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Macrophage migration inhibitory factor and gremlin-1 in patients with coronary artery disease and diabetes: patterns of expression and interaction

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ABBREVIATIONS

Ab	Antibody
ACS	Acute Coronary Syndrome
ADA	American Diabetes Association
ADP	Adenosine Diphosphate
AMPK	AMP-Activated Protein Kinase
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMI	Body-Mass Index
BMPs	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Surgery
CAD	Coronary Artery Disease
CD42a	Glycoprotein IX or Cluster of Differentiation 42a
CD62P	P-Selectin
CHD	Coronary Heart Disease
CRP	C-Reactive Protein
CURE	Clopidogrel in Unstable Angina to Prevent Recurrent Events
CV	Cardiovascular
CVDs	Cardiovascular Diseases
CXCR2	Chemokine (C-X-C Motif) Receptor 2
CXCR4	Chemokine (C-X-C Motif) Receptor 4
DALY	Disability-Adjusted Life Year
EASD	European Association for the Study of Diabetes
EDTA	Ethylenediaminetetraacetic Acid
EF	Ejection Fraction
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting

FFA	Free Fatty Acid
FITC	Fluorescein Isothiocyanate
GIF	Glycosylation-Inhibiting Factor
GPVI	Platelet Glycoprotein VI
GREM1	Gremlin-1
HbA1c	Glycated Hemoglobin A1C
HDL	High-Density Lipoprotein
HPR	High Platelet Reactivity
hsCRP	High Sensitivity C-Reactive Protein
IGF	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IHD	Ischemic Heart Disease
IL-6	Interleukin-6
IQR	Interquartile Range
IVUS	Intravascular Ultrasound
LDL-C	Low-Density Lipoprotein Cholesterol
MACE	Major Adverse Cardiac Events
MFI	Median Fluorescence Intensity
MFI	Median Fluorescence Intensity
MI	Myocardial Infarction
MIF	Macrophage Migration Inhibitory Factor
MMIF	Macrophage Migration Inhibitory Factor
MMPs	Matrix Metalloproteinases
mRNA	Messenger RNA
NSTEMI	Non-ST Segment Elevation Myocardial Infarction
OGTT	Oral Glucose Tolerance Test
OxLDL	Oxidized Low-Density Lipoprotein
P2RY12	Inergic Receptor P2Y, G-Protein Coupled, 12
PAC-1	First Procaspase Activating Compound
PAMPs	Pathogen-Associated Molecular Patterns

PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
PFA	Paraformaldehyde
PG	Plasma Glucose
PRM	Pattern Recognition Molecule
PVDF	Polyvinylidene Difluoride
ROS	Reactive Oxygen Species
SCAD	Stable Coronary Artery Disease
SD	Standard Deviation
SDF-1	Stromal Cell-Derived Factor 1
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SMCs	Smooth Muscle Cells
SR-B	Scavenger Receptor B
STEMI	ST Segment Elevation Myocardial Infarction
T2DM	Type 2 Diabetes Mellitus
TGF-β	Transforming Growth Factor Beta
TNF-α	Tissue Necrosis Factor-alpha
UA	Unstable Angina
URL	Upper Reference Limit
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
VLDL	Very Low-Density Lipoprotein
WHO	World Health Organization

1. INTRODUCTION

1.1. CORONARY ARTERY DISEASE

Coronary artery disease (CAD) also called coronary heart disease (CHD) or ischemic heart disease (IHD) is caused by the obstruction of one or more coronary arteries resulting from the accumulation of atheromatous plaques within the walls of the coronary arteries. It is the leading cause of death worldwide accounting for 7.025 million deaths yearly or 12.9 per cent of total deaths (116) and the mortality is projected to rise to 7.594 million deaths or 13.2% of deaths by 2015 and 9.245 million deaths or 13.2% by 2030 (117). In Europe ischemic heart disease is the leading cause of death with 2.245 million deaths or 24.8% of all deaths in 2011 (116). Nevertheless, ischemic heart disease does not consist a problem only when it has a fatal outcome. It is also one of the leading causes of burden of disease in the world. IHD accounted for 159.659.000 disability-adjusted life years (DALYs) or 5.8% of total DALYs in 2011. This data puts IHD on the 2nd place of leading causes of morbidity after lower respiratory infections with increasing tendency in the last decade (114).

The manifestation of CAD was first clinically described based on the angina symptoms classified by Herberden in 1772 (156). Since then there has been extended research focused on the pathophysiological mechanisms that lead to CAD. The clinical manifestations of CAD include silent ischemia, stable angina pectoris, unstable angina pectoris (UA), myocardial infarction (MI), heart failure, and sudden cardiac death.

Traditional risk factors for CAD include smoking (102, 145), obesity (121), dyslipidemia, arterial hypertension, diabetes and chronic kidney disease. Thus lifestyle modifications are suggested in CAD patients such as smoking cessation that is associated with a 36% mortality reduction in the post MI phase (37), adoption of a healthy diet such as “Mediterranean” diet (52), weight management (121) and physical activity.

At the same time pharmacological management plays an important role in relief from symptoms and prevention of cardiovascular events. For the symptomatic treatment of angina short acting nitrates, β-blockers or calcium channel blockers, ranolazin and ivabradine are indicated. For prevention of thromboischemic events a low-dose aspirin daily is recommended in all CAD patients with clopidogrel as an alternative in case of aspirin intolerance. Statins are recommended for all CAD patients and angiotensin converting enzyme inhibitors or Angiotensin II receptor blockers are recommended in the presence of comorbidities such as heart failure, diabetes or hypertension (143).

When evaluating a patient with chronic coronary artery disease, doctors must choose their strategy between catheterization with revascularization, if feasible, combined with drug therapy or initial medical management with revascularization only if drug therapy fails. Although the superiority of coronary revascularization in relieving angina has been a consistent finding in many trials, only patients with high-risk profiles have been shown to benefit by reducing rates of subsequent myocardial infarction and (20, 44, 80, 162). Diabetics though present a high-risk group even when only mild symptoms are manifest. But trials in this group have also been controversial regarding major adverse cardiac events (MACE). The largest trial in the diabetic population showed that either therapeutic decision is correct in the setting of stable CAD (40). Latest trials have focused on which of the invasive options are most beneficial for diabetics with multivessel coronary artery disease, without reaching a unanimous verdict (55, 151).

In patients with ACS, guidelines are clearer with an invasive treatment recommended for all patients without contraindications for revascularization. In STEMI patients the revascularization should be prompt, whereas in UA and NSTEMI patients without recurrent ischemia in the first 24 hours the invasive risk stratification can be reserved for a later time point, depending on their cumulative risk (e.g. calculated by the GRACE-score) between 2 and 72 hours (72, 143, 144).

1.2. DIABETES MELLITUS AND CARDIOVASCULAR DISEASE (CVD)

Data from multiple epidemiological studies show that diabetes mellitus type 2 (T2DM) is a major risk factor contributing to the development of all manifestations of cardiovascular disease, including fatal and non-fatal myocardial infarction and heart failure (5). Recent data support an increasing burden of cardiovascular disease attributable to diabetes mellitus (61). Along with the globally increasing prevalence of obesity and the aging population, the incidence and prevalence of T2DM is anticipated to rise. From the year 2000 to 2030 increase of the prevalence of T2DM is projected to 100%, with those older than 65 years of age contributing the biggest share (155). CVD and cardiovascular events are major contributors to morbidity and mortality of diabetics, with estimations holding CVD responsible for at least 50% of all deaths in T2DM patients (15, 24, 38, 69, 115, 124).

In the Norfolk cohort of European Prospective Investigation Into Cancer and Nutrition (EPIC-Norfolk) trial an increase of 1% in glycated hemoglobin (HbA1c) was associated with a 40% increase in CVD mortality in T2DM patients and with a 28% increase in risk of death independent of age, blood pressure, serum cholesterol, body mass index, and cigarette smoking habit (87). A meta-analysis of almost 700.000 subjects from 102 different prospective studies reported that diabetics presented with a near 2-fold increase in CVD risk, independent from other known CVD risk factors (50). Intensive glycemic control and its impact on CV outcomes has been a controversial subject for at least 2 decades (67). Therefore it is clear that metabolic control is essential in treating diabetics with CVD.

A goal HbA1c of less than 7.0% has been proposed by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) for most of T2DM patients (81). The same position paper advises towards less stringent goals for patients with a history of severe hypoglycemia, limited life expectancy, advanced microvascular or macrovascular disease, or

those with long-standing diabetes (13). It has also been suggested that insulin may be atherogenic in a dose dependent fashion (42).

CVD risk in T2DM patients most likely owes a multifactorial etiology. In T2DM patients there is often a clustering of additional CV risk factors closely associated with insulin resistance, including hypertension and central obesity (2, 41, 112).

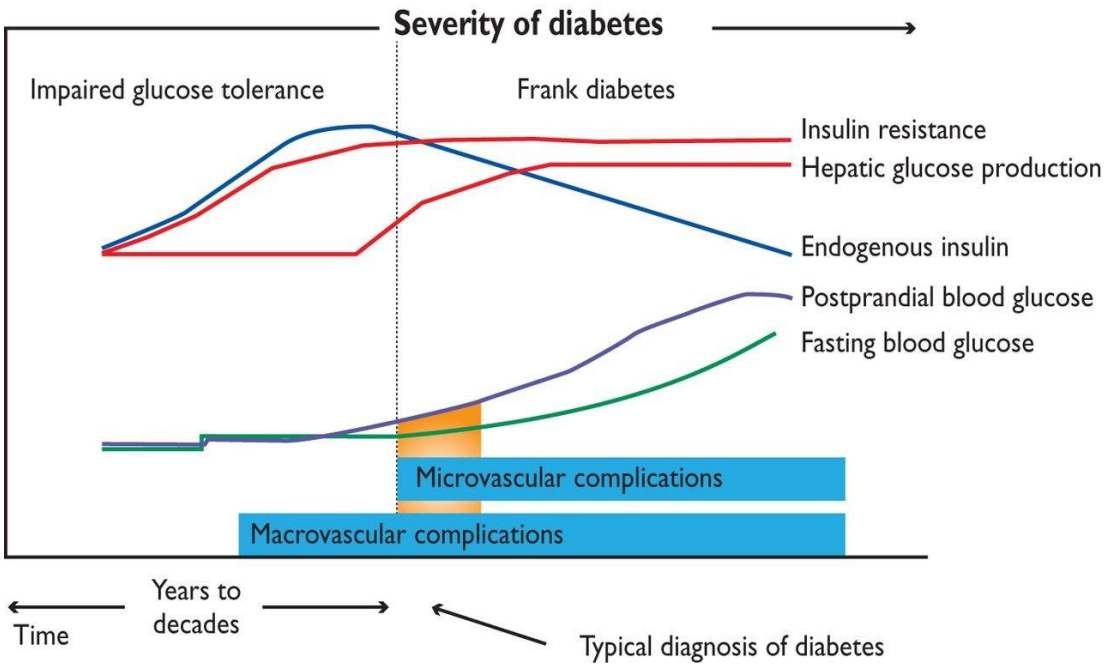


Figure 1.1 Glycemic continuum and cardiovascular disease.
Diabetes mellitus type 2 is a state of combined IR, compensatory hyperinsulinemia and elevated PG, which lead to a higher cardiovascular risk and rapid development of macrovascular disease prior to diagnosis of diabetes (13).

1.3. ATHEROSCLEROSIS AND MANIFESTATIONS OF CAD

Epicardial coronary arteries are a major site of clinically relevant atherosclerotic disease (91, 129). Atherosclerosis is a chronic, systemic, lipid-associated, immuno-inflammatory disease of the medium and large-sized arteries resulting in plaque development. Plaques are principally formed at predilection sites that

are characterized by high endothelial stress (32). Atherosclerosis begins with our birth and the lesions develop over the course of our live, one of the longest incubation periods among human diseases. Despite the chronic character of the disease, its most dangerous effects occur suddenly. CAD includes two pathophysiological pathways that determine the clinical manifestations of the disease. One chronic, non-reversible process of progressive stenosis of the lumen of the coronary arteries and a very dynamic but potentially reversible process that leads to a hemodynamically significant occlusion of the coronary artery (131).

Most plaques stay asymptomatic, some become obstructive leading to stable angina and a few are prone to thrombosis and disruption (vulnerable). When the progression of atherosclerosis has led to a high-grade stenosis, a platelet thrombus can occlude the vessel completely and cause ST-segment elevation myocardial infarction. When only incomplete or transient obstruction of flow occurs, the result is an acute coronary syndrome without persisting ST-segment elevation. In the complex process of plaque vulnerability and disruption inflammation plays an important pathophysiological role. Rarely, ACS may have a non-atherosclerotic etiology, e.g. thoracic trauma, dissection of arteries, thromboembolism, cocaine abuse or iatrogenic.

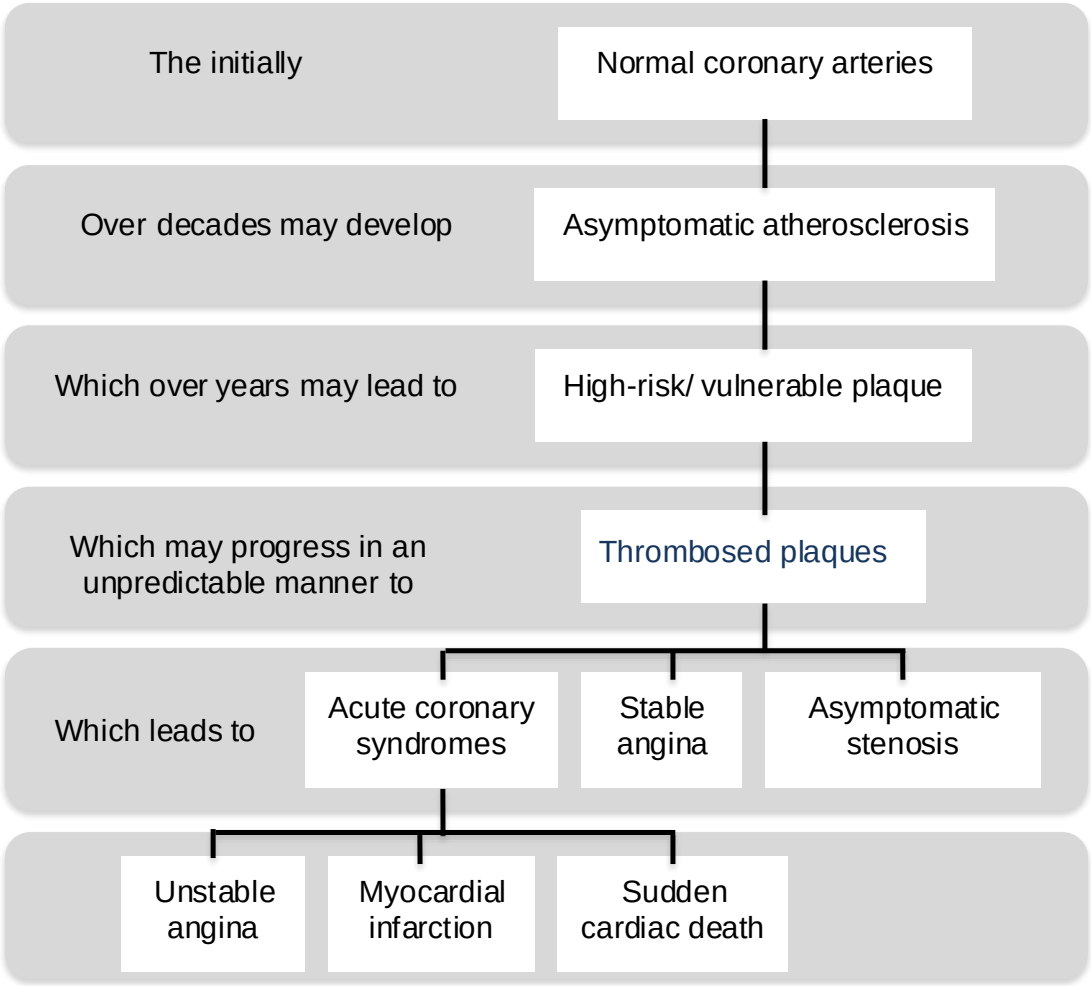


Figure 1.2: Development of atherosclerosis and possible progression to thrombosis and corresponding clinical events (131).

1.3.1. ENDOTHELIAL DYSFUNCTION, VASCULAR INFLAMMATION: THE VALNURABLE PLAQUE THEORY

According to the prevalent theory sudden and unpredictable changes in symptoms of atherosclerosis appear to be associated with plaque disruption. Genesis of ACS is the plaque rupture and not every plaque poses the same

danger of rupture. Patients with unstable angina or NSTEMI often enough have multiple plaques vulnerable to disruption (6, 68).

Pathological studies in humans showed that thrombotic coronary occlusion after rupture of a lipid-rich atheroma seems to be the trigger for myocardial infarction in 73% of the cases (54, 56, 57).

One of the seminal clinical studies outlining this is the collaborating PROSPECT Trial, in which Stone and colleagues investigated 697 patients undergoing elective PCI and systematic interventional imaging of the entire coronary artery tree using intravascular ultrasound (IVUS) to try to characterize the risk that individual lesion characteristics at baseline conferred regarding the recurrence of coronary events. This study identified three characteristics associated with plaque vulnerability:

1. High plaque burden >70% (hazard ratio (HR) 5.03)
2. Small minimal lumen area (<4 mm^2, HR 3.21)
3. Classification as a thin-cap fibroatheroma (HR 3.35) (139)

The notion that cells mostly associated with the inflammatory response play an important role in the pathogenesis of ACS found fertile ground in the scientific community (74, 92). Currently it’s a common ground that the activation of inflammatory cells in the culprit lesion triggers the coronary plaque instability (73, 92). With imaging techniques available today investigation of arterial wall inflammation is indirectly possible e.g. by using FDG-PET (60).

The dysfunctional endothelium cannot properly regulate the recruitment of leukocytes. Macrophage foam cells serve as lipid reserve and also induce decreased production of nitric oxide, aggravate the endothelial dysfunction (163) and lead to thickening of the intima media (59). They also serve as a source of pro-inflammatory mediators, such as cytokines and chemokines,

reactive oxygen intermediates or platelet activating factor that lead to a prolonged inflammatory activation of the endothelium (66).

This type of antigen-stimulation independent amplified inflammatory response is described as innate immunity. Proliferation of smooth muscle cells (SMCs) furthers promotes atherosclerosis. Extracellular matrix makes up most of the volume of an atherosclerotic plaque in advanced stages. The imbalance in its production lies with the disrupted breakdown, which is catalyzed in part by matrix metalloproteinases (MMPs).

The fibrous cap is a dynamic structure undergoing active remodeling. With its high concentration of type I collagen it can resist high amounts of stress. The collagen de novo synthesis is modulated by growth factors and supported by degradation through proteases produced from activated macrophages. The local apoptosis of SMCs on the cap tissue can also contribute to rupture (142).

The lipid-rich core of the fibroatheroma when exposed after plaque rupture is highly thrombogenic and the thrombosis can lead to total or subtotal occlusion of the artery that cannot be compensated for through positive remodeling. This thrombus can fragment into smaller pieces, creating emboli that may cause necrosis in the area of the myocardium supplied by the culprit vessel (39, 53).

1.3.2. MECHANISMS THAT CONTRIBUTE TO THE INCREMENTAL RISK FOR CAD IN DIABETICS

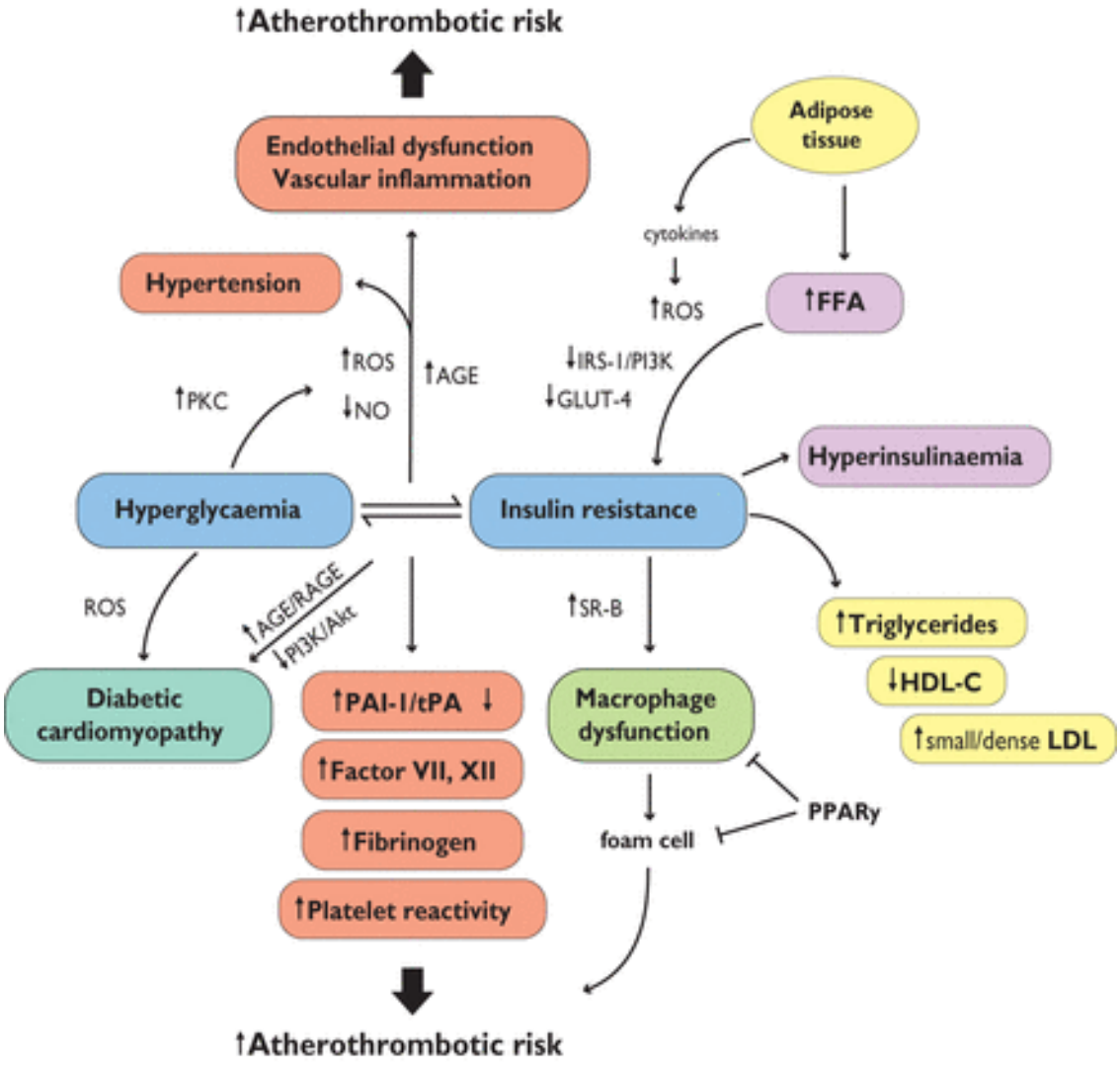
The atheroma in type 2 diabetics is more prone to inflammation, thrombosis and has a higher lipid concentration than that of non-diabetics. T2DM patients are obese (78) and adipose tissue at a hyperglycemic state intensively accumulates macrophages, that form foam cells and promote atherosclerosis through oxidized low-lipoprotein (LDL) scavenger receptor B (SR-B) and releases substances that further impair insulin sensitivity (128). At the same time

endothelial dysfunction and vascular remodeling is promoted involving overproduction of reactive oxygen species (ROS) (13, 36).

The free fatty acids (FFA) induce increased production of very low-density lipoprotein (VLDL), which is more susceptible to oxidative stress (119, 164). At the same time the function of high-density lipoprotein (HDL) as protective factor is lost in diabetics (8, 62, 90).

T2DM is a prothrombotic state with hypofybrinolytic abnormalities (70) and hyper-reactive, dysfunctional platelets. Hyperglycemia-induced up-regulation of platelet glycoproteins (Ib and IIb/IIIa), P-selectin and enhanced $P2Y_{12}$ signaling are associated with the atherothrombotic risk in diabetics. Furthermore, the cardiovascular risk burden in T2DM is not diminished by intensive glycemic control and mechanism-based therapeutic strategies are needed such as inhibition of key-enzymes involved in protein-production and activation of pathways. There is also need for biomarkers aiding in early detection of CAD in asymptomatic patients and prediction of CV risk (13).

Figure 1.3: Interaction of the pathophysiological mechanisms in diabetes and the resulting atherothrombotic risk.
More than 90% of people withT2DM are obese and the release of free fatty acids (FFAs) and cytokines from adipose tissue directly impairs insulin sensitivity. The picture presents the interaction of the pathophysiological mechanisms in diabetes and the resulting atherothrombotic risk. Connecting arrows depict the mediators of such interactions.
AGE = advanced glycated-products; FFA = free fatty acids; GLUT-4 = glucose transporter 4; HDL-C = high-density lipoprotein cholesterol; LDL = low-density lipoprotein particles; NO = nitric oxide; PAI-1 = plasminogen activator inhibitor-1; PKC = protein kinase C; PPARy = peroxisome proliferator-activated receptor y; PI3K = phosphatidylinositide 3-kinase; RAGE = AGE receptor; ROS = reactive oxygen species; SR-B = scavenger receptor B; tPA = tissue plasminogen activator (13).



1.4. BIOMARKERS IN ACS

1.4.1. HIGH-SENSITIVITY C-REACTIVE PROTEIN

The acute-phase reactant CRP has emerged as a predictor for long-term events in ACS (125). CRP belongs to the superfamily of pentraxins and more specifically to the classic short pentraxins. It is a secreted endogenous PRM (pattern recognition molecule) and is synthesized by the liver (64). Limited number of germline-encoded PRMs are used by the innate immune system to detect conserved molecular structures known as pathogen-associated

molecular patterns (PAMPs) exposed on pathogens but absent from healthy host cells (82, 120, 123).

In humans, CRP is a major acute-phase plasma protein, in which the serum concentration can rapidly increase in response to infection or tissue injury. It is the principal downstream mediator of the acute phase response and is primarily derived via IL-6- dependent hepatic biosynthesis. During the acute phase response, levels of CRP will increase within 2 hours of acute insult, reaching a peak at 48 hours and start declining relatively rapidly with a half-life of 18 hours.

CRP levels can increase as a response to a wide variety of biological insults, infections, autoimmune inflammatory conditions or malignant processes. While CRP has multiple pro-inflammatory and proatherogenic properties, recent studies, one using a mendelian randomization approach (30) and a large genomics study (49), both reached the conclusion that C-reactive protein does not seem to be the cause of coronary heart disease, although it is a risk marker for it (49, 146). Various medications have been proved to reduce serum CRP levels, e.g. aspirin and clopidogrel or statins and that in turn reduce the incidence of major cardiovascular events (83, 127).

After Liuzzo's et al. first report on the prognostic role of CRP in acute coronary syndromes (94), several trials and meta-analysis have put that finding in clinical context (49) and now serum CRP levels are used in scores to determine one-year mortality and the 10-year cardiovascular risk (122, 126, 157). The American Heart Association and the American College of Cardiology gave a recommendation for testing of CRP in asymptomatic intermediate-risk men 50 years of age or younger or women 60 years of age or younger as a tool for CV risk assessment (Level of Evidence: B) (71). Elevation of CRP-levels is not only a predictor for CV-events, but also for the onset of T2DM. This might be because CRP correlates with characteristics of the metabolic syndrome, including insulin sensitivity, endothelial dysfunction, and hypofibrinolysis, as noted earlier.

Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) study showed no association of CRP levels with $P2Y_{12}$ inhibition and diminished levels of CRP 30days after the event (161). The concept of use of statins to reduce the CV-risk in patients with elevated hs-CRP independent from LDL-Cholesterol levels has been confirmed in the A-to-Z clinical trial (107) and the JUPITER trial (127).

1.4.2. MACROPHAGE MIGRATION INHIBITORY FACTOR

Macrophage migration inhibitory factor (MIF or MMIF) also known as glycosylation-inhibiting factor (GIF), L-dopachromeisomerase/tautomerase, or phenylpyruvatetautomerase is a very pleiotropic and widely studied inflammatory cytokine, an evolutionarily highly conserved molecule with an almost ubiquitous expression pattern and an extensive regulatory activity in humans. MIF was first isolated back in the 60's, as a protein from the supernatant of activated lymphocytes, however little was then known about its role. More light in its function was shed after the introduction of molecular biology. More specifically, MIF was categorized as a chemokine like factor in humans (43).

MIF is expressed on the endothelium and macrophages and in humans it is involved in the innate immune response to bacterial pathogens and its expression at sites of inflammation suggests a role as mediator in regulating the function of macrophages in host defense. MIF counteracts the anti-inflammatory activity of glucocorticoids. Serum levels of MIF are elevated in patients with severe sepsis or septic shock and high levels of MIF are correlated with poor survival and drugs that inhibit tautomerase activity attenuate the risk of death due to sepsis (17, 18, 26, 27, 47, 51, 89, 104, 154). Data is widely available on the functions of this versatile molecule including its role in glomerulonephritis (159) and arthritis (16).

Regarding cardiovascular diseases, the expression and activity of MIF was recorded in the onset of atherogenesis and advanced lesions of hypercholesterolemic rabbits and humans (93). MIF is an inflammatory cytokine with a chemokine-like function, promoting leukocyte recruitment, adhesion and atherosclerotic lesion formation via the chemokine receptors CXCR2 und CXCR4 (23). It has also been shown that MIF deficiency considerably reduces atherogenesis in LDLr-/- (118), but no data is available on serum levels of MIF in CVD. Despite its wide tissue distribution, the secretion of MIF is tightly regulated, with triggers such as hypoxia/ischemia or oxidized low-density lipoprotein (9). Schmeisser et al. showed that MIF is expressed by advanced plaques and mainly in areas of enhanced instability (132); nevertheless the current studies are in dispute regarding how MIF affects the plaque size and stability of the vulnerable plaque (22, 134).

Latest studies have shown a correlation of levels of MIF with cardiac dysfunction in diabetic patients (97, 160). In a known background of elevated MIF levels in diabetics and the correlation of MIF with the progression of glucose resistance to diabetes (88, 150, 152) the MONICA/KORA Augsburg Case-Cohort Study tried to include multiple inflammation-related biomarkers into a basic risk assessment model for cardiovascular events in type 2 diabetics (75).

Pilot studies of our group demonstrated correlation of MIF levels with established inflammatory markers, the extent of cardiac necrosis marker release after PCI and ACS (110).

1.4.3. GREMLIN-1

Gremlin-1 is a secreted, highly conserved glycoprotein with an atomic mass of 20.7 kDa and a structure shared by members of the TGF-β superfamily and Vascular Endothelial Growth Factors (106, 148, 149). Gremlin-1 plays a part

during lung, limb, urethra and kidney formation and neural crest cell differentiation through regulation of BMPs (33, 96, 103, 135, 140). Through its interaction with Slit proteins Gremlin-1 functions as an inhibitor of monocyte chemotaxis and through binding of VEGFR-2 acts as a proangiogenic agonist with a role in vascular development, angiogenesis-dependent diseases, and tumor neovascularization (106).

Gremlin-1 is expressed in endothelial cells that are exposed to disturbed flow in mice aortas as well as in human coronary arteries (31, 45). An up-regulation of Gremlin-1 in pericytes in response to elevated glucose levels was reposted, suggesting a role in diabetic retinopathy (85). Additionally, a role in tubulointerstitial fibrosis in diabetic nephropathy has been suggested (46).

In studies of our group we could demonstrate that Gremlin-1 regulates foam cell formation in vitro, is an endogenous antagonist of MIF and binds with high affinity to MIF. Administration of a dimeric recombinant fusion protein mGremlin-1-Fc reduced the content of macrophages in atherosclerotic plaques, and limits atheroprogression and lesion instability (109). One can speculate that GREM1 plays a pivotal role in proliferation of atherosclerotic lesions and plaque vulnerability and consequently instability.

Pilot studies of our group in patients with ACS suggest a potential role of Grem1/MIF ratio to indicate acuity of CAD and the grade of plaque stability (108).

1.5. SCOPE OF THE INVESTIGATION

In this study we aimed to evaluate the expression of MIF and GREM1 in patients with symptomatic IHD with major focus in diabetics with CAD. Following research questions were pursued:

1. Are MIF and GREM1 expressed from human platelets, and if yes at what degree?
2. Is the expression of MIF and GREM1 from human platelets dependent from platelet count, platelet activation or platelet aggregation?
3. Is there a correlation between MIF and GREM1 levels in human platelets
4. Do patients with stable ischemic heart disease express MIF and GREM1 at the same degree as patients with acute coronary syndromes?
5. Do diabetics with symptomatic ischemic heart disease have the same MIF and GREM1 platelet levels compared to non-diabetics?

2. METHODS

2.1. STUDY DESIGN

The study was conducted at the UniversitätsklinikumTübingen, Medizinische Universitätsklinik, Department für Innere Medizin III, Kardiologie und Kreislauferkrankungen. The institutional review board approved the study (270/2011BO1).

2.2. STUDY PATIENTS AND PROTOCOLS

Patients with stable CAD or acute coronary syndromes were enrolled at the time of percutaneous coronary intervention (PCI). The inclusion and exclusion criteria are as listed in Table 2.1. ACS Patients that did not undergo an intervention, either because of ACS without significant coronary stenosis or because they were referred for coronary artery bypass surgery (CABG) or because they were considered too frail/critical for intervention were not included in this study. All patients provided written informed consent. The study was approved by the institution ethics committee (270/2011BO1) ad complies with the declaration of Helsinki and the good clinical practice guidelines (1, 3, 4).

Table 2.1: Inclusion and exclusion criteria in our study

Clinical inclusion criteria

1. Age ≥18 years

2. Symptomatic CAD undergoing PCI

3. Patient provides written, informed consent.

Clinical exclusion criteria

1. Pregnant or nursing patients

Angiographic inclusion criteria

1. Lesions in at least 1 epicardial coronary artery requiring reperfusion therapy.
2. Successful PCI of at least one lesion.

Levels of serum creatinine, fasting lipids, glucose, glycated hemoglobin, and C-reactive protein were measured at baseline. Blood was collected in EDTA tubes at the time of angiography, centrifuged at room temperature (1500xg) for 15 min and the supernatants were stored as EDTA-plasma at -80°C for the ELISA. At the same time citrate phosphate dextrose adenine (CPDA)-tubes were collected for the fluorescence-activated cell sorting (FACS). 1.5 mL blood was collected in hirudin tubes for Multiplate measurements of agonist induced platelet aggregation.

2.3. CRITERIA FOR ACUTE MYOCARDIAL INFARCTION

Acute myocardial infarction was defined according to the third universal definition (147). Detection of a rise and/or fall of cardiac troponin with at least one value above the 99th percentile URL and with at least one of the following: (i) symptoms of ischemia, or (ii) new or presumed new significant ST-segment–T wave (ST–T) changes or new left bundle branch block, or (iii) development of pathological Q waves in the electrocardiogram, or (iv) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality, or (v) identification of an intracoronary thrombus by angiography or autopsy according

to the third universal definition of myocardial infarction. In our laboratory the cut-off value for troponin I was 0.04 µg/l.

2.4. DEFINITION OF DIABETES MELLITUS TYPE 2

Diabetes mellitus type 2 was defined according to current diagnostic criteria of the American Diabetes Association (ADA) (7) and the world health organization (WHO) (113) as the following:

Table 2.2: Major Diagnostic Criteria for Diabetes and Prediabetic or At-Risk States

Measure	American Diabetes Association		World Health Organization	
	Diabetes	Prediabetes	Diabetes	Impaired Glucose Regulation
Fasting plasma glucose	≥126 mg/dl	100–125 mg/dl (IFG)	≥126 mg/dl	110–125 mg/dl (IFG)
2h plasma glucose∗	≥200 mg/dl	140–199 mg/dl (IGT)	≥200 mg/dl	140–199 mg/dl (IGT)
Casual (or random) plasma glucose∗∗	≥200 mg/dl		≥200 mg/dl	
Glycated hemoglobin	≥6.5%	5.7–6.4%	≥6.5%	

∗ During an OGTT with a loading dose of 75 g
∗∗ In a patient with classic hyperglycemic symptoms

2.5. WESTERN BLOT

For immunodetection of MIF and GREM1 in platelet lysates were purified from platelets from peripheral blood sample of control subject with normal peripheral blood platelets. A standard immunodetection was performed on blotted proteins directly after electrotransfer. Protein concentration was determined using Biorad Protein Assay with protein standard BSA (Sigma) and measurement of absorption at 495 nm. The samples were diluted with Lämmli buffer (5x, +5% mercaptoethanol) and heated for 10 min up to 95°C. 30µg of total protein were separated on a 15% SDS-polyacrylamide gel electrophoresis (PAGE) (Invitrogen). Blotting of the protein onto a polyvinylidenedifluoride membrane (PVDF, Immibilon, Millipore) was performed using Semi Dry Transfer Cell System (Peqlab). As primary antibody Recombinant Human MIF polyclonal antibody from RD Systems was used in 1:7500 dilution in 5% milk/ Phosphate buffered saline (PBS) was used for detection of MIF and purified rabbit polyclonal GREMLIN (C-Term) antibody from Abgent for detection of GREMLIN1. ß-actin antibody (Sigma-Aldrich, Steinheim, Germany) and α-actin antibody (polyclonal) (Abcam, Cambridge, UK) were used as internal loading control. For detection of antibody binding, corresponding secondary fluorescence labeled antibodies and the Odyssey infrared imaging system (LI-COR, Bad Homburg, Germany) were used. Bands were quantified using ImageJ software (National Institutes of Health, USA).

2.6. FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Fluorescence-activated cell sorting was used to quantify the expression of MIF and GREM1 from platelets of the subjects. We aimed to quantify the expression of the proteins in both stimulated (with 20 µmol/l ADP) and naïve platelets

collected from full blood in sodium citrate tubes. 20µL blood were diluted in 980 µL PBS and 40 µL were aliquoted in every tube.

Activation

The activation of the platelets was performed with 20 µmol/l ADP in a 1:5 ADP: blood volume concentration. After pipetting ADP, the dilute was gently swirled to mix and was incubated in a dark room at room temperature. After 30 minutes 2.5 µL 10% paraformaldehyde (PFA) solution was used to fixate the cells.

Multicolour Direct Immunofluorescence Staining

This protocol followed the same steps for unstimulated and activated fixed whole blood suspension. After the 2.5 µL 10% PFA solution was added, 5 µL of CD42a platelet-specific antibody conjugate were used to threshold data acquisition to analyze only platelets. 5µL 1% Triton X-100 ($C_{14}H_{22}O$ $(C_2H_4O)_n$) was used to permeabilize the platelet cell membranes for the tubes, where MIF and GREM1 expression was investigated, as preliminary tests had shown better results with permeabilized cells in flow cytometry. Another antibody conjugated to a different fluorochrome, fluorescein isothiocyanate (FITC), was used to simultaneously assess the binding of platelet-associated antibodies. For the scope of this study following antibodies were used:

1. Activation-dependent antibodies alpha2b-beta3 (PAC-1) (BD Biosciences), P-selectin (CD62P) (R&D Systems) and Stromal Cell-Derived Factor 1 (SDF-1) (Abcam)
2. MIF (Abcam)
3. GREM1 (Abcam)

After an incubation period of 30 minutes in a dark room at room temperature the conjugated platelets were fixated with 300 µL PFA 0.5% and the tubes were stored at 4° C, protected from light for at least 30 minutes, but not more than 48

hours. Right before flow cytometry, samples were vortexed for at least 10 seconds to gain a proper suspension.

2.7. IMPEDANCE PLATELET AGGREGOMETRY

The Multiplate® analyzer, a whole blood platelet function assay, was used to study the platelet aggregation level. 900µL blood acquired in hirudinized tubes (Sarstedt) was needed to perform ADPtest, ASPItest and TRAPtest in each patient. The Multiplate® test principle is based on an advancement of Cardinal and Flower's 1979 impedance aggregometry method, the Multiple Electrode Aggregometry (MEA)(86). We used the area under the aggregation curve (AUC) to express the overall platelet aggregation. U was used as unit for AUC (1 Unit * 10 AUC). Tests were performed at least 30 minutes after blood acquisition, but not longer than 3 hours later.

Table 2.3: Reagents used in impedance platelet aggregometry.

Reagent	Description
ADPtest	ADP induced platelet activation sensitive to clopidogrel, prasugrel and other ADP receptor antagonists
ASPItest	Cyclooxygenase dependent aggregation (using arachidonic acid) sensitive to Aspirin®, NSAIDs and other inhibitors of platelet cyclooxygenase
TRAPtest	Platelet stimulation via the thrombin receptor (using TRAP-6), sensitive to IIb/IIIa receptor antagonists (95)

3. RESULTS

3.1. MIF IS EXPRESSED IN HUMAN PLATELETS

3.1.1. DETECTION OF MIF BY WESTERN BLOT

To determine whether MIF is expressed in normal platelets protein was harvested from cell lysates and western blot was performed (Fig. 3.1). Blots were probed with an anti-MIF rabbit polyclonal antibody and an anti-β actin antibody. A specific anti-MIF antibody recognized a single band of approximately 12.5 kDa in all specimens tested in resting and activated platelets. Peripheral-blood platelets from healthy blood donors were used as controls.

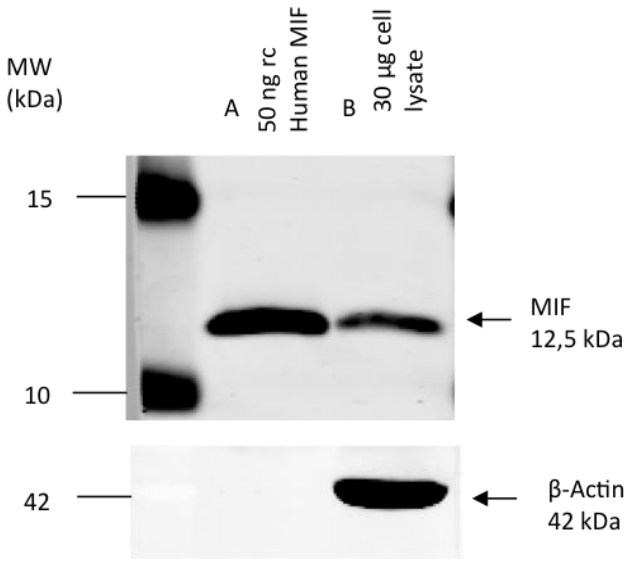


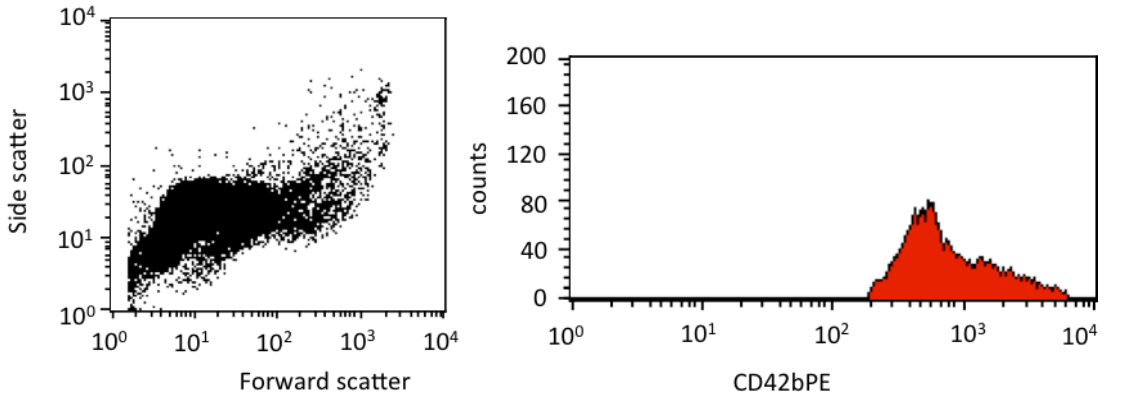
Figure 3.1: Qualitative study of MIF expression in platelets

Western blot analysis of lysates derived from purified platelets from a peripheral blood sample from a control subject with normal peripheral blood platelets for the expression of MIF. Reducing conditions, 15% SDS-Page, blocking in 5 % milk/PBS,(A) 1st Ab Anti-MIF (RD Systems) 1:100 in 5% milk/PBS, (B) 2nd Ab 1:7500 in 5% milk/PBS.

3.1.2. IMMUNOFLUORESCENCE MEASUREMENTS OF MIF BY FACS

Quantitative platelet MIF expression was analyzed by flow cytometry. Flow cytometry analysis confirmed the expression of MIF in the three main blood cell lineages and a two-color analysis revealed that MIF was highly expressed on platelets that were stained with CD42b. CD42b positive cells demonstrated prominent surface staining (Figure 3.2.). Before the double staining procedure one sample was activated with ADP and one was left in the resting state. The total level of fluorescence on a per cell basis was not significantly altered.

A



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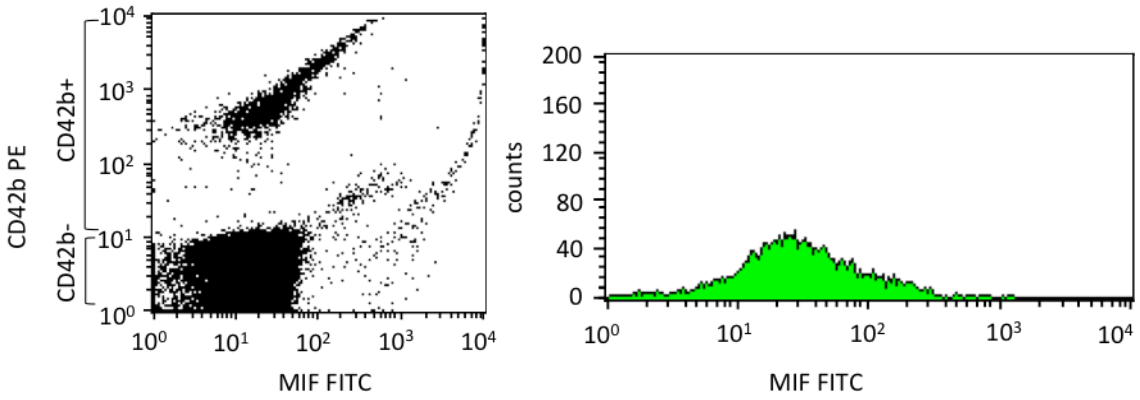


Figure 3.2: Flow cytometric analysis of the expression of MIF

Panel A shows flow cytometric plots of whole blood. In the left-hand plot, forward and side scatter is demonstrated. The histogram on the right represents the expression of CD42b by the three main blood cell lineages.

Panel B The left-hand plot shows the expression of CD42b on gated MIF cells, with two regions showing the CD42+ and CD42b- cell subpopulations. The

histogram on the right represents the expression of MIF by the CD42b+ subpopulation.

3.2. GREM1 IS EXPRESSED IN HUMAN PLATELETS

3.2.1. DETECTION OF GREM1 BY WESTERN BLOT

To determine qualitative expression of GREM1 in platelets, protein was harvested from cell lysates and western blot was performed (Fig. 3.3.). Blots were probed with an anti-GREM1 rabbit polyclonal antibody and an anti-β actin antibody. A specific anti-GREM1 antibody recognized a single band of approximately 20.7 kDa followed with a double band of approximately 25 kDa in all specimens tested in resting and activated platelets.

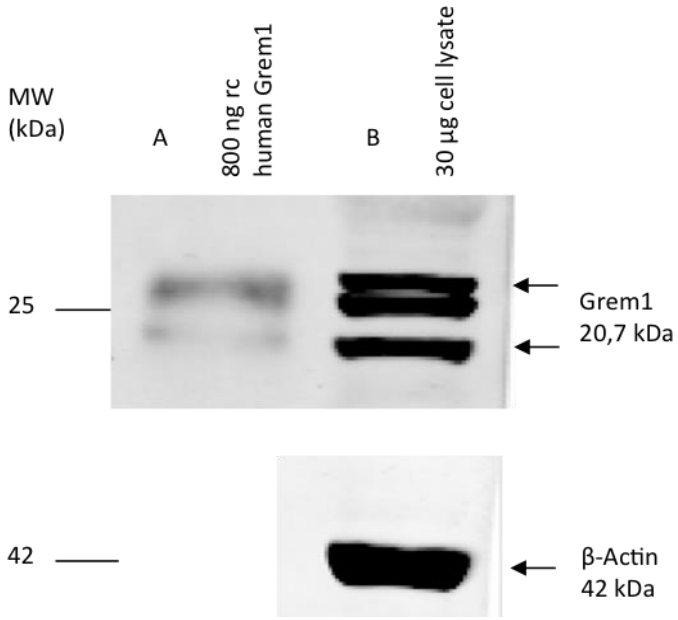


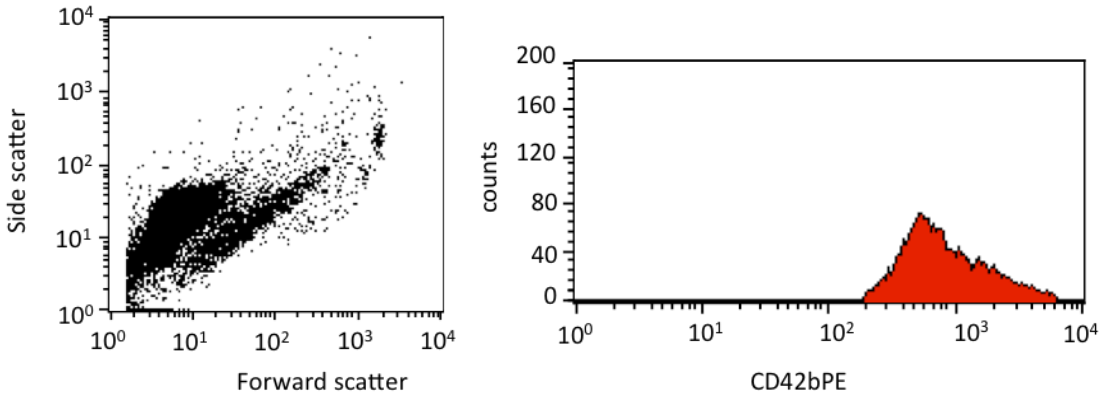
Figure 3.3: Qualitative expression of GREM1 in platelets

Western blot analysis of lysates derived from purified platelets from a peripheral blood sample from a control subject showing expression of GREM1 in platelets. Reducing conditions, 15% SDS-Page, blocking in 5 % milk/PBS, (A) Ab Anti-Gremlin (Abgent) 1:100 in 5% milk/PBS, (B) Ab 1:7500 in 5% milk/PBS.

3.2.2. IMMUNOFLUORESCENCE MEASUREMENTS OF GREM1 BY FACS

Platelet GREM1 expression was analyzed by two-colour flow cytometry with simultaneous staining with CD42b. Platelets were studied in both resting and activated state without an alteration of total level fluorescence on a per cell basis.

A



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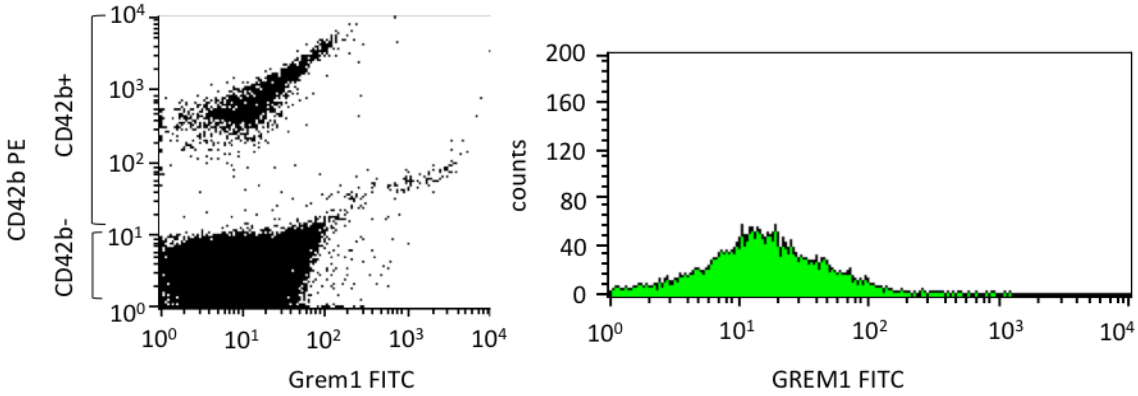

Figure 3.4: Flow cytometric Analysis of the Expression of GREM1

Panel A shows flow cytometric plots of whole blood. In the left-hand plot, forward and side scatter of blood is demonstrated. The histogram on the right represents the expression of CD42b by all cell subpopulations.

Panel B The left-hand plot shows the expression of CD42b on gated GREM1 cells, with two regions showing the CD42+ and CD42b- cell subpopulations. The histogram on the right represents the expression of GREM1 by the CD42b+ subpopulation.

3.3. STUDY SUBJECTS' CHARACTERISTICS

The study included 300 CAD patients. Mean study participant age was 67.7 years and more than fifty per cent were of age older than 70 years. More than three quarters were men, with women on average being older than men with a mean age of 71.8 (± 10.4) years versus 66.4 (± 11.6) years. Mean body-mass index was 29.2 (± 18.9). One quarter of the subjects were active smokers when enrolled in the study, while 54.3% of them had never been smokers. Diabetes mellitus was common between subjects, with 31.7% having some form of insulin resistance. Amongst the diabetics those under therapy with oral anti-diabetic agents had the poorest control of blood glucose levels and those under insulin the best one (HbA1c: 8.63 (± (2.3) g/dL versus 8.09 (± 0.96) g/dL). From the 95 diabetics 51 (54%) had a HbA1c over 7 g/dL. The mean total cholesterol levels were 176.4 (± 41.8) mmol/L with LDL 129.5 (± 39.2) mmol/L. Fifty five subjects suffered from chronic kidney disease with a mean serum creatinine value of 1.54 (± 1.5) mg/dL and mean serum Urea value of 55.69 (± 25.4) mg/dL. Patients without known chronic kidney disease had a mean serum creatinine value of 0.93 (± 0.2) mg/dL. More than 25% of the subjects had some family history of coronary artery disease and 78.7% had hypertension requiring medication. Table 3.1. shows the patients' characteristics.

Table 3.1. Baseline characteristics of the patient cohort

Characteristic	Total (300)
Age	
30–59 years	72
60–69 years	71
≥70 years	155

Mean age — yr.	67.7 (±11.6)
Sex	
Male sex — no. (%)	228 (76)
Female sex — no. (%)	72 (24)
BMI † — mean kg/m²	29.2 (±18.9)
Smoking status	
Current smoker — no. (%)	75 (25)
Former smoker — no. (%)	62 (20.7)
Never smoker — no. (%)	163 (54.3)
History of diabetes — no. (%)	
No known diabetes — no. (%)	205 (68.3)
Type 1 diabetes — no. (%)	0
Type 2 diabetes — no. (%)	95 (31.7)
Low glycemic index diet — no. (%)	20 (6.7)
Oral anti-diabetic medication — no. (%)	38 (12.7)
Insulin — no. (%)	37 (12.3)
HbA1c (g/dL), median (IQR‡)	7.2 (±4.2)
HbA1c > 7 g/dL	51
Total cholesterol — mg/dL	176.4 (±41.8)
LDL cholesterol	129.5 (±39.2)
Chronic kidney disease — no. (%)	55 (18.3)
Serum creatinine value (mg/dL), median (IQR‡)	1.05 (±0.7)
Family history of coronary artery disease — no. (%)	79 (26.3)

Hypertension requiring medication — no. (%)	236 (78.7)
Clinical presentation	
Stable angina pectoris — no. (%)	131 (43.6)
Unstable angina pectoris — no. (%)	67 (22.3)
NSTEMI — no. (%)	69 (23)
STEMI — no. (%)	33 (11)
History of known coronary artery disease — no. (%)	248 (82.7)
NYHA I	140 (46.7)
NYHA II	101 (33.7)
NYHA III	41 (13.7)
NYHA IV	18 (6)
Ejection fraction	47.14 (±10.6)
Atrial fibrillation	58 (19.3)
Log_e CRP‡ — mg/liter	
CRP Day 0 (mg/dl), median (IQR‡)	1.62 (±3.9)
Biomarkers of cardiac injury	
Troponin I_{max} (µg/dL)	5 (±28.4)
Creatinine kinase (mg/dL)	276.9 (±668.2)
Medication on presentation	
Aspirin	179 (59.7)
Anticoagulants	27 (9)
Clopidogrel	37 (12.3)
Prasugrel	7 (2.3)

Ticagrelor	12 (4)

* Values represent means ±SD.

† BMI=body-mass index

‡IQR, Interquartile range

ŦCRP, C-reactive protein

131 elective patients with stable CAD were included in this cohort. 40 of them had a myocardial infarction in their medical history at a mean of 5.5 years before their current presentation. The mean left ventricular ejection fraction in this group was 45.9 (± 10.6) % and the mean age was 68.23 (± 10.6) years. The distribution of weight was normal with a mean weight of 83.3 kg and standard deviation of 13.6 kg. Their mean BMI was 28.7 (± 4.37) kg/m^2, their mean LDL 127.56 (± 44.7) mmol/L, their Creatinine 0.97 (± 0.26) mg/dL and 43 of them were diabetics. Furthermore they had a Creatinine kinase within the normal with 107.23 (± 62.7) U/L and a CRP of 0.6 (± 0.88) mg/dL.

The remaining 169 patients presented with an acute coronary syndrome. 67 patients or 22.3% were diagnosed with an unstable angina pectoris, 69 or 23% with a non ST-Segment Elevation Acute Coronary Syndrome and 33 patients or 11% presented with ST-segment elevation acute myocardial infarction. Patients with unstable angina pectoris were the oldest group with a mean age of 70.78 (± 10.49) years and patients with STEMI were the youngest group with a mean age of 61.29 (± 12.27) years. Furthermore, left ventricular ejection fraction was most impaired in the STEMI group with a mean EF of 47.85 (± 6.53) % and less impaired in the unstable angina group, where the patients had a mean EF of 49.48 (± 11.33) %. Overall the STEMI group included the least multimorbid patients in the ACS-group, including better renal function (mean Creatinine of 1 (± 0.64) mg/dL versus 1.2 (± 1.3) mg/dL in the unstable angina pectoris group

and 1.04 (± 0.35) mg/dL in the NSTEMI-group), better glucose control (with mean glycated hemoglobin of 5.19 (± 1.6) % versus 6.46 (± 1.01)% in the unstable angina group and 7.44 (± 1.74)% in the NSTEMI group) and less obese patients (with mean BMI of 25.55 (± 1.84) kg/m^2 versus 27.18 (± 3.64) kg/m^2 in the unstable angina group and 28.06 (± 5.28) kg/m^2).

Depending on their diabetes status patients were classified in non-diabetics, patients with diabetes that requires lifestyle changes to control, diabetics who need oral agents to control their glucose levels and patients who require treatment with insulin. There were 95 diabetics included in this study. Diabetics were in general older with a mean age of 70.8 (± 10.2) years versus 66.3 (± 11.9) years for the non-diabetics, without any statistical significance (p= 0.54). They had a slightly worse ejection fraction with 49.25 (± 11.2)% versus 51.25 (± 11.5)%, also with no statistical significance (p= 0.585). Furthermore diabetics had a slightly higher BMI with a mean of 28.95 (± 5.26) kg/m^2 versus 27.57 (± 3.8) kg/m^2 (p= 0.009). Their LDL-Cholesterol levels were better controlled, since more of them were under therapy with statins (124.34 (± 32.4) mg/dL in diabetics versus 131.99 (± 42.1) mg/dL in non-diabetics, p= 0.143). The levels of C-reactive protein in diabetics were slightly higher in comparison with non-diabetics (2.04 (± 4.7) mg/dL in diabetics versus 1.42 (± 3.4) mg/dL in non-diabetics, p= 0.44). Mean serum creatinine levels did not differ significantly with diabetics having a mean creatinine of 1.09 (± 0.42) mg/dL and non-diabetics 1.03 (± 0.8) mg/dL (p= 0.381). The following table sums up the characteristics of the cohort according to their diabetes status.

Table 3.2. Baseline Characteristics according to diabetes status

Characteristic	Diabetics (95)	Nondiabetics (205)
Mean age — yr.	70.82 (± 10.2)	66.3 (± 11.9)
Male sex — no. (%)	66 (69.5)	162 (79)

BMI † — mean kg/m²	28.9 (± 5.3)	27.6 (± 3.8)
Smoking status		
Current smoker — no. (%)	14 (14.7)	61 (29.8)
Former smoker — no. (%)	21 (22.1)	41 (20)
Never smoker — no. (%)	60 (63.2)	103 (50.2)
HbA1c (g/dL), median (IQR‡)	8.3 (± 5.4)	5.8 (± 0.5)
Total cholesterol — mg/dL	169 (±34)	179.9 (44.7)
LDL cholesterol — mg/dL	124.34 (±32.4)	132 (±42.1)
Chronic kidney disease — no. (%)	30 (31.6)	25 (12.2)
Serum creatinine value (mg/dL), median (IQR‡)	1.09 (±0.4)	1.03 (± 0.8)
Family history of coronary artery disease — no. (%)	20 (21.1)	59 (28.8)
Hypertension requiring medication — no. (%)	82 (86.3)	154 (75.1)
Clinical presentation		
Stable angina pectoris — no. (%)	13 (45.3)	88 (42.9)
Unstable angina pectoris — no. (%)	20 (21.1)	47 (22.9)
NSTEMI — no. (%)	25 (26.3)	44 (21.5)
STEMI — no. (%)	7 (7.4)	26 (12.7)
History of known coronary artery disease — no. (%)	86 (90.5)	162 (79)
NYHA I	40 (42.1)	100 (48.8)
NYHA II	34 (35.8)	67 (32.7)

NYHA III	12 (12.6)	29 (14.1)
NYHA IV	9 (9.5)	9 (4.4)
Ejection fraction	49.25 (± 11.2)	51.21(± 11.5)
Atrial fibrillation	32 (33.7)	26 (12.7)
Baseline CRP (mg/dl), median (IQR‡)	2.04 (±4.7)	1.42 (± 3.36)
Biomarkers of cardiac injury		
Troponin I_{max} (µg/dL)	11.26 (± 21.73)	24.49 (± 51.05)
Creatinine $kinase_{max}$ (mg/dL)	526.8 (± 1177)	712.6(± 1009)
Medication on presentation		
Aspirin	68 (71.6)	111 (54.1)
Anticoagulants	13 (13.7)	14 (6.8)
Clopidogrel	12 (12.6)	4 (2)
Prasugrel	3 (3.2)	4 (2)
Ticagrelor	7 (7.4)	5 (2.4)

* Plus–minus values are means ±SD.

† BMI=body-mass index

‡ IQR, Interquartile range

ŦCRP, C-reactive protein

3.4. PLATELET MIF AND GREM1 EXPRESSION ARE INDEPENDENT OF PLATELET COUNT

In our cohort the mean platelet count was 250 (± 74) thousands/µL. We found that expression of MIF in platelets was independent from platelet count with a mean MFI value of 17.76 (± 9.7) and a p value of 0.222. In activated state the mean MFI value for the expression of MIF was 16.63 (± 11.38) (p= 0.419). For GREM1 from resting platelets a mean MFI of 15.38 (± 7.49) was found (p= 0.530), whereas for GREM1 from platelets activated with ADP the mean MFI value was 16.14 (± 9.48) (p= 0.338).

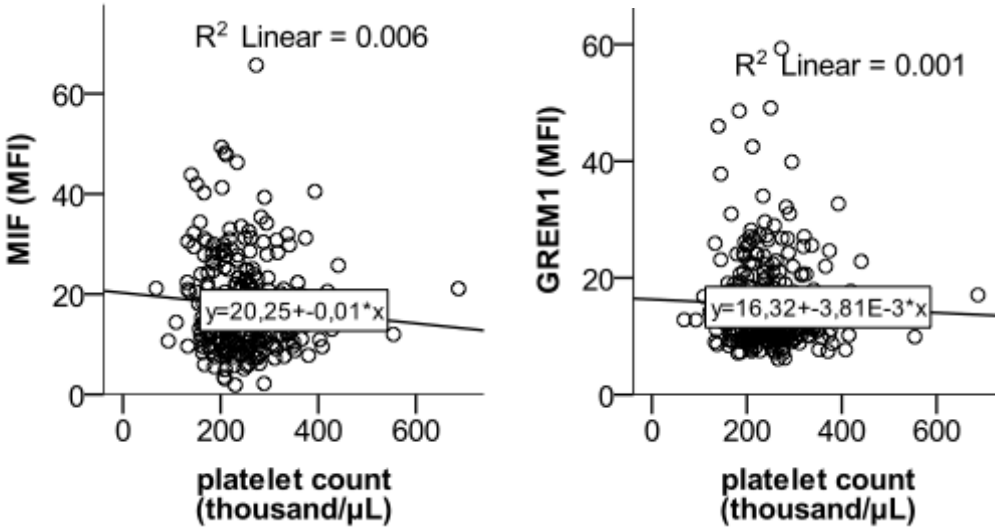

Figure 3.5: Scatterplot presenting the correlation of the mean values of MFI of MIF (above) and GREM1 (below) with the platelet count in our cohort.

3.5. PLATELETS STIMULATED WITH ADP PRODUCE DIFFERENT AMOUNTS OF MIF AND GREM1

We compared the expression of MIF and GREM1 in resting and activated platelets. The platelets were activated with ADP before staining as described above. MIF levels in resting platelets were 17.98 (± 9.8), while in activated ones

16.9 (± 11.7). GREM1 levels in resting platelets were at mean 15.58 (± 7.7) and in activated ones 16.53 (± 10.6) MFI. The levels of MIF and MIF + ADP correlated significantly with a p value of 0.006 and a correlation coefficient of 0.706. The levels of GREM1 and GREM1 + ADP also correlated significantly with a p value of 0.004 and a correlation coefficient of 0.743.

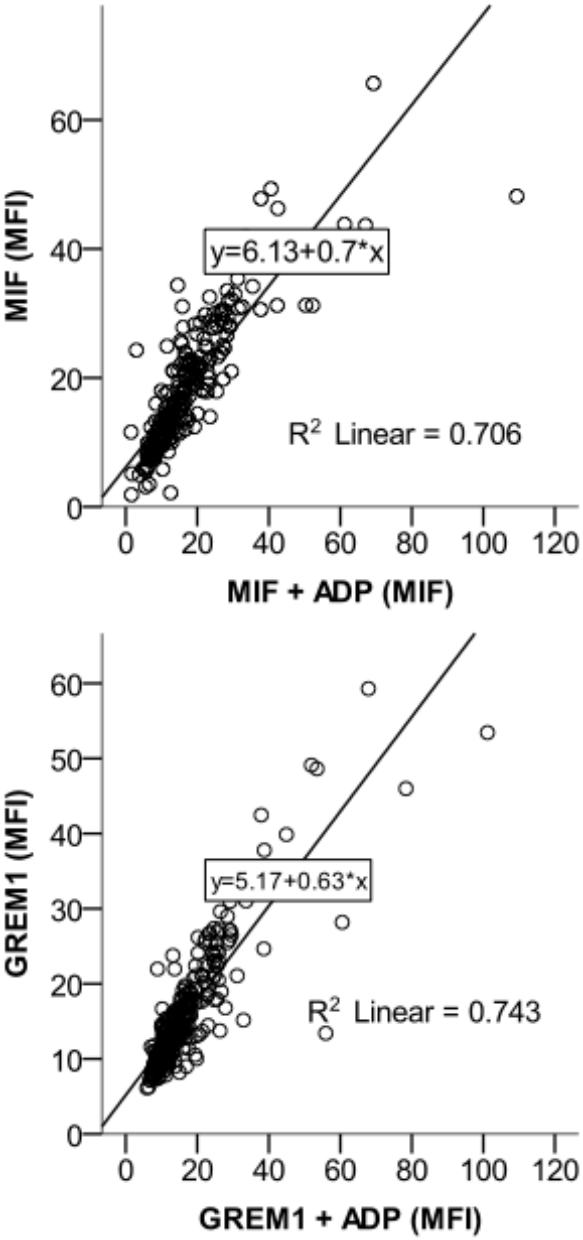

Figure 3.6: Scatterplots presenting the correlation of the mean values of MFI of MIF with MIF in platelets stimulated with ADP (above) and GREM1 in resting platelets and GREM1 in platelets stimulated with ADP (below) in our cohort. R^2 coefficient of determination was 0.706 for MIF and 0.743 for GREM1.

3.6. THE LEVELS OF MIF EXPRESSED BY PLATELETS CORRELATES WITH THE EXPRESSION OF GREM1 IN THE SAME PATIENT

Our cohort expressed MIF from platelets with a mean MFI value of 17.98 (± 9.77) and GREM1 with a mean MFI value of 15.65 (± 8.08). These mean values correlated significantly in our patients with a p value <0.001.

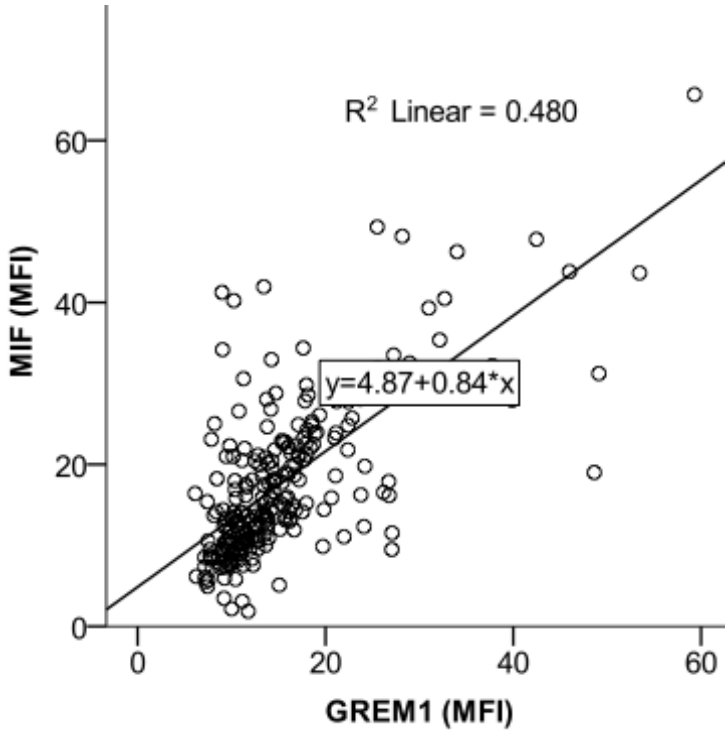

Figure 3.7: Scatterplot presenting the correlation of the mean values of MFI of MIF and GREM1 in our cohort. R^2 coefficient of determination was 0.48.

3.7. P-SELECTIN PLATELET EXPRESSION CORRELATES WITH MIF AND GREM1 EXPRESSION

We compared the expression of P-selectin with the expression of MIF. MIF levels were found higher in patients with high CD62P-expression (18.55 ± 9.9 versus 13.28 ± 6.42, p= 0.044). Platelets that showed a high expression of P-selectin appeared to also express higher amounts of Gremlin-1 (14.9 ± 6.2 versus. 10.4 ± 2.6, p=0.03).

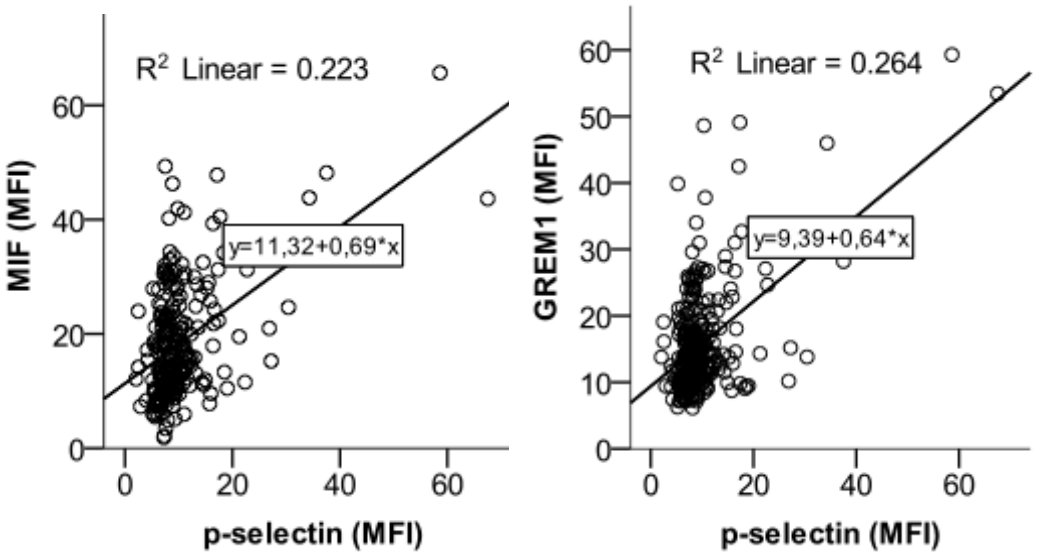

Figure 3.8: Scatterplots presenting the correlation of the mean fluorescence intensity of MIF (left) and GREM1 (84) with values for P-selectin in our cohort. R2 coefficient of determination was 0.223 for MIF (p=0.044) and 0.264 for GREM1 (p=0.03).

3.8. PLATELET REACTIVITY AS MEASURED WITH MULTIPLATE® ANALYSER WAS INDEPENDENT FROM MIF AND GREM1 EXPRESSION

MIF and GREM1 expression in resting and activated platelets did not correlate with platelet aggregation induced with ADP, ASPI or TRAP-6 as measured with the Multiplate® analyzer. For MIF and ADP-test the Pearson correlation was 0.021 (p= 0.747) for resting and 0.049 (p= 0.447) for stimulated platelets. For GREM1 and ADP-test the Pearson correlation coefficient was 0.051 (p= 0.383) for resting and 0.067 (p= 0.256) for stimulated platelets. The ASPI and TRAP-6 tests yielded comparable results with ASPI-test units showing no correlation neither with MIF or GREM1 levels (p= 0.933 and 0.675 in resting platelets and p= 0.606 and 0.870 in stimulated platelets, respectively). P values for correlation with TRAP-induced aggregation were 0.89 for MIF in resting platelets and 0.931 in stimulated ones and for GREM1 0.491 and 0.672, respectively.

When 40 AUC (Area Under the Curve) was used as cut-off value for high platelet reactivity (HPR) in patients on both aspirin and an ADP-receptor inhibitor, no correlation of HPR with GREM1 and MIF levels was found. This cut-off value was selected according to the consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate state of art paper and the experience of our institution (86, 137, 138, 141)

Patients with HPR had a mean MIF value of 16.66 (± 10.6) versus 16.59 (± 6.98), p= 0.038. GREM1 levels were found with a mean value of 17.91 (± 9.67) in patients with HPR and 14.58 (±7.19) in patients with and less than 40 AUC at ADP-test (p= 0.077).

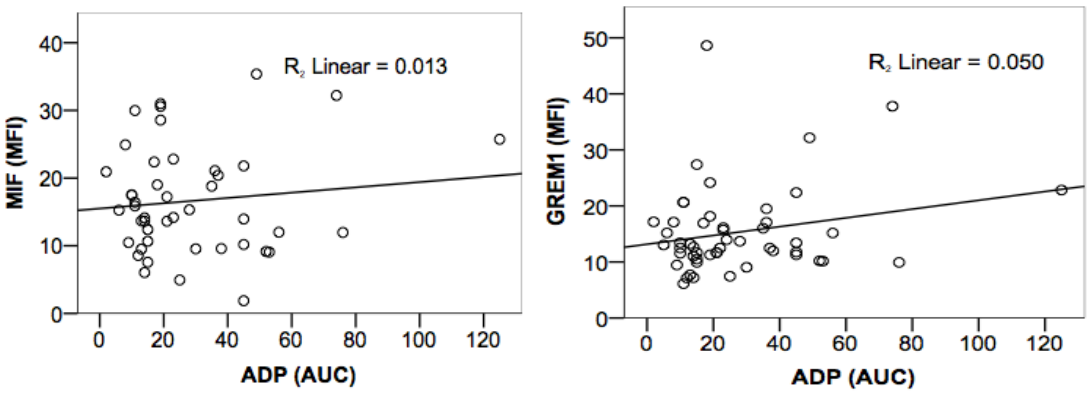

Figure 3.9: Scatterplots presenting the correlation of the mean fluorescence intensity of MIF and GREM1 with values of platelet aggregation as measured after stimulation with ADP (AUC) in our cohort.

3.9. LEVELS OF MIF AND GREM1 EXPRESSION FROM PLATELETS CORRELATE WITH THE LEVELS OF GLYCOPROTEIN VI EXPRESSION FROM PLATELETS

There was a weak correlation between platelet MIF and Glycoprotein VI expression as shown with a Pearson-test value of 0.249 or p<0.001 in resting

platelets and 0.148 or p= 0.023 in stimulated platelets. Correlation between GREM1 and Glycoprotein VI was also found to be statistically significant with p values of <0.001 for resting and 0.003 for activated platelets.

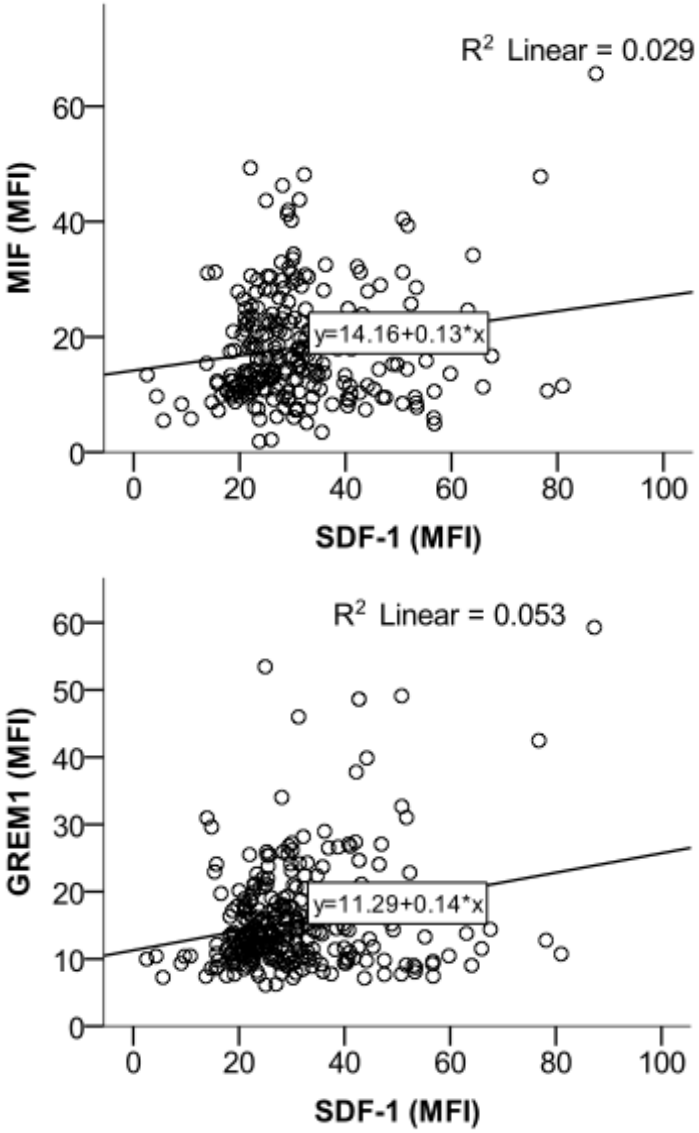

Figure 3.10: Scatterplots presenting the correlation of the mean values of MFI of MIF (above) and GREM1 (below) with a mean fluorescence intensity of SDF-1 in our cohort.

3.10. LEVELS OF MIF AND GREM1 EXPRESSION FROM PLATELETS CORRELATE WITH THE LEVELS OF PAC-1 EXPRESSION FROM PLATELETS

There was a correlation between platelet MIF and PAC-1 expression as shown with a Pearson-test value of 0.498 or $p<0.001$ in resting platelets and 0.517 or $p<0.001$ in stimulated platelets. Correlation between GREM1 and PAC-1 was also found to be statistically significant with p values of <0.001 for resting ($r_s=0.364$) and activated ($r_s=0.417$) platelets. Platelets with high amount of PAC-1 had a high amount of MIF and GREM1 (MIF: 24.6 (± 11.4) MFI versus 15.8 (± 8) MFI, GREM1: 19.7 (± 11.4) MFI versus 14.1 (± 5.4) MFI).

3.11. LEVELS OF GREM1 EXPRESSION FROM PLATELETS AND PLASMA LEVELS CORRELATE WITH CARDIAC NECROSIS MARKER CREATININE KINASE

Levels of platelet bound GREM1 showed a weak correlation with the maximum creatinine kinase values with a correlation coefficient of 0.174 and a p value of 0.047. Levels of plasma GREM1 showed a correlation with the creatinine kinase levels with a correlation coefficient of 0.158 and a p value of 0.038.

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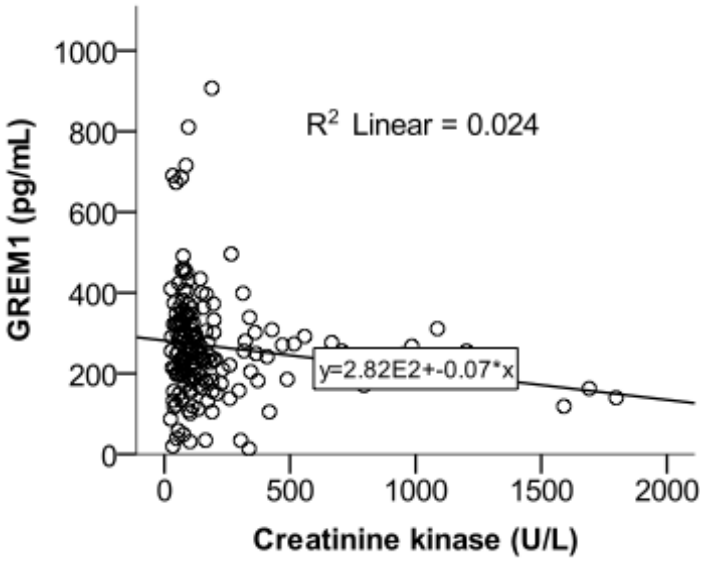

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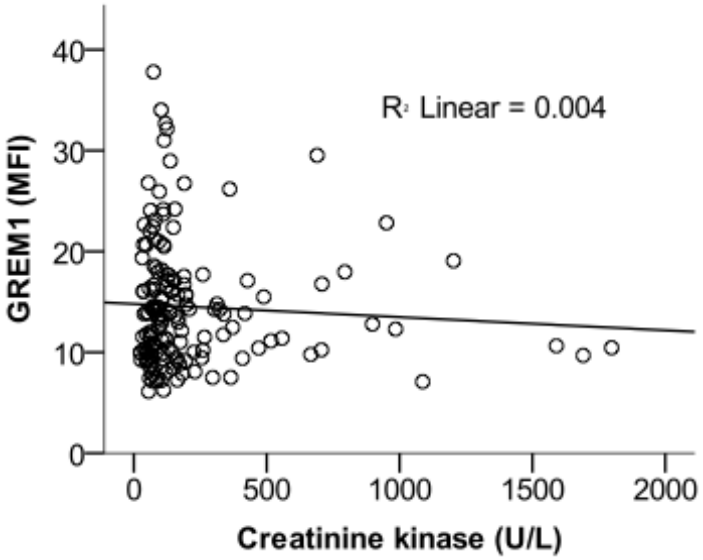

Figure 3.11: Scatterplot presenting the correlation of the values of plasma levels of GREM1 (A) and mean MFI values of platelet GREM1 (B) with creatinine kinase in our cohort.

3.12. LEVELS OF GREM1 AND MIF EXPRESSION FROM PLATELETS CORRELATE WITH TROPONIN I

Levels of GREM1 showed a statistical correlation with the maximum values of Troponin I with a correlation coefficient of 0.243 (p= 0.006), while MIF and Troponin levels had a correlation coefficient of 0.233 (p= 0.014). Patients with positive troponin tests (cut-off 0.004 *μg/dL*) showed lower expression of MIF and GREM1 in their platelets with mean MFI values of 16.59 (± 9.8) versus 18.96 (± 9.8) for MIF and 14.42(± 7) versus 16.21 (± 7.9) for GREM1.

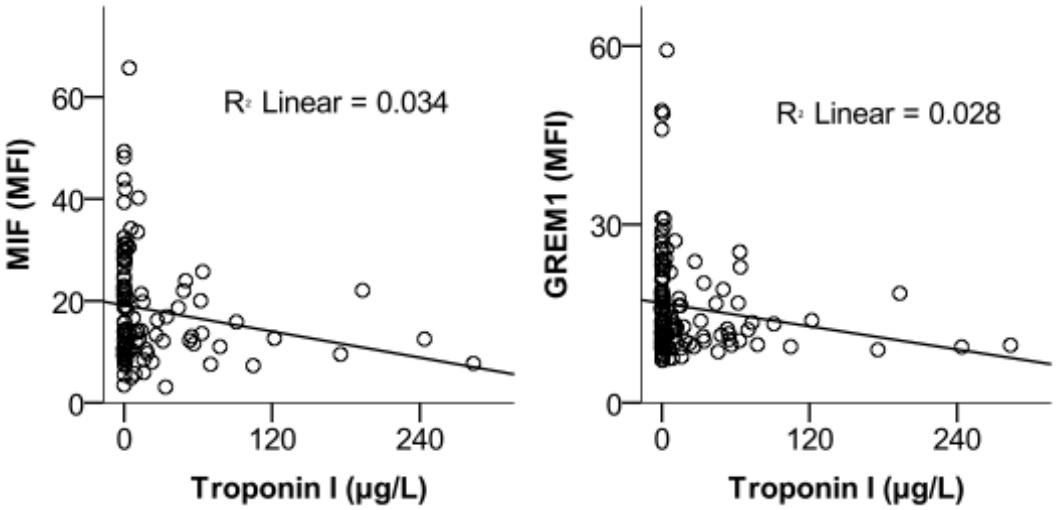

Figure 3.12: Scatterplots presenting the correlation of the mean MFI values of MIF (left) and GREM1 (84) to Troponin values in patients with positive Troponin-test in our cohort.

3.13. LEVELS OF SERUM CRP, MIF AND GREM1 EXPRESSION FROM PLATELETS ARE INCREASED IN DIABETICS

We observed that C-reactive protein levels are increased in diabetics in comparison to non-diabetics. Diabetics had a mean CRP in serum of 2.04 (± 4.715) mg/dL in comparison to non-diabetics that had a mean serum CRP of 1.42 (± 3.356) mg/dL. This difference was found statistically significant with a p value of 0.044. Platelets of diabetics expressed MIF significantly more with a

mean MFI value of 18.67 (± 11.257) in comparison to non-diabetics, whose platelets expressed MIF with a mean MFI value of 17.66 (± 9.03) (p= 0.004). GREM1 was also expressed stronger in platelets of diabetics with a mean MFI value of 15.78 (± 8.399) versus 15.49 (± 7.428) in those of non-diabetic patients. (p=0.022).

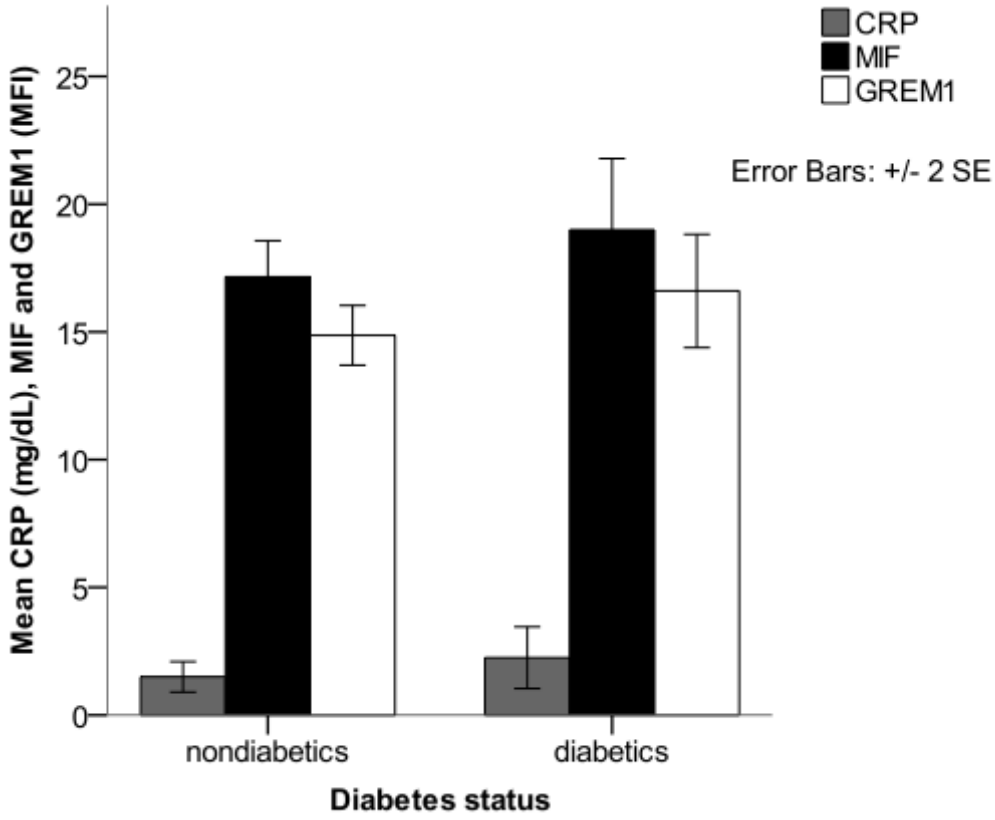

Figure 3.13: Clustered bar presenting the mean values of CRP, MIF and GREM1 and the standard error of the mean in correlation with diabetic status of the subjects.

3.14. ONGOING THERAPY REGIMENT FOR DIABETES DID NOT CORRELATE WITH THE EXPRESSION OF MIF FROM PLATELETS

The expression of MIF showed no significant intergroup differences in platelets of diabetics undergoing lifestyle changes to control their glucose levels and platelets of diabetics requiring oral antidiabetic drugs (mean MFI 16,65 (± 11,2) versus 18.6 (± 9.7), p= 0.366). MIF expression levels of diabetics requiring therapy with insulin were as high as 19.8 (± 12.7), but the difference showed no statistical significance with a p value of 0.201 when compared to diabetics using oral antidiabetic drugs and a p value of 0.773 when compared with those undergoing lifestyle changes. A trend of increased MIF expression from platelets depending to the therapeutic regiment required was just arithmetically apparent.

3.15. MIF EXPRESSION FROM PLATELETS CORRELATES WITH THE SUCCESS IN CONTROLLING GLUCOSE LEVELS IN CAD PATIENTS

A comparison of MIF levels with the quality of glucose level controls, using HbA1c, showed a statistical correlation. MIF was expressed stronger in platelets of patients with a poor control of their diabetes with a mean MFI value of 20.94 (± 12.32) versus 13.65 (± 4.1) in those with an HbA1c lower than 7% (p= 0.003).

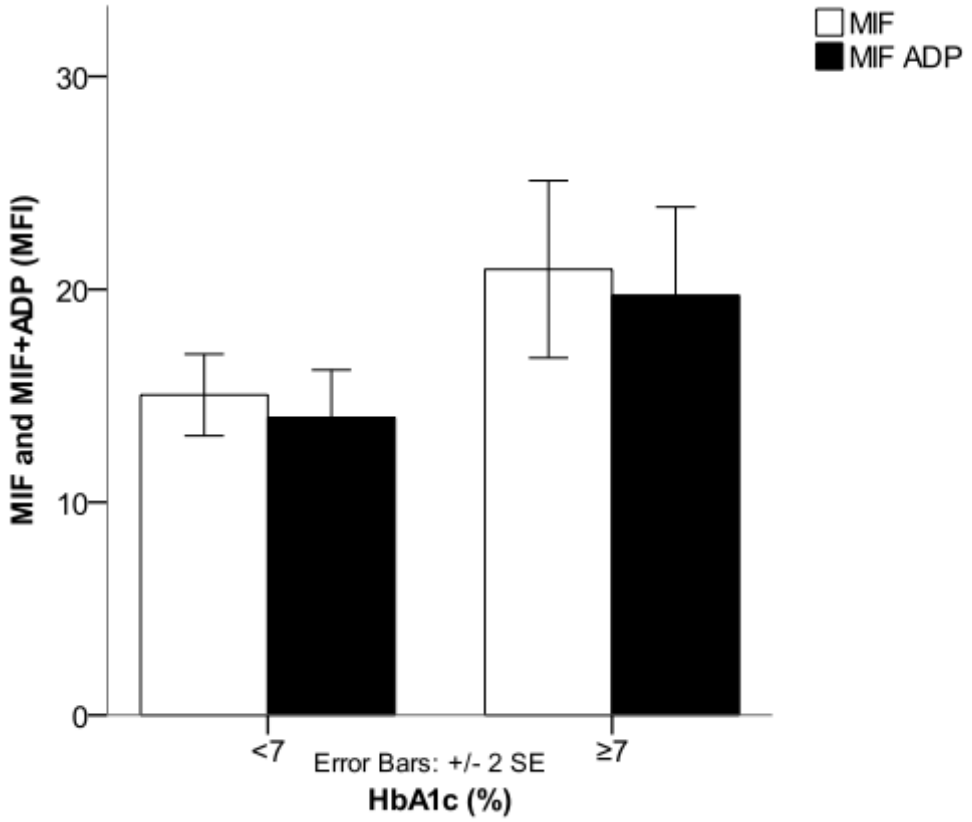

Figure 3.14: Bar chart representing MFI of MIF from resting and MIF from activated platelets with HbA1c as the discrete data set, stratifying patients to those with well controlled diabetes (HbA1c <7%) and those with poor control over their average plasma glucose concentration (HbA1c ≥7%).

3.16. GREM1 EXPRESSION IN PLATELETS CORRELATES WITH THE SUCCESS IN CONTROLLING GLUCOSE LEVELS IN CAD PATIENTS

The expression of GREM1 in platelets showed a statistical increase in patients with poorly controlled diabetes mellitus independent of the therapy. This increase was present in both resting and activated platelets. For patients with an HbA1c ≥7% GREM1 expression in resting platelets had a mean MFI value of 17.98 (± 10.82) versus 13.65 (±4.1) in those with control over their average plasma glucose concentration (p= 0.005). In activated platelets of patients with poorly controlled diabetes GREM1 was expressed with a mean MFI value of

19.82 (± 13.56) versus 13.23 (± 5.4) in those with an HbA1c under 7% (p= 0.001).

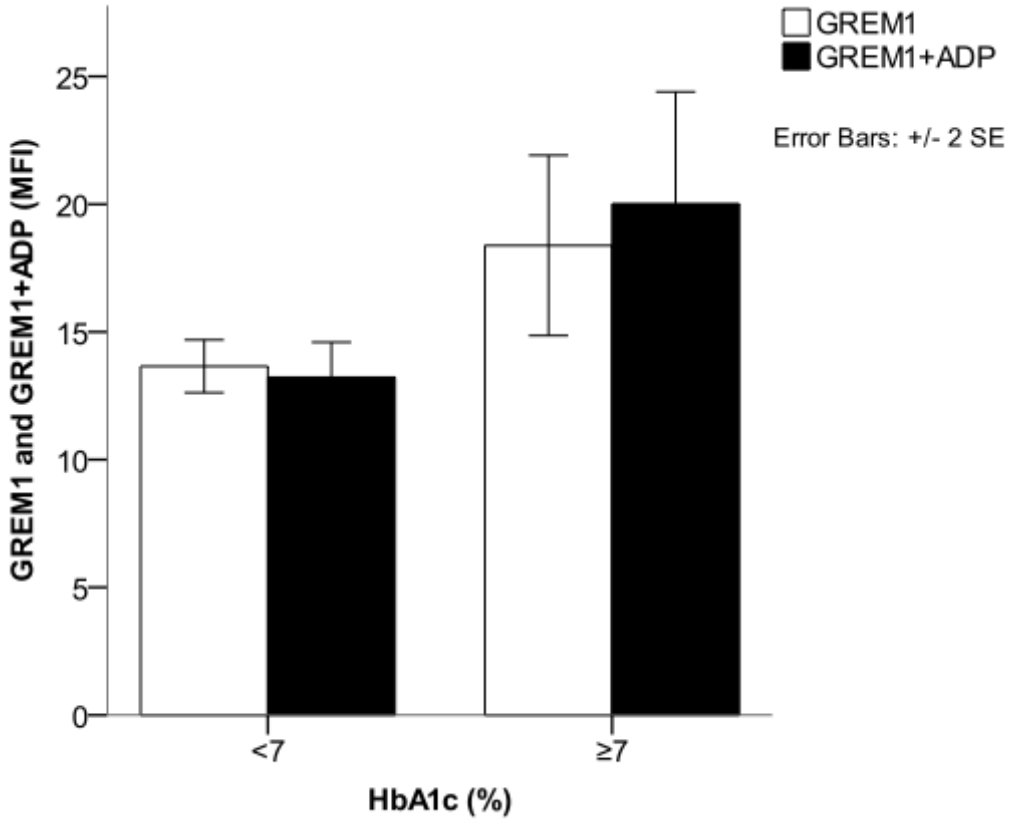

Figure 3.15: Bar chart representing MFI of GREM1 from resting and GREM1 from activated platelets with HbA1c as the discrete data set, stratifying patients to those with well controlled diabetes (HbA1c <7%) and those with poor control over their average plasma glucose concentration (HbA1c ≥7%).

3.17. MIF AND GREM1 PLATELET LEVELS ARE LOWER IN PATIENTS WITH IMPAIRED EJECTION FRACTION

The ejection fraction as assessed by contrast ventriculography correlated with the mean MIF levels in resting platelets and activated ones. Quiescent platelet from patients with left ventricular dysfunction (ejection fraction (<45%) express less MIF with a mean MFI of 3.4 (± 0.7) versus 4.1 (± 1) MFI in those without a relevant impairment (p< 0.001). Activated platelets also expressed less MIF in

patients with left ventricular dysfunction with a mean MFI of 3.2 (± 0.8) versus 3.9 (± 1) mean MFI in those without a relevant impairment ($p<0.001$).

GREM1 expression in platelets of patients with left ventricular dysfunction was decreased in comparison with patients with no relevant left heart failure, with mean MFI 3.5 (± 0.4) versus 3.9 (± 0.8) ($p=0.005$) for resting platelets and mean MFI 3.6 (± 0.6) versus 3.9 (± 1) for activated platelets ($p=0.046$).

3.18. MIF AND GREM1 PLATELET LEVELS ARE SIGNIFICANTLY LOWER IN ACS PATIENTS WITH IMPAIRED EJECTION FRACTION

Patients suffering from systolic heart failure during an ACS have significantly lower levels of platelet MIF and GREM1. Patients in heart failure were considered patients with an ejection fraction under 45% as assessed by cardiac ventriculography.

Platelet MIF expression in patients with left ventricular dysfunction during an ACS was decreased in comparison with patients with no relevant left heart failure, with a mean MFI 3.35 (± 0.8) versus 4 (± 1) ($p=0.004$) for resting platelets and mean MFI 3.1 (± 0.8) versus 3.9 (± 1) for activated platelets respectively ($p=0.001$). Platelet GREM1 expression in patients with left ventricular dysfunction during an ACS was decreased in comparison with patients with no relevant left heart failure, with a mean MFI 3.4 (± 0.4) versus 3.9 (± 0.9) respectively ($p=0.008$).

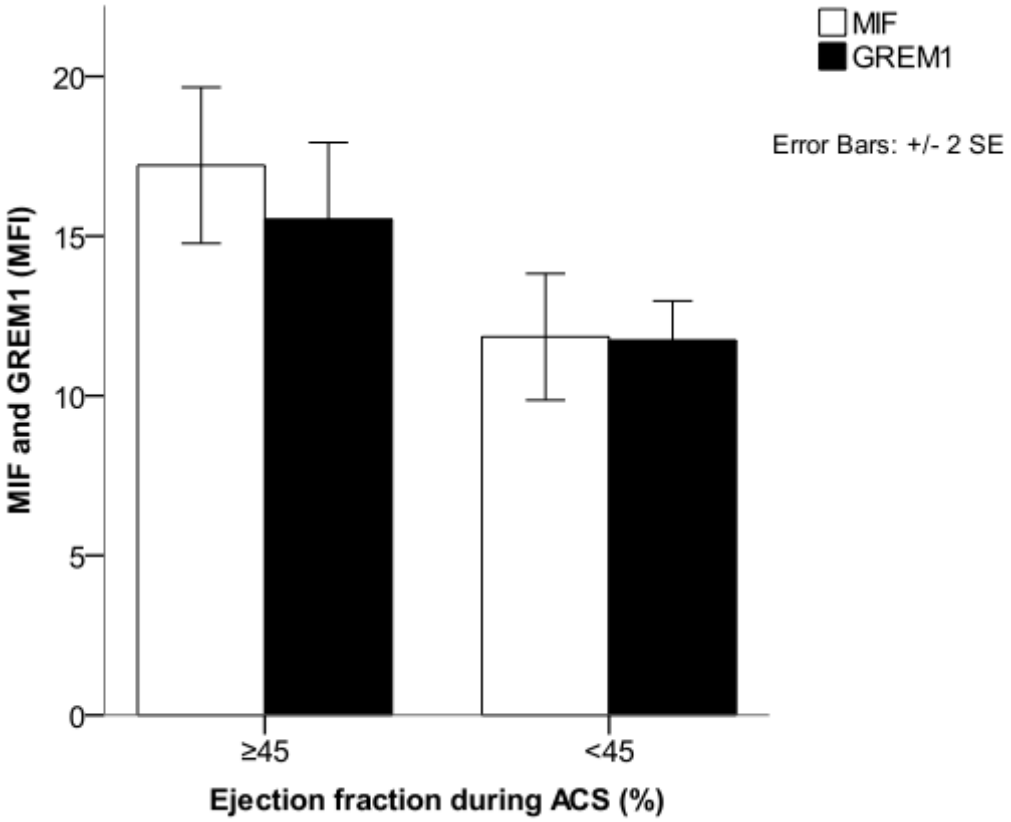

Figure 3.16: Bar chart presenting the correlation of MIF and GREM1 in platelets with the ejection fraction assessed by ventriculography. We classified impaired left ventricular function as left ventricular ejection fraction <45 percent and as non-impaired EF ≥45%.

3.19. THE CLINICAL PRESENTATION OF IHD CORRELATES WITH THE LEVELS OF MIF AND GREM1 EXPRESSED BY PLATELETS

The different clinical presentations of ischemic heart disease were found to correlate highly with MIF and GREM1 levels in resting and activated platelets. Platelet levels of MIF decrease as the acuteness of the ischemic disease increases. Mean MFI value of MIF in patients with stable angina was 4.3 (± 1), in patients with unstable angina 4.1 (± 1.2), in patients with non-STEMI infarction was 3.8 (± 1.1) and in patients presenting with an ST-elevation ACS was found the lowest with a mean value of 3.8 (± 0.9). The difference between

expression of MIF in stable CAD and heart attacks was statistically significant with a p value of 0.006 for stable CAD and NSTEMI and 0.034 for stable CAD and STEMI.

GREM1 levels in stable ischemic heart disease were found at a mean value of 3.9 (± 0.8) MFI when in STEMI patients 3.6 (± 0.7) MFI (p=0.028).

MIF levels in platelets of patients with ACS were 3.9 (± 1.1) MFI versus 4.3 (± 1) MFI in patients with a stable angina (p= 0.008). GREM1 platelet expression in patients with ACS was found lower with a mean MFI of 15.3 (± 8.4) versus 16 (± 6.8) in patients with a stable CAD (p=0.029).

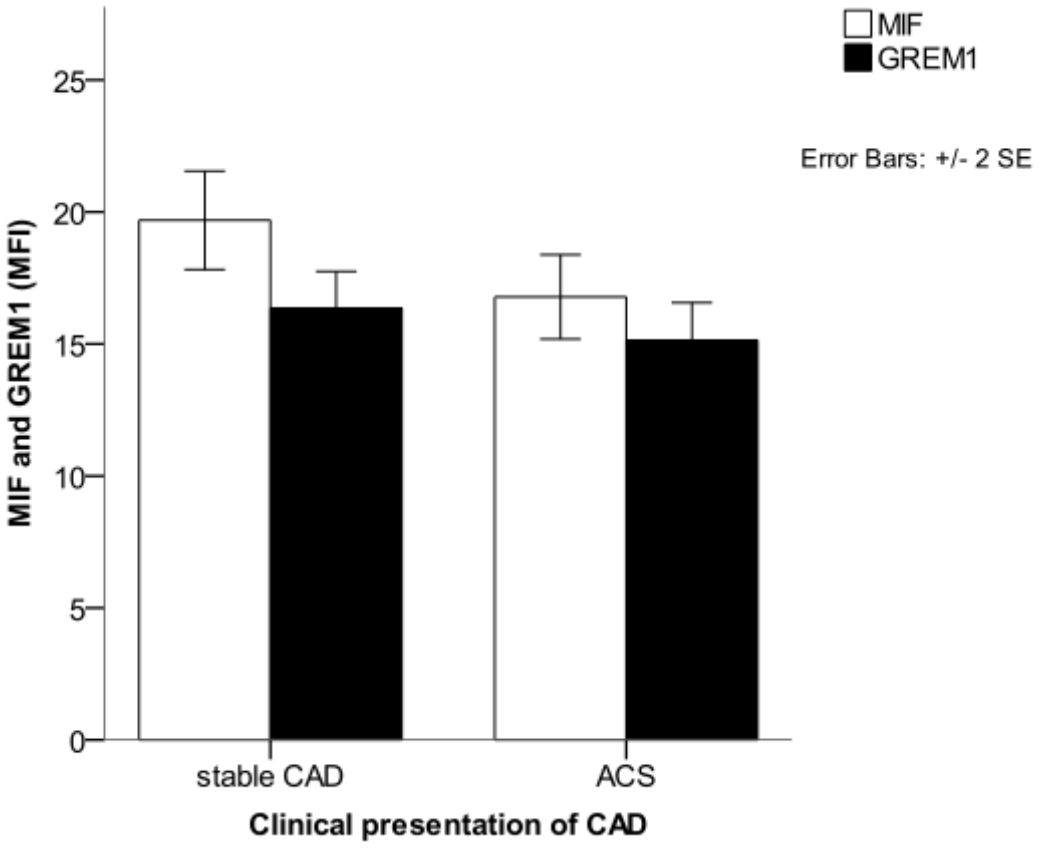

Figure 3.17: Bar chart representing the mean MFI for MIF (white) and GREM1 (black) depending on the presence of ACS.

3.20. PLASMA LEVELS OF GREM1 CORRELATE WITH PLASMA CRP LEVELS

GREM1 levels in serum as evaluated with ELISA showed a correlation with C-reactive protein levels with a Spearman's rho of 0.179 and a p value of 0.014. A non-parametric test was used because CRP values had a skewed distribution and also allows for inclusion of outliers. The cut-off value used for CRP was 0.5 mg/dL. Patients with positive CRP had a mean GREM1 of 288 (± 137) pg/mL and patients with CRP values under 0.5 mg/dL had a mean GREM1 value of 249 (± 132) pg/mL.

3.21. PLASMA LEVELS OF GREM1 AND MIF WERE INDEPENDENT OF WHITE BLOOD CELL COUNT

Total white blood cell count did not affect plasma levels of both GREM1 and MIF with a correlation coefficient for MIF of 0.47 (p= 0.532) and for GREM1 of 0.85 (p= 0.250).

3.22. PLASMA LEVELS OF GREM1 CORRELATED WITH RENAL FUNCTION MARKER

Plasma levels of GREM1 showed a weak correlation with the values for serum creatinine with a correlation coefficient of 0.178 and p-value of 0.015 and with the serum urea levels with a correlation coefficient of 0.216 with a p-value of 0.006.

3.23. PLATELET BOUND GREM1 AND MIF EXPRESSION IS INDEPENDENT OF CONCOMITANT MEDICATION

The platelet expression of MIF in patients under aspirin-therapy was compared to the MIF expression of aspirin-naïve patients and no significant difference was found (p= 0.105). The platelet expression of MIF in patients under dual antiplatelet regiment with aspirin and a $P2Y_{12}$-inhibitor was compared to patients' naïve to both medications and no significant difference was found (p= 0.154 for clopidogrel, p= 0.790 for prasugrel and p= 0.618 for ticagrelor).
The platelet expression of GREM1 in patients under aspirin-therapy was compared to the GREM1 expression of aspirin-naïve patients and no significant difference was found (p= 0.203). The platelet expression of GREM1 in patients under dual antiplatelet regiment with aspirin and a $P2Y_{12}$-inhibitor was compared to patients' naïve to both medications and no significant difference was found (p= 0.998 for clopidogrel, p= 0.859 for prasugrel and p= 0.405 for ticagrelor).

4. DISCUSSION

4.1. MIF IN CORONARY ARTERY DISEASE AND TYPE 2 DIABETES MELLITUS

In this study we concentrated on the atherogenic function of MIF. The role of monocytes, T-cells and activated endothelium in the circle of MIF-expression has already been broadly implicated in atherosclerosis. Our group has managed to show that plasma MIF expression is enhanced in ACS, is associated with various markers of inflammation and that it correlated with cardiac necrosis markers after PCI (110). In other studies it has been shown that genetic manipulation and deletion of MIF in atherosclerosis susceptible mice protected the heart from severe ischemia-reperfusion injury through suppression of inflammatory responses (63).

This study provides new knowledge concerning the clinical role of platelet bound MIF in cardiovascular disease. Many mechanisms of MIF in atherosclerosis have been proposed; promotion of atherogenic leukocyte recruitment processes has been recognized as a major underlying mechanism of the role of MIF in plaque instability (23, 136).

Vascular injury might precede atherothrombosis and MIF has been proved to regulate the biological response to injured tissue. In a study model of carotid artery injury in atherosclerosis-susceptible mice MIF was shown to promote thickening of the neointima through accumulation of inflammatory cells and proliferation of the media and intima layer (34). A study about the interaction of MIF with AMP-activated protein kinase (AMPK) suggested a critical role for MIF in the cardiomyocyte response to ischemia, implicating MIF-release genes in ATP-regulation in cardiomyocytes and suggesting MIF genes as a diagnostic target in risk stratification for CAD (105).

Platelets are recruited to the site of vascular injury to fulfill their hemostatic role, but also at sites of activated, inflamed but still intact endothelium and promote atherosclerosis (21). Platelets are a major part of thrombi in acute myocardial infarctions and their proinflammatory mediators might contribute to fibrosis and systolic dysfunction following myocardial infarctions (101). Thus, the expression and regulation of the expression of MIF from platelets poses an interesting investigational target.

In several clinical studies serum MIF levels correlated with insulin resistance and metabolic syndrome, providing support for a role of MIF in the development of insulin resistance and T2DM (76, 158). However, in the large Finnish Diabetes Prevention Study which tested lifestyle intervention in patients with BMI> 25 kg/m^2 with impaired glucose tolerance, MIF was not associated with the risk of T2DM in the control group and subjects with high MIF had lower risk for T2DM (77). Clinical studies so far have suggested an important link between MIF, obesity, insulin resistance and T2DM, but it is not clear if the MIF expression irregularities are epiphenomena or casual factors. Animal studies showed that MIF-Knockout mice had increased glucose uptake into white adipose tissue, with MIF inhibiting insulin signal transduction, overall suggesting an important role for MIF in the regulation of systemic glucose metabolism during infection and tissue invasion (10).

There is a growing body of evidence on platelets and their role in the progression of the atherosclerotic procedure through modulation of immune responses. Many pathways for that interaction have been suggested such as the OxLDL-mediated platelet-monocyte aggregate formation theory highlighting the role of platelets in atherosclerotic plaque development and plaque destabilization (11, 14). This project is based on an observation that platelets contain significant amounts of MIF and GREM1.

Chronic low-grade inflammation is a hallmark of T2DM, a process guided from cytokines, including the macrophage migration inhibitory factor. The role of MIF

in T2DM has been extensively studied. Several clinical studies have found slightly elevated serum MIF levels in patients with T2DM (150). Higher MIF levels were found in patients in pro-diabetic states spawning the theory of MIF level elevation preceding the onset of T2DM (58, 76, 88). These higher levels of MIF were shown to be a risk factor for coronary events in diabetics and especially to mediate myocardial damage (97).

Hence, MIF is one of the chemokines that directly relate to T2DM and risk for coronary disease. It has been suggested that it contributes to coronary disease development in diabetics indirectly through stimulation of the production of other cytokines. It also stimulates certain inflammatory adipocytokines promoting insulin resistance and creating a vicious circle (130).

4.2. GREMLIN-1 AS AN ENDOGENOUS ANTAGONIST OF MIF AND ITS ROLE IN ATHEROSCLEROSIS

Gremlin is a highly conserved 184 amino acid protein (20.7 kDa), which contains a cysteine-rich region and a cysteine knot motif, a structure shared by members of the TGF-β superfamily. Gremlin exists in both secreted and cell associated forms. This pattern of expression permits its binding to extracellular BMP-2, BMP-4 and BMP-7 and inhibition of smad-1, smad-5 and smad-8 signaling, when expressed on membranes of renal cells (165) or alternatively binding to the slit1 and slit2 receptor when expressed on monocyte membranes to act as negative modulator of monocyte chemotaxis (79). These are some of many possibly existing mechanisms of gremlin regulation of cellular behavior. High glucose levels have been showed to increase gremlin mRNA in pericytes and mesangial cells, implicating GREM1 in the pathogenesis of diabetic nephropathy and retinopathy (85, 100, 153).

The role of GREM1 in diabetes and coronary heart disease has not been adequately investigated. However, since it has been shown that it acts as an antagonist of MIF (109) it was interesting to investigate the correlation of

GREM1 and MIF levels in patients with IHD and diabetes. Regarding the role of GREM1 in atherosclerosis, gremlin-1 expression has been identified in humans at sites of arterial injury and has been shown that GREM1 inhibits MIF-induced foam cell formation and monocyte and macrophage adhesion on the atherosclerotic vessel wall, and reduces substantially plaque size and plaque foam cell content in ApoE-/- mice in vivo (109).

It has also been shown that Gremlin1 induces a proinflammatory response in endothelial cells causing reactive oxygen species and cyclic adenosine monophosphate production and the upregulation of proinflammatory molecules involved in leukocyte extravasation like vascular cell adhesion molecule-1 (VCAM-1), results that suggest a cross-talk between angiogenesis and inflammation and demonstrate a role of gremlin in the proinflammatory/ proangiogenic response (35).

4.3. EXPRESSION OF GREM1 AND MIF IN OUR GROUP OF PATIENTS WITH IHD

We managed to detect substantial levels of GREM1 and MIF proteins in lysates of platelet populations of healthy individuals, recognizing human platelet MIF as a 12.5 kDa protein and GREM1 as a band of approximately 20.7 kDa followed by a double band of approximately 25 kDa. Substantial levels of MIF and GREM1 were found through flow cytometry in platelet populations of patients with CAD.

Platelet bound MIF and MIF plasma levels correlated significantly in our study suggesting that platelets are probably a relevant source of plasmatic MIF and suggesting a cross talk between cells producing MIF and a complex regulation of its expression. The plasma levels of MIF were found to be independent of the white blood cell count; the same applied for GREM1 plasma levels. Furthermore GREM1 and MIF platelet bound levels were also independent from platelet

count in peripheral blood. Of note was that plasma levels of GREM1 correlated significantly with plasma levels of CRP.

We also managed to show that the levels of the two investigated proteins in human platelets correlate to each other statistically significantly with a p value of 0.0001. These findings are in accordance with a theory of variability of expression of these proteins depending on the inflammation status of the human organism and not the cell count itself. However, such findings suggest that platelets are an important vehicle for MIF and GREM1 through the cardiovascular system and that GREM1 and MIF production are regulated through the interaction of the two proteins.

Platelet-bound expression of MIF and GREM1 were found independent of platelet count and platelet reactivity status. High platelet reactivity can be considered a risk factor for post-PCI stent thrombosis and myocardial infarction, while patients with T2DM consist a high-risk group as they exhibit increased platelet reactivity (98, 133), but the clinical usefulness of platelet function testing and its role as an independent risk factor is still under question (48, 65, 86).

The expression and induction of CD62p indicating release reaction of α-granules and PAC-1 directed against activation-dependent epitopes on GPIIb/IIIa and GPVI expression correlated with the levels of platelet bound MIF and GREM1 proteins. P-selectin, a known recruitment and aggregation factor of platelets, has been found to correlate with the severity of acute coronary syndromes in patients with IHD, most probably through plaque disruption, rupture and thrombus formation (12, 19, 86). Increased expression of platelet glycoprotein VI (GPVI) correlated with increased MIF and GREM1 platelet expression. GPVI is a transmembranous glycoprotein that forms a complex with the Fc receptor at the platelet surface (111). Fc receptor expression correlates with collagen-induced aggregation and has been found increased in diabetics (28, 29). GPVI has been implicated in platelet-mediated arterial thrombosis (99), whereas it has been shown that platelets of patients with T2DM express GPVI

on their surface more than those of nondiabetics (25). The platelet binding of PAC-1, another monocolonal antibody that selectively interacts with activated GP IIb/IIIa, was also used as a platelet activation marker (86) and we found an up-regulation of expression correlates with up-regulated MIF and GREM1 platelet expression.

Such findings suggest a cross talk between activated leukocytes and platelets and regulation of platelet activation through inflammation and thus a role for GREM1 and MIF in atherothrombosis and acute myocardial infarctions.

In concordance with findings of previous studies plasma levels of MIF were found slightly elevated in patients with diabetes (76, 158), whereas the plasma levels of GREM1 in the same patient group were also found slightly elevated. When we compared levels of platelet bound MIF and GREM1 in diabetics and nondiabetics we found them significantly higher in diabetics in comparison to nondiabetics, while a more traditional marker for inflammation CRP, was also found increased. These results support the theory of platelet mediated GREM1 and MIF expression as important mediator in vascular inflammation in diabetics and the progression of CAD. Furthermore, levels of MIF and GREM1 expressed from platelets showed no correlation with the various therapy schemes, but only to the HbA1c levels, suggesting a direct correlation of MIF and GREM1 expression with the glucose levels and the progression of inflammatory mediated atherosclerosis in diabetics.

Only the levels of GREM1 correlated well with the maximum of creatinine kinase, while both the levels of GREM1 and MIF correlated with the most sensitive marker for cardiac injury at the moment, troponin. The higher the troponin levels were the lower the expression of MIF and GREM1 was. In patients with ACS expression of MIF and GREM1 was significantly lower than in patients with stable CHD. MIF and GREM1 levels also correlated with the ejection fraction of patients undergoing a cardiac ventriculography with down-regulation of MIF and GREM1 expression in patients with a left ventricular

dysfunction as consequence of a myocardial infarction. In those patients the plasma levels of GREM1 and MIF were found slightly higher. These findings highlight the connection of platelet bound MIF and GREM1 with the settings of ACS and the prognostic factors of CHD.

These finding could extend the role of GREM1 and MIF as markers for imminent myocardial ischemia in IHD and prognostic markers of the severity and the stadium of the disease. It could be suggested that ongoing ischemia causes platelets to release MIF and GREM1 produced in platelets to the adjacent tissues and into plasma, thus propagating atherothrombotic processes, myocardial fibrosis and post-infarction heart failure.

With the results of our current study are taken into account, one could conclude that platelets serve as carriers of MIF and GREM1 in patients with coronary artery disease and seem to release GREM1 and MIF during ACS. Patients at risk for a rapid progression of atherosclerosis, such as diabetic patients or patients with a high platelet activation status show a significant up-regulation of GREM1 and MIF. GREM1 and MIF might therefore be useful biomarkers in the clinical routine to assess individual risk. Future studies including follow-up measurements of MIF and GREM1 levels in CAD patients and studies with MACE endpoints are warranted to assess MIF and GREM1 as biomarker.

5. ZUSAMMENFASSUNG

Die Progression der Atherosklerose ist ein langjähriger Prozess und Diabetes mellitus stellt einen der wichtigsten Risikofaktoren der koronaren Herzkrankheit dar. Thrombozyten spielen eine wichtige Rolle bei der Hämostase, aber auch bei inflammatorischen Prozessen und bei der Atherosklerose sind sie wesentlich beteiligt, unter anderem, durch die zahlreiche Zytokine die sie beherbergen und die Bildung von Thromben auf rupturierten Plaques, was zum akuten Verschluss und damit zum akuten Myokardinfarkt führt. Makrophage migration inhibitory factor (MIF) ist ein ubiquitärer Mediator mit proinflammatorischer Wirkung. Seine Interaktion mit GREM1 in der Atherosklerose in APO-Mäusen wurde durch unsere Gruppe beschrieben und die Rolle von GREM1 als endogener Inhibitor von MIF erläutert.
Um die Expression, Funktion und Interaktion von MIF und GREM1 in der Atherogenese zu analysieren, untersuchten wir in 300 KHK-Patienten die Expression von MIF und GREM1 von Thrombozyten mittels Western Blotting und Durchflusszytometrie und deren Plasma-Spiegel durch ELISA. Die MIF und GREM1 Expression war unabhängig von Thrombozytenzahl, Leukozytenzahl und begleitender medikamentöser Therapie, inklusive Thrombozytenaggregationshemmer. Bei Diabetikern waren Thrombozytenaktivierungsmarker signifikant höher und korrelierten sich mit der thrombozytären Expression von MIF und GREM1. Zudem waren bei Diabetikern die MIF- und GREM1-Konzentration im Serum höher und die thrombozytenassozierte Expression von MIF und GREM1 korrelierten signifikant mit dem HbA1c-Wert, mit dem Troponin und mit der Ejektions-Fraktion.
Unsere Ergebnisse lassen darauf schließen, dass Thrombozyten als Quelle von MIF und GREM1 dienen, die als Mediatoren der vaskulären Inflammation bei Diabetikern agieren. Diese Erkenntnisse bringen wichtige Informationen über mögliche Interaktionen von MIF und GREM1 und die damit verbundene mögliche Entwicklung spezifischer Inhibitoren, womit neue Therapieansätze in der Behandlung der vaskulären Komplikationen des Diabetes mellitus geschaffen werden können.

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7. PUBLICATIONS

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reactivity and outcome in patients with stable coronary artery disease (PREDICT-STABLE). PLoS One. 2015;10(3):e0121620.
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Posters and oral presentations

DGK-Jahrestagung

2012 Clin Res Cardiol. 2012 Apr;101 Suppl 1:1. Impact factor (2014): 4.56

I.I. Muller, A. Karathanos, M. Schneider, K. A. L. Muller, H. Schonleber, P. Seizer, M. Gawaz, T. Geisler (2012) “Macrophage migration inhibitory factor” (MIF) is carried by platelets of patients with stable and unstable angina pectoris and its amount correlates with the stability of coronary artery disease. Oral presentation (78. Jahrestagung)

I.I. Muller, M. Schneider, A. Karathanos, K. A. L. Müller, H. Schönleber, P. Seizer,M. Gawaz, T. Geisler (2012) The pro-angiogenic BMP-antagonists Gremlin 1 and -2 are expressed in platelets of patients with acute coronary syndrome and correlate with platelet activation. Poster (78. Jahrestagung)

P. Seizer, K. Mueller, I. Mueller, A. Karathanos, B. Bigalke, M. Gawaz, T. Geisler, A.May (2012) Platelet-boundCyclophilin A in patients with acute coronary syndrome. Poster (78. Jahrestagung)

T. Geisler, A. Karathanos, K. Mueller, I. Mueller, F. Stimpfle, B. Bigalke, P.Seizer, M. Gawaz (2012) Indidualized Anti-platelet therapy guided by genotype analysis in patients with acute coronary syndromes. Oral presentation (78. Jahrestagung)

2013 Clin Res Cardiol. 2013 Apr;102 Suppl 1:1. Impact factor (2014): 4.56

A. Kilias, P. Berlitz, A. Karathanos, R. J. Sauter, P. Seizer, K. A. L. Müller, C. S. Zürn, T. Geisler, M. Gawaz, J. Schreieck. Event-triggered anticoagulation of patients after catheter ablation of atrial fibrillation guided by implantable cardiac monitors – early experience. Oral presentation (79. Jahrestagung)

I. I. Müller, K. A. L. Müller, A. Karathanos, H. Schönleber, C. Chakkalakal, M. Haas, D. Eppler, M. Schneider, M. Gawaz, T. Geisler. The MIF-antagonist Grem1 is strongly up-regulated in patients with coronary artery disease and the Grem1/MIF-ratio correlates significantly with the occurrence of acute plaque rupture. Oral presentation (79. Jahrestagung)

A. Karathanos, K. A. L. Müller, M. Schmid, M. Schneider, M. Chatterjee, M. Gawaz, T. Geisler, I. I. Müller. Macrophage migration inihibitory factor and its endogenous antagonist Gremlin-1 are carried by platelets and the stored

amount of both proteins correlates with the stability of coronary artery disease. Oral presentation (79. Jahrestagung)

M. Droppa, D. Tschernow, K. A. L. Müller, E. Tavlaki, A. Karathanos, F. Stimpfle, M. Gawaz, T. Geisler. Evaluation of clinical risk factors to predict high on-treatment platelet aggregability and outcome in patients with stable coronary artery disease (PREDICT-STABLE). Poster (79. Jahrestagung)

E. Tavlaki, J. Metzger, A. Valera, K. A. L. Müller, A. Karathanos, F. Stimpfle, M. Gawaz, T. Geisler. Variability of on-treatment platelet reactivity under prasugrel and ticagrelor and association with pre-treatment platelet reactivity in a large real-world cohort of ACS patients undergoing PCI. Poster (79. Jahrestagung)

P. Seizer, J. Schwille, A. Karathanos, H. Sturhan, S. v. Ungern Sternberg, O. Borst, T. Geisler, A. May. Extracellular Cyclophilin A activates platelets in vitro and in vivo. Poster (79. Jahrestagung)

K. A. L. Müller, A. Karathanos, S. Breuning, M. Gawaz, T. Geisler, I. I. Müller. Plasma ratio of Gremlin-1 and Macrophage Migration Inhibitory Factor indicates a higher risk for vulnerable plaque formation and acute coronary syndrome in patients with type 2 diabetes mellitus. Poster (79. Jahrestagung)

2014 Clin Res Cardiol 103, Suppl 1 (2014) Impact factor (2014): 4.56

A. Karathanos, K.A.L. Müller, M. Schmid, M. Gawaz, T. Geisler, I.I. Müller. Gremlin-1 is released by platelets and its expression level correlates with the degree of platelet activation in patients with coronary artery disease. Oral presentation (80. Jahrestagung)

M. Haas, A. Karathanos, D. Rath, KAL. Mueller, E. Tavlaki, M. Droppa, F. Stimpfle, II. Mueller, M. Gawaz, T. Geisler. Prognostic impact of platelet macrophage migration inhibitory factor in patients with symptomatic coronary artery disease. Oral presentation (80. Jahrestagung)

I.I. Müller, K.A.L. Müller, A. Karathanos, H. Schönleber, M. Chatterjee, M. Schmid, M. Haas, P. Seizer, H.-F. Langer, M. Gawaz, T. Geisler. Impact of counterbalance between macrophage migration inhibitory factor (MIF) and its endogenous inhibitor Gremlin-1 in patients with symptomatic coronary artery disease. Poster (80. Jahrestagung)

E. Tavlaki, A. Karathanos, M. Droppa, D. Rath, K. A. L. Müller, M. Gawaz, J. Booth, S. Davidson, R. Stables, A. Zaman, W. Banya, M. Flather, M. Dalby, T. Geisler. Benefit of Additional Platelet Inhibition in pre-treated ACS patients with high platelet reactivity undergoing PCI (APACS HPR) – a randomized controlled trial. Poster (80. Jahrestagung)

K.A.L. Müller, A. Karathanos, M. Schmid, M. Gawaz, T. Geisler, I.I. Müller. Plasma ratio of Gremlin-1 and Macrophage Migration Inhibitory Factor indicates a higher risk for plaque instability and acute coronary syndrome in patients with type 2 diabetes mellitus. Poster (80. Jahrestagung)

A. Kilias, P. Berlitz, A. Karathanos, K. Rizas, P. Seizer, K. Müller, C. S. Zürn, T. Geisler, M. Gawaz, J. Schreieck. Discontinuation of anticoagulation in patients after “successful” catheter ablation of atrial fibrillation – a study of event-triggered anticoagulation guided by implantable cardiac monitors. Poster (80. Jahrestagung)

I.I. Müller, K.A.L. Müller, A. Karathanos, H. Schönleber, M. Chatterjee, M. Schmid, M. Haas, P. Seizer, H.-F. Langer, M. Gawaz, T. Geisler. Impact of counterbalance between macrophage migration inhibitory factor (MIF) and its endogenous inhibitor Gremlin-1 in patients with symptomatic coronary artery disease. Poster (80. Jahrestagung)

ESC-Jahrestagung:

2013
Geisler T, Müller KA, Karathanos A, Gawaz M, Deliargyris E, Bernstein D, Lincoff AM, Mehran R, Dangas G, Stone G. Short term outcomes of patients undergoing PCI of the left main and correlations with the type of adjunctive antithrombotic therapy: pooled analysis from REPLACE-2, ACUITY, and HORIZONS-AMI trials. Poster

2014
A. Karathanos, K.A.L. Müller, M. Schmid, M. Gawaz, T. Geisler, I.I. Müller. Gremlin-1 is released by platelets and its expression level correlates with the degree of platelet activation in patients with coronary artery disease. European Heart Journal (2014) 35 (Abstract Supplement), 1059. Oral presentation

A. Karathanos, M. Haas, D. Rath, KAL. Mueller, E. Tavlaki, M. Droppa, F. Stimpfle, II. Mueller, M. Gawaz, T. Geisler. Prognostic impact of platelet macrophage migration inhibitory factor in patients with symptomatic coronary artery disease. European Heart Journal (2014) 35 (Abstract Supplement), 486-487. Poster

8. ERKLÄRUNG ZUM EIGENANTEIL DER DISSERTATIONSSCHRIFT

Die Arbeit wurde in der Klinik für Innere Medizin III – Kardiologie und Kreislauferkrankungen unter Betreuung von Herrn Professor Dr. med. T. Geisler dürchgeführt.

Die Konzeption der Studie erfolgte durch Herrn Professor Dr. med. T. Geisler.

Die Versuche wurden nach Einarbeitung durch Frau L. Laptev von mir eigenständig durchgeführt.

Die statistische Auswertung erfolgte nach Anleitung durch Herrn Professor Dr. med. T. Geisler durch mich.

Ich versichere, das Manuskript selbständig nach Anleitung durch Herrn Professor Dr. med. T. Geisler verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Düsseldorf, den 05.12.2016

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10. CURRICULUM VITAE

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BERUFLICHE ERFAHRUNG	
Seit 11. 2014	Universitätsklinikum Düsseldorf *Assistenzarzt Klinik für Kardiologie, Pneumologie und Angiologie, Ärztlicher Direktor Univ.-Prof. Dr. med. Malte Kelm*
2/2012 – 10/2014	Universitätsklinikum Tübingen *Assistenzarzt Innere Medizin III – Kardiologie und Kreislauferkrankungen, Ärztlicher Direktor Univ.-Prof. Dr. Meinrad Paul Gawaz*
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SONSTIGE KENNTNISSE UND QUALIFIKATIONEN	
Sprache	- Griechisch: Muttersprache - Deutsch: Fließend - Englisch: Fließend
EDV-Kenntnisse	- fortgeschrittene Kenntnisse der gängigen Microsoft-Office-Anwendungen - sicherer Umgang mit SPSS - sicherer Umgang mit SAP und Medico (KIS)
Sonstige	- GCP-Training AMG-Studien (8. Februar 2012, CenTrIaL) - Zusatztraining MPG-Studien (3. Dezember 2012, CenTrIaL) - Universität Tübingen Summer course: complementary skills (11.-15. 6. 2012)