytoskeleton reorganization and cell migration- PI3K also triggers, by Rac, the phosphorylation of mitogen activated protein kinase (MAPK) that, once translocated to the nucleus, promotes the expression of immediate early genes; the genes activated by TF encode for transcriptional factors, growth factors, receptors, regulators of cellular motility and some cytokines.

The intracellular domain of TF can interact directly with the cytoskeleton through the protein filamin-1, forming a protein complex involved in the remodelling of actin filaments, responsible for morphology and cell motility [98].

TF is expressed in subendothelial cells such as in smooth muscle cells and fibroblasts; endothelial cells do not express TF under physiological conditions but it may be stimulated by various cytokines. TF has been shown to be present in atherosclerotic plaques [99]; vessel-wall associated TF, however, does not entirely explain the thrombogenic potential of vascular lesions when they are exposed to flowing blood. It has been proposed that thrombus growth might be promoted by circulating (ie, microparticles or platelet-associated) TF [100].

Platelet-associated Tissue Factor

At the beginning of 2000, Rauch et al. proposed that thrombosis occurring upon a plaque rupture does not necessarily require the exposure of vessel wall-TF [101]. We now know that also platelets are a source of active TF, the so called "blood-borne-TF" or "circulating TF", which can sustain the activation of blood coagulation cascade on the edge of a growing thrombus. In the 2000, Rauch and Nemerson published the finding that TF-positive microparticles (MPs) released by activated monocytes were up taken by activated platelets through a CD15-P-selectin transfer mechanism. The authors suggest that platelet-associated TF, resulting from TF-positive MPs transfer, is an alternative source of procoagulant activity [102].

In 2003 Camera et al [103] and Muller et al [104] showed the evidence that human platelets from healthy donors express TF antigen, using different approaches (western blotting, confocal and immune electron microscopy). Moreover, Camera and colleagues reported, by using flow cytometry, that platelet activation results in the expression of TF besides other platelet activation markers such as P-selectin, on platelet surface. The presence of TF protein and its functional activity was further confirmed showing the bindings of this protein to its physiologic ligand FVIIa and the capacity to generate FXa.

In the same year, TF mRNA was found in a sample of resting platelets depleted from leukocytes contamination, suggesting that platelets could perform de novo synthesis [103].

Three years later, two independent group provided the evidence that platelet activation results in the translation of TF mRNA, increasing the membrane-bound TF protein [105, 106].

At present, published data suggest that three are the possible mechanisms responsible for the presence of TF in human platelets: 1) the transfer from TF-positive MPs released by activated endothelial cells or leukocytes; 2) the storage of TF protein within the alpha granules [99]; 3) de novo synthesis from specific TF mRNA. All these mechanisms are not mutually esclusive (Figure 6).

As stated above, studies recently carried out in our laboratory showed that in vitro platelet activation by classical agonists results in expression of functionally active TF on the cell membrane [103]. Furthermore non-ST elevation ACS (NSTE-ACS) patients have a greater number of TF positive platelets and platelet-monocyte aggregates than stable CAD patients or healthy subjects, providing additional insight into the prothrombotic potential of CAD platelets [107].

The finding that both TF mRNA and protein are significantly higher in platelets from NSTE-ACS patients than in stable CAD patients supports the idea that platelets from stable CAD and ACS patients are potentially preconditioned to a different degree of reactivity on the transcriptional level [87, 107].

In conclusion, platelet activation not only results in thrombus formation, but also leads to de novo protein synthesis through rapid and highly regulated translation of pre-existing megakaryocyte-derived mRNAs [108]. Although specific changes in the expression/distribution or amount of platelet mRNAs and proteins are associated with CAD, no information is still available on the changes that may occur in CAD with or without T2DM.
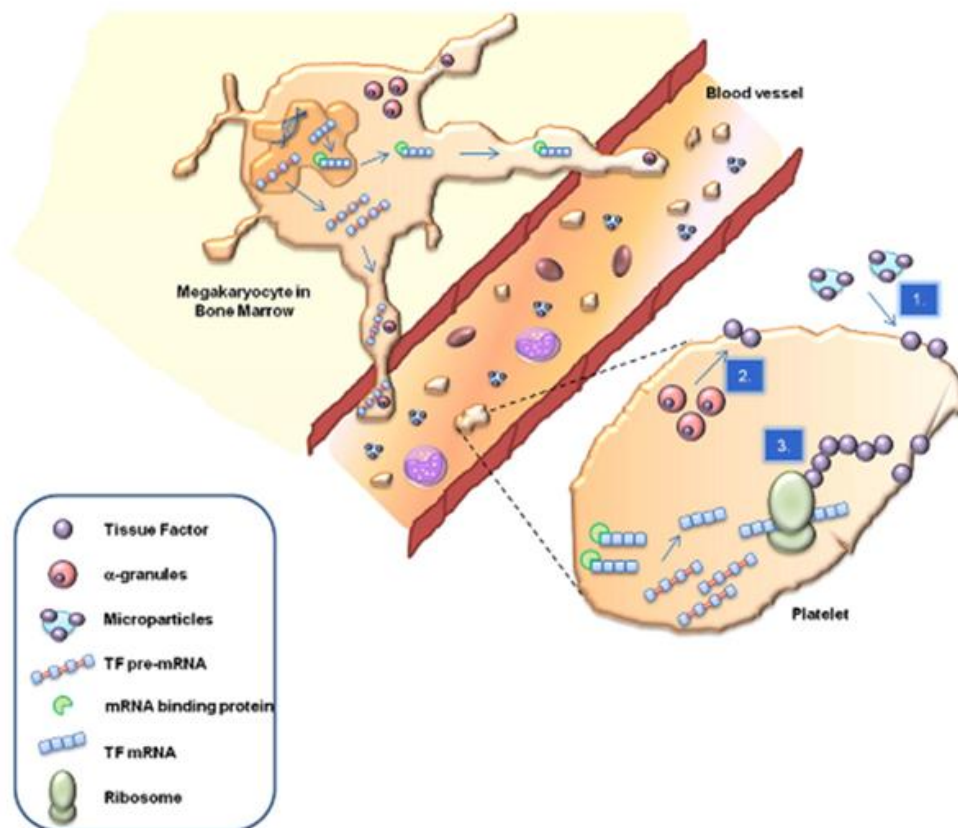
Figure 6. Schematic view of the three possible mechanisms responsible for the presence of TF in human platelets.

Contribution of platelets to atherothrombosis

Atherogenesis

Atherosclerosis is the leading cause of morbidity and mortality in the industrialized word. Atherosclerosis is a diffuse process that starts early in childhood and progresses, mostly in the absence of symptoms, throughout the lifespan. This systemic arterial disease preferentially affects the intima of large- and medium-sized systemic vessels, including the carotid, aorta, coronary, and peripheral arteries. As a long-term process, atherosclerosis progression results in atherothrombosis; the principal clinical manifestations are sudden cardiac death, myocardial infarction (MI), ischaemic stroke and peripheral arterial ischemia. These events are mostly secondary to atherosclerotic plaques disruption and subsequent thrombus formation [109].

The main components of atherothrombotic plaques are [110]:

- connective tissue extracellular matrix such as collagen, proteoglycans, and fibronectin elastic fibres;
- crystalline cholesterol, cholesteryl esters, and phospholipids;
- cells such as monocyte-derived macrophages, T-lymphocytes, and smooth-muscle cells;
- thrombotic material with platelets and fibrin deposition.

Furthermore, atherothrombotic events result from a complex inflammatory response to a multifaceted vascular pathology. Key inflammatory factors in atherothrombosis include activated endothelial cells; inflammatory leukocytes (which are a source of thrombogenic stimuli); smooth muscle cells (which act as a source of procoagulants and an amplifier of inflammatory responses during thrombosis); and platelets (which act as an important source of further inflammatory mediators and are involved in thrombus formation by aggregating) [110].

The endothelium is a dynamic autocrine and paracrine organ which covers the inner surface of all blood vessels. Important bioactive substances such as nitric oxide, prostaglandins, endothelin, and angiotensin II, regulating blood vessel function and structure, are synthesized by endothelial cells. Endothelial dysfunction is a systemic, reversible disorder considered as the earliest pathological process of atherosclerosis [111]. It is defined as the partial or complete loss of balance between vasoconstrictors (endothelin, angiotensin II) and vasodilators (nitric oxide, prostacycline), growth-promoting and -inhibiting factors, proatherogenic and antiatherogenic factors, and procoagulant and anticoagulant factors. It is involved in the recruitment of inflammatory cells into the vessel wall and in the initiation of atherosclerosis. Endothelial cells produce cytokines, express adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and selectins, and assist leukocytes and other blood-derived cells in atheroma infiltration. A dysfunctional endothelium facilitates vessel wall entry and oxidation of circulating lipoproteins, monocyte entry and internalization or inflammation, smooth-muscle cell proliferation and deposition of extracellular matrix, vasoconstriction, as well as a prothrombotic state within the vessel lumen.

Activation of the arterial endothelium may arise from a number of cardiometabolic derangements, including oxidative stress (e.g. from smoking or formation of advanced glycation end products in the setting of hyperglycaemia), mechanical stress due to hypertension, or reduced endothelial function secondary to insulin resistance.

The activated endothelium expresses cell adhesion molecules (CAMs), such as ICAM and VCAM, or proteins from the selectin family such as E- or P-selectin, which bind monocytes loosely to the endothelial surface. The chemotactic factor monocyte chemoattractant protein-1

(MCP-1) mediates tight binding of monocytes and infiltration into the vessel wall where they readily take up lipids. The interaction of low-density lipoprotein (LDL) with proteoglycans is of major concern in early atherosclerosis; intravascular accumulation of LDL leads to chemical modification (oxidation) and induction of inflammation. Monocytes then differentiate into macrophages under the influence of macrophage colony-stimulating factor and other chemokines. These macrophages, in turn, become foam cells, and then break down to form fatty streaks, thus providing the beginnings of the lipid core of the mature atherosclerotic plaque. Typically, this accumulation of lipids drives the growth of the atherosclerotic plaque. However, the inflammatory cells that invade the arterial wall during atherogenesis also secrete a range of inflammatory substances and growth factors which profoundly influence the properties of the arterial wall. Thus, in the later steps of atherosclerosis development, proliferation of smooth muscle cells and deposition of collagen contribute increasingly to the overall formation of the plaque. The contents of the plaque are contained within a collagen-rich fibrous cap which stabilizes the plaque and prevents access of its thrombogenic core to the bloodstream. This cap itself is continually remodelled, with simultaneous removal and replacement of collagen. Clearly, any reduction in the strength of the fibrous cap during this process may increase the likelihood of plaque rupture, which is believed to be the most common precipitating event for coronary thrombosis and MI, and an important cause of unstable angina pectoris where coronary occlusion is incomplete. One key event besides inflammation in atherosclerotic progression is the production of reactive oxygen species, which lead to oxidative stress.

The role of platelets

Platelet activation plays a pivotal role in the pathogenesis of atherogenesis and its thrombotic complications and increased in vivo platelet activation has been reported in patients with coronary artery disease (CAD) [112]. The presence of platelet-derived chemokines and growth factors has been observed in atherosclerotic plaque [113]; a relation between platelet activation and carotid wall thickness [114] and increased urinary excretion of thromboxane metabolites in association with the main cardiovascular risk factors which accelerate atherogenesis have been reported [71, 115, 116].

Platelets are activated upon stimulation with arachidonic acid, adenosine diphosphate (ADP), thrombin, advanced glycation end products, or when in contact with surface molecules of the subendothelial layer. Although activation is caused by a variety of substances, platelets respond with the same series of distinguishable actions: a) change of shape from a discoid to a pseudopodial structure; b) aggregation with platelets and other blood-derived cells (leukocytes);

c) release of substances from the three secretory granules; and d) liberation of arachidonic acid, which is rapidly converted to prostaglandins and lipoxygenase products [117].

The interaction between a platelet-activating agonist and its receptor causes rapid mobilization of signalling molecules within the platelet, mainly calcium, diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP3), which are sufficient to initiate and complete shape change and aggregation responses.

Platelets are involved in the very early phase of atherothrombosis. Endothelial inflammatory conditions lead to an activation of the endothelial monolayer, to which platelets adhere. Platelet adherence occurs through several steps involving platelet tethering, rolling, and finally firm adhesion to the vessel wall [118]. These processes involve selectins, integrins, and immunoglobin-like receptors, which induce receptor-specific activation signals in both platelets and endothelial cells. Selectins mediate the rolling of platelets on the endothelial surface and are present on the platelet surface as well as on the endothelial cell. Platelets interact with the inflamed endothelium with an endothelial ligand via P-selectin. Moreover, P-selectin expressed on activated platelets triggers the formation of platelet-leukocyte aggregates and the recruitment of leukocytes to the endothelium surface [119].

The GPIIb/IIIa receptor plays a key role in platelet accumulation on activated endothelium, as it is the major integrin on platelets.

Activated platelets show an increased adhesiveness and aggregation in response to collagen, thrombin, and platelet-activating factor. Thus, abnormalities in platelet function may exacerbate the progression of atherosclerosis and the consequences of plaque rupture. Platelet activation may lead to increased microembolism in the capillaries and local progression of pre-existing vascular lesions [120].

Patients with diabetes mellitus frequently have hypercoagulable blood, as evidenced by increased plasmatic procoagulant factors, depressed fibrinolysis, reduced endothelial thromboresistance, and platelet hyperreactivity.

Summarizing, in atherothrombosis platelets contribute to the amplification of atherosclerotic lesions, to thrombogenesis and to distal embolization into the microvasculature.

Stable angina is associated with smooth, fibrous coronary artery plaques, whereas unstable angina, acute MI, and sudden cardiac death are invariably associated with irregular or ruptured plaques. In the coronary arteries, small plaques tend to be lipid-rich and prone to disruption. Markedly stenotic plaques tend to be fibrotic and stable. Features of vulnerability are: a large lipid core, a thin fibrous cap, and an inflammatory filtrate rich in monocytes and macrophages. Physical forces may disrupt the thin foam-cell-infiltrated cap. Vulnerability of the plaque

depends on several factors, including circumferential wall stress or cap fatigue, blood-flow characteristics, location, size, and consistency of the atheromatous core.

The atherosclerotic process in patients with diabetes mellitus in not really distinct from that in nondiabetic people. However, it starts earlier and progresses faster [121].

Altered platelet functions in type-2 diabetes mellitus

Platelets from diabetic patients are characterized by a variety of abnormalities including dysregulation of several signalling pathways, both receptor and intracellular downstream signalling, higher expression of adhesion molecules on the surface as well as increased release of vasoconstrictors, which lead to increased platelet reactivity [122]. The etiology of these abnormalities is attributed to several changes in the diabetic environment: endothelial dysfunction contributing to less endogenous platelet inhibition and increased sensitivity to platelet agonists, oxidative stress, inflammation, insulin resistance, hyperglycaemia and advanced glycation end products, which either directly influence metabolism in platelets or worsen structural changes in the vessel wall (Figure 7). This may play a role not only in the higher risk of developing CAD and the worse outcome observed in T2DM, but also in the larger proportion of diabetic patients with inadequate response to antiplatelet agents compared with non-T2DM subjects [72]. Increased platelet reactivity has been suggested as a potential mechanism contributing to the accelerated atherosclerosis seen in diabetic patients, by detrimental effects such as capillary microembolisation, local progression of vascular lesions and triggering of acute arterial thrombosis [120]. Increasing levels of glucose have been identified as independent predictors of platelet-dependent thrombosis in patients with coronary artery disease [123]. Platelet degranulation in patients with diabetes is associated with progression of pro-atherosclerotic vessel wall modification [124]. Platelet activation is increased in disease state with impaired nitric oxide (NO) bioavailability such as acute coronary syndromes, diabetes and hypercholesterolemia [125-127]. Therefore, activated platelets are not only a consequence of dysfunctional endothelium but also contribute to further damage to the endothelium.
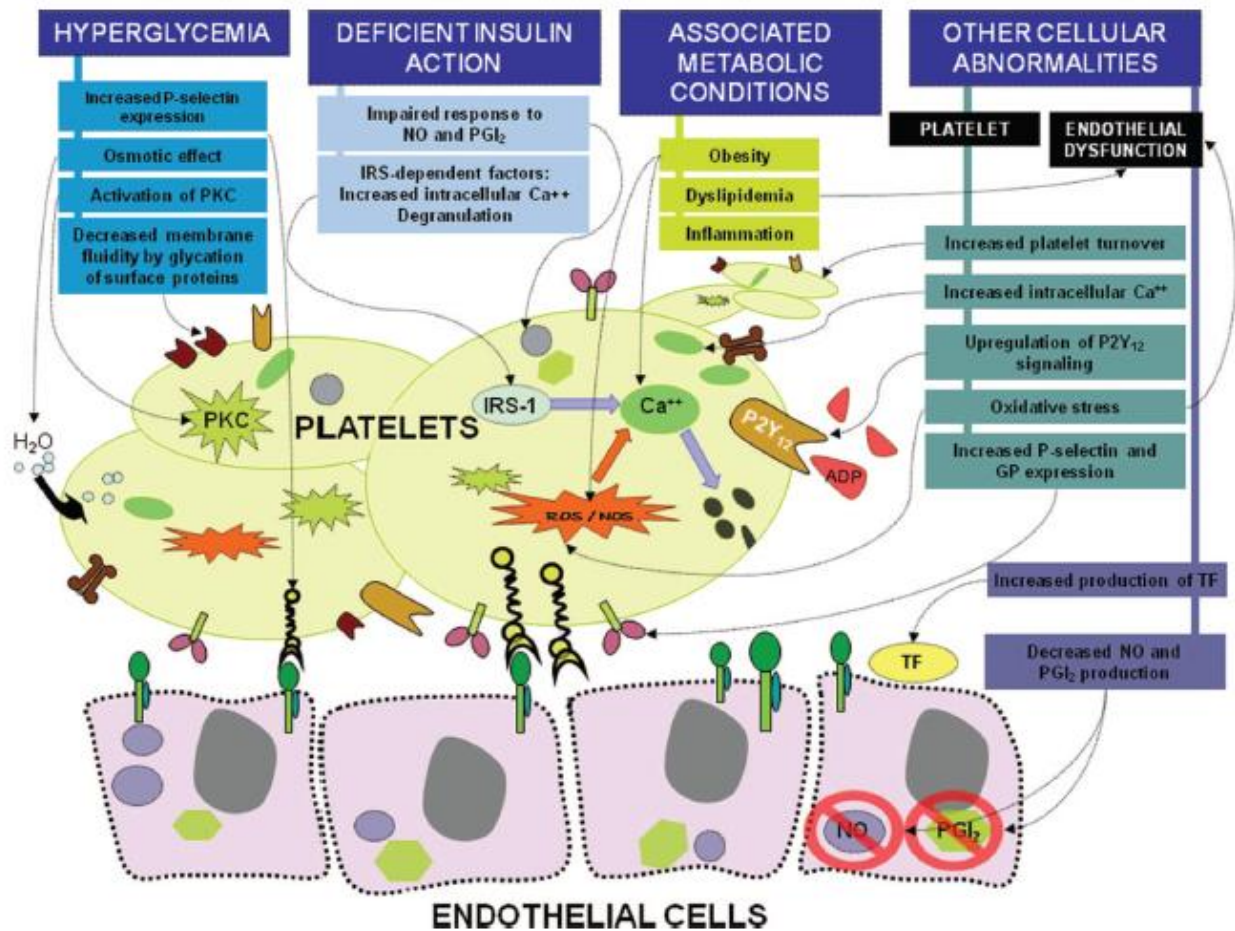
Figure 7. Mechanisms involved in platelet dysfunction in patients with T2DM. Several mechanisms contribute to platelet dysfunction in diabetes mellitus, including hyperglycaemia, insulin deficiency, associated metabolic conditions and other cellular abnormalities. From Ferreiro, J.L. et al., *Circulation* 2011.

Platelets of T2DM patients have been proven to be hyperreactive with intensified adhesion, activation and aggregation. In the hyperglycaemic milieu, there is an increased platelet surface expression of glycoprotein Ib (GPIb), which mediates binding to von Willebrand factor (vWF), and GPIIb/IIIa, which acts as a receptor for fibrinogen and mediates platelet-fibrin interaction leading to platelet aggregation [128]. The increased expression of GPIIb/IIIa is consistent with the enhanced fibrinogen binding and aggregability seen in platelets from diabetics subjects [129]. Induction of hyperglycaemia has been shown to increase platelet P-selectin expression, a surface adhesion molecule, in patients with T2DM [130]. McDonagh et. al have shown that P-selectin expression is significantly increased in patients with ischemic heart disease (IHD) compared to normal subjects and the expression is further and significantly increased in diabetic patient with IHD; they found a same trend for GPIIb/IIIA. Aspirin treatment leads to a reduced P-selectin expression and to a significant decrease of GPIIb/IIIa expression in the diabetic group with IHD;

thus, when comparing diabetic patients with IHD to nondiabetic patients with IHD, both on aspirin therapy, the differences in expression of these molecules are lost [131].

Patients with diabetes have higher levels of sCD40L compared to healthy controls and elevated levels are associated with progression of atherosclerotic diseases [132]. Neubauer et al has demonstrated that T2DM is associated with up regulation of the platelet-bound CD40-CD40 ligand (CD40L) system, enhanced P-selectin expression, as well as increased sCD40L levels compared to healthy volounteers; the improvement of glycaemic control helps to correct the abnormal platelet activation via down-regulation of CD40-CD40L system and P-selectin [133]. CD40L is absent on unstimulated platelets and appears on the platelet surface after platelet activation. Surface platelet CD40L induces the release of proinflammatory cytokines, procoagulant tissue factor and the expression of adhesion molecules. Subsequently, surface-expressed CD40L is split and generates a soluble fragment, the soluble CD40 ligand (sCD40L); sCD40L is predominantly derived from platelets, hence reflects platelet activation. Hyperglycaemia, one of the most characteristic features of T2DM, may play an independent role in the abnormalities found in platelets of DM patients. Proposed mechanisms by which hyperglycaemia may increase platelet reactivity are glycation of platelet surface proteins that decreases membrane fluidity [134], which may increase platelet adhesion; osmotic effect of glucose [135] and activation of protein kinase C, a mediator of platelet activation [136].

Platelets from diabetic patients exhibit reduced membrane fluidity which may reflect changes in the lipid composition of the membrane or glycation of membrane proteins [137].

Using both in vitro and in vivo mouse models, Zhu et. al demonstrated direct and specific interactions of advanced glycation end products (AGEs) generated under hyperglycaemic conditions with platelet CD36, first described as platelet glycoprotein IV. CD36 is expressed constitutively at high levels on platelets and modulates platelet function by ligand-dependent triggering of a signalling pathway that involves specific Src family kinases and JNK family MAPK. The finding that CD36-signalling pathway is activated cronically by hyperglycaemia represents a novel mechanism by which hyperglycaemia lead to platelet hyperreactivity [138].

Platelets from diabetic subjects produce less NO and prostacyclin (PGI$_2$); the concentration of NO synthase in platelets from patients with T2DM is less than half that from nondiabetic individuals [139].

NO and PGI$_2$ normally enhances the levels of cGMP and cAMP, leading to smooth muscle cells relaxation (vasodilation), inhibition of platelet-endothelium interactions and prevention of platelet aggregation. PGI$_2$ binds to a transmembrane receptor that is linked to a G-protein which

interacts with adenylate cyclase, while NO directly activates guanylate cyclase; both pathways culminate in cAMP and cGMP protein kinase phosphorylation, respectively.

Platelets in diabetes have reduced sensitivity to NO and PGI_2, two mediators that under physiologic condition, are potent platelet inhibitor via insulin-mediated platelet sensitization. A proposed mechanism of resistance to PGI_2 in the setting of T2DM involves decreased levels of G protein leading to decreased levels of cAMP and cGMP [140]. Another mechanism is due to insulin deficiency (inadequate insulin secretion) and insulin resistance (diminished tissue response), cardinal factors of T2DM which contribute to platelet dysfunction. Platelets express both insulin receptors and insulin-like growth factor-1 (IGF-1) receptors. Among other effects, the binding of insulin to platelets increases surface expression of adenylate cyclase–linked prostacyclin receptor [141]. Moreover, insulin stimulates NO synthesis in platelets [142].

However, insulin receptor expression is relatively low because the majority of its subunits heterodimerize with those of the IGF-1 receptor to form an insulin/IGF-1 hybrid receptor, which avidly binds IGF-1 but not insulin [143]. However, IGF-1 is present in the granules of platelets, and its receptor is expressed on the platelet surface, which may contribute to the amplification of platelet responses and the pathogenesis of cardiovascular disease. IGF-1 stimulation of platelets results in dose-dependent phosphorylation of the IGF receptor. Furthermore, IGF-1 stimulates tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 and their subsequent binding with the p85 subunit of phosphoinositide-3 kinase, leading to phosphorylation of protein kinase B, which is involved in several cellular responses to insulin and IGF-1, including modulation of platelet reactivity [144].

Among IRS-independent factors, insulin resistance causes impairment in platelet sensitivity to nitric oxide (NO) and prostacyclin leading to platelet hyperreactivity. Among IRS-dependent factors, insulin resistance provokes an increase in intracellular calcium concentration, leading to enhanced platelet degranulation and aggregation [145]. However, the precise mechanism by which calcium concentration is increased is not yet fully elucidated [146].

An increase in calcium mobilization from intracellular storage pools, resulting in increased intracellular calcium levels, has been correlated with the reduction in membrane fluidity [137]. Moreover, since intraplatelet calcium regulates platelet shape change, TXA_2 formation and platelet aggregation, disordered calcium regulation may contribute significantly to abnormal platelet reactivity in diabetes [147]. In addition to alterations in platelet calcium homeostasis, intracellular magnesium concentrations are reduced, consistent with an increase in platelet hyperaggregability and adhesiveness [148].

Arachidonic acid metabolism is increased in platelets from diabetic patients; this leads to enhanced TXA_2 production and may contribute to increased platelet sensitivity [149]. In 1990, Davì et al. demonstrated enhanced thromboxane biosynthesis in T2DM and provided evidence for its platelet origin [71]; tight metabolic control led to a reduction of its level.

T2DM is also associated with oxidative stress, in particular with an overproduction of reactive oxygen and nitrogen species, as well as reduced platelet antioxidant levels. The excessive generation of potent oxidants such as superoxide anions and hydrogen peroxide increases platelet reactivity and enhances the production of advanced glycation end products [150]. Platelets from diabetic patients show low intracellular levels of glutathione and other antioxidant and this, in turn, has been linked to an increased production of TXA_2. Furthermore, hyperglycaemia-induced oxidative stress is responsible for enhanced peroxidation of arachidonic acid to form biologically active F2-isoprostanes; among them, 8-iso-$PGF_{2\alpha}$ which induces vasoconstriction and may modify aspects of platelet function such as adhesive reaction and activation by low concentrations of other agonists. 8-iso-PGF2α formation correlates with the rate of TXA_2 biosynthesis in T2DM patients and improvement of metabolic control is accompanied by significant reduction in urinary 11-dehydro-TXB_2 (a stable enzymatic metabolite of TXB_2). Thus, changes in the rate of arachidonate peroxidation to form 8-iso-$PGF_{2\alpha}$ may represent an important biochemical link between altered glycaemic control, oxidant stress and platelet activation in T2DM [151].

T2DM is also associated with systemic inflammation; expression of platelet FcgammaRII receptor is enhanced in diabetic patients and it is involved in platelet activation and modulate by inflammation [152]; therefore, systemic inflammation may contribute to increased platelet reactivity in T2DM subjects.

Different condition associated with inflammation and endothelial dysfunction, such as diabetes, promote the conjugation of leukocytes with platelets. Platelets and leukocytes may form platelet-leukocyte aggregates mainly via platelet P-selectin and its receptor P-selectin glycoprotein ligand-1 (PSGL-1), resulting in inflammatory tissue damaging processes. Monocyte-platelet aggregates (MPA) are increased in patients with T2DM compared to healthy subjects. More severe diabetes is associated with an even higher number of MPA [153, 154].

Excess risk of atherothrombosis in diabetic patients is also associated with procoagulant microparticles (MPs); MPs are submicron membrane-coated vesicles that emerge by budding from their parental cells upon activation or apoptosis and retain some functions of their cell of origin. Increased levels of platelet-derived MPs have been reported in diabetic patients with macrovascular complications and have been suggested to play a role in these complications

[155]. Interestingly, elevated levels of TF-bearing MPs correlate with the components of the metabolic syndrome in uncomplicated patients with T2DM [156].

As far as platelet Tissue Factor is concerned, Gerrits et. al characterized the extra- and intracellular mechanisms that couple surface activation to TF synthesis in adhering platelets. They show that different adhesive proteins induce different levels of TF synthesis and that ADP release and thromboxane A(2) production followed by activation of P2Y12 and thromboxane receptors mediate surface-induced TF synthesis, since the treatment with a mimetic of active clopidogrel metabolite and an aspirin-like inhibitor reduced TF synthesis. Interference with intracellular pathways revealed inhibition by agents that raise cAMP and interfere with phosphatidylinositol 3-kinase/protein kinase B. Insulin is known to raise cAMP in platelets and inhibited collagen III-induced TF premRNA splicing and reduced TF activity. In healthy individuals, TF synthesis is inhibited by insulin, but in patients with T2DM inhibition is impaired resulting in an approximately 1.6-fold higher TF synthesis than in matched control subjects. This leads to the novel finding that platelets from type 2 diabetic patients produce more TF than platelets from matched control subjects [157]; but there are still no information about circulating platelet TF expression in diabetic patient with CAD.

Furthermore, compared with nondiabetic patients, those with T2DM tend to have platelets of increased size and volume, a similar pattern to that observed in patients at the time of an acute coronary syndrome event, and all associated with increased platelet activation and aggregation thereby reflecting platelet hyperreactivity and a prothrombotic milieu [158, 159]. Mean platelet volume (MPV) and immature platelet fraction (IPF) are markers of platelet activity; high levels of IPF result in lower response to antiplatelets therapies and are associated with increased residual platelet aggregation in stable CVD patients receiving antiplatelet therapy [160, 161]. IPF, besides being an alternative marker of platelet activity replacing MPV, provides additional information on thrombopoiesis or platelet turnover. IPF or reticulated platelets are newly formed platelets; these young platelets are larger and characterized by a higher number of dense granules and an increased platelet volume than older platelets [162]. Finally, larger platelets have been shown to be enzymatically and metabolically more active and to have a higher thrombotic potential than smaller platelets [163]. IPF and MPV are convenient measured with an automated hematology analyzer; reticulated platelets can be also identified and quantified by staining for messenger ribonucleic acid (mRNA) using flow cytometry, since they contain great amount of megakaryocyte-derived mRNA. Lee et al. found that IPF is elevated in T2DM patients compared to controls and is associated with failure of proper glycemic control and the presence of

cardiovascular complications in these patients. In fact, IPF is higher in DM patients complicated by CVD compared to those without CVD. Moreover, IPF was higher in poor glycemic control group than intermediate or strict glycemic control groups; IPF and MPV, as platelet activity markers, were not different between the intensive and standard therapy groups, thus, excessive glucose lowering is not related to further benefit in controlling platelet activity [164].

The underlying mechanism of higher IPF or MPV in T2DM is not clear. Osmotic effect of hyperglycaemia has been shown to pose dual effects both on platelet activity and size. Intriguing effect of insulin to promote larger platelets from megakaryocyte in mice has also been reported [165]. Kraakman et al have reported [135] that, in an animal model, increased reticulated platelets in diabetes are due to enhanced proliferation and expansion of megakaryocytes in the bone marrow. This is driven by increased thrombopoietin production, likely due to IL-6 release by Kupffer cells downstream of receptor for AGEs (RAGE) signaling. Thrombopoietin is a marker of thrombopoiesis and it has been found to be increased in diabetic patients [166].

Finally, results obtained by Hernandez Vera et. al indicate that megakaryopoiesis is altered in the bone marrow of diabetic rats, giving rise to hyperreactive platelets with altered expression of endoplasmic reticulum stress protein, 78-KDa glucose-regulated protein (GRP78) and protein disulphide isomerase (PDI), which can increase the amount of active TF, thus contributing to increase thrombotic risk in diabetic animals independently of blood glucose levels.

GRP78 is a multifunctional protein usually found in the lumen of the ER that has also been described to be present in the cell surface. In fact, cell-surface GRP78 has been proven to exert an atheroprotective function in platelets and endothelial cells inhibiting TF procoagulant activity by direct binding [167]. On the other hand, PDI is a ubiquitously expressed thiol isomerase and oxidoreductase that has been described to contribute to TF decryption and to increase TF procoagulant activity even though the mechanism still remains unclear.68–73 Thus, the decreased levels of GRP78 observed in platelets from ZD animals together with the increased levels of PDI seem to contribute to the increased TF procoagulant activity observed in such animals [168].

AIMS OF THE STUDY

Type-2 diabetes mellitus (T2DM) is a major cardiovascular risk factor and has recently become a high profile public health concern. Atherosclerosis and coronary artery disease (CAD) are the leading causes of premature mortality in diabetic patients; indeed, diabetic patients show a higher incidence of cardiovascular (CV) events compared to non-diabetic subjects, together with a platelet hyperreactive phenotype and a reduced platelet responsiveness to aspirin and efficacy of antiplatelet agents [72, 73].

Platelet activation plays a pivotal role in the pathogenesis of atherosclerosis and its thrombotic complications and increased *in vivo* platelet activation has been reported in patients with CAD [112].

Several biochemical abnormalities have been found to be associated with diabetic platelet hyperreactivity, which lead to increased platelet adhesion, activation and aggregation [122, 137, 147, 149].

Although hyperglycemia has been associated with cardiovascular disease [169, 170], recent clinical trials have shown that intensive glycemic control is not sufficient to counteract all these abnormalities and to reduce cardiovascular event presentation [171-173]. Moreover, the finding that the current antiplatelet therapies appear less effective in T2DM patients as compared to non-diabetics with a similar risk profile, both in primary and secondary CV prevention [72, 73], suggests a disease-associated change in platelet responsiveness to current antiplatelet and antidiabetics strategies, or the presence of additional T2DM-specific mechanisms, not targeted by current drugs, contributing to thrombus formation (e.g., haemostatic adhesive molecules such as von Willebrand factor, TF-related coagulation pathways, inflammatory or oxidative mechanisms).

Activated platelets express a variety of molecules relevant for the atherothrombotic processes, including membrane glycoproteins involved in adhesion, aggregation, and coagulation. We reported that *in vitro* platelet activation by classical agonists results in expression of functionally active Tissue Factor (TF), a major player in the coagulation cascade and thrombotic complications of atherosclerosis [103]. Furthermore, we also provided evidence that patients with non-ST elevation acute coronary syndrome (NSTE-ACS) had a greater number of TF-positive platelets and platelet-monocyte aggregates than stable CAD patients or healthy subjects, providing additional insight into the prothrombotic potential of CAD platelets [107, 140].

Several studies have likewise described the platelet contribution in the onset and progression of vascular complications observed in T2DM affected patients. However, no previous study has focused on platelet-associated TF contribution to the higher thrombogenicity observed in CAD patients with T2DM.

In the last ten years we have also learned that platelets contain between 2000 and 7000 transcripts derived from megakaryocytes and that platelets can use their transcriptome to perform new protein synthesis in response to activation, thus modifying their proteome and, as a consequence, their function. Specific mRNAs may vary in different clinical conditions. We have previously showed that specific changes in the expression/distribution or amount of platelet mRNAs and proteins are associated with CAD, suggesting that platelet from patients in different clinical conditions are potentially preconditioned to a different degree of reactivity on the transcriptional levels [87]. However no information is still available on the changes that may occur in platelet transcript profile in CAD patients with T2DM.

Although our understanding of vascular pathology has greatly improved in recent years, the cellular and molecular mechanisms that influence and regulate the platelet function and the platelet response to stimuli during atherogenesis and that are involved in the enhanced thrombotic propensity in T2DM remain incompletely characterized.

Based on these premises the general purpose of this study is to provide insights into the enhanced thrombotic propensity of CAD patients with T2DM.

In particular, the specific aims of the present project are to elucidate whether the T2DM-related metabolic unbalance affects:

platelet-associated TF expression which may contribute to the increased thrombogenicity in stable CAD patients with T2DM, bridging coagulation cascade, inflammation and platelet activation in this setting;

the procoagulant potential of whole blood, studying parameters that describe clot properties during its formation and evaluating the contribution of platelet-TF to thrombin generation, in order to understand if platelet-associated TF is functionally active and involved in the increased thrombogenicity related to T2DM;

platelet transcriptome composition, to gain insights into the molecular pathways associated with and/or triggering persistent platelet activation. This could allow identification of a gene signature that predicts phenotype membership, i.e. predictive platelet biomarkers for classifying patients into disease groups (stable CAD and/or T2DM) based on expression levels.

METHODS

Study design

The present project is an observational, case-control, and cross-sectional study. It was carried out at Centro Cardiologico Monzino IRCCS after approval by the Ethical Committe. All patients and control subjects gave their written, informed consent to participate in this study after receiving oral and written information. 97 consecutive patients were enrolled from March 2012 to June 2014. Of these, 85 patients (53 patients with SA without T2DM and 32 patients with SA and T2DM) met all the inclusion criteria and were included in the study. Moreover, to assess the contribution of T2DM *per se*, 28 patients without SA with T2DM (enrolment in collaboration with Università G. D'Annunzio, Chieti, Dipartimento di Medicina e Scienze dell'Invecchiamento) were recluted; finally, to characterize the "baseline" platelet phenotype and relate the relative gene abundance in T2DM/CAD patients to a reference expression level, 37 healthy subjects were also enrolled.

Inclusion criteria

• T2DM: diagnosis of diabetes was made according to the ADA criteria (fasting glycaemia $\geq$ 126 mg/dl or 7 mmol/L, for two measurements). A recent (less than 30 days) determination of HbA1c was recorded at study entry.
• Stable CAD: diagnosis was made, according to the AHA criteria, based on typical chest pain on exertion associated with ST segment depression >1.0 mm on an exercise test, and a coronary angiography that confirms the presence of significant coronary artery stenosis ($\geq$75%).
• Treatment with low-dose aspirin (100 mg/die) for at least 1 month in all patients.
• Negative electrocardiogram (ECG) for those subjects enrolled as healthy subjects or diabetic subjects without CAD.
• Age between 40 and 85 years old both for patients and healthy subjects.
• Absence of hypertension or dyslipidemia pharmacologically treated in healthy volounteers.

Exclusion criteria

Chronic atrial fibrillation, cardiac disease other than stable CAD, myocardial infarction within the last 30 days, major non-cardiac diseases (anemia, infection, cancer, collagen disease, thyrotoxicosis, major liver or kidney disorders), heparin and/or oral anticoagulant treatment, other antiplatelet drugs, steroidal and non-steroidal anti-inflammatory drugs, cigarette smoking

pregnancy, platelet count <150000 platelets/μl, major bleeding, presence of hypertension or dyslipidemia not pharmacologically treated in patients.

At study admission, glycaemia, HbA$_{1c}$ only for diabetics, lipid profile (total cholesterol, HDL, LDL, triglycerids), liver enzymes, blood pressure and body mass index were measured for all patients and healthy subjects.

All healthy subjects enrolled had no clinical evidence of inflammatory, neoplastic or cardiovascular disease based on clinical history, examination, routine laboratory tests and resting ECG.

Biological material collection

At study admission, peripheral venous blood samples were collected without stasis, with a large bore needle (19G), discarding the first 4 mL, from each patient in order to:

- performe fresh whole blood flow cytometry analysis of platelet activation and/or prothrombotic phenotype;
- assess the hemochrome and the immature platelet fraction (IPF);
- study the global haemostatic function by Rotem;
- isolate platelets for transcriptome analysis and thrombin generation assay;
- separate plasma and serum for biochemical determinations.

Assessment of platelet phenotype by flow-cytometry

Platelet activation and/or prothrombotic phenotype was evaluate by whole blood flow cytometry with a fluorescence-activated-cell sorter (FACS) Calibur (Becton Dickinson), using specific fluorochrome-labeled monoclonal antibodies; the immunostaining was performed as previously described [103, 107], within 15 minutes after blood sampling to avoid artificial platelet activation. The sensitivity of fluorescence detectors was set and monitored using CaliBRITE beads (Becton Dickinson) according to the manufacturer's instruction. For this analysis it was used blood collected into citrate-containing (1/10 volume of 0.129 mol/L sodium citrate) vacuum tubes (vacutainer, Becton Dickinson).

The expression of the following platelet activation markers was assessed: TF, P-selectin (CD62P), GPIIb/IIIa complex (PAC-1), CD36 as well as annexin V binding. Saturating concentrations of the following mouse anti-human monoclonal antibodies were used: CD61-PerCP (Becton Dickinson, BD), also known as integrin β3 and used as a marker of platelet

population; PAC-1-FITC, BD; CD62-APC,BD; CD36-PE, BD, also known as glycoprotein IV; annexin-V-PE, BD; TF4507CJ-FITC (American Diagnostica, AD).

FITC-, PE-, PerCP-, APC-conjugated isotype controls were used in all the experiment to quantify the background labeling.

Briefly, 5 µl of whole blood was added to phosphate buffer saline (PBS, pH 7.4, Life Technologies) to a final volume of 100 µl in presence of saturating concentration of the above described antibodies (a sample was prepared for each marker of platelet activation and co-stained with CD61). For SA patients (with or without diabetes), a sample was also prepared to assess surface tissue factor expression upon stimulated condition (prepared following the same labeling of the resting sample except for co-incubating ADP 10 µM, Sigma, together with antibodies, blood and PBS in a final volume of 100 µl). Samples were incubated at room temperature for 15 minutes (kept away from light). Then, they have been diluted with 600 µl of paraphormaldeid (PFA, final concentration 1%; Sigma) to obtain fixation. Finally they were kept in the dark until analysis at FACSCalibur. Platelets positive to activation markers were determined in 10000 CD61 positive events per sample.

All the data were analyzed by CELLQuest software (Becton Dickinson) and the results will be expressed as percentage of positive cell, upper right (UR), and as mean fluorescence intensity (MFI), calculated for the gated population.

Assessment of platelet intracytoplasmic TF was performed on 50 µl of fixed whole blood with PFA1% in SA patients with or without T2DM. Fixed blood was washed with 500 µl of PBS, centrifuged at 1500 g for 5 minutes; pellet were resuspended with 100 µl of PBS-Triton 0,1% (Triton X-100, Carlo Erba) to obtain permeabilization of cell membrane and antibodies access to the antigen in the intracytoplasmic compartment. After 15 minutes, blood was added with saturating concentration of the following mouse anti-human monoclonal antibodies: TF4508CJ-FITC, BD and CD61-PerCP, BD. Then incubation in the dark for 15 minutes, washing in PBS, centrifugation as before described and resuspension in PBS were performed.

TF-positive platelets were determined in 10000 CD61 positive events per sample.

All the data were analyzed by CELLQuest software (Becton Dickinson) and the results will be expressed as percentage of positive cell, upper right (UR), and as mean fluorescence intensity (MFI), calculated for the gated population.

Evaluation of platelet turnover

Blood count was performed on blood collected into a EDTA-containing vacutainer. In order to evaluate platelet turnover, platelet counts, mean platelet volume (MPV), platelet distribution width (PDW) and IPF was measured with a hematology analyzer (Sysmex XE-2100, Dasit) equipped with upgraded software allowing flow cytometric detection of immature platelets. Circulating biomarkers of thrombopoiesis (Interleukin-6, IL-6; and thrombopoietin, TPO) was also determined using commercial ELISA assay.

Platelet isolation from whole blood

Blood collected into three acid citrate dextrose-containing vacutainers was processed within 15 minutes from sampling and platelets were isolated by centrifugation and filtration. Particular care was taken in using an isolation technique that minimizes non-specific platelet activation and leukocyte contamination. Briefly, whole blood was centrifuged at 120 x g for 15 minutes, at room temperature, without break, in order to obtain platelet rich plasma (PRP). PRP was transferred into a new tube, added with 4 µM prostaglandin (PG)E1 and 10 µM EDTA. All PRP obtained was then diluted 1:3 in HEPES-Tyrode's buffer (by adding 2 volumes of HEPES Tyrode's buffer to PRP volume). HEPES-Tyrode's buffer's composition is the following: 10 mmol/L HEPES, 134 mmol/L NaCl, 2,9 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0,4 mmol/L Na_2HPO_4, 0,1% glucose, 5 mmol/L EDTA, 1 µM PGE1); all reagents are purchased from Sigma. Diluted PRP was filtered through Filter Pall Purecell^{TM}, which captures residual contaminating leukocytes whereas platelets pass through the filter and are collected in a new tube. Filter is washed with 2 volume of HEPES-Tyrode's buffer in order to recover all platelets.

Filtered PRP is then divided, since a volume corresponding to at least 800 million of platelet is necessary for transcriptome studies and a volume corresponding to at least 2 million of platelets (divided into 2 aliquots of 1 million of platelets each) is used to prepare dry pellet. Filtered PRP is analyzed by hematologic analyzer Sysmex to obtain the platelet count and to assess the eventual presence of contaminating leukocytes. A samples of this PRP is used for the assessment of platelet activation (staining with CD62P-APC (BD), and CD61PerCP(BD), and comparison of P-selectin value in this sample with the value in fresh whole blood to confirm that the procedure does not lead to non-specific platelet activation) and leukocyte contamination by flow cytometry (staining with CD45 PerCP, BD); only those preparations with less than 5 leukocytes per 105 platelets, without sign of activation are used for genomic analysis.

The sample volume for transcriptome analysis is centrifuged at 11000 x g for 3 minutes and lysed in 500 µL of QIAzol Lysis Reagent (QIAGEN), immediately frozen till RNA extraction.

To obtain dry pellet from the other sample volume, diluted PRP is centrifuged at 1000 x g, 10 minutes; pellets are washed in Tyrode's buffer and centrifuged again at 11000 x g for 3 minutes. After removing surnatant, pellet was frozen until thrombin generation assay was performed.

Serum and plasma preparation for subsequent measurements of plasma biomarkers of inflammation, coagulation and platelet turnover

Serum

Blood collected into serum tube (vacutainer Z, BD) was incubated at 37°C for 1 hour.

Blood was centrifuged at 1700 x g for 10 minutes at 4°C.

Serum is placed in a fresh tube, aliquotated in small volumes and frozen at -80°C.

Plasma

Blood collected into EDTA- and citrate-containing tubes was centrifuged at 1700 x g for 10 minutes at 4°C.

Plasma from the 2 vacutainers is placed in fresh tubes separately, aliquotated in small volumes and frozen at -80°C.

Measurements

Serum was used for TXB_2 dosage; TXB_2 was measured with previous validated, non-commercial EIA assay [174];

EDTA plasma was used to dose TPO, IL-6 and hsCRP by ELISA;

other aliquots will be used to measure vWF multimers by Western Blot in agarose gel electrophoresis and, by ELISA, TAT, F1+2, and esRAGE (the endogenous soluble receptor for advanced glycation-end-products; decreased levels of esRAGE result in ligand-RAGE pathway hyperactivity), data not obtained yet.

Citrate plasma will be used to measure fibrinogen (by Clauss method), sCD40L (by ELISA) and vWF antigen and activity (by immunometric methods). Data not obtained yet.

A comprehensive profile of relevant soluble factors bridging inflammation and coagulation to platelet hyperreactive phenotype will be obtained.

Thromboelastometry (ROTEM system)

Global haemostatic function was assessed by using Rotem coagulation analyzer (Tem International Gmbh, Munich, Germany) in SA patients with or without T2DM; in particular we performed Natem assay, which provides a very sensitive assessment of the equilibrium of coagulation activation or inhibition.

ROTEM is a system for the study of haemostasis that is based on the measurement of the elasticity of the blood through constant graphic recording of the firmness and the size of the clot during its formation and subsequent fibrinolysis.

ROTEM system sets a reaction curve and kinetic parameters derived from the analysis of the curve, describing the kinetics of clot formation.

Blood collected in a sodium citrate-containing vacutainer, BD, was put into a cuvette and added with 20 μL of 0.2 mol/L $CaCl_2$ to triggers coagulation, according to the manufacturer's instruction. The cuvette was placed on a rod applied to the lower end of a vertical axis that rotates from left to right with a defined angle. The rotation was detected optically. In case of clot formation, the clot adheres to the surface of the rod and cuvette; the movement is hindered and the amplitude of the angle decreases to the increase of clot firmness.

All measurements were performed at 37°C.

The following parameters were recorded:

- Clotting Time (min), CT, is the time required to form a clot of 2 mm of amplitude from the beginning of the assay;

- Clot Formation Time (min), CFT, is the time to form a clot of 20 mm amplitude from a clot of 2 mm;

- Maximum Clot Firmness (mm), MCF, (mm) represents the maximum amplitude of the curve before fibrinolysis reduce the size of the clot.

- Maximum Clot Firmness Time (min), MCF-t, is the time necessary to reach MCF starting from the 2 mm amplitude time-point;

- Alpha Angle is the angle between the tangent to the curve at the 2 mm amplitude time -point and the baseline;

- Maximum Velocity, V max, and the Area Under the Curve, AUC, are two parameters obtained from the first derivative of the clot curve which respectively represent the maximum degree of clot formation and an indirect measurement of thrombin generation [175].

Thrombin generation assay

Dry pellets of 1 million of platelets prepared as described above (from SA patients with or without T2DM) were solubilized with 15 mM octyl-β-D-glycopyranoside at 37°C for 10 minutes, diluted with 25 mM HEPES–saline buffer (β-octyl-Hepes buffer) and tested for their capacity to promote thrombin generation using the Calibrated Automated Thrombogram (CAT) assay. Twenty µl of cell sample were incubated for 10 minutes with 20 µL of platelet-free normal pooled plasma (Pool Norm) in round-bottomed 96-well microliter plates (Immulon 2HB). To assess TF dependent contribution to thrombin generation, the CAT assay was performed after pre-incubation of cell samples with a neutralizing anti-TF antibody (AD4501, 100 µg/ml final concentration selected on the basis of dose-finding experiments). Thrombin generation was started by the addition of a $CaCl_2$/fluorogenic substrate mixture (FluCa Kit) and fluorescence was read for 60 minutes in a Fluoroskan Ascent® reader (Thermo Labsystems) equipped with a 390/460 filter set. In order to correct for inner filter effects, ageing of the lamp and filters, donor-to-donor variability in color of plasma and substrate consumption, each thrombin generation measurement was calibrated against the fluorescence curve obtained in the same sample to which a fixed amount of thrombin-α_2-macroglobulin complex was added (Thrombin Calibrator). Thrombin generation curves were analyzed by dedicated software (Thrombinoscope BV).

The main parameters used to describe thrombin generation were the flollowing:

- Lag Time (LagT, minutes), defined as the time needed to start thrombin generation upon triggering coagulation;

- The height of the thrombin peak (peak height) which correspond to the time in which the maximum amount (nM) of thrombin is generated;

- The time to peak (ttPeak, minutes), time needed to reach the maximum thrombin generation;

- The Area under the curve, defined as Endogenous Thrombin Potential (ETP, nM thrombin*minutes); it is the total amount of active thrombin generated from activation of coagulation. It represents the plasmatic balance between the action of pro- and anti-coagulants.

Total RNA extraction

RNA extraction was performed using matherials RNasi free and always wearing gloves to avoid the presence of RNasi, enzymes that degrade RNA; reagents were purchased from Sigma Aldrich. Total RNA was isolated from platelet samples lysed in phenol/guanidine-based Qiazol Lysis Reagent (sample preparation was described above).

The combination of organic extraction and chaotropic disruption contributes to efficient lysis and higher yields of total RNA.

Each platelet sample in 500 μL of lysis reagent was added with 50 μL of 2M, pH4, sodium acetate and with 100 μL chloroform. After shaking by vortex, sample were kept in ice for 15 minutes. After this, the homogenate was separated into aqueous and organic phases by centrifugation at 9000 x g, 4°c for 20 minutes. RNA partitions to the upper, aqueous phase while DNA partitions to the interphase and proteins and lipids to the lower, organic phase. Aqueous phase was placed in a new tube and RNA was precipitated from the aqueous phase by adding 1 volume of isopropanol and incubating over night, at 4°C. Sample was centrifuged at 9000 x g, 4°C, for 15 minutes and, after removing isopropanol, the pellet was then washed with 75% ethanol, 500 μL; sample was kept for ten minutes at room temperature and then centrifuged again at 9000 x g, 4°C, for 5 minutes. After removal of ethanol, pellet was dissolved in 15 μL RNase-free water.

RNA Quality control

RNA quantification was performed by Infinite M200 Pro Tecan, an instrument that calculates RNA concentration and purity, measuring absorbance at 230, 260 and 280 nm and giving the ratio of the absorbances 260/280, index of protein contamination if higher than 2, and the ratio of the absorbances 260/230, index of solvent contamination if lower than 2.2.

To evaluate the quality and integrity of extracted RNA the Agilent RNA 6000 Pico kit and reagents and the Bioanalyzer 2100 Agilent Technologies were used.

Genomic analysis

The primary objective of this study is the search for genes that are differentially expressed (DE) in distinct phenotype (*class comparison*), performing genome-wide transcriptional analysis in SA^+T2DM^+ *vs.* SA^+T2DM^- platelets. To do so, we enrolled 32 SA^+T2DM^+ patients, 53 SA^+T2DM^- patients, and 16 healthy subjects (controls), matched for age and sex. DE platelet transcripts were assessed by microarray profiling.

RNA was reverse transcribed, labeled, and linearly amplified using the Total Prep RNA Amplification Kit (Life Technologies, Carlsbad, CA), in order to be hybridized to HumanHT-12 v.4 Expression BeadChip microarrays (Illumina, San Diego, CA), according to manufacturers' instructions. This platform provides genome-wide transcriptional coverage of annotated genes, gene candidates and splice variants (>25000 annotated genes, about 42000 unique transcripts, with >47000 50-mer gene-specific probes derived from the NCBI RefSeq Release 38 and the UniGene Build 99 databases). To estimate technical variability, 10% of the samples were

hybridized in duplicate. Arrays were read using the high resolution confocal scanner iScan (Illumina) and signal quantification and quality control (data mining) were performed with the Genome Studio v. 1.9.0 software (Illumina).

Primary analyses were performed using BRB-ArrayTools v. 4.4.0β2 developed by Dr. R. Simon and BRB-ArrayTools Development Team (http://linus.nci.nih.gov/BRB-ArrayTools.html). Data variance stabilizing transformation and robust spline normalization were conducted with the *lumi* R package. Probes were filtered out under any of the following conditions: when the 95th percentile of intensities showed a detection P-value greater than 0.01 as calculated by Genome Studio (*i.e.*, the 5th percentile of intensities did not significantly differ from the background level) and/or the P-value of the log-ratio variation was greater than 0.01 (*i.e.*, probes that showed minimal variation across the entire set of arrays). Multiple probes were reduced to one per gene symbol by using the most variable probe measured by interquartile range across arrays. The number of genes that passed these filtering criteria was 5797.

Replicate arrays were averaged and genes that were differentially expressed among classes were identified using random-variance univariate F-test (when comparing healthy *vs.* SA$^+$T2DM$^+$ *vs.* SA$^+$T2DM$^-$ platelets) or *t*-test (when comparing SA$^+$T2DM$^+$ *vs.* SA$^+$T2DM$^-$ platelets). Genes were considered statistically significant if their parametric P-value was less than 0.005. To correct for multiple comparisons, in case of the F-test we used a multivariate permutations test, computed based on 1000 random permutations, setting the maximum allowed false discovery rate (FDR) at 0.05 (5% of false discoveries in the list of DE genes) with a confidence level of FDR assessment of 80%. With probability of 80% the number of genes containing no more than 5% of false discoveries was 510. A fold-change (FC) cut-off of ±1.2 (healthy *vs.* SA) was applied to this list to focus on most meaningful DE genes: thus the list of DE genes was reduced to 319. For the univariate *t*-test, permutation testing (based on 10000 random permutations) was used to compute the significance of the rank assigned to each gene. The number of genes significant at 0.005 level of the univariate test was 35 (SA$^+$T2DM$^+$ *vs.* SA$^+$T2DM$^-$ platelets). DE genes were clustered using Pearson's correlation (centered) and average linkage method.

Gene-annotation enrichment analysis for Gene Ontology (GO) biological processes and cellular components was performed using the web-based applications GOrilla (Gene Ontology enRIchment anaLysis and visuaLizAtion tool) or WebGestalt (WEB-based GEne SeT AnaLysis Toolkit), which perform hyper-geometric enrichment tests to analyze whether groups of DE genes in each comparison were enriched for (associated with) a particular biological function or cellular compartment. A GO term was considered significantly enriched if its P-value was lower than 0.001 for the healthy *vs.* SA comparison or 0.01 for the SA$^+$T2DM$^+$ *vs.* SA$^+$T2DM$^-$

comparison. Redundant GO terms were removed from the results of the above analyses using the web-based tool REVIGO (REduce and VIsualize Gene Ontology).

Statistical analysis

Baseline categorical and continuous variables were expressed as n (%), and as mean ± SD, respectively. Platelet activation markers, as well as plasma variables, were summarized as mean ± SEM and were log transformed before analysis. Differences among groups were analyzed by using chi-square test for categorical variables and Kruskaal Wallis test for continuous variables. Only $p<0.05$ was regarded as statistically significant. The effect of T2DM in SA patients was evaluated by ANCOVA with log-transformed data after adjustement for age and gender.

RESULTS

Patient enrollment

In order to assess the contribution of type-2 diabetes (T2DM) in platelet hyperreactivity in patients with cardiovascular disease (stable angina, SA) we prospectively recruited 97 consecutive patients admitted at Centro Cardiologico Monzino IRCCS in Milan from March 2012 to June 2014. Of these, 85 patients (53 patients with SA without T2DM and 32 patients with SA and T2DM) met all the inclusion criteria and were included in the study. Moreover, 28 patients without SA with T2DM (enrolment in collaboration with Università G. D'Annunzio, Chieti, Dipartimento di Medicina e Scienze dell'Invecchiamento) and 37 healthy subjects were also enrolled.

Clinical characteristics of the patients included in the study matched the inclusion criteria previously described.

The three groups of patients are comparable in term of age, risk factors, past medical history and treatment medications, with the exception of:

- the presence of diabetes and consequent treatment with hypoglycaemic drugs only in the two groups of diabetic patients and not in the group of patients with SA but without T2DM;

- the treatment with nitrates in the two groups with SA and not in the group of patients with T2DM, without SA ($p<0,05$);

- the treatment with beta-blockers (less diabetic patients were taking beta-blockers compared to SA patients with or without diabetes), $p<0.05$.

The first two differences were expected on the basis of the study design (Table 1).

Of note, according to the established enrolment criteria, all patients were treated with low-dose aspirin (100 mg/once a day) and they did not undergo a therapy with any other antiaggregant drug.

Healthy volunteers were characterized by a younger age and a prevalence of female compared to all the patients groups ($p<0.001$); this has been taken into account in the data analysis.

In healthy subjects, body mass index is significant lower compared to three groups of patients ($p<0.001$) (Table 1).

VARIABLES mean ± SD or n° of patients (%)	SA+ PATIENTS		SA- PATIENTS	HEALTHY SUBJECTS
	NO T2DM	T2DM	T2DM	
n°	53	30	28	37
Age (years) *	65±9	68±7	69±6	50±9
Gender, male	44 (83)	28 (88)	21 (75)	18 (48)
Risk factors/past medical hystory				
Body mass index (BMI) *	27±4	29±5	26±9	24±3,5
Family history of CAD	21 (40)	14 (44)	10 (36)	6 (16)
Hypertension	46 (87)	28 (88)	22 (78)	2 (5)
Hyperlipidemia	34 (64)	22 (69)	18 (64)	3 (8)
Type 2 diabetes	0 (0)	32 (100)	28 (100)	0 (0)
Prior IMA	12 (23)	9 (28)	0 (0)	0 (0)
Prior CABG	4 (8)	3 (9)	0 (0)	0 (0)
Prior PCI	20 (38)	11 (34)	0 (0)	0 (0)
Treatment medications				
Aspirin	53 (100)	32 (100)	28 (100)	0 (0)
Beta-Blockers **	32 (60)	18 (56)	13 (46)	0 (0)
ACE inhibitors	16 (30)	7 (22)	7 (24)	0 (0)
Calcium-channel blockers	21 (40)	14 (44)	10 (36)	0 (0)
Ipoglycaemic medication	0 (0)	28 (88)	24 (86)	0 (0)
Nitrates **	16 (30)	12 (38)	0 (0)	0 (0)
Lipid-lowering agents	34 (64)	22 (69)	19 (67)	0 (0)

Table 1. Clinical characteristics of enrolled patients and healthy subjects.
*$p<0,001$ among the 4 groups; **$p<0.05$ among the 3 groups of patients. Data analysed by Chi Square or Kruskaal Wallis tests.

Biochemical laboratory investigations and hematologic parameters

Metabolic parameters were measured in all patients and healthy subjects enrolled (Table 2).

As expected, glycaemia and glycated haemoglobin were higher in the two groups of diabetic patients compared to the other two groups ($p<0.0001$ and $p=0.06$ among the three groups of patients for glycaemia and glycated haemoglobin, respectively).

Lipid profiles were well controlled by therapy with lipid-lowering agents and renal function was preserved in all groups.

VARIABLES mean ± SD	SA+ PATIENTS		SA- PATIENTS	p value	HEALTHY SUBJECTS
	NO T2DM	T2DM	T2DM		
Fasting glycaemia (mg/dL)	106±14	143±20	127±23	0,00001	84±10
HbA1c (%)	5,2±0,5	7±1	7±0,8	0,06248	5,5±0,4
Total Cholesterol (mg/dL)	178±45	155±26	179±33	0,08135	189±50
HDL Cholesterol (mg/dL)	49±12	43±10	50±10	0,20908	61±13
LDL Cholesterol (mg/dL)	106±38	82±28	99±28	0,11655	123±35
Triglycerides (mg/dL)	126±76	134±57	145±53	0,22738	101±38
S-Creatinine (mg/dL)	0,9±0,1	1±0,2	0,9±0,1	0,14727	0,8±0,2

Table 2. Metabolic parameters measured in enrolled patients and healthy subjects. Data are shown as mean ± SD. P values are calculated by Kruskaal Wallis test among the 3 groups of patients.

Also hematologic parameters were measured in patients and healthy subjects recluted in the study. Most of them showed comparable values among the four groups (Table 3). When comparing the three groups of patients, all the hematologic parameters were comparable except for the percentage of immature platelet fraction (IPF), which was significantly different among them ($p > 0.05$); SA patients with T2DM showed the highest value (Table 3 and Figure 8B). Moreover a trend towards higher percentage of IPF was observed also in T2DM patients compared to SA patients and healthy subjects. Mean platelet volume (MPV) was found comparable among groups, although both in SA patients with T2DM and in T2DM patients without SA a weak trend towards an increased MPV was found (Figure 8C and table 3).

| VARIABLES mean ± SD	SA+ PATIENTS		SA- PATIENTS	p values	HEALTHY SUBJECTS
	NO T2DM	T2DM	T2DM		
Red blood cell count ($10^6/\mu L$)	4,8±0,5	4,7±0,4	4,8±0,5	0,29871	4,7±0,5
Hematocrit (%)	42,4±4,1	40,3±4,4	41,3±3,2	0,22794	41,6±3,7
Haemoglobin (g/dL)	14,2±1,7	13,4±1,8	14,2±1,2	0,30880	14,1±1,4
White blood cell count ($10^3/\mu L$)	6,8±1,2	7,2±1,7	6,87±1,5	0,91355	5,9±1,2
Neutrophil count ($10^3/\mu L$)	4,2±1,2	4,4±1,3	4,1±1,2	0,80835	3,4±1,1
Lymphocyte count ($10^3/\mu L$)	2±0,6	2±0,6	2,1±0,7	0,73464	2±0,5
Monocyte count ($10^3/\mu L$)	0,44±0,1	0,50±0,1	0,44±0,14	0,42227	0,35±0,1
Platelet count ($10^3/\mu L$)	203±44	218±54	212±45	0,50351	210±51
Mean platelet volume (fL)	10,6±1,1	11,3±1,0	11,3±0,9	0,08547	10,9±0,8
Immature platelet fraction (%)	2,4±0,8	4,3±1,1	2,9±0,4	0,03985	2,4±0,7

Table 3. Hematologic parameters measured in enrolled patients and healthy subjects. Data are shown as mean ± SD. P values are calculated by Kruskaal Wallis test among the 3 groups of patients.
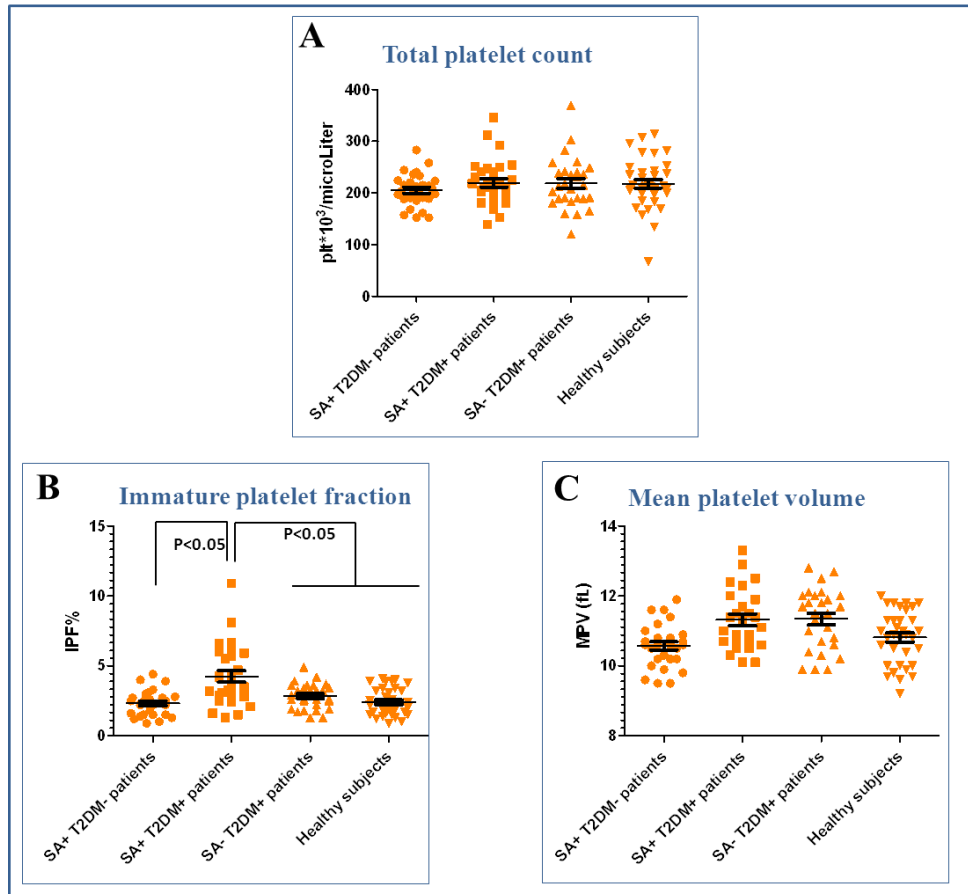
Figure 8. Distribution of platelet parameters derived from the blood count of the 4 groups enrolled. **A**, Platelet count expressed as platelets$*10^3$/µL); **B**, Immature platelet fraction (IPF), expressed in percentage; **C**, Mean platelet volume (MPV), expressed in fL.

As expected, a positive correlation was found between IPF% and MPV (R=0,6, p<0,05) (Figure 9), whereas no correlation was found between IPF and platelet count and between MPV and platelet count. No association was observed between glycaemia or HbA1c with MPV, IPF and platelet count.
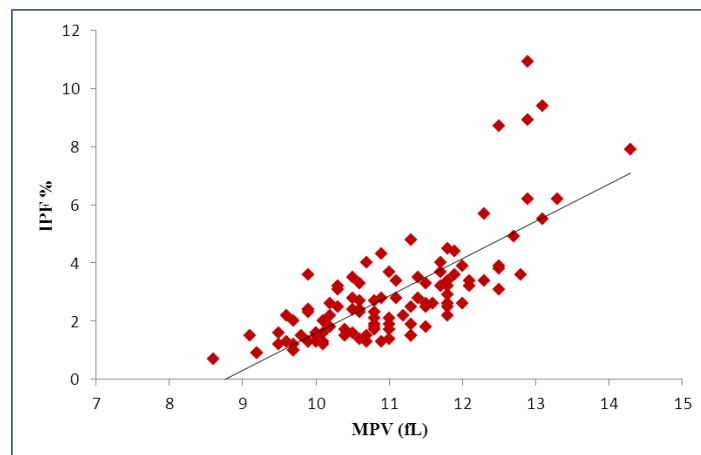
Figure 9. Correlation between MPV and IPF in the enrolled patients**.**

Evaluation of plasma biomarkers of platelet turnover and inflammation

Plasma levels of thrombopoietin (TPO), a marker of platelet turnover since it controls thrombopoiesis in the bone marrow, were found comparable among the four groups (figure 10A, table 4). Moreover, in accordance with this finding, TPO levels did not show any relation with platelet count, MPV, IPF, glycaemia and HbA1c; considering only the group of SA patients with diabetes, a weak inverse relation between TPO levels and MPV and IPF ($r= -0,2$; $p>0.05$) was observed. The regulation of thrombopoietin is complex and is not fully understood. Under normal physiologic conditions, decreased platelet production and turnover rate result in increased levels of unbound thrombopoietin, thereby enabling a compensatory response of megakaryocytes to the increased demand for peripheral blood platelets [176]. One may hypothesize that the inverse relation between thrombopoietin and platelet turnover parameters found in our study could be explained by an inclination of immature platelets to take up circulating thrombopoietin, thus decreasing free thrombopoietin levels in the setting of increased platelet turnover. Authors have suggested the megakaryocyte mass as the major determinant of thrombopoietin levels as opposed to circulating platelet count and size [176, 177]. Plasma interleukin-6 (IL-6), which regulates TPO production, was measured in all patients and healthy subjects (figure 10B, table 4). In agreement with data about TPO levels, IL-6 values were not significantly different among the groups of patients and healthy volunteers and did not correlate with any of the platelet turnover parameters (IPF, MPV, platelet count, TPO) and with any glycemic control parameters (glycaemia and glycosylated haemoglobin).
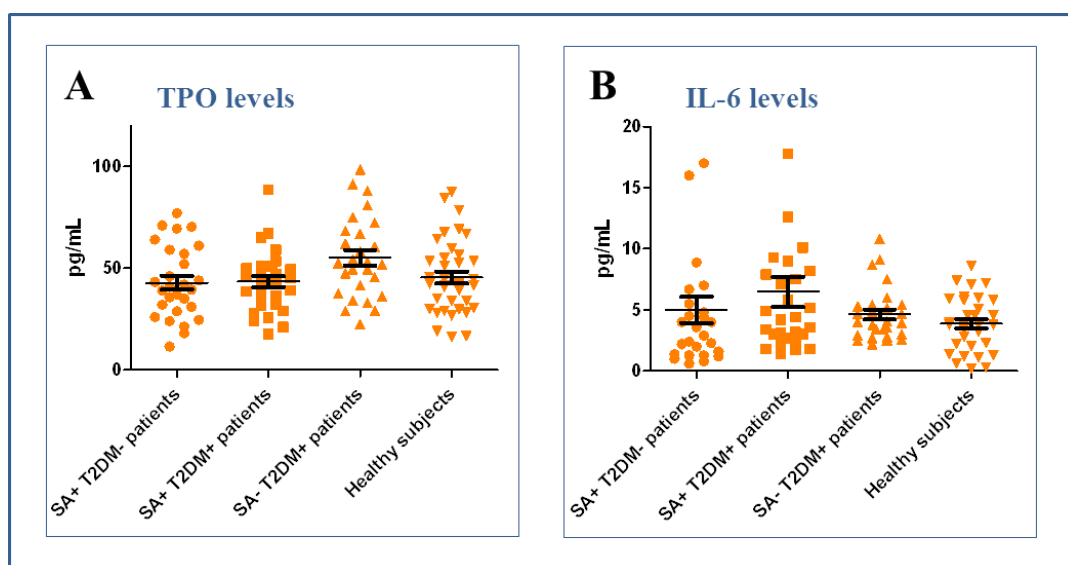

Figure 10. Plasma levels of thrombopoietin (TPO; **A**) and interleukin-6 (IL-6; **B**) in the enrolled population.

Measurements of hsCRP (high sensitive C reactive protein), which reflects chronic low-grade inflammation and is a marker of cardiovascular risk, revealed significantly increased levels in T2DM patients with SA compared to healthy volunteers (6,21±1,3 *versus* 2,43±0,4 respectively, p<0,01) and in T2DM patients without SA compared to healthy volunteers (4,48±0,75 *versus* 2,43±0,4 respectively, p<0,05); a moderate but significant difference was found comparing T2DM patients with SA with nondiabetic patients with SA (6,21±1,3 *versus* 3,18±1,73 respectively, p<0,05). No significant differences were observed comparing SA patients without T2DM and healthy subjects, but only a trend towards an higher value is SA patients (Figure 11). This suggests a role for diabetes in the increase in hsCRP levels, thus diabetes influences the inflammatory state of patients.

| VARIABLES mean ± SD	SA+ PATIENTS		SA- PATIENTS	HEALTHY SUBJECTS
	NO T2DM	T2DM	T2DM	
TPO (pg/mL)	40,2±18	44±15	52±20	42±18
IL-6 (pg/mL)	4,99±1	5,47±0,7	4,63±0,4	3,86±2,24
hsCRP (µg/mL)	3,18±1,73*b	6,21±1,3**	4,48±0,75*	2,43±0,4

Table 4. Plasma levels of biomarkers of platelet turnover and inflammation. TPO= thrombopoietin; IL-6= interleukin-6; hsCRP= high sensitive C reactive protein.*p<0,05 compared to healthy subjects; *b p<0,05compared to SA patients with T2DM; **p<0,01 compared to healthy subjects.
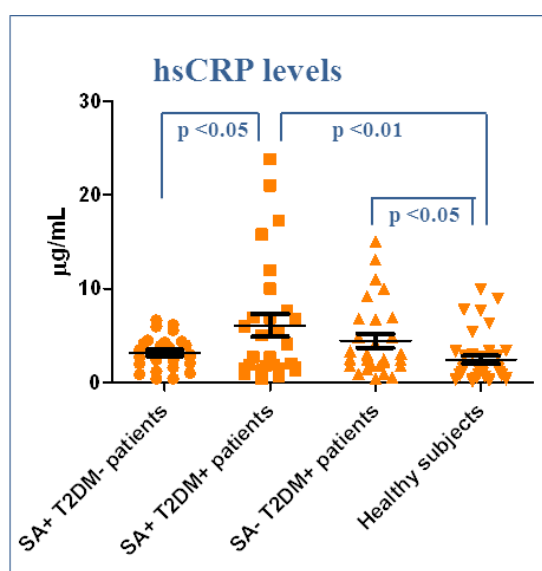
Figure 11. Plasma levels of high sensitive C reactive protein (hsCRP) in the enrolled population.

Compliance to aspirin therapy

Patient compliance to therapy with low-dose aspirin (100 mg/day) was assessed through the evaluation of serum thromboxane B_2 (TxB_2) levels. As a reference value, TxB_2 was measured in healthy subjects too. A good compliance was observed in the three groups of patients, which showed levels of serum TxB_2 similar among them, but significantly lower compared to those measured in healthy subjects, $p<0,001$ (Figure 12).
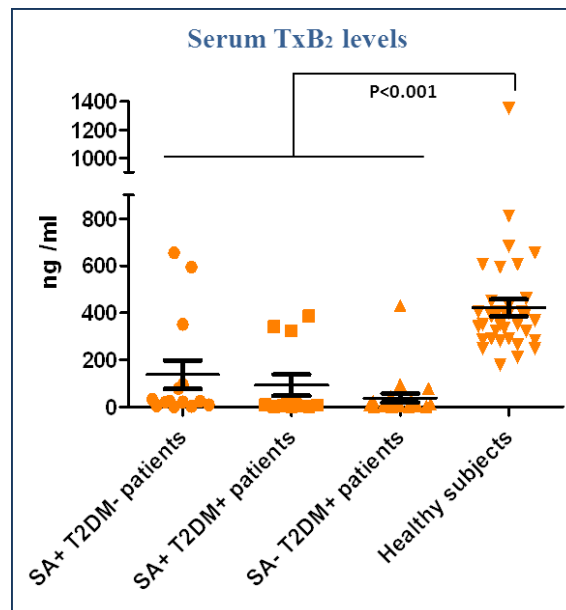

Figure 12. Serum TxB2 levels. A significant difference was found between patients and healthy subjects.

Assessment of platelet activation marker expression by flow cytometry

Surface expression of platelet activation markers was assessed by whole blood flow cytometry. Classic platelet activation markers, such as P-selectin, activated glycoprotein IIb/IIIa (aGPIIb/IIIa) and annexin V binding to phosphatidylserine, as well as glycoprotein IV (CD36), a constitutively glycoprotein, were evaluated. Furthermore, a novel marker of platelet activation, Tissue Factor, was assessed.

As far as P-selectin is concerned (Figure 13A), diabetes does not influence its expression since the two diabetic patient groups (T2DM patients with SA and T2DM patients without SA) showed values comparable to those observed in the two groups without diabetes (SA patients and healthy volunteers). However a significant higher P-selectin expression was found in the two groups of SA patients (with or without diabetes) compared to the two groups without SA

(diabetic patients and healthy subjects), both in term of percentage of positive cells (1,73±0,22 and 1,65±0,21 *versus* 1,12±0,14 and 1,19±0,09 respectively, p<0,05) and in term of mean amount of antigen expressed on each cells, MFI (3,76±0,69 and 3,76±0,69 *versus* 2,50±0,24 and 2,82±0,5 respectively, p<0.001), suggesting that the cardiovascular disease rather than the diabetes influences P-selectin expression.
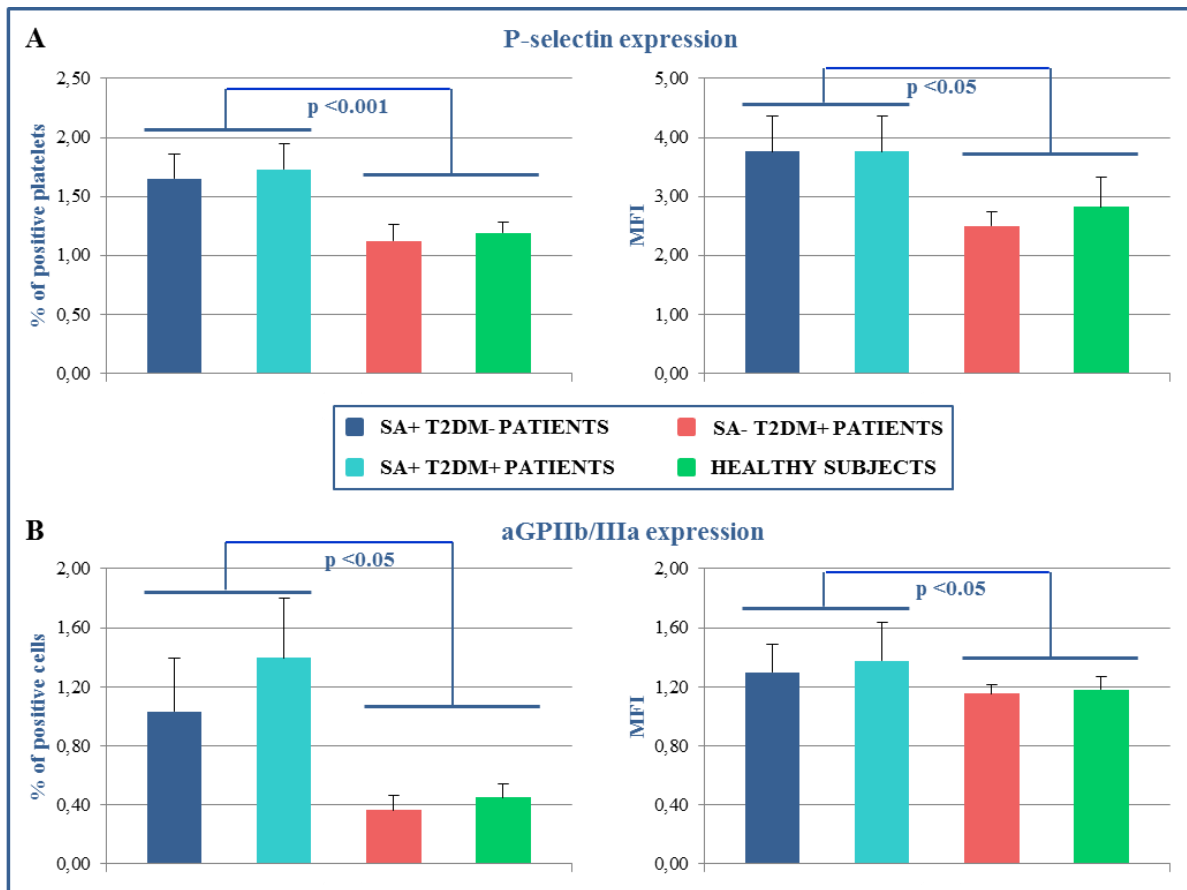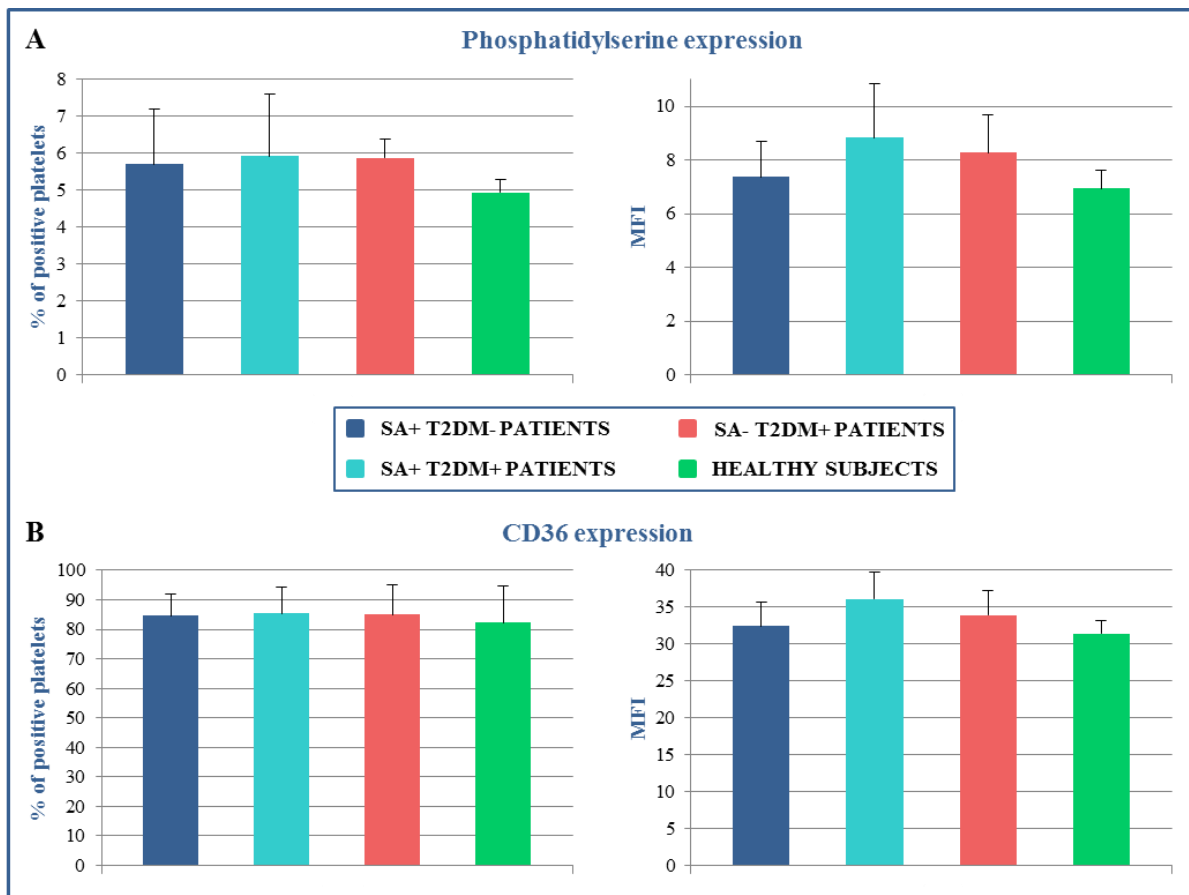

Figure 13. P-selectin (**A**) and aGPIIb/IIIa (**B**) expression on platelet surface by flow cytometry. Values are reported both as % of positive platelets as well as Mean fluorescence Intensity (MFI).

The same trend was found studying aGPIIb/IIIa platelet surface expression (figure 13B); SA patients with or without diabetes showed significant higher values compared to diabetic patients without SA and healthy subjects, both in term of percentage of positive platelets (1,40±0,60 and 1,03±0,36 *versus* 0,36±0.10 and 0,45±0,09 respectively, p<0,05) and in term of MFI (1,37±0,36 and 1,30±0,19 *versus* 1,15±0,06 and 1,18±0,09 respectively, p<0,05). Since a significant difference in aGPIIb/IIIa was detected between the two groups of diabetic patients, with or without SA, whereas no difference from the comparison of diabetic and nondiabetic SA groups, we could speculate again that the enhanced expression of aGPIIb/IIIa is due to the cardiovascular disease rather than to diabetes.

No significant differences were found in the annexin V binding to phosphatidylserine and in the CD36 expression among the four groups of patients. Only a mild trend towards higher value of phosphatidylserin and CD36 expression in patients with diabetes, with or without SA, compared to SA patient without diabetes and healthy volunteers could be observed (figure 14, A and B).


Figure 14. Platelet phosphatidylserine (**A**) and CD36 (**B**) expression assessed by flow cytometry. Values are reported both as % of positive platelets as well as Mean fluorescence Intensity (MFI).

On the contrary, TF expression on platelet surface appeared to be increased in the two groups of diabetic patients compared to the two groups without diabetes, suggesting that diabetes affects the expression of this antigen. In particular a clear trend towards an higher, although statistically not significant, percentage of TF-positive platelets was found in diabetic patients without SA (5,0±0,9) compared to SA patients (2,69±0,7) and healthy subjects (2,75±0,8). Comparison between two groups of SA patients resulted in a significant difference, with an increased percentage of TF-positive platelets found in T2DM patients compared to those without it (5,43±1 *versus* 2,69±0,7, p<0,05). In accordance with previously published data from our group [107], no statistically significant differences were observed between SA patients and healthy subjects (Figure 15). T2DM patients were characterized not only for a higher number of TF-positive

platelets but also for a trend towards a higher platelet TF Mean Fluorescence Intensity (MFI), which represents the amount of TF antigen expressed on each single platelet (Figure 16).
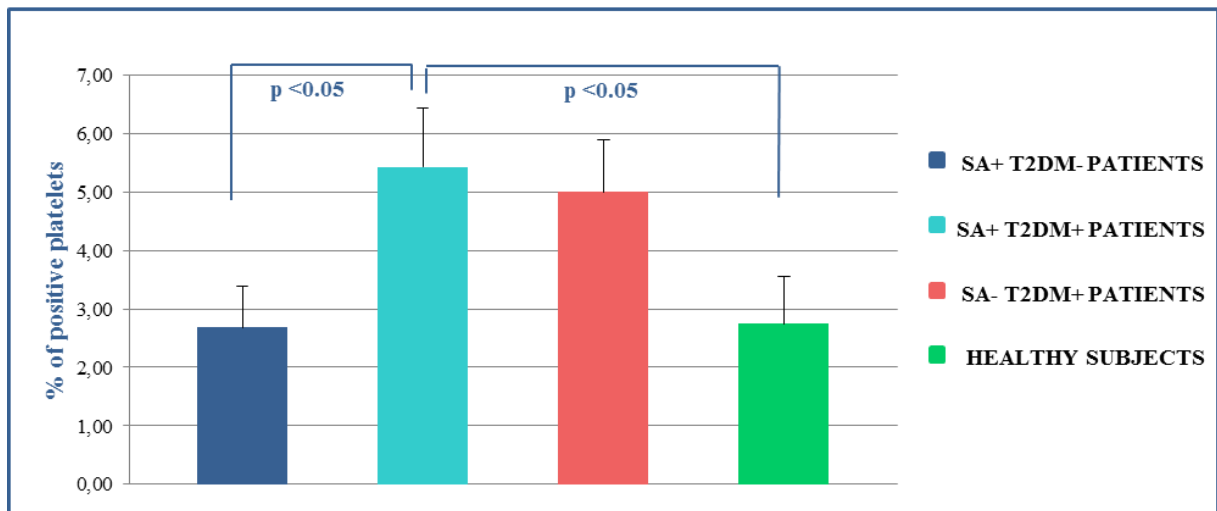
Figure 15. Percentage of Tissue Factor-positive platelets, assessed by flow cytometry, in the four groups of enrolled patients. Values are reported as % of positive platelets.
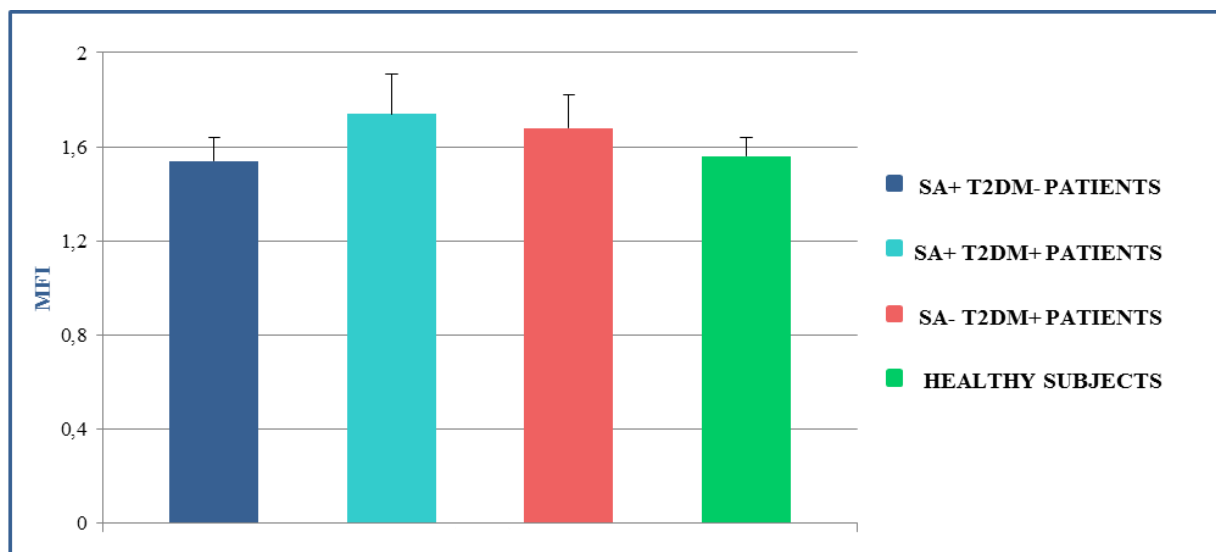
Figure 16. Platelet-associated Tissue Factor assessed by flow cytometry in the four groups of enrolled patients and expressed as Mean Fluorescence Intensity (MFI), which represents the average amount of antigen expressed on each cell per group.

We then focused our further analysis on platelet-associated TF expression on the comparison between SA patients with or without T2DM. In order to assess whether the different percentage of TF-positive platelets was the result of a global higher number of TF-positive platelets in T2DM patients or was due to a different platelet activation state, flow cytometry analysis was performed on permeabilized samples (Figure 17, A and B). Intracytoplasmic staining showed that a significantly higher number of TF-positive platelets was present in SA T2DM patients

compared to SA patients without T2DM (27,53±2,8 versus 14,98±1,34, p<0,001). Also the amount of TF antigen present in each single platelet showed a trend towards a higher value, although without reaching a statistically significance, in SA patients with T2DM compared to SA patients without T2DM.

Platelet associated TF expression was studied also in blood samples stimulated with 10 μM ADP. In agreement with the data just described above, upon stimulation, a higher number of platelets from SA patients with T2DM exposed TF on the surface compared to platelets from SA patients without T2DM (Figure 18, A and B); in fact, we found a significant difference (p<0,05) in the percentage of TF-positive platelets in SA patients with T2DM (26,60±4,98) compared to that of SA patients without diabetes (16,47±2,73). A trend towards an increased amount of TF expressed on the membrane of each platelet (represented by the MFI) was also observed upon stimulation.
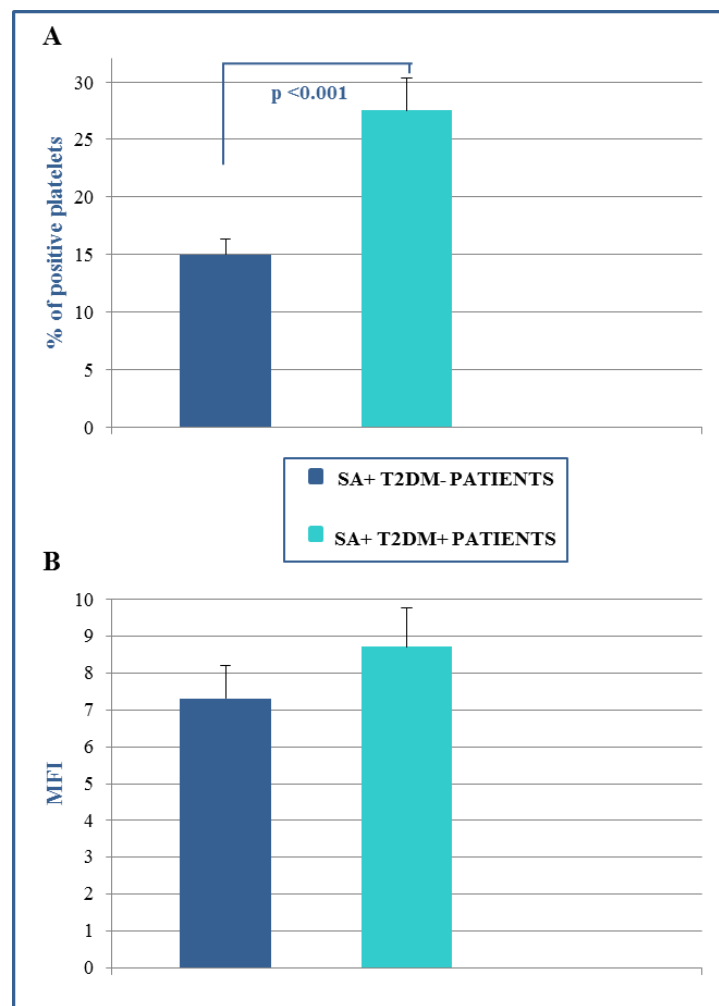
Figure 17. Intracellular platelet-associated Tissue Factor in patients with SA, with or without T2DM, evaluated by flow cytometry in permeabilized blood samples. **A**, percentage of intracytoplasmic TF-positive platelets; **B**, Intracytoplasmic TF-MFI.
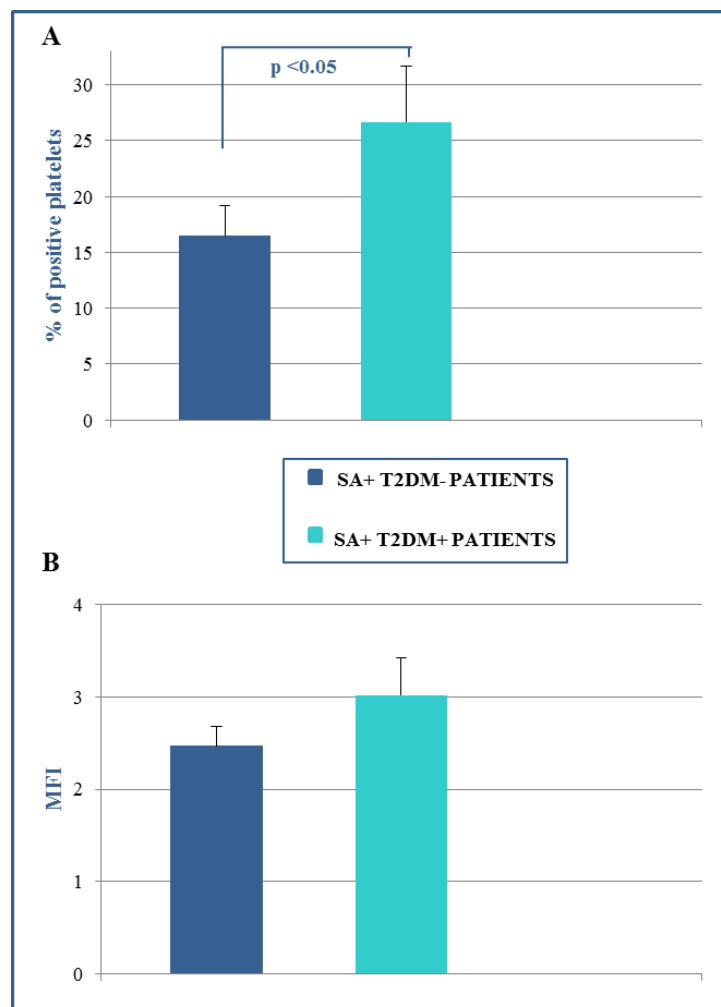
Figure 18. Surface expression of platelet-associated Tissue Factor in patients with SA, with or without T2DM, upon ADP stimulation, evaluated by flow cytometry. **A**, percentage of surface TF-positive platelets; **B**, Surface TF-MFI.

Evaluation of the thombin generation capacity of platelet isolated from the SA patients

In order to assess whether the platelet-associated TF described in SA patients with and without T2DM was functionally active, we evaluated the thrombin generation capacity of platelet lysates obtained from a subset of patients (n=10) of the two groups by means of the Calibrated Automated Thrombogram (CAT) assay.

All the parameters extrapolated by the thrombograms at the end of the thrombin generation assay were different between SA patients without diabetes and SA patients with diabetes (Table 5). In SA patients with T2DM an increase in the peak height, which represents the amount of thrombin generated, in the endogenous thrombin potential (ETP), index of the total amount of active thrombin generated from the activation of coagulation, and in the velocity index, which

represents the kinetics of thrombin generation, were observed compared to SA patients without T2DM. In agreement with these data, a decrease in lag time, which is the time needed to start thrombin generation, and a decrease in time to peak, which is the time necessary to reach the maximum of generated thrombin, were observed in SA patients with T2DM compared to SA patients without T2DM. Although the comparison does not reach the statistical significance, perhaps due to the limited number of comparison performed, these data are suggestive that platelets from T2DM patients show a higher thrombin generation capacity.

		Lag time (min)	ETP (nM•min)	Peak (nM)	ttPeak (min)	Vel Index (nM/min)
SA+ T2DM-	Media	12,1	1229,4	134,2	16,8	42,3
	St. dev.	1,5	18,6	7,3	2,7	2,6
SA+ T2DM+	Media	9,8	1233,9	158,3	12,4	66,3
	St. dev.	0,8	20,7	6,8	1,8	3,9

Table 5. Main parameters calculated from thrombin generation assay used to determine thrombin generation capacity of platelets from SA patients, with or without T2DM.

In order to verify the direct contribution of platelet-associated TF to thrombin generation, isolated platelet samples were treated with a specific anti-TF antibody in order to inhibits TF activity and to investigate if this inhibition led to changes in thrombin generation capacity. Moreover, a comparison between TF inhibition effect in SA patients with T2DM with the effect produced in SA patients without T2DM was performed in order to elucidate if the TF contribution to thrombin generation is increased in diabetic patients which were those patients having higher number of TF-positive platelets. In SA patients without T2DM, the treatment with the anti-TF antibody led to a moderate increase both in lag time and in time to peak; the difference in lag time and in time to peak obtained performing the assay in presence and in absence of the antibody was defined "delta lag-time" and "delta time-to-peak" respectively, and was calculated for each patients. In SA patients without T2DM, the mean of delta lag time was 2,67±0,87 min and the mean of delta time to peak was 3,88±1,14 min. In SA patients with T2DM, the treatment with the anti-TF antibody caused a strong increase both in lag time and in time to peak: the mean of delta lag time was 8,29±1,31 min and the mean of delta time to peak was 9,18±1,30 min (Figure 19 and Table 6).

The delay caused by the TF-antibody was significantly higher in diabetic patients, in fact both the delta lag-times and the delta times-to-peak were significantly increased in SA patients with T2DM compared to SA patients without T2DM ($p < 0,01$ and $p < 0,05$ respectively) (Table 6).

The enhanced kinetics of thrombin generation and the greater delay caused by TF inhibition in T2DM patients suggest an increased overall coagulability potential and a higher contribution of TF to thrombin generation in SA patients with T2DM compared to SA patients without T2DM.
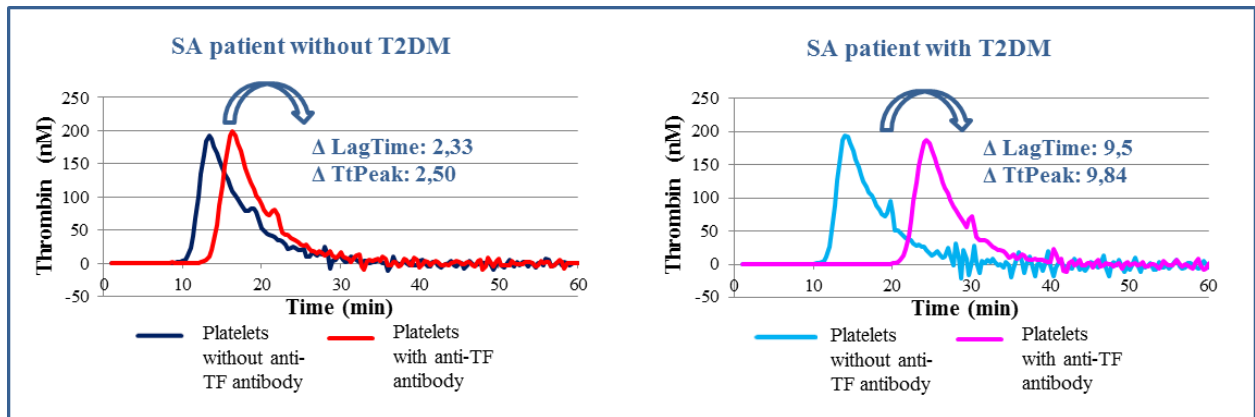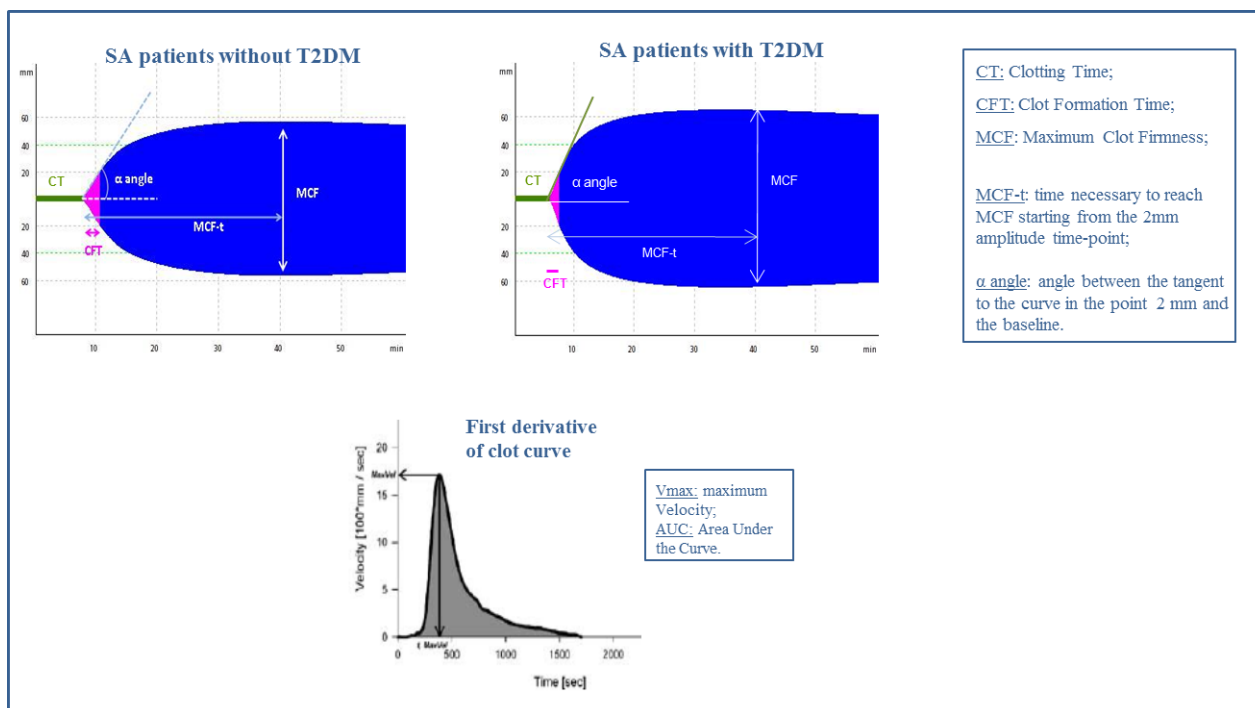

Figure 19. Representative thrombin generation curves from platelets of a SA patient without T2DM, on the left, and of a SA patient with T2DM, on the right, in the presence and in the absence of a specific anti-TF antibody.

A

	Delta LagTime (min)	
	SA+ T2DM-	SA+ T2DM+
Mean	2,67	8,29
st.dev.	0,87	1,31
T-test	0,009	

B

	Delta Time to peak	
	SA+ T2DM-	SA+ T2DM+
Mean	3,88	9,18
st.dev.	1,14	1,3
T-test	0,035	

Table 6. Comparison of the delay in lag time (**A**) and in time to peak (**B**) caused by the anti-TF antibody treatment in the two groups of SA patients with or without T2DM.

Assessment of global hemostatic function by thromboelastometry

The capacity to form a clot, defined as global hemostatic function, was studied by thromboelastometry, a technique that assesses in whole blood the kinetics of clot formation as well as the size of the clot. Significant differences were found in the main parameters describing clot formation between SA patients with and without T2DM; these differences could be appreciated also looking at the clot curves of SA patients with or without T2DM: diabetic patients had a reduced clotting time (CT) and reached a higher maximum amplitude of the curve (Figure 20).

Figure 20. Representative thrombin generation curves from platelets of a SA patients without T2DM, on the left, and of a SA patient with T2DM, on the right, in the presence and in the absence of a specific anti-TF antibody

In particular a significant decrease in CFT, index of the kinetic of clot formation since it represents the time needed to form a clot of 2 mm, was found in SA patients with T2DM compared to SA patients without T2DM; furthermore, significant increases in MCF, a parameter that describes clot firmness, in alpha angle, index of the rate of clot formation, in Vmax, which represents the maximum degree of clot formation, and in AUC, which is an indirect measurements of generated thrombin, were found in T2DM patients compared to nondiabetic patient (Table 7).

		CFT	MCF	alpha	MAXV	AUC
SA with T2DM	median	**122,0**	**61,0**	**66,0**	**11,0**	**6147,0**
	min-max	69-189	53-71	55-76	7-20	5284-7065
SA noT2DM	median	**148,0**	**58,0**	**61,5**	**9,5**	**5889,0**
	min-max	78-302	46-71	42-74	5-16	4751-7064
	p value *versus* T2DM	0,0095	0,0013	0,0077	0,0083	0,0017

Table 7. Thromboelastometric parameters measured in SA patients with T2DM and in SA patients without T2DM by Rotem system.

Quality controls for platelet isolation and RNA extraction

Platelet preparations intended for RNA isolation were all checked for the absence of platelet activation by comparing platelet P-selectin expression in whole blood with that of isolated platelets. Thanks to the developed technique and to the care in handling the samples, all the isolated platelet preparations were not activated by the separation procedure. To be sure that transcriptome analysis was performed only on platelet-derived RNAs and not on leukocyte-derived RNAs, all preparations were also checked for absence of contaminating leukocytes both by flow cytometry (Figure 21) or by Hematologic analyzer (the two instruments gave comparable results). Only platelet preparations with less than 50 leukocytes per 10^6 platelets were considered suitable for RNA analysis. Only 1,6% of the preparations resulted contaminated from leukocytes and, as a consequence, they were discarded for transcriptome analysis; 98,4% were suitable, in particular 34,4% of them were completely free of leukocyte contamination (Figure 22).
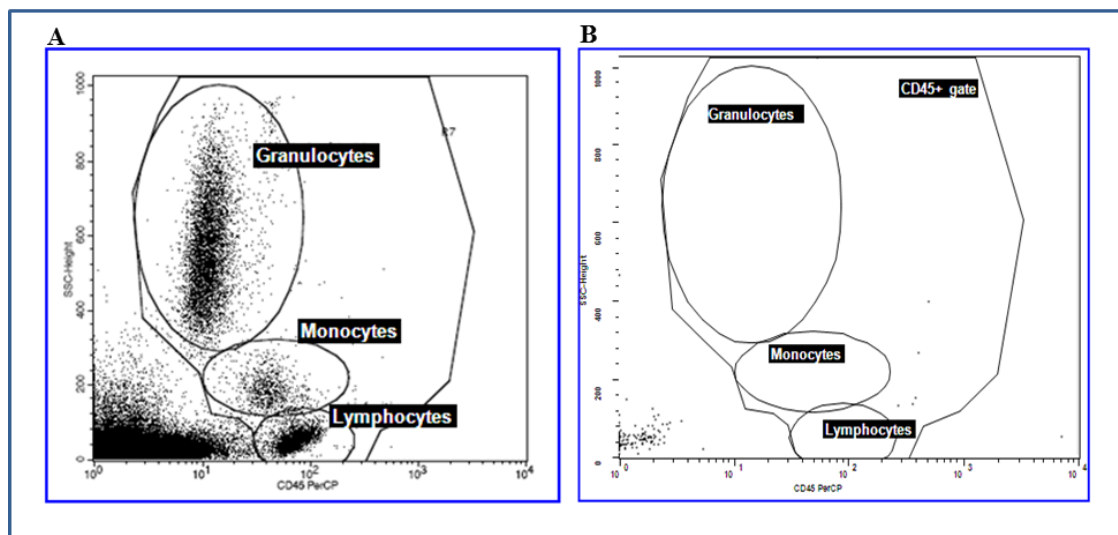
Figure 21. Control of leukocyte contamination in isolated platelets by flow cytometry. **A**, leukocytes detection in whole blood on the basis of side scatter characteristics and positivity to CD45; **B**, detection of leukocyte contamination in isolated platelet samples applying the same setting used for leukocytes acquisition in whole blood and the same strategy of gating and analysis.

RNA was extracted from pure platelet preparations and quantified by a highly sensitive absorbance reader. RNA quality control and quantitation is essential before any downstream application. A positive correlation was found between the platelet number in each sample and the amount of recovered RNA suggestive of the good optimization of the method applied (r=0,78) (Figure 23). Generally, we obtained 1 µg of RNA from 1 billion of platelets. We checked the quality of these RNAs by using Agilent RNA 6000 Pico bioanalyzer and the obtained profiles

showed the typical platelet RNA pattern, with the 18S ribosomal RNA more abundant than the 28S ribosomal RNA. The 260/280 ratio indicated absence of contaminant proteins (Figure 24).
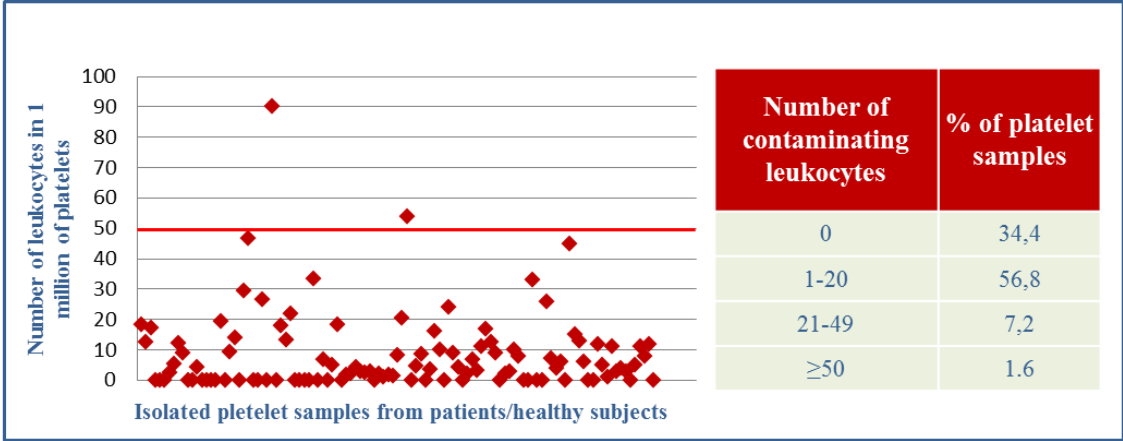
Figure 22. Results of leukocyte contamination controls in isolated platelets.The cut off of 50 leukocytes in 1 million of platelets was used to discriminate pure and contaminated preparations.
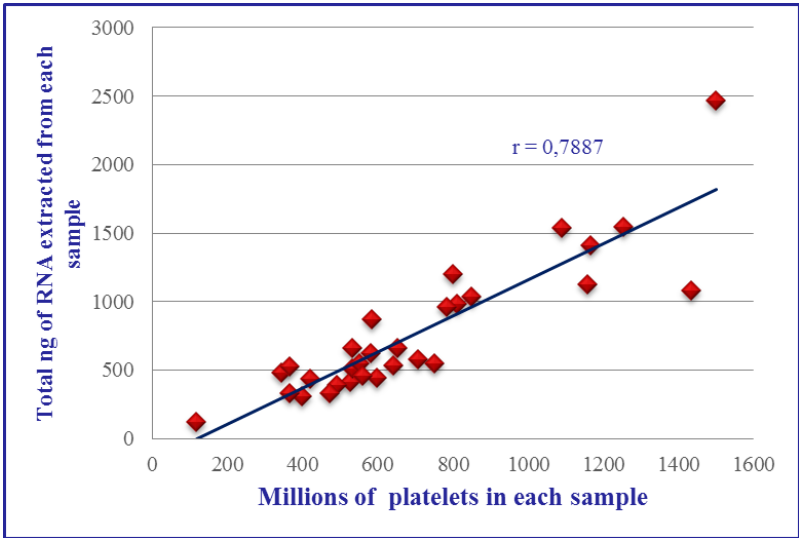
Figure 23. Correlation between the number of platelets isolated from a patient and the amount of recovered RNA in a representative subset of samples.
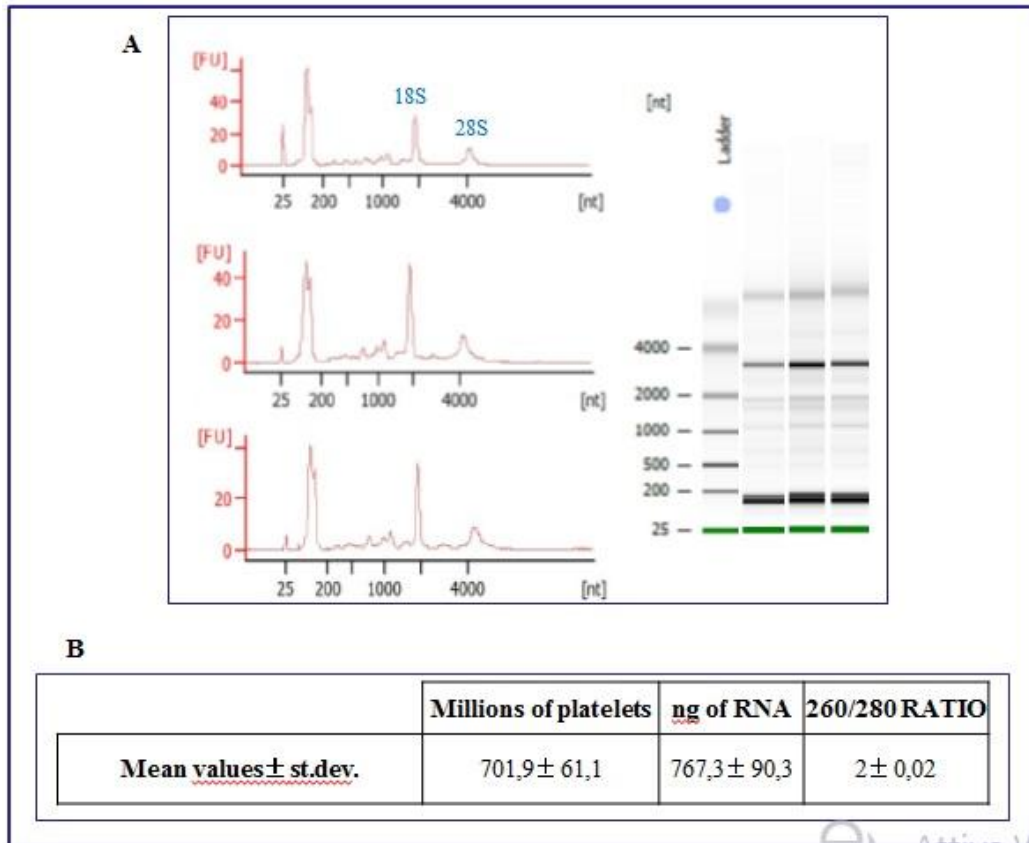
Figure 24. A, RNA elettropherograms by Agilent bioanalyzer to assess quality of recovered platelet RNAs; **B**, quantification and quality control by Infinite M200Pro.

Platelet transcriptome profiles

Gene profiling by microarray analysis of RNA samples detected more than 6000 distinct transcripts as being present in SA and healthy platelets (≈13% of the printed probes). One-way ANOVA followed by correction for multiple testing to obtain a FDR < 0.05 with a confidence level of 80% identified 319 unique mRNAs that showed a significant ± 1.2-fold or greater difference in expression between healthy and SA platelets (Figure 25): 156 of these genes were over-expressed, whereas 163 had a lower expression in healthy compared to SA (either T2DM-positive or not) platelets. Healthy *vs*. SA mean FC ranged from −2.5 to +3.0, and p-values from 0.003 to 0.0000001. Unsupervised hierarchical clustering was used to group healthy subjects and SA patients and platelet transcripts on the basis of similarities of gene expression (Figure 25). The differential gene expression profiles allowed a partial discrimination of healthy from SA platelets.
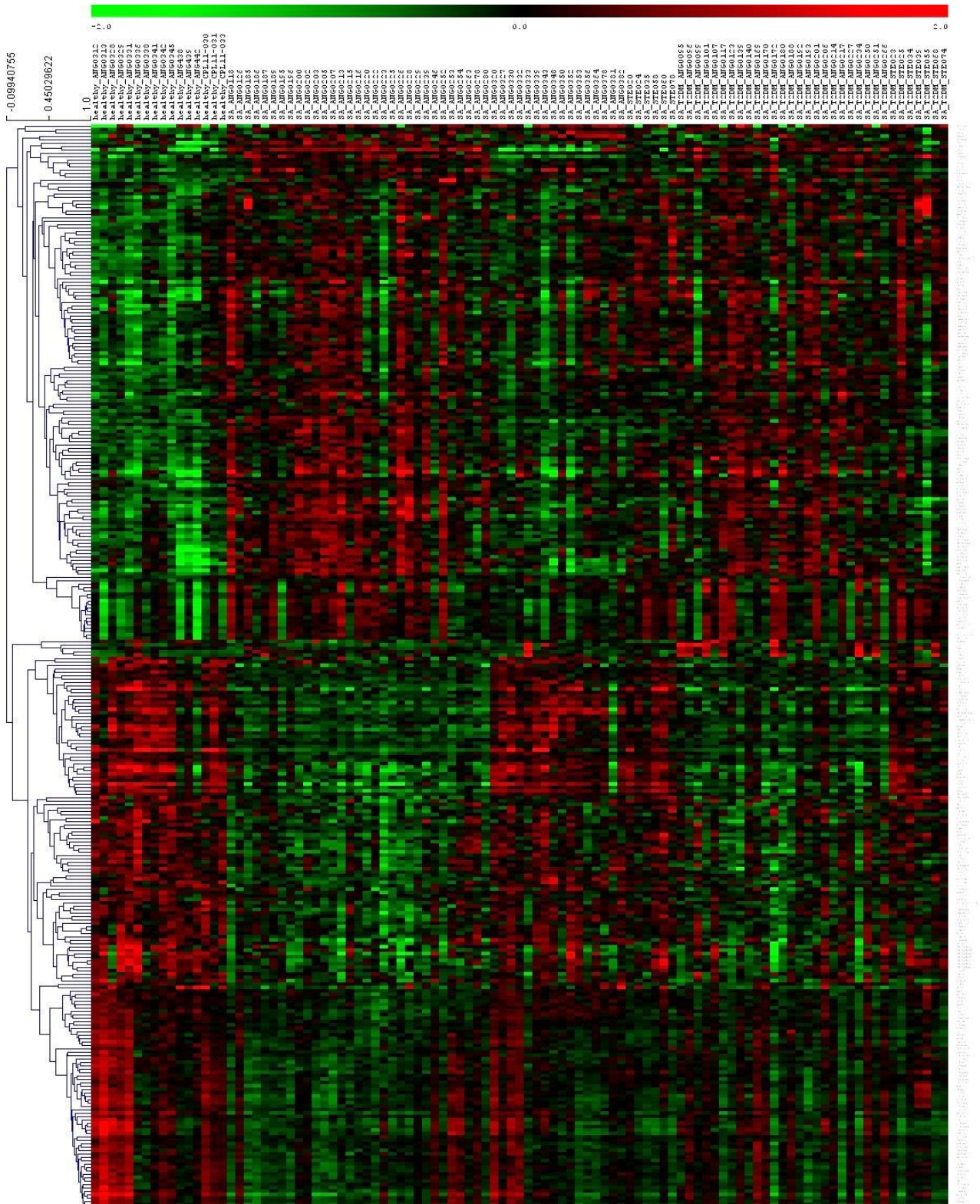
Figure 25. Heatmap of DE genes healthy *vs.* SA platelets

The univariate *t*-test followed by permutation testing identified 35 genes significantly different between SA$^+$T2DM$^+$ and SA$^+$T2DM$^-$ platelets: 24 of them were over-expressed, whereas 11 showed a decreased expression in SA$^+$T2DM$^+$ compared to SA$^+$T2DM$^-$ platelets (Table 8).

Parametric p-value	Permutation p-value	Geom mean of intensities SA	Geom mean of intensities SA_T2DM	Fold-change T2DM/SA	Name	Accession	Symbol
4,92E-04	3,00E-04	143,02	216,8	1,52	CD69 molecule	NM_001781	CD69
1,93E-03	2,30E-03	1626,54	2248,7	1,38	ribosomal protein L21	NM_000982	RPL21
4,89E-03	4,60E-03	238,77	321,73	1,35	Sad1 and UNC84 domain containing 2	NM_015374	SUN2
3,76E-03	4,10E-03	230,96	293,13	1,27	immunoglobulin lambda-like polypeptide 1	NM_020070	IGLL1
1,71E-03	2,20E-03	158,11	196,22	1,24	mal, T-cell differentiation protein	NM_022440	MAL
4,65E-03	2,50E-03	162,18	193,17	1,19	CDC-like kinase 1	NM_004071	CLK1
1,61E-03	1,30E-03	151,72	178,52	1,18	stathmin-like 3	NM_015894	STMN3
3,20E-03	3,00E-03	191,94	224,46	1,17	AHNAK nucleoprotein	NM_001620	AHNAK
1,06E-03	4,00E-04	168,6	196,83	1,17	DNA-damage-inducible transcript 4	NM_019058	DDIT4
4,26E-03	3,50E-03	371,43	432,43	1,16	catenin (cadherin-associated protein), alpha-like 1	NM_003798	CTNNAL1
3,33E-03	3,10E-03	155,15	180,4	1,16	arginine and glutamate rich 1	NM_018011	ARGLU1
2,31E-03	2,60E-03	168,67	195,57	1,16	IL2-inducible T-cell kinase	NM_005546	ITK
3,22E-03	1,30E-03	170,38	196,28	1,15	ZFP36 ring finger protein-like 2	NM_006887	ZFP36L2
5,40E-04	4,00E-04	137,75	156,95	1,14	natural killer-tumor recognition sequence	NM_005385	NKTR
2,22E-03	2,80E-03	135,12	152,06	1,13	ATPase, aminophospholipid transporter, class I, type 8B, member 2	NM_020452	ATP8B2
5,43E-04	3,00E-04	157,06	175,48	1,12	small nucleolar RNA host gene 1 (non-protein coding)	NR_003098	SNHG1
3,19E-03	3,10E-03	219,13	244,76	1,12	BTB (POZ) domain containing 1	NM_001011885	BTBD1
3,50E-03	3,10E-03	142,68	158,41	1,11	DnaJ (Hsp40) homolog, subfamily B, member 1	NM_006145	DNAJB1
3,56E-03	2,70E-03	152,16	164,2	1,08	tumor protein p53 inducible protein 13	NM_138349	TP53I13
2,81E-03	2,60E-03	153,36	164,87	1,08	Fc receptor-like 3	NM_001024667	FCRL3
4,57E-03	3,30E-03	166,32	178,53	1,07	Rab geranylgeranyltransferase, beta subunit	NM_004582	RABGGTB
1,58E-03	1,40E-03	158,07	169,63	1,07	RNA binding motif protein 33	NM_001008408	RBM33
9,58E-04	7,00E-04	173,66	185,19	1,07	cullin 1	NM_003592	CUL1
2,24E-03	3,00E-03	153,48	163,6	1,07	SMG1 phosphatidylinositol 3-kinase-related kinase	NM_015092	SMG1
3,99E-03	4,00E-03	162,01	154,19	-1,05	crumbs homolog 3 (Drosophila)	NM_139161	CRB3
3,31E-03	3,70E-03	171,75	162,44	-1,06	Sjogren syndrome nuclear autoantigen 1	NM_003731	SSNA1
5,95E-04	3,00E-04	171,06	161,6	-1,06	PHD finger protein 16	NM_001077445	PHF16
2,29E-03	2,50E-03	159,41	149,49	-1,07	Kruppel-like factor 7 (ubiquitous)	NM_003709	KLF7
7,60E-04	1,20E-03	182,46	170,02	-1,07	protein phosphatase 1, regulatory (inhibitor) subunit 14B	NM_138689	PPP1R14B
4,67E-03	5,70E-03	171,08	159,4	-1,07	interferon, gamma-inducible protein 30	NM_006332	IFI30
6,37E-04	8,00E-04	179,35	167,08	-1,07	COMM domain containing 4	NM_017828	COMMD4
4,88E-03	4,90E-03	215,56	188,4	-1,14	dual specificity phosphatase 6	NM_022652	DUSP6
4,97E-03	5,10E-03	504,1	408,58	-1,23	myosin light chain kinase	NM_053026	MYLK
9,91E-04	6,00E-04	6194,37	4873,55	-1,27	tubulin, alpha 4a	NM_006000	TUBA4A
3,50E-03	2,50E-03	646,1	446,15	-1,45	prothymosin alpha-like	XM_001126659	LOC728026

Table 8. DE genes in SA[+]T2DM[+] *vs*. SA[+] T2DM[-] platelets.

Unsupervised hierarchical clustering showed that these differential expression profiles partially discriminated T2DM$^+$ from T2DM$^-$ SA platelets (Figure 26). Specifically, SA$^+$T2DM$^-$ platelets presented expression profiles that were more similar to each other than SA$^+$T2DM$^+$ and, thus, most of the T2DM$^-$ patients fell into one distinct sub-cluster (on the right in Figure 26). Conversely, SA$^+$T2DM$^+$ gene expression profiles were only partially similar to each other, specifically for those genes with a reduced expression in comparison with SA$^+$T2DM$^-$ platelets (gene cluster on the bottom of the heatmap in Figure 26). On the contrary, the genes that were over-expressed in SA$^+$T2DM$^+$ platelets were divided in two sub-clusters (gene clusters on the top of the heatmap in Figure 26) and drove the partition of SA$^+$T2DM$^+$ preferentially in two sub-clusters of patients (subjects on the left of Figure 26). Finally, graph analysis of the mean expression levels of the 8 top hit DE genes in SA$^+$T2DM$^+$, SA$^+$T2DM$^-$ and healthy subjects (Figure 27) showed different expression hierarchies: SA$^+$T2DM$^+$ > SA$^+$T2DM$^-$ > healthy for two *SUN2* and *RPL21*, SA$^+$T2DM$^+$ < SA$^+$T2DM$^-$ < healthy for *MYLK*, SA$^+$T2DM$^+$ > SA$^+$T2DM$^-$ = healthy for *CD69* and *MAL*, SA$^+$T2DM$^+$ < SA$^+$T2DM$^-$ = healthy for *LOC728026*, SA$^+$T2DM$^+$ > SA$^+$T2DM$^-$ < healthy for *IGLL1*, and SA$^+$T2DM$^+$ < SA$^+$T2DM$^-$ > healthy for *TUBA4A*.
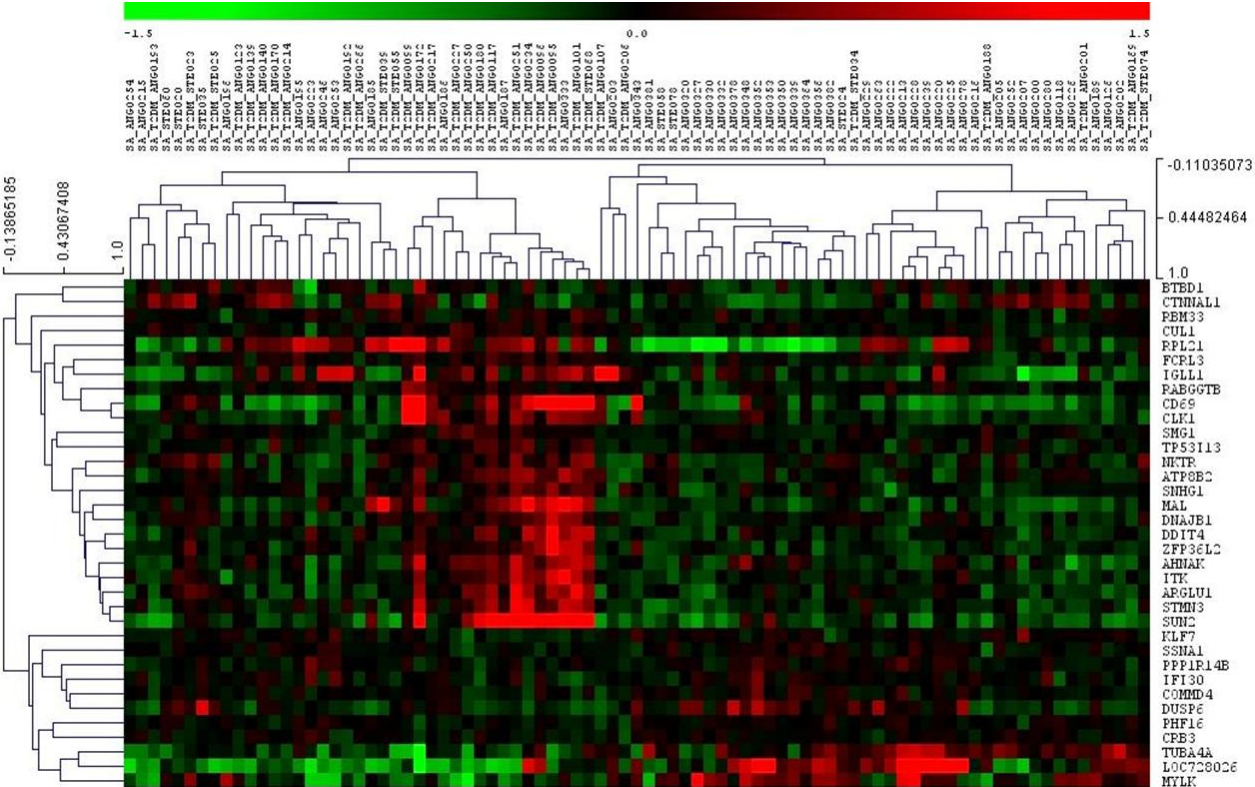
Figure 26. Heatmap of DE genes SA$^+$T2DM$^+$ *vs*. SA$^+$ T2DM$^-$ patients.
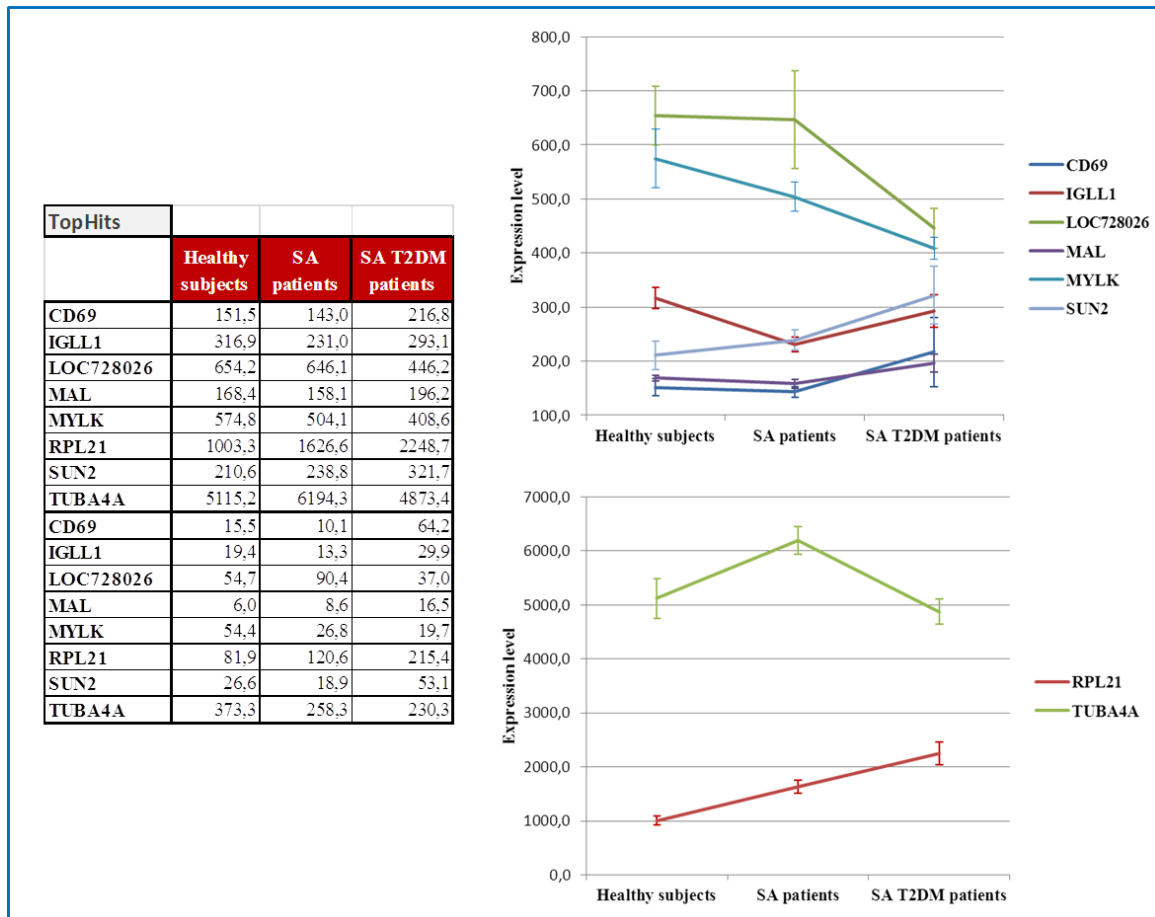
Figure 27. Comparison of the mean expression levels of top hits genes in SA+T2DM+, SA+T2DM- and healthy platelets.

Enrichment analysis of GO categories allowed identifying biological processes and cellular components whose expression was significantly altered in SA in comparison with healthy platelets (Table 9, A and B). Gene expression changes in a number of strictly related functions/processes were up-regulated in SA patients: platelet degranulation and activation, cell adhesion, negative regulation of endocytosis, and integrin complex (Figure 28, A and B). Conversely, a few GO categories were apparently down-regulated in SA platelets, namely protein-DNA complex subunit organization, regulation of protein localization, transmembrane receptor protein tyrosine kinase signaling pathway, and extracellular structure organization (Figure 29, A and B).

A similar enrichment analysis of GO terms allowed identifying biological processes whose expression was significantly altered in SA+T2DM+ in comparison with SA+T2DM- platelets (Table 10 and Figure 30). In particular, peptidyl-serine phosphorylation and nuclear-transcribed mRNA catabolic process appeared up-regulated in SA+T2DM+ platelets.

Up	GO Term Description	P-value	Enrichment	N	Genes
	platelet degranulation	9,03E-05	5,44	8	[TUBA4A - tubulin, alpha 4a, CD63 - cd63 molecule, VWF - von willebrand factor, APP - amyloid beta (a4) precursor protein, CALM1 - calmodulin 1 (phosphorylase kinase, delta), PSAP - prosaposin, ITGB3 - integrin, beta 3 (platelet glycoprotein iiia, antigen cd61), ITGA2B - integrin, alpha 2b (platelet glycoprotein iib of iib/iiia complex, antigen cd41)]
	platelet activation	1,23E-04	3,55	12	[GP1BA - glycoprotein ib (platelet), alpha polypeptide, TUBA4A - tubulin, alpha 4a, MGLL - monoglyceride lipase, CD63 - cd63 molecule, GNB1 - guanine nucleotide binding protein (g protein), beta polypeptide 1, VWF - von willebrand factor, APP - amyloid beta (a4) precursor protein, CALM1 - calmodulin 1 (phosphorylase kinase, delta), PSAP - prosaposin, ITGB3 - integrin, beta 3 (platelet glycoprotein iiia, antigen cd61), SRF - serum response factor (c-fos serum response element-binding transcription factor), ITGA2B - integrin, alpha 2b (platelet glycoprotein iib of iib/iiia complex, antigen cd41)]
	cell-substrate adhesion	1,84E-04	5,71	7	[ITGB5 - integrin, beta 5, CD63 - cd63 molecule, VWF - von willebrand factor, ITGB1 - integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen cd29 includes mdf2, msk12), ITGB3 - integrin, beta 3 (platelet glycoprotein iiia, antigen cd61), SRF - serum response factor (c-fos serum response element-binding transcription factor), ITGA2B - integrin, alpha 2b (platelet glycoprotein iib of iib/iiia complex, antigen cd41)]
BP	cellular component morphogenesis	3,07E-04	3,45	11	[PANK2 - pantothenate kinase 2, GP1BA - glycoprotein ib (platelet), alpha polypeptide, DLG4 - discs, large homolog 4 (drosophila), PNPT1 - polyribonucleotide nucleotidyltransferase 1, RILPL2 - rab interacting lysosomal protein-like 2, PICALM - phosphatidylinositol binding clathrin assembly protein, PACSIN2 - protein kinase c and casein kinase substrate in neurons 2, APP - amyloid beta (a4) precursor protein, ITGB1 - integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen cd29 includes mdf2, msk12), DBNL - drebrin-like, ITGB3 - integrin, beta 3 (platelet glycoprotein iiia, antigen cd61)]
	regulation of amyloid precursor protein catabolic process	3,73E-04	18,35	3	[GSAP - gamma-secretase activating protein, PICALM - phosphatidylinositol binding clathrin assembly protein, FLOT2 - flotillin 2]
	cell adhesion	5,35E-04	2,62	15	[INPPL1 - inositol polyphosphate phosphatase-like 1, SYMPK - symplekin, F11R - f11 receptor, ITGA2B - integrin, alpha 2b (platelet glycoprotein iib of iib/iiia complex, antigen cd41), ITGB5 - integrin, beta 5, GP1BA - glycoprotein ib (platelet), alpha polypeptide, CD63 - cd63 molecule, FLOT2 - flotillin 2, VWF - von willebrand factor, B4GALT1 - udp-gal:betaglcnac beta 1,4- galactosyltransferase, polypeptide 1, APP - amyloid beta (a4) precursor protein, ITGB1 - integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen cd29 includes mdf2, msk12), ITGB3 - integrin, beta 3 (platelet glycoprotein iiia, antigen cd61), SRF - serum response factor (c-fos serum response element-binding transcription factor), PTPRF - protein tyrosine phosphatase, receptor type, f]
	negative regulation of endopeptidase activity	5,67E-04	4,19	8	[BST2 - bone marrow stromal cell antigen 2, PICALM - phosphatidylinositol binding clathrin assembly protein, APP - amyloid beta (a4) precursor protein, ITIH5 - inter-alpha-trypsin inhibitor heavy chain family, member 5, SPINT2 - serine peptidase inhibitor, kunitz type, 2, CDKN2D - cyclin-dependent kinase inhibitor 2d (p19, inhibits cdk4), PRDX5 - peroxiredoxin 5, IFI6 - interferon, alpha-inducible protein 6]
	negative regulation of endocytosis	9,55E-04	8,63	4	[DLG4 - discs, large homolog 4 (drosophila), PICALM - phosphatidylinositol binding clathrin assembly protein, PACSIN2 - protein kinase c and casein kinase substrate in neurons 2, CNN2 - calponin 2]
CC	integrin complex	3,12E-04	11,29	4	[ITGB5 - integrin, beta 5, ITGB1 - integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen cd29 includes mdf2, msk12), ITGB3 - integrin, beta 3 (platelet glycoprotein iiia, antigen cd61), ITGA2B - integrin, alpha 2b (platelet glycoprotein iib of iib/iiia complex, antigen cd41)]

Table 9. A. GO categories significantly upregulated in SA platelets in comparison with healthy platelets; BP= biological process; CC= cellular component.

Down	GO Term Description	P-value	Enrichment	N	Genes
	protein-DNA complex subunit organization	6.73E-05	4.46	10	[PIAS1 - protein inhibitor of activated stat, 1, SMARCA4 - swi/snf related, matrix associated, actin dependent regulator of chromatin, subfamily a. member 4, GTF2H5 - general transcription factor iih, polypeptide 5, HIST1H2BC - histone cluster 1, h2bc, TCF4 - transcription factor 4, HIST1H2BE - histone cluster 1, h2be, HIST1H2BF - histone cluster 1, h2bf, HIST1H1C - histone cluster 1, h1c, HIST1H2BH - histone cluster 1, h2bh, HIST1H2BN - histone cluster 1, h2bn]
BP	regulation of protein localization	3.21E-04	2.64	16	[CNST - consortin, connexin sorting protein, LYPLAL1 - lysophospholipase-like 1, STX10 - syntaxin 10, NKD2 - naked cuticle homolog 2 (drosophila), NFKBIA - nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha, RHOQ - ras homolog family member q, TNFSF4 - tumor necrosis factor (ligand) superfamily, member 4, LEPROT - leptin receptor overlapping transcript, LYN - v-yes-1 yamaguchi sarcoma viral related oncogene homolog, FZD7 - frizzled family receptor 7, DAB2 - dab, mitogen-responsive phosphoprotein, homolog 2 (drosophila), RASSF5 - ras association (ralgds/af-6) domain family member 5, MED1 - mediator complex subunit 1, FYN - fyn oncogene related to src, fgr, yes, GSK3B - glycogen synthase kinase 3 beta, CREBRF - creb3 regulatory factor]
	transmembrane receptor protein tyrosine kinase signaling pathway	6.62E-04	2.39	17	[PDGFA - platelet-derived growth factor alpha polypeptide, PSENEN - presenilin enhancer 2 homolog (c. elegans), EIF4EBP2 - eukaryotic translation initiation factor 4e binding protein 2, NFKBIA - nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, alpha, TNRC6A - trinucleotide repeat containing 6a, CNKSR1 - connector enhancer of kinase suppressor of ras 1, DDR1 - discoidin domain receptor tyrosine kinase 1, EPHA8 - eph receptor a8, RHOQ - ras homolog family member q, ARF4 - adp-ribosylation factor 4, REPS2 - ralbp1 associated eps domain containing 2, LYN - v-yes-1 yamaguchi sarcoma viral related oncogene homolog, MEF2C - myocyte enhancer factor 2c, TNRC6C - trinucleotide repeat containing 6c, FYN - fyn oncogene related to src, fgr, yes, GSK3B - glycogen synthase kinase 3 beta, PDGFC - platelet derived growth factor c]
	extracellular structure organization	6.80E-04	3.68	9	[PDGFA - platelet-derived growth factor alpha polypeptide, RAMP2 - receptor (g protein-coupled) activity modifying protein 2, SMARCA4 - swi/snf related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4, ITGA7 - integrin, alpha 7, COL8A2 - collagen, type viii, alpha 2, AGRN - agrin, LTBP1 - latent transforming growth factor beta binding protein 1, DDR1 - discoidin domain receptor tyrosine kinase 1, FBLN1 - fibulin 1]
	cellular response to fluid shear stress	7.78E-04	14.72	3	[NFE2L2 - nuclear factor (erythroid-derived 2)-like 2, MEF2C - myocyte enhancer factor 2c, ASS1 - argininosuccinate synthase 1]
CC	protein-DNA complex	6.47E-05	4.98	9	[HIST1H2AM - histone cluster 1, h2am, HIST1H2BC - histone cluster 1, h2bc, HIST1H2AE - histone cluster 1, h2ae, HIST1H2BE - histone cluster 1, h2be, MED1 - mediator complex subunit 1, HIST1H2BF - histone cluster 1, h2bf, HIST1H1C - histone cluster 1, h1c, HIST1H2BH - histone cluster 1, h2bh, HIST1H2BN - histone cluster 1, h2bn]

Table 9. B. GO categories significantly down regulated in SA platelets in comparison with healthy platelets; BP= biological process; CC= cellular component.
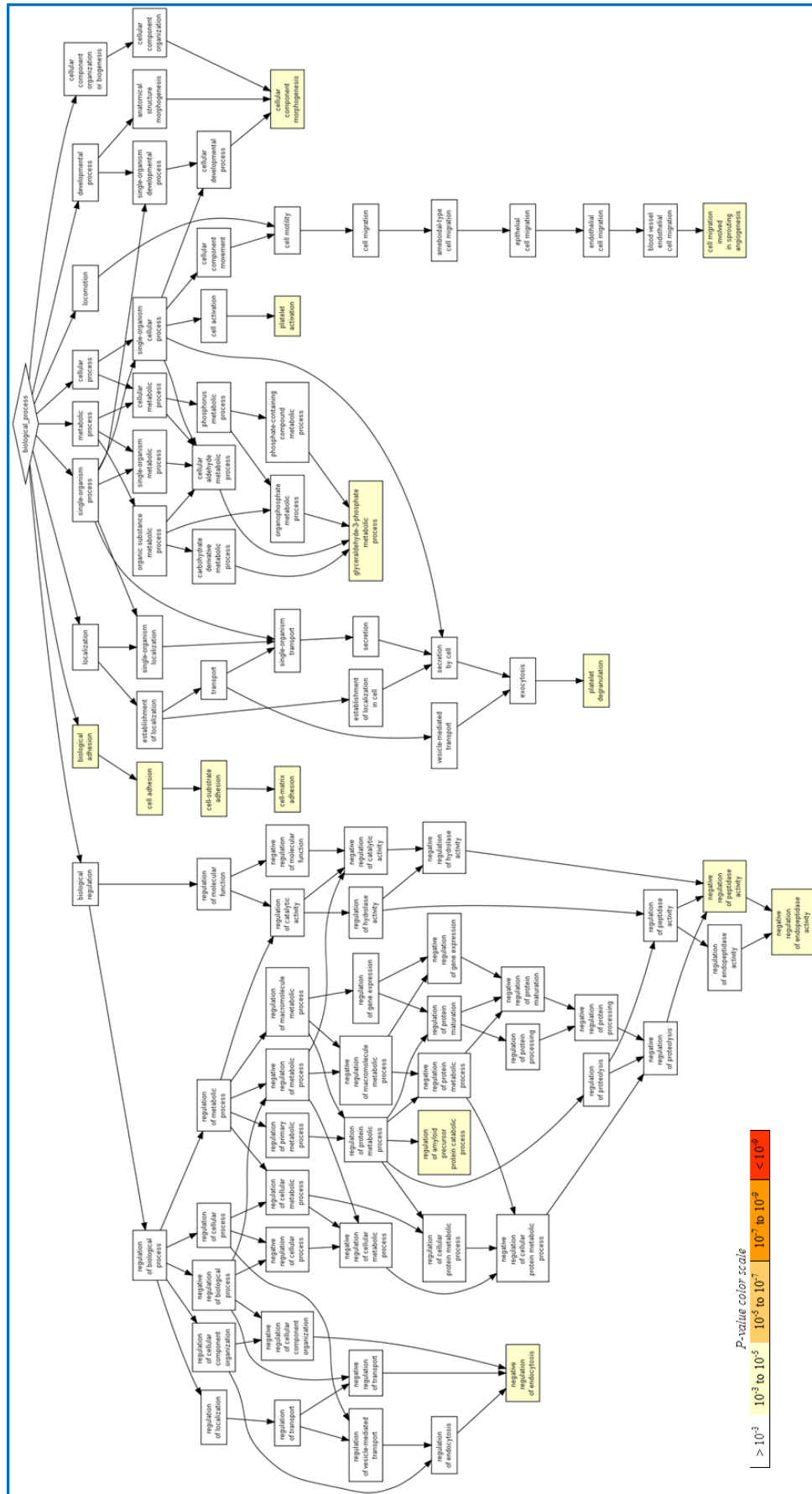
Figure 28. Directed acyclic graph of GO biological processes and cellular components up-regulated in SA+ platelets.
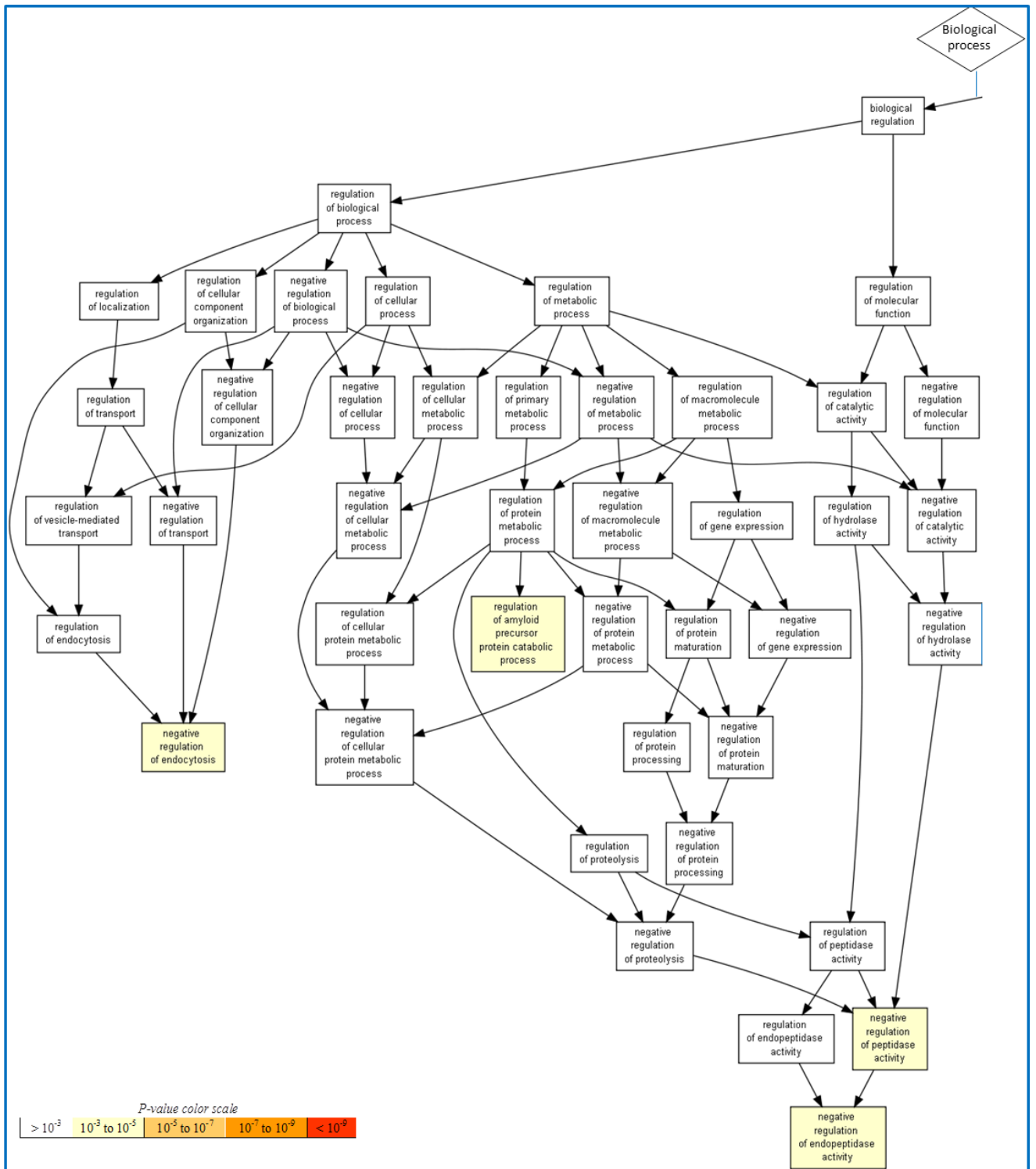
Figure 28. A Enlargement of left part of figure 22 which shows GO biological processes and cellular components up-regulated in SA[+] platelets.
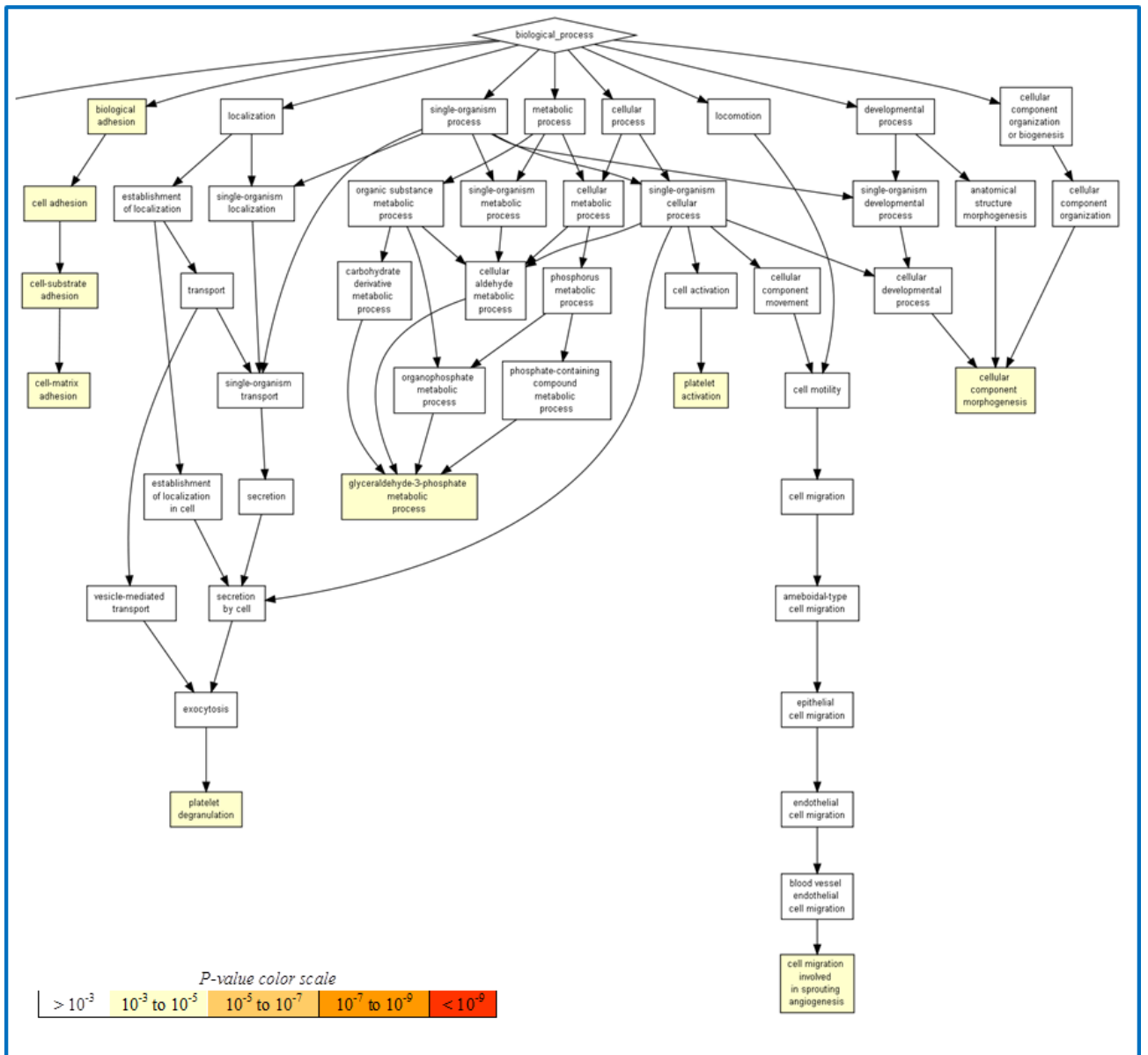
Figure 28. B Enlargement of right part of figure 22 which shows GO biological processes and cellular components up-regulated in SA$^+$ platelets.
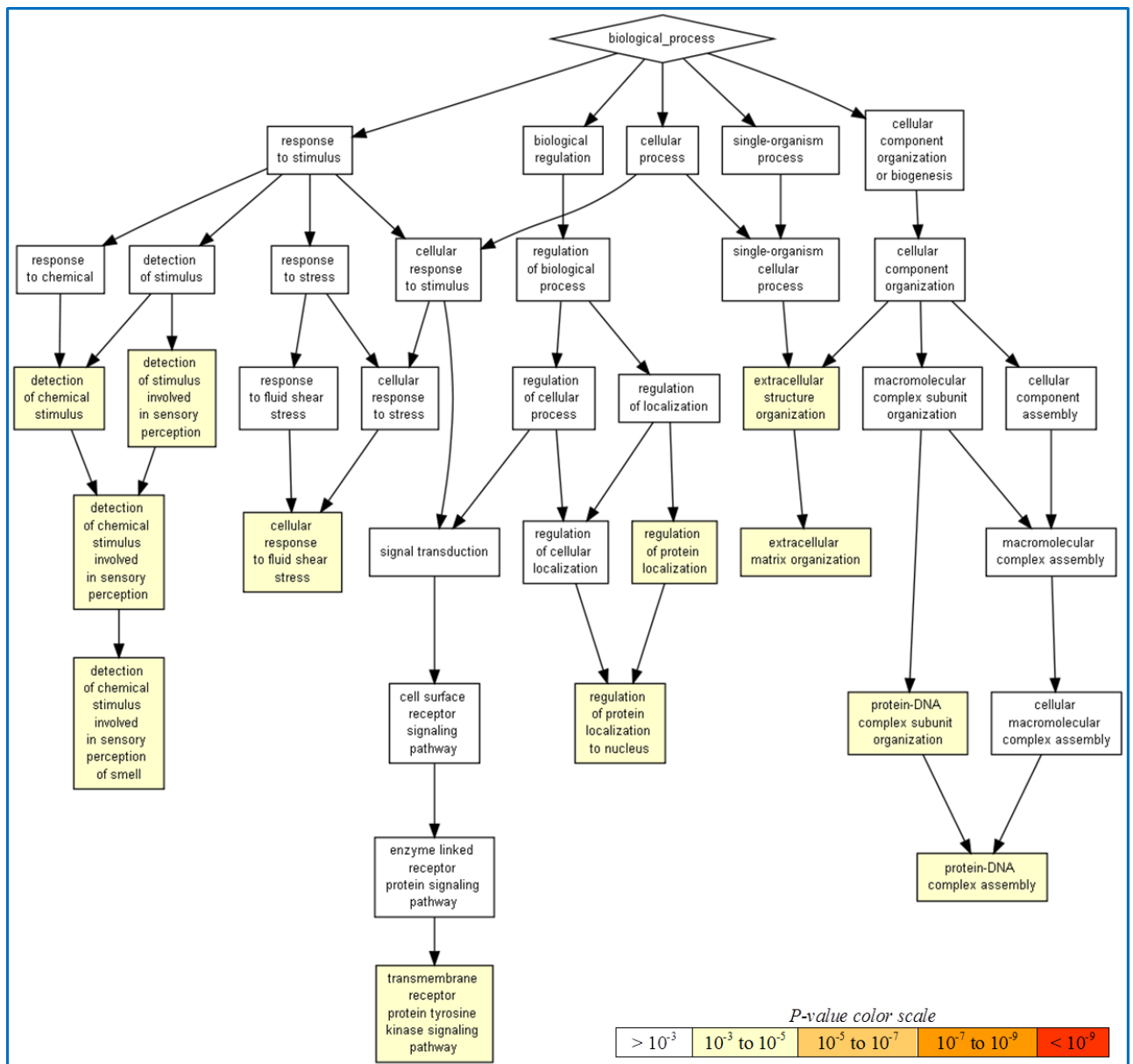
Figure 29. A Directed acyclic graph of GO biological processes down-regulated in SA$^+$ platelets.
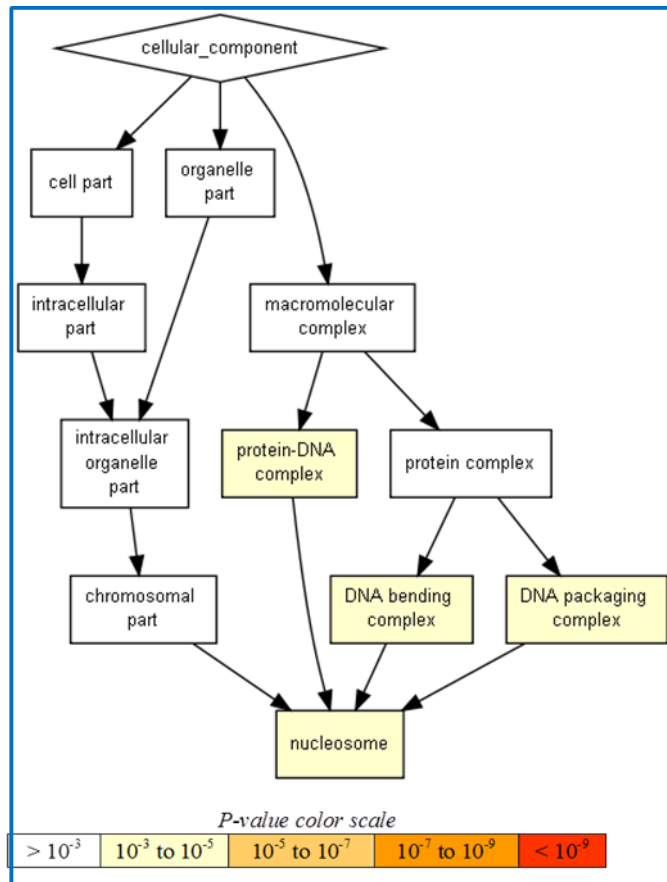
Figure 29. B Directed acyclic graph of GO cellular components down-regulated in SA$^+$ platelets.

	GO Term Description	P-value	Enrichment	N	Genes
BP	peptidyl-serine phosphorylation	0,0015	13,05	3	CLK1, SMG1, DDIT4
	nuclear-transcribed mRNA catabolic process	0,0047	8,75	3	ZFP36L2, SMG1, RPL21
	cellular protein metabolic process	0,0058	2,01	13	BTBD1, CLK1, CUL1, DDIT4, DNAJB1, NKTR, RABGGTB, RPL21, SMG1, DUSP6, MYLK, PHF16, TUBA4A
	protein folding	0,0090	6,94	3	NKTR, DNAJB1, TUBA4A

Table 10. GO categories significantly altered in SA$^+$T2DM$^+$ in comparison with SA$^+$T2DM$^-$ platelets. Red colour= up-regulated; green colour= down-regulated.
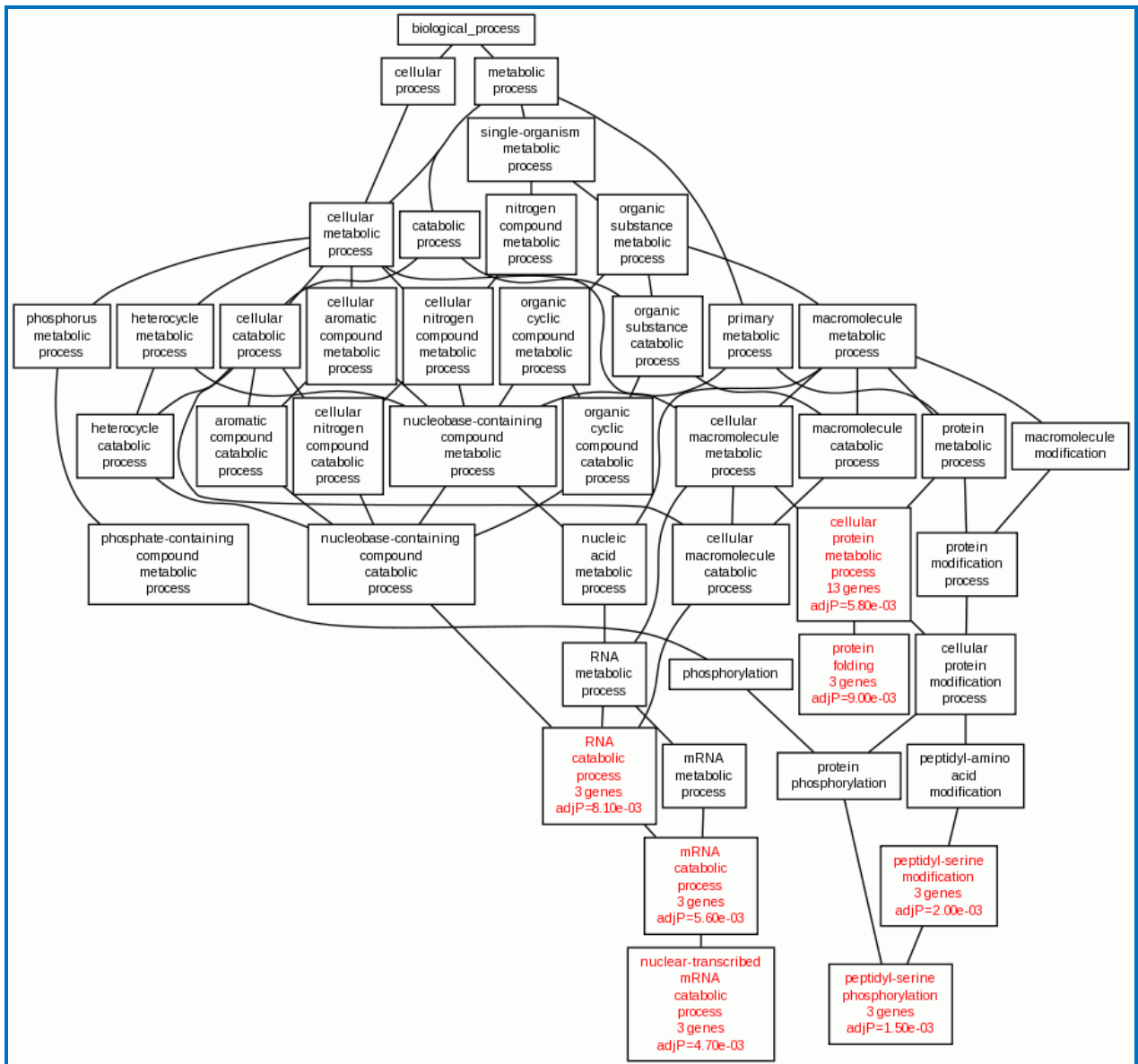
Figure 30. Directed acyclic graph of GO biological processes altered in SA[+]T2DM[+] platelets.

DISCUSSION

Patients with diabetes mellitus (DM) are at high risk for several cardiovascular disorders such as coronary heart disease, stroke, peripheral arterial disease, and congestive heart failure [35-37]. DM has reached epidemic proportions and its strong association with coronary artery disease is responsible for increased cardiovascular morbidity and mortality. DM patients are characterized by platelet hyperreactivity, which contribute to the enhanced atherothrombotic risk of these subjects. Furthermore, a large proportion of DM patients show inadequate response to standard antiplatelet treatments and high rate of adverse recurrent cardiovascular events despite compliance with standard antiplatelet treatment regimens. Several mechanisms are involved in the hyperreactive platelet phenotype characterizing DM patients, and they lead to platelet abnormalities such as, among the others, increased adhesion and activation, amplified agonist-receptor coupling, enhanced generation of reactive oxygen species, etc. [178]. In order to gain insights into the enhanced thrombotic propensity of CAD patients with T2DM, in the present study we have focused our attention on the expression of platelet-associated TF, a more recently described marker of platelet activation. Using a whole blood flow cytometry approach, which allowed us to assess cell antigen expression virtually in the absence of sample manipulation, we found that in patients treated with low dose aspirin the expression levels of the classical platelet activation markers (P-selectin, aGpIIbIIIa, CD36 and surface expression of phosphatidyl serine) were comparable in SA patients with or without T2DM. By contrast, T2DM significantly increased platelet Tissue Factor expression. Several approaches have been used to support this finding and the results can be summarized as follows: 1) the number of circulating platelets carrying TF in the cytosol of SA patients with T2DM is twice the amount found in SA patients without T2DM; 2) in SA patients with T2DM the number of circulating platelets expressing, under resting conditions, TF on the platelet membrane is twice the amount found in SA patients without T2DM; 3) upon *in vitro* activation by ADP the number of TF positive platelets is significantly higher in SA patients with T2DM compared to the other group.

Of note, the platelet-associated TF was functionally active, and thus relevant from a pathophysiological point of view, being able to trigger thrombin generation, which was indeed affected when a specific anti-TF antibody was used. The contribution of platelet-associated TF to thrombin generation was higher in T2DM patients with SA compared to SA patients without T2DM, and this was in accordance with the flow cytometry data. In order to translate these data into a clinically relevant finding, we took advantage from the use of thromboelastography -which provides global information on the dynamics of clot development, stabilization and dissolution

that reflect *in vivo* hemostasis- showing that blood from CAD patients with T2DM had a significantly higher global haemostatic potential compared to CAD patients without T2DM. Our data fit well with a recent work by Neergaard-Petersen et al [106] where it has been shown, by confocal and electron microscopy, that CAD patients with T2DM are characterized by a more compact clot structure compared to CAD patients without T2DM.

The findings that CAD patients with T2DM are characterized by higher levels of circulating (platelet-derived) TF shed new light on the potential mechanisms responsible for the increased prothrombotic propensity of these patients. The presence of TF in platelets has been proposed first in 2000 by Giesen et al. who postulated that thrombus formation and propagation, upon atherosclerotic plaque rupture, could take advantage from blood-born tissue factor: circulating platelets, carrying TF derived from microparticles, may themselves trigger the activation of the coagulation cascade [100].. Since then, several papers have documented the presence of TF in human platelets, suggesting that at least three mechanisms are involved in the presence of TF in platelets: 1) the microparticle-transfer mechanism; 2) the storage within the α-granules and the open canalicular system and 3) the *de novo* protein synthesis from the TF specific messenger RNA (mRNA). In 2003 our group provided the evidence that human CD34^{+}-derived megakaryocytes express the TF mRNA [103]. Thus, although it is commonly believed that the only mechanism responsible for the presence of TF in platelets is through the uptake of TF-positive microparticles released by activated endothelial cells or leukocytes, it can be speculated that the TF mRNA and protein detectable in platelets could be the result of a direct transfer from megakaryocytes. While patients' enrollment for the present project was ongoing, data in favors of this hypothesis have been accumulated in our laboratory. Indeed, using an *in vitro* cell culture model able to recapitulate megakaryocyte differentiation and platelet biogenesis, we provided consistent evidence that TF is an endogenously synthesized protein that characterizes megakaryocyte maturation. Since the cell system used allowed us to study mRNA and protein expression in the absence of any crosstalk with other cell or microparticle, we provided also the evidence for the direct transfer of both TF mRNA and protein from megakaryocytes to a subset of platelets where it contributes to their thrombin generation capacity. Of interest, the percentage of TF positive platelets that we observed *in vitro* (both with Meg-platelets and with CD34+-derived platelets) was virtually identical to the amount found in blood from healthy individuals. This striking data suggests that a fine-tuned mechanism, which deserves further investigation in order to dissect the molecular pathways involved in its regulation, is responsible for the controlled delivery of TF from megakaryocytes to platelets. All together these data support the

concept that, under physiological conditions, megakaryocytes are committed to release in the bloodstream a well-defined and programmed number of TF-carrying platelets (Brambilla et al, manuscript under revision). It can be speculated that under pathological conditions, such as in the presence of low grade inflammation as present in CAD patients with T2DM (as evidenced by the increased CRP levels measured in plasma samples of the enrolled patients), alterations in the megakaryocyte transcriptome and proteome as well as in the release of new platelets may occur. As a result, a higher number of TF positive platelets may reach the bloodstream. Interestingly, and in accordance with the literature [164], we found in SA patient with T2DM, compared to SA without T2DM, a higher number of reticulated platelets (IPF), which are the immature platelets circulating in blood, reflecting the activity of megakaryopoiesis in the bone marrow. Although further studies are needed to prove this hypothesis in the contest of T2DM, we have recently reported that in spontaneously hypertensive stroke-prone rats the percentage of circulating TF positive platelets directly correlated with blood pressure and is the results of an increased number of TF positive megakaryocytes which release in the bloodstream a higher number of TF positive platelets (M. Brambilla, P. Gelosa, L. Rossetti, B. Perancin, L. Castiglioni, L. Sironi, E. Tremoli, M. Camera. Captopril downregulates circulating Tissue Factor expression in stroke-prone rats. *Manuscript in preparation*).

As previously mentioned, although platelets do not have a nucleus, they contain ~2000-7000 transcripts [80, 81, 179, 180]. Microarray analysis of the platelet transcriptome from healthy human subjects identified mRNAs that encode for cell surface receptors and glycoproteins, as well as proteins involved in metabolism, signalling, inflammation, and immunity [80, 181, 182]. The finding that platelets can use their mRNA pool to perform new protein synthesis in response to cellular activation is of great importance, since these mechanisms allow them to modify their protein phenotype and, as a consequence, their functions [84].

It has been reported that specific mRNAs may vary in clinical conditions such as sickle cell disease [85], ST-elevation myocardial infarction [86] and NSTE-ACS [87]. Identification of disease-associated platelet-specific transcripts is of particular relevance in platelet pathophysiology, since it may lead to the discovery of novel therapeutic targets. No data are so far available on the platelet transcriptome profiling associated with T2DM in CAD patients. We first compared the platelet gene profiling of SA patients and healthy subjects and found 319 unique mRNAs that were differentially expressed in the two groups. Of interest, genes involved in cell adhesion, platelet degranulation and activation, and integrin complex formation such as, among the most known, von Willebrand factor, CD63, CD41, CD61 and tubulin alpha were upregulated in SA patients; by contrast, genes involved in extracellular matrix organization,

transmembrane receptor protein tyrosine kinase signaling pathway, cellular response to shear stress and protein-DNA complex such as PDGFA, collagen type 8 alpha 2, histone cluster 1 were downregulated. When comparison of platelet gene profiling was performed between SA patients with or without T2DM, only 35 genes were found differently expressed. CD69 resulted the most upregulated transcript. Interestingly CD69, together with myeloid-related protein-14, has been previously reported as the strongest discriminators of STEMI vs CAD patients [86].. CD69 is of particular interest in generating disease hypotheses. Indeed, CD69 is widely expressed in hematopoietic cells [183], and engagement of CD69 on platelets results in thromboxane production and aggregation [184].

The most downregulated gene was prothymosin alpha (ProT alpha), which is a highly acidic protein widely distributed in mammalian cells. Since its discovery in 1984, the biological role of this protein has been controversial. Initially, ProT alpha was considered a thymic factor with a hormonal-like role in the maturation of T-lymphocytes. However, molecular and cellular analyses led to conclude that ProT alpha is a nuclear protein required in proliferation events while failing to show a clear immunological effect. The involvement of ProT alpha in changes in the compaction state of chromatin has been recently elucidated with the demonstration that this protein induces the unfolding of chromatin fibers in a process that seems to be mediated by the interaction of ProT alpha with histone H1 [185]. Furthermore, the relationship between ProT alpha and apoptosis as well as with proliferation rendered this protein an attractive target in the search for modulators of cell death and tumor growth. No data are available in the literature on the function of ProT in platelets, but considering that histones are among the most abundant transcripts within platelets and extracellular histones have been shown to activate platelets [186] further studies are warranted to gain insights into the role of this protein in platelets.

In conclusion all these data shed new light on an additional mechanism involved in the enhanced prothrombotic phenotype associated with T2DM in SA patients providing for the first time evidence for the presence of a significant higher number of circulating TF positive platelets which are able to efficiently trigger thrombin generation. Efforts aimed to modulate megakaryocyte/platelet-associated TF pathway may be a promising approach for reducing the burden of the atherosclerotic complications in T2DM patients. Although the transcriptomic data have still to be validated by quantitative PCR analysis and assessment of parallel changes in the protein content has also to be performed, they are suggesting potential new mechanisms/pathways involved in the platelet hyperreactivity described in diabetic patients.

The information related to the 'molecular anatomy' of platelets (transcriptome analyses) might be of great value to identify the complex mechanisms regulating platelet function and its pharmacological inhibition in CAD patients with T2DM.

REFERENCES

1. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2012. **35 Suppl 1**: p. S64-71.
2. Naka, K.K., et al., *Determinants of vascular function in patients with type 2 diabetes.* Cardiovasc Diabetol, 2012. **11**: p. 127.
3. Shaw, J.E., R.A. Sicree, and P.Z. Zimmet, *Global estimates of the prevalence of diabetes for 2010 and 2030.* Diabetes Res Clin Pract, 2010. **87**(1): p. 4-14.
4. Roglic, G., et al., *The burden of mortality attributable to diabetes: realistic estimates for the year 2000.* Diabetes Care, 2005. **28**(9): p. 2130-5.
5. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2010. **33 Suppl 1**: p. S62-9.
6. Brambilla, P., et al., *Normal fasting plasma glucose and risk of type 2 diabetes.* Diabetes Care, 2011. **34**(6): p. 1372-4.
7. Prentki, M. and C.J. Nolan, *Islet beta cell failure in type 2 diabetes.* J Clin Invest, 2006. **116**(7): p. 1802-12.
8. Stefan, N., et al., *Identification and characterization of metabolically benign obesity in humans.* Arch Intern Med, 2008. **168**(15): p. 1609-16.
9. Medici, F., et al., *Concordance rate for type II diabetes mellitus in monozygotic twins: actuarial analysis.* Diabetologia, 1999. **42**(2): p. 146-50.
10. Pierce, M., H. Keen, and C. Bradley, *Risk of diabetes in offspring of parents with non-insulin-dependent diabetes.* Diabet Med, 1995. **12**(1): p. 6-13.
11. Dupuis, J., et al., *New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk.* Nat Genet, 2010. **42**(2): p. 105-16.
12. Herder, C. and M. Roden, *Genetics of type 2 diabetes: pathophysiologic and clinical relevance.* Eur J Clin Invest, 2011. **41**(6): p. 679-92.
13. Rajpathak, S.N., et al., *The role of iron in type 2 diabetes in humans.* Biochim Biophys Acta, 2009. **1790**(7): p. 671-81.
14. Barrett, H. and A. McElduff, *Vitamin D and pregnancy: An old problem revisited.* Best Pract Res Clin Endocrinol Metab, 2010. **24**(4): p. 527-39.
15. Yajnik, C.S., et al., *Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study.* Diabetologia, 2008. **51**(1): p. 29-38.
16. Casals-Casas, C. and B. Desvergne, *Endocrine disruptors: from endocrine to metabolic disruption.* Annu Rev Physiol, 2011. **73**: p. 135-62.
17. Arroyo, C., et al., *Depressive symptoms and risk of type 2 diabetes in women.* Diabetes Care, 2004. **27**(1): p. 129-33.
18. Williams, E.D., et al., *Health behaviours, socioeconomic status and diabetes incidence: the Australian Diabetes Obesity and Lifestyle Study (AusDiab).* Diabetologia, 2010. **53**(12): p. 2538-45.
19. Unger, R.H. and L. Orci, *Paracrinology of islets and the paracrinopathy of diabetes.* Proc Natl Acad Sci U S A, 2010. **107**(37): p. 16009-12.
20. Weir, G.C., et al., *Beta-cell adaptation and decompensation during the progression of diabetes.* Diabetes, 2001. **50 Suppl 1**: p. S154-9.
21. Nolan, C.J., P. Damm, and M. Prentki, *Type 2 diabetes across generations: from pathophysiology to prevention and management.* Lancet, 2011. **378**(9786): p. 169-81.
22. Zraika, S., et al., *Toxic oligomers and islet beta cell death: guilty by association or convicted by circumstantial evidence?* Diabetologia, 2010. **53**(6): p. 1046-56.
23. Butler, A.E., et al., *Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes.* Diabetes, 2003. **52**(1): p. 102-10.
24. Nauck, M., et al., *Reduced incretin effect in type 2 (non-insulin-dependent) diabetes.* Diabetologia, 1986. **29**(1): p. 46-52.

25.   Nauck, M.A., et al., *Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: what is up, what is down?* Diabetologia, 2011. **54**(1): p. 10-8.

26.   Suzuki, K., et al., *The role of gut hormones and the hypothalamus in appetite regulation.* Endocr J, 2010. **57**(5): p. 359-72.

27.   Morton, G.J., et al., *Central nervous system control of food intake and body weight.* Nature, 2006. **443**(7109): p. 289-95.

28.   Guilherme, A., et al., *Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 367-77.

29.   Shah, P., A. Basu, and R. Rizza, *Fat-induced liver insulin resistance.* Curr Diab Rep, 2003. **3**(3): p. 214-8.

30.   Schenk, S., M. Saberi, and J.M. Olefsky, *Insulin sensitivity: modulation by nutrients and inflammation.* J Clin Invest, 2008. **118**(9): p. 2992-3002.

31.   Ono, Y., et al., *Increased serum levels of advanced glycation end-products and diabetic complications.* Diabetes Res Clin Pract, 1998. **41**(2): p. 131-7.

32.   Ohgami, N., et al., *CD36, a member of class B scavenger receptor family, is a receptor for advanced glycation end products.* Ann N Y Acad Sci, 2001. **947**: p. 350-5.

33.   *Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group.* Lancet, 1998. **352**(9131): p. 837-53.

34.   Semenkovich, C.F. and J.W. Heinecke, *The mystery of diabetes and atherosclerosis: time for a new plot.* Diabetes, 1997. **46**(3): p. 327-34.

35.   Luscher, T.F., et al., *Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part II.* Circulation, 2003. **108**(13): p. 1655-61.

36.   Schramm, T.K., et al., *Diabetes patients requiring glucose-lowering therapy and nondiabetics with a prior myocardial infarction carry the same cardiovascular risk: a population study of 3.3 million people.* Circulation, 2008. **117**(15): p. 1945-54.

37.   McGuire, D.K., et al., *Association of diabetes mellitus and glycemic control strategies with clinical outcomes after acute coronary syndromes.* Am Heart J, 2004. **147**(2): p. 246-52.

38.   Nathan, D.M., *Long-term complications of diabetes mellitus.* N Engl J Med, 1993. **328**(23): p. 1676-85.

39.   Hu, F.B., et al., *The impact of diabetes mellitus on mortality from all causes and coronary heart disease in women: 20 years of follow-up.* Arch Intern Med, 2001. **161**(14): p. 1717-23.

40.   Gerstein, H.C., et al., *Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals.* JAMA, 2001. **286**(4): p. 421-6.

41.   Brunner, E.J., et al., *Relation between blood glucose and coronary mortality over 33 years in the Whitehall Study.* Diabetes Care, 2006. **29**(1): p. 26-31.

42.   American Diabetes, A., *Standards of medical care in diabetes--2008.* Diabetes Care, 2008. **31 Suppl 1**: p. S12-54.

43.   de Gaetano, G. and P. Collaborative Group of the Primary Prevention, *Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project.* Lancet, 2001. **357**(9250): p. 89-95.

44.   Colwell, J.A., *Vascular thrombosis in type II diabetes mellitus.* Diabetes, 1993. **42**(1): p. 8-11.

45.   Tripodi, A., et al., *Hypercoagulability in patients with type 2 diabetes mellitus detected by a thrombin generation assay.* J Thromb Thrombolysis, 2011. **31**(2): p. 165-72.

46.   Morel, O., et al., *Prothrombotic changes in diabetes mellitus.* Semin Thromb Hemost, 2013. **39**(5): p. 477-88.

47.   Morishita, E., et al., *Hypercoagulability and high lipoprotein(a) levels in patients with type II diabetes mellitus.* Atherosclerosis, 1996. **120**(1-2): p. 7-14.

48.   Yamada, T., et al., *Importance of hypercoagulability over hyperglycemia for vascular complication in type 2 diabetes.* Diabetes Res Clin Pract, 2000. **49**(1): p. 23-31.

49.   Bembde, A.S., *A study of plasma fibrinogen level in type-2 diabetes mellitus and its relation to glycemic control.* Indian J Hematol Blood Transfus, 2012. **28**(2): p. 105-8.

50.   Undas, A., et al., *Hyperglycemia is associated with enhanced thrombin formation, platelet activation, and fibrin clot resistance to lysis in patients with acute coronary syndrome.* Diabetes Care, 2008. **31**(8): p. 1590-5.

51.   Hong, L.F., et al., *Association of fibrinogen with severity of stable coronary artery disease in patients with type 2 diabetic mellitus.* Dis Markers, 2014. **2014**: p. 485687.

52.   Carr, M.E., *Diabetes mellitus: a hypercoagulable state.* J Diabetes Complications, 2001. **15**(1): p. 44-54.

53.   Carmassi, F., et al., *Coagulation and fibrinolytic system impairment in insulin dependent diabetes mellitus.* Thromb Res, 1992. **67**(6): p. 643-54.

54.   Collier, A., et al., *Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria.* Diabetes, 1992. **41**(8): p. 909-13.

55.   Kohler, H.P., *Insulin resistance syndrome: interaction with coagulation and fibrinolysis.* Swiss Med Wkly, 2002. **132**(19-20): p. 241-52.

56.   Yurekli, B.P., et al., *Global assessment of the coagulation status in type 2 diabetes mellitus using rotation thromboelastography.* Blood Coagul Fibrinolysis, 2006. **17**(7): p. 545-9.

57.   Neergaard-Petersen, S., et al., *The influence of type 2 diabetes on fibrin clot properties in patients with CAD.* Thromb Haemost, 2014. **112**(6).

58.   Hess, K., et al., *A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3.* Diabetologia, 2012. **55**(4): p. 1103-13.

59.   Esposito, K., et al., *Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress.* Circulation, 2002. **106**(16): p. 2067-72.

60.   Yudkin, J.S., et al., *High levels of circulating proinflammatory cytokines and leptin in urban, but not rural, Indians. A potential explanation for increased risk of diabetes and coronary heart disease.* Diabetes Care, 1999. **22**(2): p. 363-4.

61.   Kanemaki, T., et al., *Interleukin 1beta and interleukin 6, but not tumor necrosis factor alpha, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes.* Hepatology, 1998. **27**(5): p. 1296-303.

62.   Moreno, P.R., et al., *Coronary composition and macrophage infiltration in atherectomy specimens from patients with diabetes mellitus.* Circulation, 2000. **102**(18): p. 2180-4.

63.   Souza, J.R., et al., *Serum levels of interleukin-6 (Il-6), interleukin-18 (Il-18) and C-reactive protein (CRP) in patients with type-2 diabetes and acute coronary syndrome without ST-segment elevation.* Arq Bras Cardiol, 2008. **90**(2): p. 86-90.

64.   Evans, J.L., et al., *Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes.* Endocr Rev, 2002. **23**(5): p. 599-622.

65.   Hink, U., et al., *Mechanisms underlying endothelial dysfunction in diabetes mellitus.* Circ Res, 2001. **88**(2): p. E14-22.

66.   Guzik, T.J., et al., *Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase.* Circulation, 2002. **105**(14): p. 1656-62.

67.   Wagenknecht, L.E., et al., *Diabetes and progression of carotid atherosclerosis: the insulin resistance atherosclerosis study.* Arterioscler Thromb Vasc Biol, 2003. **23**(6): p. 1035-41.

68.   Davi, G., A. Falco, and C. Patrono, *Lipid peroxidation in diabetes mellitus.* Antioxid Redox Signal, 2005. **7**(1-2): p. 256-68.

69.   Morrow, J.D., T.A. Minton, and L.J. Roberts, 2nd, *The F2-isoprostane, 8-epi-prostaglandin F2 alpha, a potent agonist of the vascular thromboxane/endoperoxide receptor, is a platelet thromboxane/endoperoxide receptor antagonist.* Prostaglandins, 1992. **44**(2): p. 155-63.

70.   Davi, G., et al., *In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation.* Circulation, 1999. **99**(2): p. 224-9.

71.   Davi, G., et al., *Thromboxane biosynthesis and platelet function in type II diabetes mellitus.* N Engl J Med, 1990. **322**(25): p. 1769-74.

72.   Angiolillo, D.J., *Antiplatelet therapy in diabetes: efficacy and limitations of current treatment strategies and future directions.* Diabetes Care, 2009. **32**(4): p. 531-40.

73.   Natarajan, A., A.G. Zaman, and S.M. Marshall, *Platelet hyperactivity in type 2 diabetes: role of antiplatelet agents.* Diab Vasc Dis Res, 2008. **5**(2): p. 138-44.

74.   Ogawa, M., *Differentiation and proliferation of hematopoietic stem cells.* Blood, 1993. **81**(11): p. 2844-53.

75.   Patel, S.R., J.H. Hartwig, and J.E. Italiano, Jr., *The biogenesis of platelets from megakaryocyte proplatelets.* J Clin Invest, 2005. **115**(12): p. 3348-54.

76.   Saving, K.L., P.E. Mankin, and M.J. Gorman, *Differences in adhesion receptor expression between immature and older platelets and red blood cells of neonates and adults.* J Pediatr Hematol Oncol, 2002. **24**(2): p. 120-4.

77.   Clemetson, K.J., *Platelets and primary haemostasis.* Thromb Res, 2012. **129**(3): p. 220-4.

78.   Italiano, J.E., Jr. and R.A. Shivdasani, *Megakaryocytes and beyond: the birth of platelets.* J Thromb Haemost, 2003. **1**(6): p. 1174-82.

79.   Cecchetti, L., et al., *Megakaryocytes differentially sort mRNAs for matrix metalloproteinases and their inhibitors into platelets: a mechanism for regulating synthetic events.* Blood, 2011. **118**(7): p. 1903-11.

80.   Gnatenko, D.V., et al., *Transcript profiling of human platelets using microarray and serial analysis of gene expression.* Blood, 2003. **101**(6): p. 2285-93.

81.   Bugert, P. and H. Kluter, *Profiling of gene transcripts in human platelets: an update of the platelet transcriptome.* Platelets, 2006. **17**(7): p. 503-4.

82.   Herrera-Galeano, J.E., et al., *A novel variant in the platelet endothelial aggregation receptor-1 gene is associated with increased platelet aggregability.* Arterioscler Thromb Vasc Biol, 2008. **28**(8): p. 1484-90.

83.   Goodall, A.H., et al., *Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function.* Blood, 2010. **116**(22): p. 4646-56.

84.   Weyrich, A.S., et al., *Change in protein phenotype without a nucleus: translational control in platelets.* Semin Thromb Hemost, 2004. **30**(4): p. 491-8.

85.   Raghavachari, N., et al., *Amplified expression profiling of platelet transcriptome reveals changes in arginine metabolic pathways in patients with sickle cell disease.* Circulation, 2007. **115**(12): p. 1551-62.

86.   Healy, A.M., et al., *Platelet expression profiling and clinical validation of myeloid-related protein-14 as a novel determinant of cardiovascular events.* Circulation, 2006. **113**(19): p. 2278-84.

87.   Colombo, G., et al., *Gene expression profiling reveals multiple differences in platelets from patients with stable angina or non-ST elevation acute coronary syndrome.* Thromb Res, 2011. **128**(2): p. 161-8.

88.   Kondkar, A.A., et al., *VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA.* J Thromb Haemost, 2010. **8**(2): p. 369-78.

89.   Morrissey, J.H., H. Fakhrai, and T.S. Edgington, *Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade.* Cell, 1987. **50**(1): p. 129-35.

90.   van der Wal, A.C., X. Li, and O.J. de Boer, *Tissue factor expression in the morphologic spectrum of vulnerable atherosclerotic plaques.* Semin Thromb Hemost, 2006. **32**(1): p. 40-7.

91.   Hoffman, M., et al., *Factors IXa and Xa play distinct roles in tissue factor-dependent initiation of coagulation.* Blood, 1995. **86**(5): p. 1794-801.

92.   Ott, I., *Tissue factor in acute coronary syndromes.* Semin Vasc Med, 2003. **3**(2): p. 185-92.

93.   Browder, T., J. Folkman, and S. Pirie-Shepherd, *The hemostatic system as a regulator of angiogenesis.* J Biol Chem, 2000. **275**(3): p. 1521-4.

94.   Huang, X., et al., *Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature.* Science, 1997. **275**(5299): p. 547-50.

95.   Kim, J.K., *Inflammation and insulin resistance: an old story with new ideas.* Korean Diabetes J, 2010. **34**(3): p. 137-45.

96.   Camerer, E., W. Huang, and S.R. Coughlin, *Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5255-60.

97. Peppelenbosch, M.P. and H.H. Versteeg, *Cell biology of tissue factor, an unusual member of the cytokine receptor family.* Trends Cardiovasc Med, 2001. **11**(8): p. 335-9.

98. Ott, I., et al., *A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280.* J Cell Biol, 1998. **140**(5): p. 1241-53.

99. Toschi, V., et al., *Tissue factor modulates the thrombogenicity of human atherosclerotic plaques.* Circulation, 1997. **95**(3): p. 594-9.

100. Giesen, P.L., et al., *Blood-borne tissue factor: another view of thrombosis.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2311-5.

101. Rauch, U. and Y. Nemerson, *Circulating tissue factor and thrombosis.* Curr Opin Hematol, 2000. **7**(5): p. 273-7.

102. Rauch, U., et al., *Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor.* Blood, 2000. **96**(1): p. 170-5.

103. Camera, M., et al., *Platelet activation induces cell-surface immunoreactive tissue factor expression, which is modulated differently by antiplatelet drugs.* Arterioscler Thromb Vasc Biol, 2003. **23**(9): p. 1690-6.

104. Muller, I., et al., *Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets.* FASEB J, 2003. **17**(3): p. 476-8.

105. Schwertz, H., et al., *Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets.* J Exp Med, 2006. **203**(11): p. 2433-40.

106. Panes, O., et al., *Human platelets synthesize and express functional tissue factor.* Blood, 2007. **109**(12): p. 5242-50.

107. Brambilla, M., et al., *Tissue factor in patients with acute coronary syndromes: expression in platelets, leukocytes, and platelet-leukocyte aggregates.* Arterioscler Thromb Vasc Biol, 2008. **28**(5): p. 947-53.

108. Pabla, R., et al., *Integrin-dependent control of translation: engagement of integrin alphaIIbbeta3 regulates synthesis of proteins in activated human platelets.* J Cell Biol, 1999. **144**(1): p. 175-84.

109. Badimon, L., et al., *Pathogenesis of the acute coronary syndromes and therapeutic implications.* Pathophysiol Haemost Thromb, 2002. **32**(5-6): p. 225-31.

110. Stary, H.C., *Composition and classification of human atherosclerotic lesions.* Virchows Arch A Pathol Anat Histopathol, 1992. **421**(4): p. 277-90.

111. Behrendt, D. and P. Ganz, *Endothelial function. From vascular biology to clinical applications.* Am J Cardiol, 2002. **90**(10C): p. 40L-48L.

112. Fitzgerald, D.J., et al., *Platelet activation in unstable coronary disease.* N Engl J Med, 1986. **315**(16): p. 983-9.

113. Pitsilos, S., et al., *Platelet factor 4 localization in carotid atherosclerotic plaques: correlation with clinical parameters.* Thromb Haemost, 2003. **90**(6): p. 1112-20.

114. Koyama, H., et al., *Platelet P-selectin expression is associated with atherosclerotic wall thickness in carotid artery in humans.* Circulation, 2003. **108**(5): p. 524-9.

115. Di Minno, G., et al., *Abnormally high thromboxane biosynthesis in homozygous homocystinuria. Evidence for platelet involvement and probucol-sensitive mechanism.* J Clin Invest, 1993. **92**(3): p. 1400-6.

116. Minuz, P., et al., *Increased oxidative stress and platelet activation in patients with hypertension and renovascular disease.* Circulation, 2002. **106**(22): p. 2800-5.

117. Willoughby, S., A. Holmes, and J. Loscalzo, *Platelets and cardiovascular disease.* Eur J Cardiovasc Nurs, 2002. **1**(4): p. 273-88.

118. Frenette, P.S., et al., *Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7450-4.

119. Furie, B., B.C. Furie, and R. Flaumenhaft, *A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion.* Thromb Haemost, 2001. **86**(1): p. 214-21.

120. Tschoepe, D., et al., *Platelets in diabetes: the role in the hemostatic regulation in atherosclerosis.* Semin Thromb Hemost, 1993. **19**(2): p. 122-8.

121. Donahue, R.P. and T.J. Orchard, *Diabetes mellitus and macrovascular complications. An epidemiological perspective.* Diabetes Care, 1992. **15**(9): p. 1141-55.

122.   Ferroni, P., et al., *Platelet activation in type 2 diabetes mellitus.* J Thromb Haemost, 2004. **2**(8): p. 1282-91.

123.   Shechter, M., et al., *Blood glucose and platelet-dependent thrombosis in patients with coronary artery disease.* J Am Coll Cardiol, 2000. **35**(2): p. 300-7.

124.   Fateh-Moghadam, S., et al., *Platelet degranulation is associated with progression of intima-media thickness of the common carotid artery in patients with diabetes mellitus type 2.* Arterioscler Thromb Vasc Biol, 2005. **25**(6): p. 1299-303.

125.   Schafer, A., et al., *Reduced vascular NO bioavailability in diabetes increases platelet activation in vivo.* Arterioscler Thromb Vasc Biol, 2004. **24**(9): p. 1720-6.

126.   Heeschen, C., et al., *Soluble CD40 ligand in acute coronary syndromes.* N Engl J Med, 2003. **348**(12): p. 1104-11.

127.   Nimpf, J., et al., *Platelet activation in normo- and hyperlipoproteinemias.* Basic Res Cardiol, 1986. **81**(5): p. 437-53.

128.   Beckman, J.A., M.A. Creager, and P. Libby, *Diabetes and atherosclerosis: epidemiology, pathophysiology, and management.* JAMA, 2002. **287**(19): p. 2570-81.

129.   Leet, H., et al., *Fibrinogen binding and ADP-induced aggregation in platelets from diabetic subjects.* Thromb Res, 1981. **24**(1-2): p. 143-50.

130.   Vaidyula, V.R., G. Boden, and A.K. Rao, *Platelet and monocyte activation by hyperglycemia and hyperinsulinemia in healthy subjects.* Platelets, 2006. **17**(8): p. 577-85.

131.   McDonagh, P.F., et al., *Chronic expression of platelet adhesion proteins is associated with severe ischemic heart disease in type 2 diabetic patients: Chronic platelet activation in diabetic heart patients.* J Diabetes Complications, 2003. **17**(5): p. 269-78.

132.   Varo, N., et al., *Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients: a novel target of thiazolidinediones.* Circulation, 2003. **107**(21): p. 2664-9.

133.   Neubauer, H., et al., *Influence of glycaemic control on platelet bound CD40-CD40L system, P-selectin and soluble CD40 ligand in Type 2 diabetes.* Diabet Med, 2010. **27**(4): p. 384-90.

134.   Winocour, P.D., et al., *Decreased platelet membrane fluidity due to glycation or acetylation of membrane proteins.* Thromb Haemost, 1992. **68**(5): p. 577-82.

135.   Keating, F.K., B.E. Sobel, and D.J. Schneider, *Effects of increased concentrations of glucose on platelet reactivity in healthy subjects and in patients with and without diabetes mellitus.* Am J Cardiol, 2003. **92**(11): p. 1362-5.

136.   Assert, R., et al., *Regulation of protein kinase C by short term hyperglycaemia in human platelets in vivo and in vitro.* Diabetologia, 2001. **44**(2): p. 188-95.

137.   Watala, C., et al., *Platelet membrane lipid fluidity and intraplatelet calcium mobilization in type 2 diabetes mellitus.* Eur J Haematol, 1998. **61**(5): p. 319-26.

138.   Zhu, W., W. Li, and R.L. Silverstein, *Advanced glycation end products induce a prothrombotic phenotype in mice via interaction with platelet CD36.* Blood, 2012. **119**(25): p. 6136-44.

139.   Martina, V., et al., *Platelet cNOS activity is reduced in patients with IDDM and NIDDM.* Thromb Haemost, 1998. **79**(3): p. 520-2.

140.   Vinik, A.I., et al., *Platelet dysfunction in type 2 diabetes.* Diabetes Care, 2001. **24**(8): p. 1476-85.

141.   Kahn, N.N., *Insulin-induced expression of prostacyclin receptors on platelets is mediated through ADP-ribosylation of Gi alpha protein.* Life Sci, 1998. **63**(22): p. 2031-8.

142.   Trovati, M., et al., *Insulin stimulates nitric oxide synthesis in human platelets and, through nitric oxide, increases platelet concentrations of both guanosine-3', 5'-cyclic monophosphate and adenosine-3', 5'-cyclic monophosphate.* Diabetes, 1997. **46**(5): p. 742-9.

143.   Hunter, R.W. and I. Hers, *Insulin/IGF-1 hybrid receptor expression on human platelets: consequences for the effect of insulin on platelet function.* J Thromb Haemost, 2009. **7**(12): p. 2123-30.

144.   Hers, I., *Insulin-like growth factor-1 potentiates platelet activation via the IRS/PI3Kalpha pathway.* Blood, 2007. **110**(13): p. 4243-52.

145.   Ishida, M., et al., *Effects of insulin on calcium metabolism and platelet aggregation.* Hypertension, 1996. **28**(2): p. 209-12.

146. Ferreira, I.A., et al., *IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi.* J Biol Chem, 2004. **279**(5): p. 3254-64.

147. Li, Y., V. Woo, and R. Bose, *Platelet hyperactivity and abnormal Ca(2+) homeostasis in diabetes mellitus.* Am J Physiol Heart Circ Physiol, 2001. **280**(4): p. H1480-9.

148. Gawaz, M., et al., *Effects of magnesium on platelet aggregation and adhesion. Magnesium modulates surface expression of glycoproteins on platelets in vitro and ex vivo.* Thromb Haemost, 1994. **72**(6): p. 912-8.

149. Halushka, P.V., et al., *Increased platelet thromboxane synthesis in diabetes mellitus.* J Lab Clin Med, 1981. **97**(1): p. 87-96.

150. Nishikawa, T., et al., *Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage.* Nature, 2000. **404**(6779): p. 787-90.

151. Davi, G., A. Falco, and C. Patrono, *Determinants of F2-isoprostane biosynthesis and inhibition in man.* Chem Phys Lipids, 2004. **128**(1-2): p. 149-63.

152. Calverley, D.C., et al., *Increased platelet Fc receptor expression as a potential contributing cause of platelet hypersensitivity to collagen in diabetes mellitus.* Br J Haematol, 2003. **121**(1): p. 139-42.

153. Kaplar, M., et al., *The possible association of in vivo leukocyte-platelet heterophilic aggregate formation and the development of diabetic angiopathy.* Platelets, 2001. **12**(7): p. 419-22.

154. Tschoepe, D., U. Rauch, and B. Schwippert, *Platelet-leukocyte-cross-talk in diabetes mellitus.* Horm Metab Res, 1997. **29**(12): p. 631-5.

155. Nomura, S., et al., *Platelet-derived microparticles may influence the development of atherosclerosis in diabetes mellitus.* Atherosclerosis, 1995. **116**(2): p. 235-40.

156. Diamant, M., et al., *Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus.* Circulation, 2002. **106**(19): p. 2442-7.

157. Gerrits, A.J., et al., *Platelet tissue factor synthesis in type 2 diabetic patients is resistant to inhibition by insulin.* Diabetes, 2010. **59**(6): p. 1487-95.

158. Inui, Y., et al., *Platelet volume and urinary prostanoid metabolites in non-insulin-dependent diabetes mellitus.* J Atheroscler Thromb, 1994. **1**(2): p. 108-12.

159. Yilmaz, M.B., et al., *Role of mean platelet volume in triagging acute coronary syndromes.* J Thromb Thrombolysis, 2008. **26**(1): p. 49-54.

160. Guthikonda, S., et al., *Reticulated platelets and uninhibited COX-1 and COX-2 decrease the antiplatelet effects of aspirin.* J Thromb Haemost, 2007. **5**(3): p. 490-6.

161. Grove, E.L., et al., *Effect of platelet turnover on whole blood platelet aggregation in patients with coronary artery disease.* J Thromb Haemost, 2011. **9**(1): p. 185-91.

162. Ault, K.A., et al., *The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis.* Am J Clin Pathol, 1992. **98**(6): p. 637-46.

163. Thompson, C.B., et al., *Size dependent platelet subpopulations: relationship of platelet volume to ultrastructure, enzymatic activity, and function.* Br J Haematol, 1982. **50**(3): p. 509-19.

164. Lee, E.Y., et al., *Immature platelet fraction in diabetes mellitus and metabolic syndrome.* Thromb Res, 2013. **132**(6): p. 692-5.

165. Watanabe, Y., M. Kawada, and B. Kobayashi, *Effect of insulin on murine megakaryocytopoiesis in a liquid culture system.* Cell Struct Funct, 1987. **12**(3): p. 311-6.

166. Larsen, S.B., et al., *Platelet turnover in stable coronary artery disease - influence of thrombopoietin and low-grade inflammation.* PLoS One, 2014. **9**(1): p. e85566.

167. Watson, L.M., et al., *Overexpression of the 78-kDa glucose-regulated protein/immunoglobulin-binding protein (GRP78/BiP) inhibits tissue factor procoagulant activity.* J Biol Chem, 2003. **278**(19): p. 17438-47.

168. Hernandez Vera, R., et al., *Platelets derived from the bone marrow of diabetic animals show dysregulated endoplasmic reticulum stress proteins that contribute to increased thrombosis.* Arterioscler Thromb Vasc Biol, 2012. **32**(9): p. 2141-8.

169.   Gerstein, H.C., et al., *The relationship between dysglycaemia and cardiovascular and renal risk in diabetic and non-diabetic participants in the HOPE study: a prospective epidemiological analysis.* Diabetologia, 2005. **48**(9): p. 1749-55.

170.   Selvin, E., et al., *Meta-analysis: glycosylated hemoglobin and cardiovascular disease in diabetes mellitus.* Ann Intern Med, 2004. **141**(6): p. 421-31.

171.   Action to Control Cardiovascular Risk in Diabetes Study, G., et al., *Effects of intensive glucose lowering in type 2 diabetes.* N Engl J Med, 2008. **358**(24): p. 2545-59.

172.   Dluhy, R.G. and G.T. McMahon, *Intensive glycemic control in the ACCORD and ADVANCE trials.* N Engl J Med, 2008. **358**(24): p. 2630-3.

173.   Duckworth, W., et al., *Glucose control and vascular complications in veterans with type 2 diabetes.* N Engl J Med, 2009. **360**(2): p. 129-39.

174.   Santilli, F., et al., *Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance".* J Am Coll Cardiol, 2009. **53**(8): p. 667-77.

175.   Sorensen, B., et al., *Whole blood coagulation thrombelastographic profiles employing minimal tissue factor activation.* J Thromb Haemost, 2003. **1**(3): p. 551-8.

176.   Deutsch, V.R. and A. Tomer, *Advances in megakaryocytopoiesis and thrombopoiesis: from bench to bedside.* Br J Haematol, 2013. **161**(6): p. 778-93.

177.   Koike, Y., et al., *Evaluation of thrombopoiesis in thrombocytopenic disorders by simultaneous measurement of reticulated platelets of whole blood and serum thrombopoietin concentrations.* Thromb Haemost, 1998. **79**(6): p. 1106-10.

178.   Rollini, F., et al., *Platelet function profiles in patients with diabetes mellitus.* J Cardiovasc Transl Res, 2013. **6**(3): p. 329-45.

179.   Hillmann, A.G., et al., *Comparative RNA expression analyses from small-scale, single-donor platelet samples.* J Thromb Haemost, 2006. **4**(2): p. 349-56.

180.   Gnatenko, D.V., et al., *Platelets express steroidogenic 17beta-hydroxysteroid dehydrogenases. Distinct profiles predict the essential thrombocythemic phenotype.* Thromb Haemost, 2005. **94**(2): p. 412-21.

181.   Bugert, P., et al., *Messenger RNA profiling of human platelets by microarray hybridization.* Thromb Haemost, 2003. **90**(4): p. 738-48.

182.   McRedmond, J.P., et al., *Integration of proteomics and genomics in platelets: a profile of platelet proteins and platelet-specific genes.* Mol Cell Proteomics, 2004. **3**(2): p. 133-44.

183.   Marzio, R., J. Mauel, and S. Betz-Corradin, *CD69 and regulation of the immune function.* Immunopharmacol Immunotoxicol, 1999. **21**(3): p. 565-82.

184.   Testi, R., et al., *CD69 is expressed on platelets and mediates platelet activation and aggregation.* J Exp Med, 1990. **172**(3): p. 701-7.

185.   Segade, F. and J. Gomez-Marquez, *Prothymosin alpha.* Int J Biochem Cell Biol, 1999. **31**(11): p. 1243-8.

186.   Carestia, A., et al., *Functional responses and molecular mechanisms involved in histone-mediated platelet activation.* Thromb Haemost, 2013. **110**(5): p. 1035-45.

PUBLICATIONS

- <u>Rossetti L</u>, Brambilla M, Negro G, Canzano P, Perancin B, Trabattoni D, Lualdi A, Tremoli E, Camera M. New insights into the prothrombotic propensity of stable angina patients with type-2 diabetes mellitus: focus on platelet Tissue Factor and platelet transcriptome alterations. *Manuscript in preparation*.

- Brambilla M, Gelosa P, <u>Rossetti L</u>, Perancin B, Castiglioni L, Sironi L, Tremoli E, Camera M. Captopril downregulates circulating Tissue Factor expression in stroke-prone rats. *Manuscript in preparation*.

- Brambilla M, Facchinetti L, Canzano P, <u>Rossetti L</u>, Ferri N, Balduini A, Abbonante V, Boselli D, De Marco L, Di Minno MND, Toschi V, Corsini A, Tremoli E, Camera M. Human megakaryocytes express Tissue Factor and deliver it to a subset of shedded platelets where it contributes to thrombin generation. *Thromb. Heamost. Manuscript under revision*.

- Camera M, Brambilla M, Boselli D, Facchinetti L, Canzano P, <u>Rossetti L</u>, Toschi V, Tremoli E. Functionally active platelets do express tissue factor. *Blood.* 2012 May 3;119(18): 4339-41.

- Camera M, Brambilla M, Facchinetti L, Canzano P, Spirito R, <u>Rossetti L</u>, Saccu C, Di Minno MN, Tremoli E. Tissue factor and atherosclerosis: not only vessel wall-derived TF, but also platelet-associated TF. *Thromb Res.* 2012 Mar;129(3):279-84.