Platelet adherence during inflammatory diseases

Quantity matters!

Bert Rutten

Cover layout: The cover of this thesis is a painting from Salvador Dali, customized by Ankie Rutten. With his paintings, Dali encourages us to question the world and to bridge the gap between the known and the unknown.

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Platelet adherence during inflammatory diseases
Quantity matters!

Bloedplaatjes adhesie tijdens ontstekingsziekten
Kwantiteit doet ertoe!

(met een samenvatting in het Nederlands)

Proefschrift

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"It is not possible to learn what we claim to know" *(Epictetus)*

therefore

"Doubt is the beginning of knowledge" *(René Descartes)*

CONTENTS PAGE

LIST OF ABBREVIATIONS

ACD	Acid citrate dextrose
ACE	Angiotensin-converting-enzyme
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13
ADP	Adenosine diphosphate
AE	Athero Express
AUC	Area under the curve
CABG	Coronary artery bypass grafting
CaCl	Calcium chloride
CAD	Coronary artery disease
CD42b	GP1bα
CD62P	P-selectin
CEA	Carotid endarterectomy
CG	Cockroft-Gault
CRP-XL	Cross linked collagen related peptide
CTMM	Center for Translational Molecular Medicine
CVD	Cardiovascular disease
DIC	Differential interference contrast
DM	Diabetes mellitus
E.coli	Escherichia coli
EC50	Half maximal effective concentration
ECST	European carotid surgery trial
EDTA	Ethylenediaminetetraacetic acid
EGM-2	Endothelial growth medium 2
ELISA	Enzyme linked immunosorbent assay
Epo	Erythropoietin
FACS	Flow cytometry activated cell sorting
FAMI	First acute myocardial infarction
FBS	Fetal bovine serum
FFR	Fractional Flow Reserve
FITC	Fluorescein isothiocyanate
GP1bα	Glycoprotein 1bα
HBS	HEPES buffered saline
HRP	Horseradish peroxidase
hsCRP	High sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cells
IgG	Immunoglobulin G
IL	Interleukin
IP-1β	Macrophage inflammatory protein 1β
IP-1β	Iloprost
IQR	Interquartile range
LPS	Lipopolysaccharide

LTA	Light transmission aggregometry
MAC-1	Macrophage antigen 1
MACE	Major adverse cardiac events
MCP-1	Monocyte chemotactic protein-1
mEPC	Myeloid endothelial progenitor cells
MFI	Median fluorescence intensity
MI	Myocardial infarction
MMP-9	Matrix metallopeptidase 9
NA	Not applicable
NASCET	North american symptomatic carotid endarterectomy trial
	non-STEMI non ST segment elevated myocardial infarction
OD	Optical density
OPG	Osteoprotegerin
OR	Odds ratio
P/M	Platelet density per monocyte
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PBSA	PBS containing 0.1% bovine serum albumin
PC	Positive control
PCI	Percutaneous coronary intervention
PDMS	Polydimethylsiloxane
PE	Phycoerythrin
PFA-100	Platelet function analysis system
PGI2	Prostaglandin analogue
PLC	Platelet-leukocyte complexes
PMA	Phorbol myristate acetate
PMC	Platelet-monocyte complexes
PNC	Platelet-neutrophil complexes
PSGL-1	P-selectin glycoprotein ligand 1
RT	Room temperature
SEM	Standard error of the mean
STEMI	ST segment elevated myocardial infarction
TEA	Triethylamine
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor necrosis factor α
TRAP-1	Thrombin receptor activating peptide 1
TRAP-4	Thrombin receptor activating peptide 4
TY	Tryptane yeast
UL-VWF	Ultra large VWF
VEGF	Vascular endothelial cell growth factor
VIF	Variance inflation factor
VWF	von Willebrand factor

CHAPTER 1
General Introduction

The curse of the mummy

It was a hot and sunny day, like most days in the Valley of Kings, when a team of Egyptologists worked relentlessly to retrieve the mummies from their tombs. The process of mummification was reserved for royal pharaohs, who ruled the vast Egyptian empire over 4000 years ago. Nowadays it preserves a wealth of information that is no longer exclusively valuable to archaeologists. Using whole body tomography scanning, researchers found evidence of atherosclerosis in 20 of the 52 investigated mummies[1]. The presence of atherosclerosis was later confirmed in common people, conserved through natural processes, from four different geographical regions[2]. These findings made clear that our view on atherosclerosis, which was believed to be a modern lifestyle disease, was incorrect. Nowadays, we understand that atherosclerosis is a complex disease in which not only a disturbed intake of lipids plays an important role, but also numerous risk factors, growth factors, cytokines, chemokines, cellular infiltration and the adherence of platelets to leukocytes. This introduction deals with the current insights in the progression of atherosclerosis, the cells that contribute to the progression of atherosclerosis, the involvement of von Willebrand factor (VWF) but also the cells that participate in repair after vascular damage.

Atherosclerosis

Atherosclerosis is a leading cause of mortalities worldwide and the primary cause of cardiovascular disease[3]. The earliest pathological manifestation of atherosclerosis includes the formation of fatty streaks, usually, however not solely, found in the aorta already at an early age[4-8]. Physical forces that result from a disturbed blood flow are suggested to reduce the protective nature of endothelial cells and contribute to the formation of fatty streaks[9]. Under the influence of monocyte chemoattractant protein 1 (MCP-1) and chemokines, monocytes are recruited and atherosclerotic plaques start to form. Infiltration of monocytes, which involves rolling, firm adhesion, spreading and trans-migration through the endothelium[10], contributes to the thickening of intima and the narrowing of the lumen[11]. Transmigrated monocytes differentiate into macrophages[12], known to scavenge large amounts of oxidized

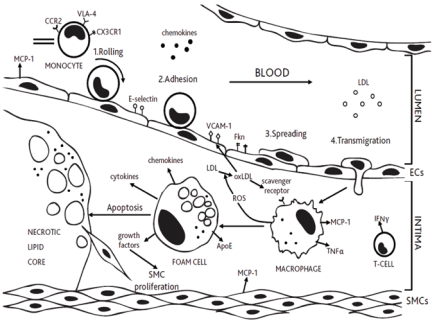

Figure 1. Monocyte recruitment into an atherosclerotic plaque. Monocytes are attracted to the inflamed endothelium under local expression of numerous chemokines and cell adhesion receptors. Following transmigration, which involves rolling, adhesion and spreading, monocytes differentiate into macrophages that are capable of scavenging large amounts of oxidized LDL (oxLDL). Eventually, macrophages differentiate into foam cells that secrete chemokines and cytokines involved in additional monocyte recruitment but also growth factors that cause smooth muscle cell (SMC) proliferation. After foam cells apoptosis, release of large amounts of cellular debris contributes to the build-up of the characteristic necrotic lipid core.

low density lipoproteins (LDL) that are present in the vessel walls of humans with atherosclerosis[13]. The presence of LDL triggers the upregulation of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells[14]. This stimulates the recruitment of additional monocytes[15] through interactions between E-selectin and VCAM-1 with the leukocyte counter receptor P-Selectin Glycoprotein Ligand-1[16-18] (PSGL-1). Eventually, the plaque residing macrophages differentiate into lipid-loaded foam-cells that are prone to cellular death[19]. Upon foam cell death, vast amounts of lipids and cellular debris are released into the lesion. This process drives the inflammatory response, weakens the fibrous cap and, eventually, may result in atherosclerotic plaque rupture[20]. Following atherosclerotic plaque rupture, circulating platelets that patrol the integrity of the endothelial lining come into contact with pro-coagulant subendothelial molecules[21]. Collagen, a major constituent of the subendothelial matrix[22], activates platelets through interaction with glycoprotein VI (GPVI)[23]. This interaction triggers a cascade of events, including the release of secondary platelet activators such as adenosine diphosphate and thromboxane[24,25], resulting in the firm adhesion of platelets to the extracellular matrix. Also, circulating activated platelets adhere to the extracellular matrix by binding to proteins like VWF26 and fibrinogen[27], which ultimately leads to the formation of a platelet plug that reduces the flow of blood[28]. Patients with reduced blood flow are diagnosed with stable or unstable angina, non-ST segment elevated myocardial infarction (non-STEMI) or ST segment elevated myocardial infarction (STEMI)[29-31]. However, the clinical diagnosis does not represent the molecular state of the disease. The multifactorial nature of atherosclerosis and the extended amount of risk factors that contribute to the severity of the disease may explain this observation. Nonetheless, clear guidelines for the diagnosis of patients with atherosclerosis have been established and percutaneous coronary intervention (PCI) followed by stent implantation and dual platelet inhibition therapy is the standard procedure to prevent the occurrence of an acute occlusive event.

Cells contributing to progression of atherosclerosis

Continuous accumulation of monocyte derived macrophages is a central hallmark of the pathogenesis of atherosclerosis[32]. Therefore, the recruitment of circulating monocytes to inflamed endothelium is considered to be a crucial event in atherosclerotic disease progression. Monocytes are recruited to the circulation from the bone marrow or the spleen[32]. Thus far, three different subsets of human monocytes are recognized based on the expression of chemokine receptors (CCR2 and CX3CR1) and cell-adhesion receptors (CD14 and CD16)[33-35] and monocyte subsets can be divided into pro-inflammatory[36] and anti-inflammatory monocytes[37]. Pro-inflammatory monocytes are characterized by high CCR2 expression levels[32,38], while anti-inflammatory monocytes have low CCR2 expression levels[39]. Both CCR2 and XC3CR1

are chemokine receptors, but CCR2 interacts with monocyte chemotactic protein-1 (MCP-1)[40], while CX3CR1 with fractalkine[41]. Both MCP-1 and fractalkine play important roles in the recruitment of monocyte from the bone marrow to the circulation[42, 43]. CD14 is a monomeric U-shaped protein[44], present on either the cell membrane of monocytes or in plasma in a soluble form (sCD14). CD14 is commonly recognized as a co-receptor for the detection of bacterial lipopolysaccharides[45], while CD16 is a receptor that is involved in antibody-dependent cellular cytotoxicity through recognition of immunoglobulin G (IgG) Fc-regions[46]. Recruitment of circulating monocytes not only depends on the expression of cytokines, chemokines and adhesion receptors on monocytes, also platelet binding to monocytes contributes to the recruitment of circulating monocytes. Leppanen et al. showed that adherence of platelets to monocytes depends on interactions between platelet P-selectin and the constitutively expressed monocyte receptor PSGL-1[47]. Engagement of PSGL-1 by P-selectin triggers tyrosine dependent intracellular signal transduction pathways[48], that cause the expression of chemokines and cytokines such as macrophage inflammatory protein 1β (MIP-1β), tissue factor, interleukin (IL)-1, IL-6, IL-8, IL-10, TNFα, matrix metallopeptidase 9 (MMP-9) and platelet activating factor (PAF)[49-54]. The adherence of platelets to monocytes not only modulates the secretion of inflammatory molecules, it also increases the adhesive capacity of platelet bound monocytes towards the endothelium. Da Costa et al. showed that platelet adherence to monocytes results in increased affinity of platelet-monocyte complexes for endothelial cells in vitro [55, 56]. In vivo, Massberg et al. showed that platelets, but not monocytes, adhere to the endothelium of 10 week old ApoE knockout mice prior to the development of atherosclerotic lesions[57]. Platelet adherence to the endothelium resulted in upregulation of endothelial cell adhesion receptors for monocytes and expression of MCP-1. These results suggest that platelets are the initiators of atherosclerosis. Prolonged infusion of platelets pre-treated with GPIb blocking antibodies resulted in a marked aberration of transient and firm platelet adhesion, while prolonged infusion of platelets pre-treated with integrin αIIbβ3 blocking antibodies caused a reduction of firm platelet adhesion. Surprisingly, it was not investigated whether infusion of platelets pre-treated with GPIb antibodies resulted in Fc-receptor mediated clearance of pre-treated platelets. However, the role of GPIIbIIIa in the reduction of firm platelet adhesion was confirmed by infusion of platelets isolated from a patient with Glanzmann's thrombasthenia, but the lack of confirmation of the adhesive role of GPIb with human platelets isolated from patients with Bernard-Soulier syndrome was a missed opportunity. Using the same GPIb blocking antibody, but now for clearance of platelets, the authors show a strong reduction in fatty streak formation and carotid plaque size. These findings confirm that platelet adhesion to endothelium plays a fundamental role in the early stages of atherosclerosis. Only 3 months later, Huo and colleagues demonstrated the significance of

platelet adherence to monocytes in the development of atherosclerosis[58]. These authors convincingly showed that infusion of activated platelets into C57BL/6 mice resulted in platelet adherence to circulating monocytes and neutrophils, in contrast to infusion of P-selectin deficient platelets. Platelet binding caused deposition of RANTES and PF-4 on the surface of platelet bound monocytes, resulting in the activation of monocytes, increased monocyte recruitment and accelerated formation of atherosclerotic lesions in mice. In contrast to the work of Massberg et al., Huo and colleagues found that only activated platelets bind to atherosclerotic carotid arteries of ApoE knockout mice, but not activated platelets lacking P-selectin. Huo and colleagues did not observe P-selectin independent platelet binding to endothelial cells, but it has to be noted that Huo et al. infused activated platelets that may have shed GPIb.

In summary, inactivated platelets express GPIb that is able to bind P-selectin expressed on activated endothelium in the early stages of atherosclerosis. Adhesion of platelets to the endothelium triggers platelet activation, which leads to the secretion of secondary platelet activators. These secondary activators mediate the activation of circulating platelets, and constitute the formation of circulating platelet-monocyte complexes with increased affinity for activated endothelium. However, the binding of circulating platelets to inflamed endothelium is also facilitated by a multimeric glycoprotein that is released by activated endothelial cells[59].

Von Willebrand factor and atherosclerosis

VWF is a multimeric glycoprotein that plays an essential role in the arrest of bleeding after vascular damage, but also causes the formation of an occlusive thrombotic plug after plaque rupture. The majority of VWF is present in the Weibel-Palade bodies of endothelial cells and in plasma, while a minor part of total VWF is present in the α-granules of platelets[60]. Unlike VWF in plasma, which circulates in an inactive cryptic form, VWF in endothelial cells is highly active[61]. Each VWF molecule has a size of 220 kDa[62], that consists of several A, B, C and D domains in the following structural order: D′-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6[63]. The A1 domain harbors the binding site for platelet GPIb[64], the A3 domain interacts with collagen type III[65] and the C1 domain contains the binding site for the platelet integrin αIIbβ3[66]. During high-shear stress dependent secretion, VWF forms elongated multimeric strings of identical monomers that capture circulating platelets through interaction between the VWF A1 domain and the platelet glycoprotein (GP)Ib[67-69]. By doing so, it facilitates platelet adhesion and supports platelet plug formation at sites of vascular damage. To control the formation of a thrombus, VWF is cleaved by the metalloprotease ADAMTS13 into multimers ranging in size from 440 to 20.000 kDa. The significance of VWF cleavage by ADAMTS13 is demonstrated by excessive micro thrombi formation in the microvasculature of patients that suffer from thrombotic thrombocytopenic purpura[70]. Besides ADAMTS13, also granzyme B[71], neutrophil derived

elastase[72, 73], thrombin[74, 75], plasmin[76] and thrombospodin-1[77] cleave VWF. Cleavage of VWF by ADAMTS13 occurs in the A2 domain[78] and results in the transition of VWF from elongated active strings to inactive globular proteins in which the GPIb binding domain (A1) is shielded by either the A2 or the D'D3 domains[79, 80]. Using x-ray crystallography, inactive VWF was shown to harbor a binding site for collagen in its A3 domain[81]. Interaction between collagen and the A3 domain converts VWF from an inactive to an active form, allowing subsequent capturing of platelets via GPIb and the re-exposed A1 domain. The interaction between GPIb and the A1 domain occurs in so called "catch and slip bonds" that allow rolling of inactive platelets over exposed subendothelial surface. Hereafter, platelets are arrested by interaction of platelet receptors such as GPIIbIIIa or α1β2 with fibronectin or collagen, respectively. Subsequent interaction between collagen and the platelet receptor GPVI result in activation of platelets and recruitment of additional platelets[82]. This results in the arrest of bleeding but also the formation of an occlusive thrombotic plug, which is the leading cause of coronary artery occlusion and subsequent vascular damage.

Platelets and monocytes contribute to vascular repair

Besides the well-known role of monocytes (CD14+) in the non-specific first line of defense against microbial invaders, they are also recognized to attribute to vascular repair in patients with atherosclerosis that suffer from ischemia. In response to ischemia, tissues restore the blood flow through the generation of novel capillaries from pre-existing arterial (angiogenesis) or through the remodeling and enlargement of pre-existing arterioles to collateral vessels (arteriogenesis). In recent years it has become clear that monocytes are critical mediators of angiogenesis and arteriogenesis[83]. Monocytes are recruited to the inflamed endothelium in response to growth factors, such as vascular endothelial cell growth factor (VEGF) or MCP-1, due to local cues of platelets or ischemic vascular cells[83-85]. Driven by these high levels of pro-angiogenic factors, CD14+ myeloid cells can differentiate into myeloid endothelial progenitor cells and act as pro-angiogenic accessory cell that adopt a pro-vasculogenic phenotype that augments re-endothelialization[86] and reduces restenosis[87]. Monocytes are also involved in vascular repair after percutaneous coronary intervention (PCI) induced local endothelial damage[88]. Plaque rupture and the occurrence of an occlusive cardiac event are prevented by PCI and stent placement, followed by dual platelet inhibition therapy. These therapies restore the blood flow, but are also associated with increased risk of restenosis after discontinuation of platelet inhibition therapy[89]. The endothelium is a potent anticoagulant surface that inhibits platelet activation through a myriad of ways. Hence, insufficient re-endothelialization may contribute to the increased risk of restenosis[90,91]. Given the previously mentioned VEGF dependent differentiation of monocytes into pro-vasculogenic cells it is well possible that enhanced recruitment of monocytes to the endothelium, after interaction with platelets, results in improved wound healing.

Outline of this thesis

We studied the influence of platelet binding to leukocytes during inflammatory processes. In **chapter 2**, we investigated the relationship between significant flow-limiting stenosis, platelet activation and platelet-leukocyte complex formation in patients with stable coronary artery disease. We found that significant flow-limiting stenosis is associated with reduced levels of platelet-neutrophil complexes. In **chapter 3**, we reported that platelet activation correlates with platelet-monocyte complex formation and macrophage levels in atherosclerotic plaques. In **chapter 4**, we investigated why Epo treatment is associated with a high risk of thrombosis. We excluded a direct role of Epo on platelet reactivity. In **chapter 5**, we studied platelet activation and platelet binding to monocytes during induced human endotoxemia. We found that endotoxemia triggers platelet activation and platelet binding to monocytes, which correlates with cytokine levels. In **chapter 6**, we combined experimental studies with clinical data and showed that the platelet density per monocyte is a novel predictor of cardiac events in patients after percutaneous coronary intervention. In **chapter 7**, we investigated markers of first myocardial infarction. We found that active VWF is a marker for first myocardial infarction in patients from 3 different ethnic groups. In **chapter 8**, we described the development of a nanobody that prevents the interaction between platelets and neutrophils and in **chapter 9** we discussed our results.

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CHAPTER 2

The relationship between fractional flow reserve, platelet reactivity and platelet- leukocyte complexes in stable coronary artery disease.

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THE RELATIONSHIP BETWEEN FRACTIONAL FLOW RESERVE, PLATELET REACTIVITY AND PLATELET- LEUKOCYTE COMPLEXES IN STABLE CORONARY ARTERY DISEASE.

Abstract

Background The presence of stenoses that significantly impair blood flow and cause myocardial ischemia negatively affects prognosis of patients with stable coronary artery disease. Altered platelet reactivity has been associated with impaired prognosis of stable coronary artery disease. Platelets are activated and form complexes with leukocytes in response to microshear gradients caused by friction forces on the arterial wall or flow separation. We hypothesized that the presence of significantly flow-limiting stenoses is associated with altered platelet reactivity and formation of platelet-leukocyte complexes.
Methods One hundred patients with stable angina were studied. Hemodynamic significance of all coronary stenoses was assessed with Fractional Flow Reserve (FFR). Patients were classified FFR-positive (at least one lesion with FFR≤0.75) or FFR-negative (all lesions FFR>0.80). Whole blood samples were stimulated with increasing concentrations of ADP, TRAP, CRP and iloprost with substimulatory ADP. Expression of P-selectin as platelet activation marker and platelet–leukocyte complexes was measured by flow cytometry. Patients were stratified on clopidogrel use. FFR positive and negative patient groups were compared on platelet reactivity and platelet-leukocyte complexes.
Results Platelet reactivity between FFR-positive patients and FFR-negative patients did not differ. A significantly lower percentage of circulating platelet-neutrophil complexes in FFR-positive patients and a similar non-significant decrease in percentage of circulating platelet-monocyte complexes in FFR-positive patients was observed.
Conclusion The presence of hemodynamically significant coronary stenoses does not alter platelet reactivity but is associated with reduced platelet-neutrophil complexes in peripheral blood of patients with stable coronary artery disease.

Introduction

For patients with stable coronary artery disease, the presence and extent of myocardial ischemia is the most important prognostic factor for myocardial infarction and death[1,2]. On the other hand, patients who have coronary artery stenoses which do not significantly obstruct blood flow and consequently do not cause ischemia have a good prognosis, annual event rates being lower than 1%[3]. The biological mechanisms that mediate the increased risk in patients with inducible myocardial ischemia are not clear. Increased platelet reactivity is associated with increased risk of myocardial infarction (MI) in patients with stable coronary artery disease[4] and antithrombotic therapy has been shown to be effective in reducing the risk of future MI[5]. Furthermore, variation in response to antithrombotic therapy is associated with increased occurrence of atherothrombotic events in patients treated with percutaneous intervention[6,7]. Increased levels of platelet-monocyte complexes and increased platelet reactivity have been found in peripheral blood of patients with stable coronary artery disease compared to healthy control subjects[8] acute coronary syndromes[9-11] and ischemic stroke[12-14]. Platelets are functionally affected by conditions of high shear stress[15,16] and platelets form larger aggregates in response to increasing microshear gradients, independent of

soluble agonists[17]. Previous reports have shown that lesion severity and calculated shear stress correlate with increasedplatelet-monocyte complexes distal to a stenosis and further in the coronary sinus, as compared with samples from the proximal coronary artery[18]. Also, experimental evidence suggests myocardial ischemia itself as a factor in platelet behavior, by secretion of pro-aggregratory substances[19]. Apart from thrombosis, it has become increasingly clear that platelets are actively involved in all stages of atherosclerosis. Platelets have been shown to interact with both endothelial cells as well as circulating leukocytes to promote atherogenesis[20]. Alpha granule fusion with the platelet membrane causes exposure of P-selectin which by interaction with P-selectin glycoprotein ligand – 1 (PSGL-1) mediates the formation of inflammatory platelet-leukocyte complexes. This facilitates a leukocyte influx into the endothelium, thereby presumably assisting in lesion development[21]. Fractional Flow Reserve (FFR) is an invasive lesion-specific index of myocardial ischemia due to epicardial coronary stenosis. FFR measures a lesion's ability to cause myocardial ischemia by measuring the pressure gradient across a stenosis during maximum induced hyperemia[22]. Moreover, patients with FFR-positive lesions benefit from revascularization and medical treatment of FFR-positive lesions is inferior to revascularization[23]. We hypothesized that in patients with stable coronary artery disease, the presence of ischemia-causing, flow-limiting coronary lesions, as measured by FFR is associated with altered platelet reactivity. Furthermore, we hypothesized that the presence of hemodynamically significant coronary lesions is associated with altered fractions of platelet-leukocyte complexes (PLCs).

Methods

lnterventional procedure

The study was approved by the local ethics committee of all participating centers (Catharina Hospital Eindhoven, Utrecht University Medical Center, Maastricht University Medical Center, Leiden University Medical Center) and all patients gave written informed consent for participation prior to coronary angiography. One hundred sixteen patients with stable angina, who were referred for angiography on basis of symptoms suggesting myocardial ischemia and/or evidence of ischemia on non-invasive testing, underwent FFR measurement were included from 3 Dutch hospitals: Catharina Hospital Eindhoven, University Medical Center Utrecht and the Maastricht University Medical Center. All patients were referred for angiography on basis of symptoms suggesting myocardial ischemia and/or evidence of ischemia on non-invasive testing. Exclusion criteria were active inflammatory state, auto-immune disease or malignancy. Fractional Flow Reserve of all coronary stenoses was performed according to established standard practice under conditions of maximal hyperemia. Evidence of myocardial ischemia was defined by the presence of at least one coronary stenosis with FFR≤ 0.75. Conversely ischemia was considered absent if none of the measured lesions had an FFR≤0.80. These cut off values have been extensively validated[24].

To clearly demarcate the presence or absence of ischemia, we did not include 16 patients with intermediate FFR-values of 0.76 to 0.80, leaving 100 patients for analysis. Decision on treatment of the coronary lesions was left at the discretion of the operator. Depending on whether PCI was expected to be performed on the basis of a previously performed angiography, patients received a loading dose of clopidogrel 600 mg on the day before the procedure, according to local protocol.

Laboratory methods

Laboratory tests were conducted in three medical centers. In order to prevent variation between the individual centers, all materials were centrally ordered and disturbed from the university medical center in Utrecht and each center was equipped with a FC500 flow cytometer (Beckman Coulter) that was centrally calibrated at the university medical center in Leiden.

Blood collection

Blood was collected from the arterial sheath into 3.2% tri-sodium citrate tubes (Greiner Bio-One), before administration of intravenous anticoagulants. All samples were processed directly after collection to prevent any potential blood storage effects.

Platelet reactivity assay

Platelet reactivity was determined with agonist concentration series of adenosine diphosphate (ADP; P2Y12 receptor agonist), cross linked collagen related peptide (CRP-XL; GPVI receptor agonist), thrombin receptor activating peptide (TRAP; PAR-1 receptor agonist), and iloprost (IP receptor agonist) with substimulatory ADP. Serial dilutions of ADP (125μM, 31.25μM, 7.8μM, 1.95μM, 488nM, 122nM, 31nM, 8nM) were prepared in 50μL HEPES buffered saline (HBS; 10mM HEPES, 150mM NaCl, 1mM MgSO4¬, 5mM KCl, pH 7.4), with 2μL phycoerythrin (PE) labeled mouse anti-human P-selectin antibodies (BD Biosciences, Breda, the Netherlands) and 2μL fluorescein isothiocyanate (FITC) labeled mouse anti-human GPIb antibodies (BD Biosciences, Breda, the Netherlands). Similarly, serial dilutions of CRP-XL (2.5μgr/mL, 625ng/mL, 156.3ng/mL, 39.1ng/mL, 9.8ng/mL, 2.4ng/mL, 600pg/mL, 153pg/mL), TRAP (625μM, 156.3μM, 39.1μM, 9.8μM, 2.4μM, 610nM, 153nM, 38nM), and iloprost (1250 ng/mL, 312.5ng/mL, 78ng/mL, 19.5ng/mL, 4.9ng/mL, 1.2ng/mL, 0.31ng/mL, 0.076ng/mL) with 5μM ADP were prepared in 50μL HBS with 2μL mouse anti-human P-selectin antibodies and 2μL mouse anti human GPIb antibodies. The platelet reactivity assay was initiated by adding 5μL whole blood to each sample of serial dilutions. Samples were incubated for 20 minutes and subsequently added with 500μL fixative (0.2% formaldehyde and 0.9% NaCl). All samples were analysed on a FC 500 flow cytometer (Beckman Coulter, FL, USA) on the same day of processing. Data acquisition was performed with the SXP SYSTEM software (Beckman Coulter, FL, USA) Single platelets were gated based on FITC signal intensity. The median fluorescence intensity (MFI) for PE was measured. Dose-response graphs for P-selectin expression were constructed and the maximum response and area under the curves (AUC) in arbitrary units was calculated.

Platelet-leukocyte complex assay

For assessment of platelet-leukocyte complexes, 50µl of citrate anticoagulated whole blood was diluted with 45ul phosphate buffered saline (PBS) and platelets were labeled by incubation with 5µl FITC labeled mouse anti human GPIb antibodies (BD Biosciences) for 30 minutes at room temperature. Triplicate samples were fixed for 10 minutes with 80µl of Optilyse B (Beckman Coulter), containing 3.4% paraformaldehyde, after which hypotonic red blood cell lysis was achieved by the addition of 820µl of demineralized water. Monocytes and neutrophils were identified by forward and sideward scatter gating. Platelet–monocyte complexes (PMCs) and platelet-neutrophil complexes (PNCs) were determined by calculating the percentage of cells in these gates that were positive for the platelet marker GPIb. All samples were analysed on a FC 500 flow cytometer (Beckman Coulter, FL, USA) on the same day of processing. Fifteen thousand cells were counted, and data acquisition was performed with the SXP SYSTEM software (Beckman Coulter, FL, USA).

Statistical analysis

Comparison of categorical variables was done using Chi square testing, while continuous variables were compared using the Student T or Mann Whitney test as appropriate. A p-value < 0.05 was considered statistically significant. We performed collinearity analysis and used the variance inflation factor (VIF), because previous research has demonstrated inhibition of clopidogrel on both platelet reactivity and platelet leukocyte complex formation[25,26]. Collinearity analysis of all independent variables showed significant collinearity of clopidogrel use and FFR (tolerance 0.037, VIF 27.4). To omit a confounding effect of clopidogrel use, we therefore stratified our patients according to clopidogrel use (either chronic or loading dose) yielding 4 groups: FFR-negative without clopidogrel (25 patients), FFR-positive without clopidogrel (9 patients), FFR-negative with clopidogrel (18 patients) and FFR-positive with clopidogrel (48 patients). All statistical analysis were performed using SPSS 18 (SPSS inc, Chicago, Ill, USA).

Results

Baseline characteristics

The baseline characteristics of the included patients are presented in table 1. As shown in table 1, baseline characteristics in general did not differ significantly between FFR-negative and FFR-positive patients, except for smoking and a family history of coronary artery disease, which were more prevalent in the FFR negative patients that were treated with clopidogrel. Use of Beta blockers was more prevalent in FFR-positive patients than in FFR-negative patients. The mean lowest FFR measured per patient was 0.55±0.14 in FFR-positive patients versus 0.87±0.04 indicating a clear distinction between ischemic and non-ischemic coronary artery disease patients.

Table 1. Baseline characteristics of FFR-positive and FFR-negative patients, stratified on clopidogrel usage.

	FFR negative Clopidogrel-	FFR positive Clopidogrel-	*P	FFR negative Clopidogrel+	FFR positive Clopidogrel+	*P
	N=25	N=9		N=18	N=48	
Age (mean±SD)	62.7 ± 8.4	64.5 ± 8.3	0.59	63.6 ± 11.7	61.3 ± 10.0	0.46
Sex – Male (%)	15 (60)	6 (66.7)	0.73	12 (66.7)	30 (62.5)	0.76
Previous MI	5 (20)	3 (33.3)	0.43	8 (44.4)	10 (20.8)	0.06
Previous PCI	7 (28)	3 (33.3)	0.77	11 (61.1)	17 (35.4)	0.07
Previous CABG	3 (12)	0 (0)	0.29	1 (5.6)	1 (2.1)	0.56
DM	4 (16)	2 (22.2)	0.69	1 (5.6)	11 (22.9)	0.11
Hypertension	11 (44)	3 (33.3)	0.26	8 (44.4)	24 (50)	0.70
Hyperlipidemia	12 (48)	4 (44.4)	0.71	8 (44.4)	25 (25.1)	0.60
Smoking	5 (20)	2 (22.2)	0.89	9 (50)	11 (22.9)	0.03
history of CAD	14 (56)	5 (55.6)	0.98	9 (50)	37 (77.1)	0.03
ASA	22 (88)	9 (100)	0.08	15 (83.3)	44 (91.7)	0.41
B-blockers	12 (48)	8 (88.9)	0.03	11 (66.1)	42 (87.5)	0.02
Statins	21 (84)	8 (88.9)	0.73	17 (94.4)	42 (87.5)	0.35
ACE-inhibitors	6 (24)	3 (33.3)	0.60	7 (38.9)	13 (27.1)	0.39
Oral anticoagulation	2 (8)	0 (0)	0.40	0 (0)	3 (6.3)	0.29
Non-significant	2 (8)	0 (0)	1.0	1 (5.6)	0 (0)	0.27
1 – vessel disease	16 (64)	3 (33.3)	0.13	7 (38.9)	28 (58.3)	0.18
2 – vessel disease	5 (20)	2 (22.2)	1.0	6 (33.3)	16 (33.3)	1.0
3 – vessel disease	2 (8)	4 (44.4)	0.03	4 (22.2)	4 (8.3)	0.2

Table 1. Baseline characteristics of FFR-positive and FFR-negative patients, stratified on clopidogrel usage. Continuous values are presented as means ± SD . Categorical values are presented as number (percentages). Non-significant indicates no lesion with stenosis > 50%.*Significant p-values are printed bold. MI=myocardial infarction, PCI= percutaneous coronary intervention, CABG= coronary artery bypass grafting, DM= diabetes mellitus, CAD= coronary artery disease.

Platelet reactivity

Platelet reactivity was determined by the maximal expression of P-selectin after stimulation and cumulative reactivity by the area under the curve (AUC) calculations determined from the dose-response curves. The use of clopidogrel resulted in a markedly lower response to ADP, and Iloprost with sub-optimal ADP stimulation for both the maximal response and the AUC. Clopidogrel was not able to significantly inhibit activation by TRAP or CRP-XL as was observed in both the maximal response and the AUC values (Figure 1 and 2). No significant difference was observed between FFR-positive and FFR-negative patients for all activation responses by either platelet reactivity measurements.

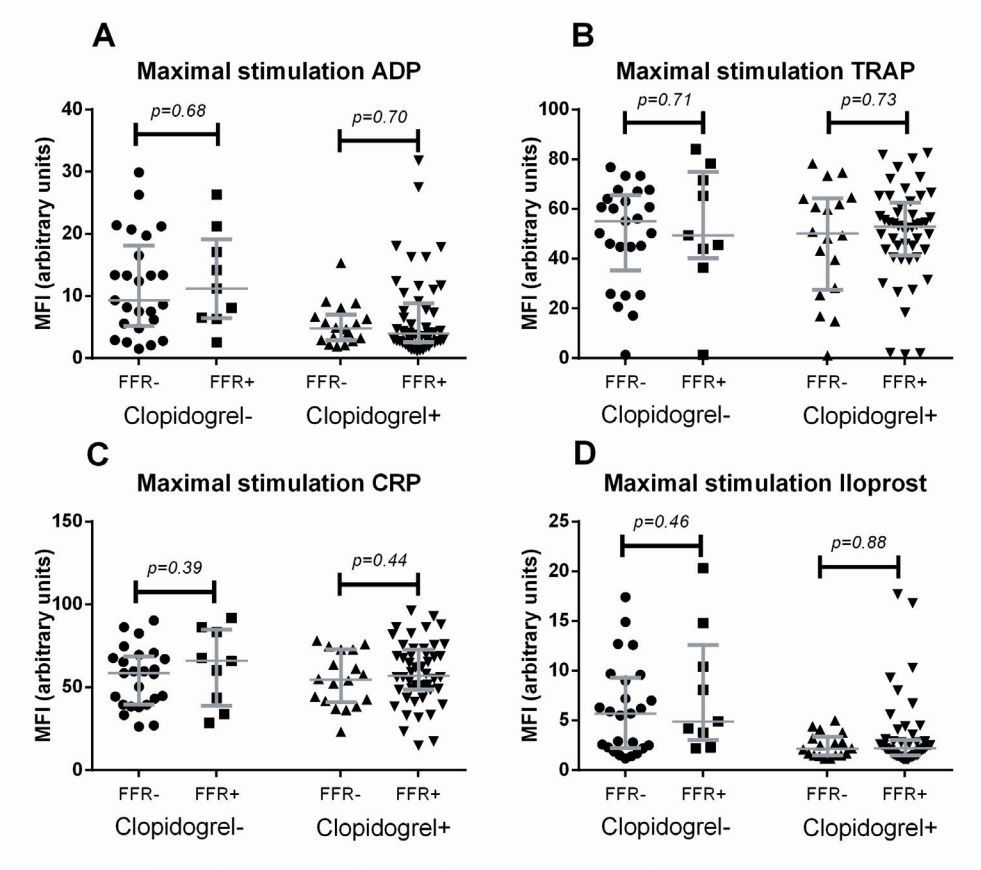

Figure 1. Maximal expression of P-selectin after stimulation with ADP (A), TRAP (B), CRP (C) and Iloprost with substimulatory ADP (D). Lines indicate median values with interquartile range.

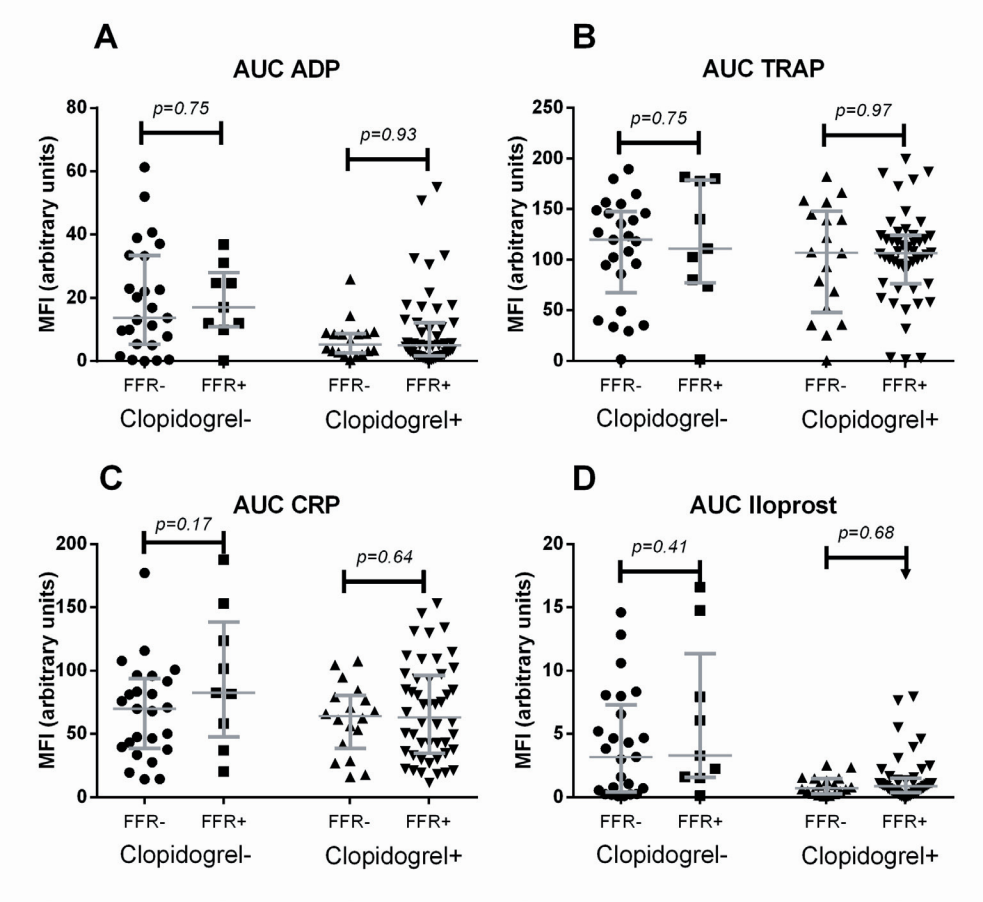

Figure 2. Cumulative expression of P-selectin after stimulation with ADP (A), TRAP (B), CRP (C) and Iloprost with substimulatory ADP (D). Lines indicate median values with interquartile range.

2

Platelet-leukocyte complexes

Percentages of platelet–monocyte complexes (PMCs) and platelet–neutrophil complexes (PNCs) in whole blood were available for 67 included patients. Patients were again stratified according to clopidogrel use and percentages of complexes were compared between FFR-positive and negative patients. The number of platelets per leukocyte, as measured by MFI of CD42b, did not significantly differ between groups (Figure 3).

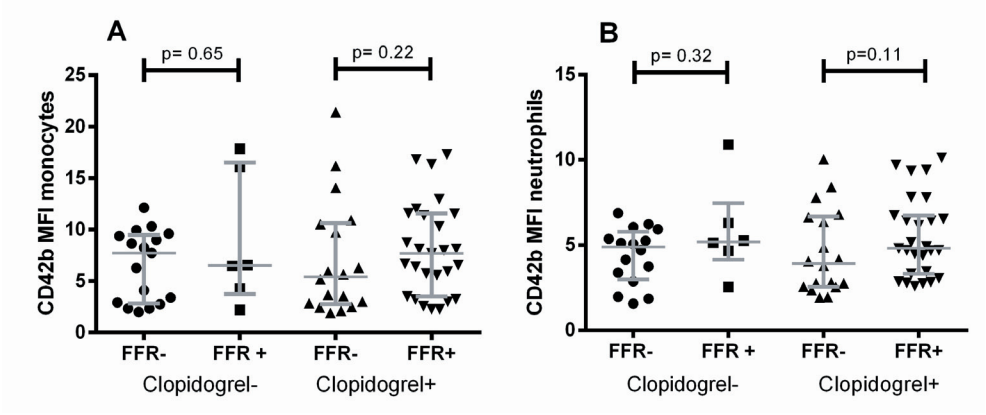

Figure 3. Platelets per monocyte (A) and neutrophil (B) measured by MFI of CD42b. Lines indicate median values with interquartile range.

We observed no significant difference in percentage of PMCs or PNCs between the FFR-positive and FFR-negative patients that were not treated with clopidogrel, although comparison is hampered by the small sample size (n=15 vs. n=5) (Figure 4A). There was a significant decrease in the percentage of PNCs (p=0.03; Figure 4B) and a trend towards a decrease in the percentage of PMCs (p=0.08; Figure 4A) in the clopidogrel treated FFR-positive patients compared to FFR-negative patients.

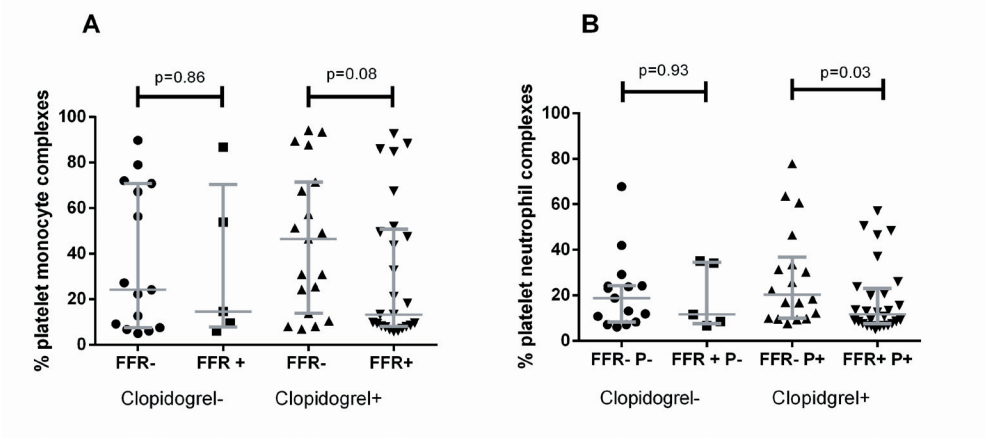

Figure 4. Percentages of platelet-monocyte (A) and platelet-neutrophil (B) complexes in FFR-negative and FFR-positive patients, identified by presence of platelet marker CD42b on monocytes and neutrophils. Lines indicate median values with interquartile range.

Discussion

In this observational study we observed no differences in both maximal and cumulative in vitro platelet reactivity between patients with a positive FFR and a negative FFR. However, we did observe a significantly lower percentage PNCs in patients with a positive FFR compared to patients with negative FFR in the clopidogrel treated group (p=0.03), and a trend towards lower percentages of PMCs in patients with a positive FFR compared to a negative FFR in the clopidogrel treated group (p=0.08).

Previous clinical studies have shown diverging results with respect to the relationship between platelet reactivity, platelet-leukocyte complexes and coronary luminal obstruction or inducible myocardial ischemia. Increased systemic platelet reactivity was described in patients with documented coronary artery disease immediately after peak exercise[27,28], however no relationship with ischemia was found[27]. In critical limb ischemia, increased PMCs and expression of P-selectin was observed, implying an ischemic mechanism of platelet activation[29]. Conversely, other investigators found an inverse relationship between coronary obstruction and platelet reactivity[30], although all patients investigated had severe single-vessel disease. Others found experimental evidence that platelet reactivity might be reduced by ischemic pre-conditioning[31], which may point to the possibility of down regulation of platelet reactivity by repeated, short-acting bouts of ischemia as occurs in stable coronary disease. We found no evidence for this.

The paradoxical lower percentage of platelet-leukocyte complexes observed in the FFR-positive group may be explained by increased activation of leukocytes due to significantly flow-limiting stenoses[14] and subsequent increased clearance of the complexes from the circulation. Da Costa et al showed that attachment of monocytes to platelets leads to enhanced transmigration of monocytes into the subendothelium[32]. Also, Huo et al showed that the interaction of infused activated platelets with leukocytes resulted in increased adherence to the endothelium. Subsequent transmigration of the complexes led to absence of detectable levels of platelet-leukocyte complexes in a time frame of 3-4 hours[33]. Acute ischemic events, like myocardial and cerebral infarction cause a strong inflammatory response and tissue damage, and are associated increased levels of peripherally detectable leukocyte-platelet formation during or shortly after the ischemic event, as previously shown[9-14]. In contrast, inducible ischemia in stable coronary disease implies relatively short, reversible episodes of ischemia (during for example exercise) without permanent damage, which may transiently increase leukocyte-platelet complexes. PLCs may subsequently be cleared from the circulation by the above mentioned mechanisms, effectively leading to decreased levels when measured in stable, non-ischemic conditions. Besides lower systemic percentages of platelet-leukocyte complexes due to increased transmigration of these complexes, neutrophils have been shown to phagocytose activated platelets in vivo[34]. Hence, lower percentages of circulating

neutrophils with platelets adhered to the cell membrane might reflect leukocyte populations that effectively phagocytosed activated platelets.

This study has several limitations. In our population, platelet reactivity and platelet – leukocyte complexes were assessed in stable, non-ischemic conditions, as opposed to the majority of previously mentioned research in which platelets and platelet – leukocyte complexes were mostly investigated during or shortly after ischemia or exercise. Thus, we cannot discount the possibility that acute changes in platelet reactivity occur during ischemic episodes, which subsequently disappear when ischemia is resolved, presumably by increased removal of formed PLCs. Another limitation is that blood samples were drawn from the arterial sheath, which may potentially dilute any local effect caused by the FFR-positive lesion. Systemic blood sampling may not recognize local PLC formation in the coronary circulation, although previous research have also found differences in PLCs in systemic blood samples of patients with localized arterial pathology[9,29].

In this observational study, patients were included before coronary angiography and FFR, which may account for differences in baseline characteristics. Importantly, use of clopidogrel before angiography differed significantly between FFR –positive and FFR-negative patient groups. The reason for this is that the majority of these patients were referred for PCI in which loading of clopidogrel is mandatory, in contrast to coronary angiography with FFR alone, in which this is not required. This introduces a bias between the FFR-positive and negative groups, since patients with severe lesions are more likely to be referred for PCI and thus be treated with clopidogrel upfront while at the same time these are more likely to have a FFR≤0.75. Given the observational nature of this study, however, we could not interfere with local regimens. We therefore chose to stratify patients, which resulted in relatively small groups and reduced statistical power. More precisely, the low sample sizes after stratification, although justified, prevented us from performing a meaningful multivariate analysis, especially since the data are non-normally distributed. Our findings therefore need further validation with larger sample sizes.

Acknowledgments

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CHAPTER 3

Increased platelet reactivity is associated with circulating platelet-monocyte complexes and macrophages in human atherosclerotic plaques

Manuscript under revision

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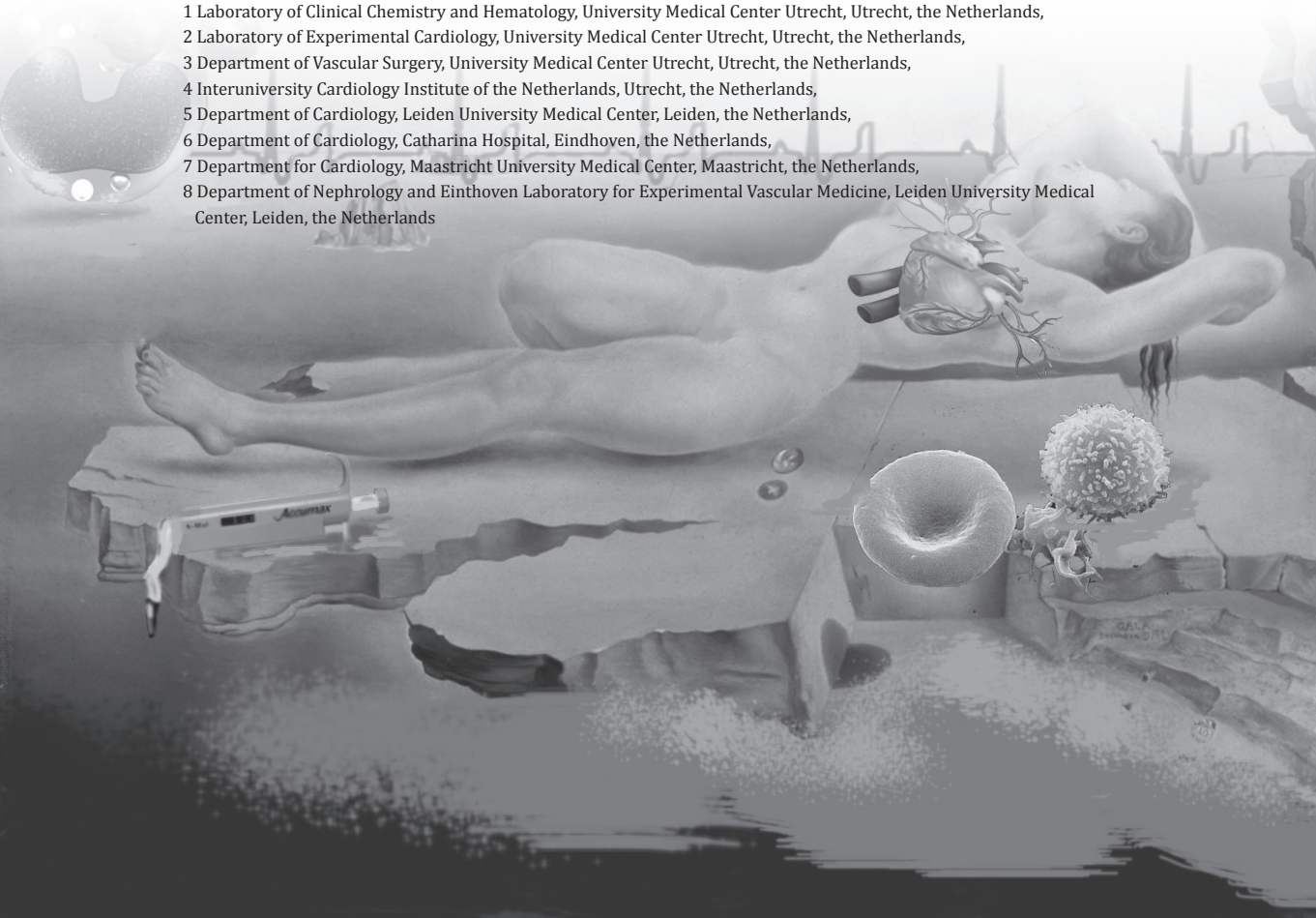

INCREASED PLATELET REACTIVITY IS ASSOCIATED WITH CIRCULATING PLATELET-MONOCYTE COMPLEXES AND MACROPHAGES IN HUMAN ATHEROSCLEROTIC PLAQUES

Abstract

Objective Platelet activation and monocyte infiltration play a detrimental role in atherosclerotic plaque progression. We investigated whether platelet reactivity is associated with levels of platelet-monocyte complexes and macrophages in human atherosclerotic carotid plaques. **Methods** Platelet reactivity was determined by measuring CD62P (P-selectin) expression on the platelet surface, after stimulation with increasing concentrations of adenosine diphosphate (ADP), in two independent cohorts: the Circulating Cells cohort (n=244) and the Athero-Express cohort (n=91). Levels of platelet-monocyte complexes were assessed by flow cytometry in blood samples of patients who were scheduled for percutaneous coronary intervention. Monocyte infiltration was semi-quantitatively determined by histological examination of atherosclerotic carotid plaques collected during carotid endarterectomy. **Results** We found increased platelet reactivity in patients with high platelet-monocyte complexes compared to patients with low platelet-monocyte complexes (median (interquartile range): 9633 (3580-21565) area under the curve (AUC) vs. 4153 (1585-11267) AUC, P<0.001). Also, we observed increased platelet reactivity in patients with high macrophage levels in atherosclerotic plaques compared to patients with low macrophage levels in atherosclerotic plaques (mean±SD; 9255±4530 AUC vs. 6995±3406 AUC, P=0.01). All associations remained significant after adjustment for age, sex and use of drugs against platelet activation. **Conclusion** Platelet reactivity is associated with levels of platelet-monocyte complexes and numbers of macrophages in human atherosclerotic carotid plaques.

Introduction

Platelets and monocytes play a crucial role in the initiation and progression of atherosclerosis[1]. Platelets tether and roll over inflamed endothelial cells through transient interactions between platelet Glycoprotein Ibα (GPIbα) and endothelial P-selectin[2,3], which also facilitates the capturing of monocytes by interaction with the constitutively expressed monocyte receptor P-Selectin Glycoprotein Ligand-1 (PSGL-1)[4]. These interactions, together with interactions between GPIbα and the integrin macrophage-1 antigen (MAC-1), results in firm cellular arrest and subsequent infiltration of monocytes into the vascular wall[5,6]. The importance of initial platelet binding to inflamed tissue was evidenced by a significant reduction in monocyte accumulation and atherosclerotic plaque progression after treatment with GPIbα blocking antibodies[7].

Monocytes migration into atherosclerotic plaques, and subsequent differentiation into macrophages[8], worsens atherosclerosis[7]. The differentiated macrophages secrete proteases and inflammatory proteins that weaken the fibrous cap, which results in increased risk of plaque rupture. Rupture-prone plaques are characterized by a thin fibrous cap covering a large lipid core that is enriched with macrophages. Stable plaques contain less macrophages and are enriched with smooth muscle

cells and high amounts of collagen[3]. After plaque rupture, platelets are activated due to interactions with the exposed pro-thrombogenic subendothelial matrix, resulting in the formation of an occlusive thrombus. Also, activated platelets bind to circulating monocytes and these circulating platelet-monocyte complexes are elevated in coronary artery disease[9-11].

The role of platelet activation in atherosclerotic disease progression has been demonstrated by a reduction of cardiovascular events after platelet inhibition therapies[12]. Moreover, P-selectin knockout mice showed decreased monocyte adherence to the vessel wall and delayed atherosclerotic plaque formation[13,14]. Platelet activation is triggered through stimulation of various receptors that stimulate integrin αIIbβ3 activation, which is crucial for platelet aggregation. Several assays, that measure platelet aggregation, are currently available, e.g.; light transmission aggregometry (LTA), VerifyNow® and platelet function analysis system (PFA-100). We have optimized a whole blood flow cytometry assay that measures platelet activation instead of platelet aggregation[15]. Our assay is based on the platelet response to increasing concentrations of adenosine diphosphate (ADP), which was quantified by the measurement of P-selectin upregulation after stimulation[16]. We used the platelet reactivity assay to investigate whether the reactivity of platelets towards ADP is associated with PMCs in circulation and macrophages in human atherosclerotic carotid plaques.

Materials and methods

Ethics statement

All research in this study was approved by the ethics committees of the four participating medical centers in the Netherlands, being: Catharina Hospital in Eindhoven, University Medical Center in Maastricht, University Medical Center in Leiden and the University Medical Center in Utrecht. The study conforms to the Declaration of Helsinki and all participants provided written informed consent prior to participation.

Study population

This report includes 2 cohorts, being the Center for Translational Molecular Medicine (CTMM) cohort and the Athero Express (AE) cohort, executed at different laboratories in The Netherlands.

We analyzed 244 blood samples from patients included in the CTMM cohort, who were scheduled for percutaneous coronary intervention, as previously described[17]. Exclusion criteria were age under 18 years, suspected alcohol or drug abuse, ST-elevation myocardial infarction, serious concomitant disease, an infection or suspected immune status elevation in six weeks prior to inclusion or absence of cooperation. Patients were included between September 2009 and April 2011.

We investigated 91 patients from the AE cohort, who were scheduled for carotid endarterectomy (CEA), as described previously[18]. Indications for CEA were reviewed by a multidisciplinary vascular team and were based on the recommended criteria of the Asymptomatic Carotid Atherosclerosis Study, the North American Symptomatic Carotid Endarterectomy Trial (NASCET), and the European Carotid

Surgery Trial (ECST)[19,20]. Patients undergoing CEA in the University Medical Center Utrecht were included between January 2011 and July 2012.

Flow cytometry

Flow cytometry measurements were performed on the BD FACS Canto II (BD Biosciences). All samples were measured once. Compensation was applied to all measurements and compensation was calibrated using FITC and PE labeled beads according to the manufacturers protocol (BD Biosciences). Samples were measured using a flow rate of 1.5 µL/sec and data was acquired in a four-decade logarithmic scale. In the platelet activation assay, a gate was applied in the SSC-H/FSC-H plot around the GPIb+ (CD42b-FITC) cells. Within this gate, we measured 10.000 events. An blood sample that was not stimulated was used as a negative control to set the threshold for P-selectin (CD62P-PE) expression. With this threshold, we measured the increase in P-selectin median fluorescence intensity after stimulation with increasing concentrations of ADP. To measure PMCs, we gated CD14+ (CD14-PE) cells within the SSC-H/FSC-H plot, and measured 15.000 events within this gate. Within this gate, the GPIb+ (CD42b-FITC) events were measured. An isotype control was used as a negative control for GPIb expression.

Blood collection and platelet reactivity assay

Arterial blood samples were collected in citrate (3.2%) anticoagulated tubes (BD Biosciences) prior to intervention and processed directly after collection to prevent any potential blood storage effects.

Platelet reactivity was quantified with an increasing agonist concentration series of ADP ranging from 8 nM to 125 µM. The serial dilutions were prepared in 50 µL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO4, 5 mM KCl, pH 7.4) to which 2 µL CD62P-PE (550561, BD Biosciences) and 2 µL CD42b-FITC (555472, BD Biosciences) mouse anti-human antibodies were added. The platelet reactivity assay was initiated by the addition of 5 µL whole blood to each sample of the serial dilution for 20 minutes at room temperature. Samples were fixated with 500 µL of 0.2% formaldehyde in 0.9% NaCl solution. Ten thousand platelets were identified by scatter gating and CD42b-FITC binding, using flow cytometry. Subsequently, CD62P-PE mean fluorescence intensity was measured, dose-response curves were produced and the area under the curve (AUC) was used to integrate the sensitivity and the maximum response of platelets towards agonist concentrations into one quantitative value. All data acquisition was performed with the CXP SYSTEM software.

Measurement of platelet-monocyte complexes

In the CTMM cohort, circulating platelet-monocyte complexes were quantified with a mixture of 40µL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO4, 5 mM KCl, pH 7.4) to which 5 µL CD14-PE (555398, BD Biosciences) and 5 µL CD42b-FITC (555472, BD Biosciences) mouse anti-human antibodies were added. Subsequently, 50 µL of whole blood was added to the mixture for 30 minutes at room temperature. Samples were fixated with 80 µL of Optilyse B

(Beckman Coulter), containing 3.4% paraformaldehyde for 10 minutes, after which red blood cells were lysed by the addition of 825 μL of demineralized water. Monocytes were identified by scatter gating and CD14 labeling. Platelet-monocyte complexes were determined by the percentage of monocytes that were positive for the platelet marker CD42b-FITC. Fifteen thousand cells were counted on the same day of processing.

Histological examination of macrophages in plaques

In the AE cohort, atherosclerotic carotid plaques were collected during CEA and immediately transported to the laboratory for processing. Using a standardized protocol, plaques were divided in 5-mm segments along the longitudinal axis, followed by fixation with 4% paraformaldehyde and embedment in paraffin. The segment with the largest plaque burden was defined as the culprit lesion. Subsequent histological plaque assessment was performed by an experienced technician, blinded for patient characteristics or clinical outcome, with a good inter- and intraobserver variability[21]. Macrophages were identified by CD68 labeling and semi-quantitatively categorized in two categories, being low and high staining, at original magnification x40. Plaques were categorized as low macrophage staining if staining was negative or clusters with <10 cells were present. Cell clusters >10 cells or abundance of positive cells were categorized as high staining.

Statistical analysis

Patients included in the CTMM cohort were dichotomized into groups with low (n=122) or high (n=122) levels of circulating platelet-monocyte complexes based on the median value of these complexes. Levels of platelet reactivity were compared between the groups using the Mann-Whitney U test. In the CTMM cohort, platelet reactivity values were not normally distributed. Therefore, the data were transformed into natural logarithmic values, which were used in univariate analysis of variance with age, sex, acetylsalicylic acid and clopidogrel use as covariates to exclude possible confounding of these variables.

Patients included in the AE cohort were divided into groups with low (n=67) or high (n=24) macrophage infiltration based on semi-quantitative analysis. Levels of platelet reactivity were compared between the groups using the Student's t-test. In addition, platelet reactivity values were adjusted for age, sex, acetylsalicylic acid and clopidogrel use in univariate analysis of variance, as above. All statistical analyses were performed using SPSS version 20.0 software (IBM Corp, IBM SPSS Statistics for Windows, Armonk, NY) and two-sided P-value <0.05 was considered statistically significant.

Results

Study population

The baseline characteristics and medication use at inclusion of patients investigated in the CTMM cohort (n=244) and in the AE cohort (n=91) are shown in table 1.

In the CTMM cohort, 189 patients (77.5%) were presenting with stable angina and 32 patients (13.3%)

with unstable angina and 23 patients (9.5%) with nSTEMI. Subjects with stable angina had lower mean cholesterol levels (P=0.03) and a higher prevalence of renal failure (P=0.01) as compared with subjects with unstable angina, while subjects with nSTEMI used nitrates less frequently (P=0.05) as compared with subjects with stable angina.

In the AE cohort, 78 patients (86%) had symptomatic carotid artery disease.No significant differences were observed in baseline characteristics between patients in the high groups versus patients the low groups within the CTMM and AE cohorts. Moreover, no significant differences in baseline P-selectin expression before platelet stimulation with ADP were observed between the two groups within the cohorts (data not shown).

Table 1. Baseline characteristics

	CTMM population (n=244)	**AE population** (n=91)
Age in years (mean ± SD)	63 ± 9.7	71.6 ± 9.5
Sex – male (%)	167/244 (69%)	66/91 (73%)
Body mass index (kg/m^2)	27.7 ± 4.5	25.4 ± 5.1
Smoking	53/244 (22%)	31/88 (35%)
Diabetes mellitus	45/244 (19%)	18/91 (20%)
Hypertension	157/244 (65%)	54/88 (61%)
Dyslipidemia	155/244 (64%)	53/75 (71%)
Total cholesterol (mmol/L)	4.4 ± 1.2	4.9 ± 1.5
History of coronary artery disease	115/244 (48%)	36/91 (40%)
Kidney disease	6/244 (3%)	NA
Glomerular Filtration Rate, CG, (mL/min)	NA	69.2 ± 24.5
Acetylsalicylic acid	198/244 (83%)	73/89 (82%)
Clopidogrel	131/244 (55%)	9/89 (10%)
Dipyridamole	Not available	62/90 (69%)
Statins	192/244 (80%)	77/90 (86%)
Nitrates	88/244 (37%)	Not available
Clinical presentation	NA	
Asymptomatic		14/89 (16%)
Amaurosis fugax		23/89 (26%)
Transient ischemic attack		26/89 (29%)
Stroke		26/89 (29%)
Bilateral carotid stenosis (>50%)	NA	33/72 (47%)
Days from event to CEA (median [IQR])	NA	18 [11-31]

Continuous values are expressed as mean ± standard deviation (unless specified otherwise). Categorical values are expressed as number of total (%).CG: Cockroft-Gault; CEA: carotid endarterectomy, IQR: interquartile range; NA: not applicable.

High platelet reactivity is associated with increased levels of platelet-monocyte complexes

We found significant positive correlations between platelet reactivity and levels of platelet monocyte complexes (R=0.27, P<0.001) and a higher AUC after platelet stimulation with ADP in patients with high levels of platelet-monocyte complexes as compared with patients with low levels of platelet-monocyte complexes (median (interquartile range): 9633 (3580-21565) AUC vs. 4153 (1585-11267) AUC, P<0.001) (Figure 1). These results remained significant after adjustment for age, sex, acetylsalicylic acid and clopidogrel (data not shown).

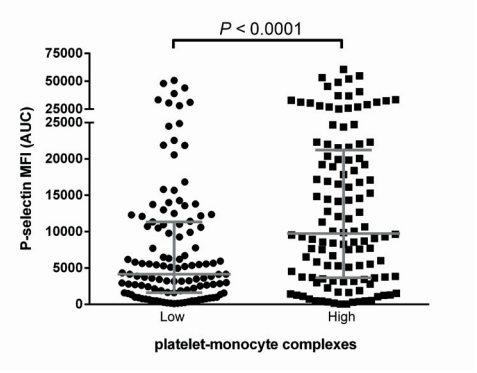

Figure 1. Platelet reactivity related to platelet-monocyte complexes. Comparison of the AUC after ADP stimulation between groups of low (n=122) versus high levels of platelet-monocyte complexes (n=122). Grey bars represent medians with interquartile ranges. AUC = area under curve, ADP = adenosine diphosphate.

High platelet reactivity is associated with high levels of macrophages in atherosclerotic carotid plaques

We observed a higher AUC after platelet stimulation with ADP in patients with high levels of macrophages in atherosclerotic carotid plaques compared to patients with low levels of macrophages in atherosclerotic carotid plaques (mean±SD, 9255±4530 AUC vs. 6995±3406 AUC, P=0.01) (Figure 2). Adjusting for age, sex, acetylsalicylic acid and clopidogrel use did not change the association of high platelet reactivity with increased levels of macrophages in plaques.

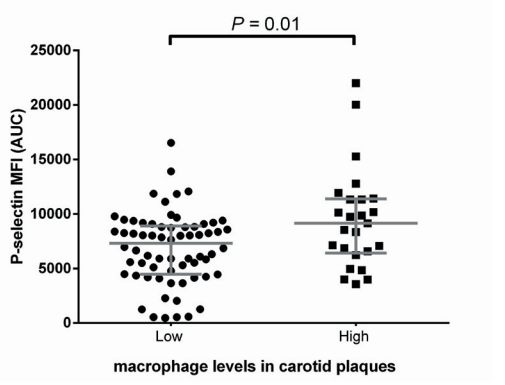

Figure 2. Platelet reactivity related to macrophage levels. Comparison of the AUC after ADP stimulation between low (n=67) versus high macrophage levels in atherosclerotic carotid plaques (n=24). Grey bars represent means with standard deviations. AUC = area under curve, ADP = adenosine diphosphate.

Figure 3 shows representative images of carotid plaques with low (Figure 3a, with higher magnification in 3b) and high (Figure 3c, with higher magnification in 3d) amounts of macrophages in the plaque.

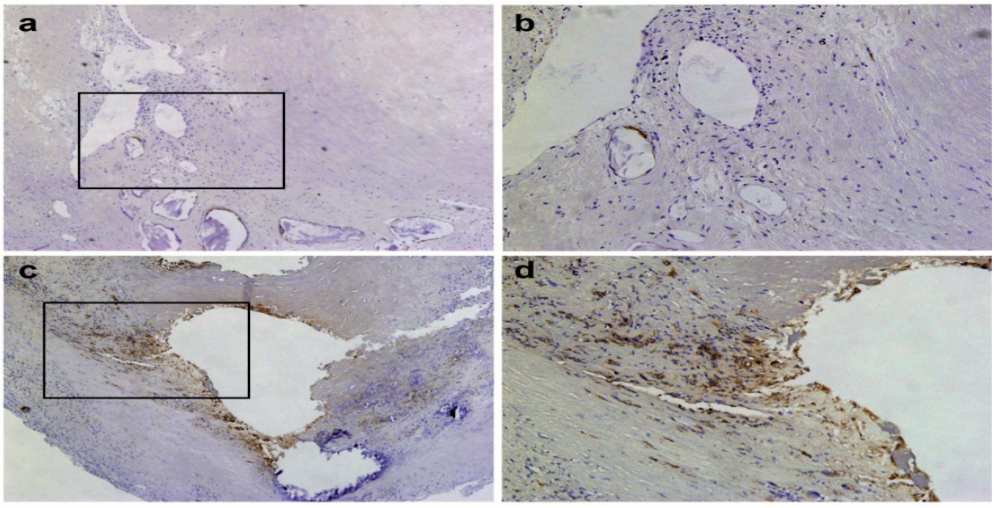

Figure 3. Histology of macrophage numbers in atherosclerotic carotid plaques. Macrophage staining (brown), nucleus staining with haematoxylin (blue). (a) Low macrophage numbers (40x magnification). (b) Higher magnification of the indicated area in (a) (100x magnification). (c) High macrophage numbers (40x magnification). (d) Higher magnification of the indicated area in (c). (100x magnification).

Discussion

In patients referred for percutaneous coronary intervention, we found that high platelet reactivity was associated with high levels of circulating PMCs. In patients undergoing carotid endarterectomy, we observed an association between high platelet reactivity and high levels of macrophages in atherosclerotic carotid plaques. All associations were found to be independent of age, sex or the use of drugs that inhibit platelet activation.

We determined platelet reactivity by measuring P-selectin upregulation after stimulation with increasing concentrations of ADP, using flow cytometry[16]. ADP activates platelets via the P2Y12 and P2Y1 receptors, which have been implicated as important mediators of atherosclerosis[22,23]. Inflamed endothelium does not induce excessive platelet activation, and, similarly, ADP is a mild activator of platelet activation. Moreover, previous work from our department shows that measurement of platelet reactivity towards a mild activator like ADP is more likely to differ between cases and controls than stronger activators like thrombin receptor activating peptide (TRAP), collagen related peptide (CRP) or convulxin (a snake venom toxin that activates platelets)[24]. Hence, we feel that ADP is a suitable agonist that represents in vivo platelet stimulation. P-selectin, which is a component of the platelet α-granules, is translocated from the inner α-granule membrane to the outer platelet cell membrane after platelet activation. Therefore, the expression of P-selectin on the platelet cell membrane is the most widely used platelet activation marker. Hence, we used

P-selectin as a marker of platelet activation.

In contrast to the majority of commercially available platelet function assays that measure platelet aggregation, which have been questioned regarding their sensitivity and reproducibility[25-28], we measure platelet reactivity. Evidence for a role of platelet reactivity in the progression of atherosclerosis was previously shown by infusion of activated platelets into ApoE knockout mice. The infused activated platelets adhered to monocytes, which resulted in increased atherosclerotic lesion size[29]. The mechanism by which platelets play a role in monocyte infiltration depends on the interaction between P-selectin on the platelet membrane and P-Selectin Glycoprotein Ligand-1 (PSGL-1) on the monocyte cell membrane. The binding of P-selectin to PSGL-1 induces the activation of integrins on the monocyte cell membrane, which results in enhanced adhesion of monocytes to inflamed endothelial cells[30,31]. Also, the release of potent inflammatory proteins from the platelet alpha-granule during activation enhances the adhesive and chemotactic properties of endothelial cells[32,33] and the release of monocyte chemoattractants, such as PF4 and RANTES, contributes to the infiltration of monocytes into atherosclerotic plaques[34-36]. The release of alpha-granule proteins is dependent on platelet reactivity towards agonists, but the relationship between platelet reactivity, PMC formation and macrophages in atherosclerotic carotid plaques has not yet been fully investigated. Previously it was shown that levels of circulating PMCs and percentages of platelets expressing P-selectin after stimulation were increased in 19 patients with stable angina compared to 19 healthy control subjects[10]. We extend these results by showing, in a population of 244 coronary artery disease patients, an independent association between high P-selectin expression after ADP stimulation and increased levels of circulating PMCs. Additionally, we show in 91 carotid endarterectomy patients that high P-selectin expression is associated with increased monocyte infiltration into atherosclerotic carotid plaques, independent of investigated covariables.

A limitation of this study is that levels of platelet-monocyte complexes and macrophages in plaques were not assessed in both cohorts, which included patients with different vascular diseases. Therefore we could not directly analyse whether platelet reactivity, levels of platelet-monocyte complexes and numbers of macrophages present in atherosclerotic carotid plaques were directly linked to each other. Nevertheless, similar findings in two different patients groups underscore the potential role of platelet reactivity in cardiovascular disease.

In conclusion, our observations suggest that platelet reactivity plays a role in monocyte infiltration into atherosclerotic plaques. Likewise, reduction of macrophage numbers in atherosclerotic plaques, which contributes to plaque destabilization, may be achieved by inhibition of platelet activation.

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CHAPTER 4

Recombinant erythropoietin has no direct effect on platelet reactivity

Manuscript submitted

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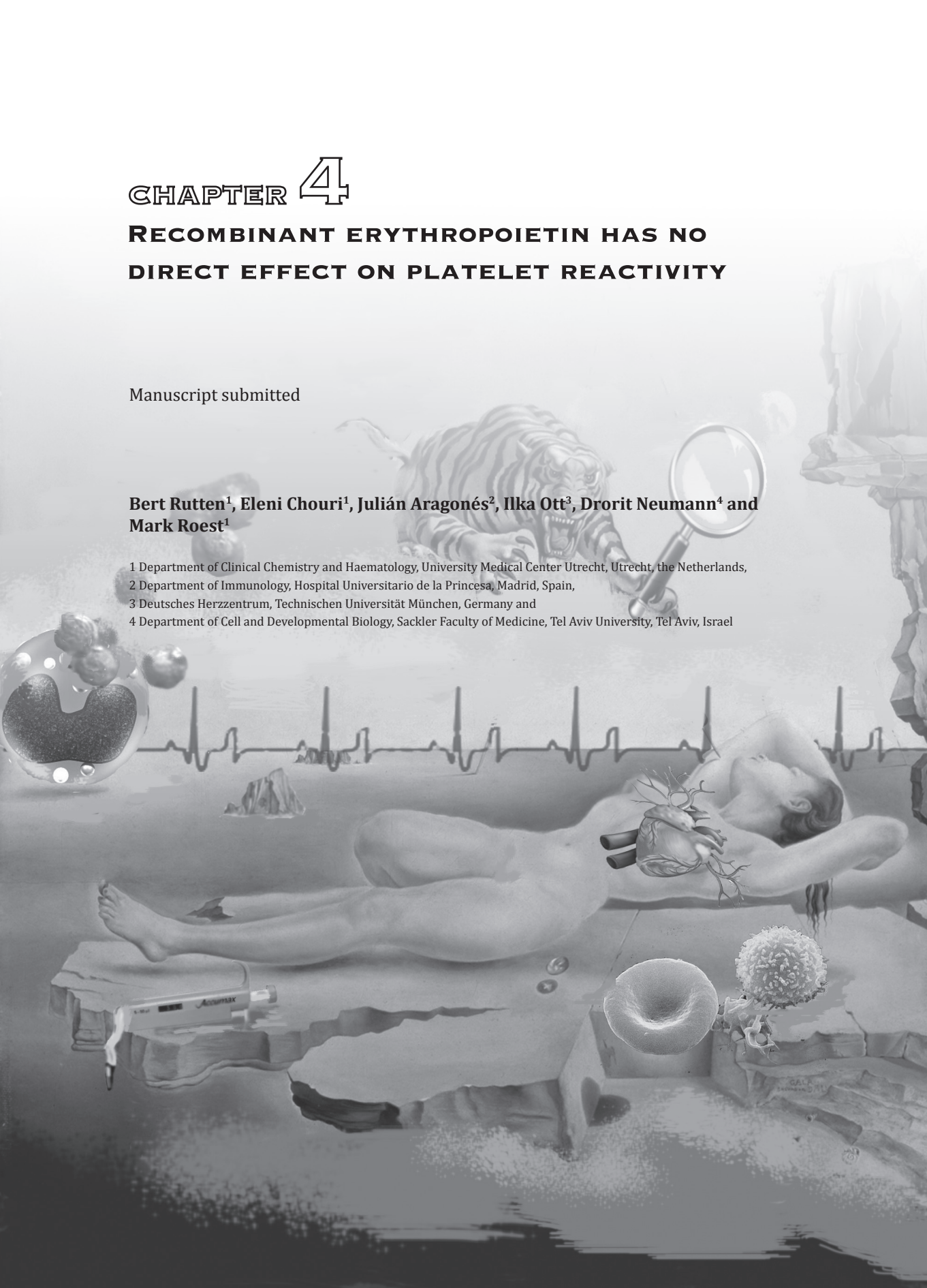


RECOMBINANT ERYTHROPOIETIN HAS NO DIRECT EFFECT ON PLATELET REACTIVITY

Abstract

Background and aim Recombinant forms of human erythropoietin (Epo) are commonly used to raise erythrocyte levels in patients with anemia due to end-stage renal disease or cancer. Recent clinical trials indicate that Epo treatment is associated with increased rates of venous thrombosis and morbidity. Since platelet reactivity is a major determinant of thrombosis, we investigated whether Epo has a direct effect on platelet reactivity. **Methods and results** Whole blood from healthy volunteers was activated for 2, 4, 8, 16, 32 and 64 minutes with the following platelet activators: TRAP-1, TRAP-1 + P2Y12 inhibitor AR-C69931MX, TRAP-4, ADP, CRP, thromboxane analogue U46619 and all agonists combined in the presence or absence of recombinant human Epo (8 U/mL). Using flow cytometry, platelet reactivity was quantified by measuring P-selectin upregulation and fibrinogen binding after agonist induced platelet activation. We observed no differences in P-selectin upregulation (N=10) and fibrinogen binding (N=10) in blood samples that were treated with Epo as compared with blood samples that were not treated with Epo. Next, we used real-time video microscopy to investigate the influence of Epo on platelet aggregate formation during whole blood perfusion over a collagen coated surface. No differences in platelet aggregate formation were observed between blood samples that were treated with Epo as compared with controls (N=5). **Conclusions** Our results show that Epo has no direct effect on platelets in vitro. This suggests that venous thrombosis in patients after Epo treatment is not mediated by a direct effect of Epo on platelets.

4

Introduction

Patients with end-stage renal disease and certain cancers are commonly treated with the recombinant form of human Epo in order to reduce anemia and fatigue[1]. The administration of Epo stimulates the production of erythrocytes, which leads to an increase in hemoglobin levels and improves the quality of life of patients1. However, findings from several clinical trials and meta-analysis studies show an increased risk of venous thrombosis, increased progression of cancer and increased mortality of patients with cancer after treatment with Epo[2-6]. This makes venous thrombosis the second most frequent cause of death among cancer patients, after cancer itself. Previous studies showed that administration of Epo to healthy volunteers increases platelet activation and plasma levels of the platelet activation marker P-selectin[7]. Despite these findings, it remains unclear whether Epo has a direct effect on platelet activation and aggregation or whether there are other factors that explain the association between thrombosis and administration of Epo to patients. Since platelet activation and aggregation are crucial steps in the development of thrombosis, we used a flow cytometry based assay to determine the upregulation of the platelet activation marker P-selectin and binding of the platelet aggregation marker fibrinogen in response to agonists of the major platelet activation pathways, being: the collagen activation pathway using collagen related peptide (CRP), the thrombin activation

pathway using thrombin receptor activated peptide-1 (TRAP-1), TRAP-4 and the protease activating peptide-4 (PAR-4), the adenosine activation pathway using adenosine diphosphate (ADP) in the presence or absence of the ADP pathway inhibitor AR-C69931MX and the thromboxane pathway using the thromboxane analogue U46619.

Considering the association of venous thrombosis with Epo treatment of patients, we questioned whether Epo may directly potentiate or modulate platelet activation and aggregation.

Materials and methods

Epo was purchased from Janssen-Cilag (Tilburg, the Netherlands), Horm collagen from Nycomed (Munich, Germany), silicone Elastomer from Dow Corning (Michigan, USA), Thrombin Receptor Activated Peptide 1 (TRAP-1) peptide from Bachem (Bubendorf, Switzerland), TRAP-4 peptide from the Dutch Cancer Institute (Amsterdam, the Netherlands), Adenosine Diphosphate (ADP) from Sigma (St. Louis, USA), thromboxane analogue U46619 from Santa Cruz Biotechnology (Santa Cruz, USA), Protease Activating Peptide 4 (PAR4) activating peptide AYPGKV was synthesized by the Dutch Cancer Institute (NKI-AVL) in Amsterdam. Collagen Related Peptide (CRP) was a generous gift from Richard W. Farndale (Cambridge, UK), while the P2Y12 inhibitor AR-C69931MX was a kind gift from Astra Zeneca (Loughborough, UK). Mouse anti-human CD62P conjugated to phycoerythrin (PE) was purchased from BD Pharmingen (San Jose, USA), rabbit anti-human fibrinogen conjugated to fluorescein isothiocyanate (FITC) from Dako (Glostrup, Denmark).

Blood collection

Blood was collected into 3.2% sodium citrate tubes, from healthy employees of the University Medical Center Utrecht, The Netherlands. They were all self-reported free of drugs known to affect VWF levels or VWF-platelet binding potential, or any other platelet function inhibiting agents (i.e.: acetylsalicylic acid or clopidogrel). Institutional ethics committee approval was obtained for this study and informed consent was obtained from all participants and all procedures were in accordance with the ethical standards of the Declaration of Helsinki.

Measurement of platelet activation in whole blood

Blood was mixed with either 8 IU/mL Epo or 10 mM HEPES buffered saline (HBS). Next, platelet activation was measured by P-selectin upregulation and fibrinogen binding at baseline and at 2, 4, 8, 16, 32 and 64 minutes after activation with the following platelet activators: 10 μM TRAP-1, or 10 μM TRAP-1 and 5 μM P2Y12 inhibitor AR-C69931MX, or 400 μM TRAP-4, or 31.25 μM ADP, or 153.69 ng/mL CRP, 16 μM thromboxane analogue U46619 or all agonists combined. All samples were fixated using 0.2% formaldehyde in 0.9% natrium chloride buffer and analyzed on the same day of processing. Ten thousand platelets were identified using forward and sideward scatter gating on a flow cytometer FACS Canto II (BD Biosciences). P-selectin and fibrinogen expression on the surface of platelets was assessed based on the median fluorescence intensity (MFI) of PE conjugated to mouse

anti-human CD62P and FITC conjugated to rabbit anti-human fibrinogen. Platelet aggregate formation in whole blood using real-time video microscopy. Whole blood was pre-incubated with physiological concentrations of Epo (8 IU/mL) or 10 mM HBS and perfused into PDMS flow chambers containing collagen coated coverslips at a shear rate of 1600 s-1, using a syringe pump (Harvard Apparatus, Holliston, USA), at a temperature of 37°C. Aggregate formation was visualized at 40x magnification using an Axio Observer Z1 microscope (Carl Zeiss B.V. Germany). Videos were recorded with a CCD camera (Zeiss Axiocam MR3, Germany) during 5 minutes and subsequently edited with AxioVision Rel 4.8 (Carl Zeiss B.V. Germany) before conversion into JPEG format. Surface coverage (pixels) of platelet aggregation was measured at 35, 60, 85, 110, 135, 160, 185, 210 and 260 seconds and quantified with Image-J software.

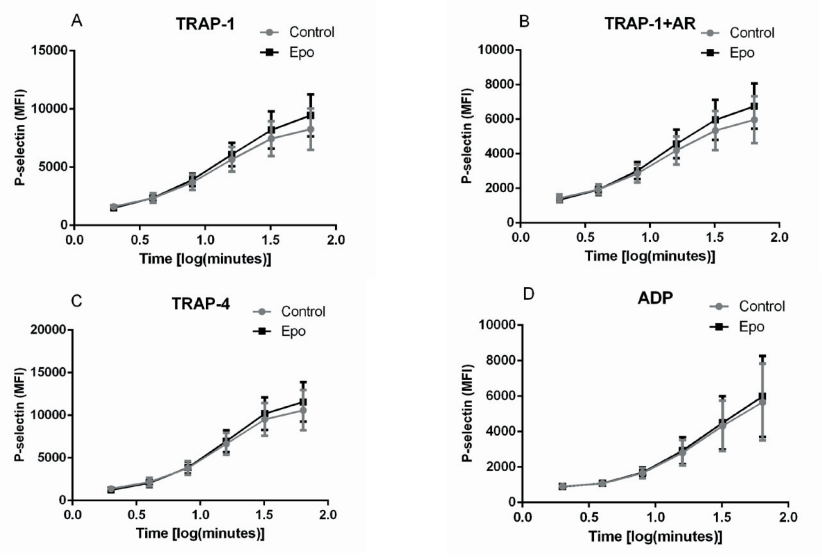
Statistical analysis

Data were presented as means ± standard error of the mean (SEM). Levels of platelet reactivity were compared between groups using the Student t-test. A P-value <0.05 was considered statistically significant.

Results

Epo has no influence on P-selectin expression or fibrinogen binding

We measured whether Epo affected P-selectin expression or fibrinogen binding after activation with TRAP-1, TRAP-1 in the presence of its inhibitor AR-compound, TRAP-4, ADP, CRP, the thromboxane analogue U46619, all agonists together or no agonists. We observed no difference in agonist induced P-selectin expression in Epo treated blood samples as compared with controls (N=10) (Figure 1).



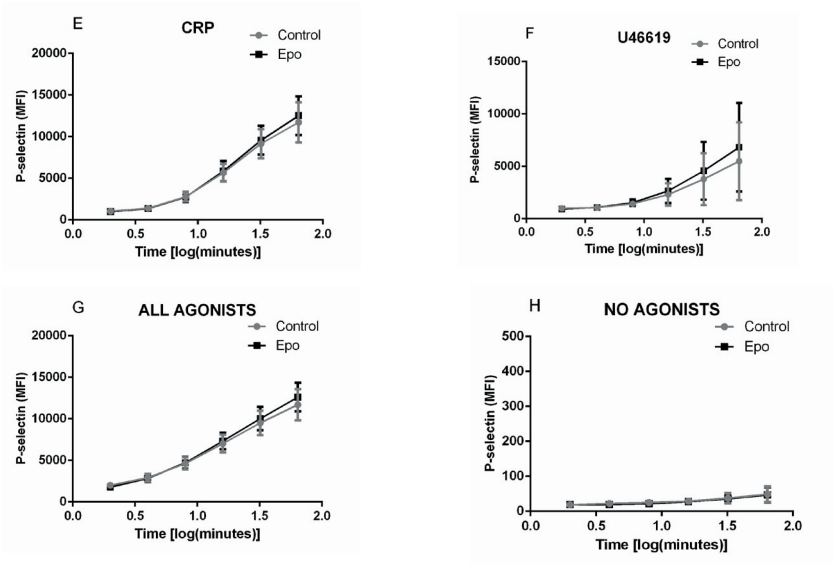
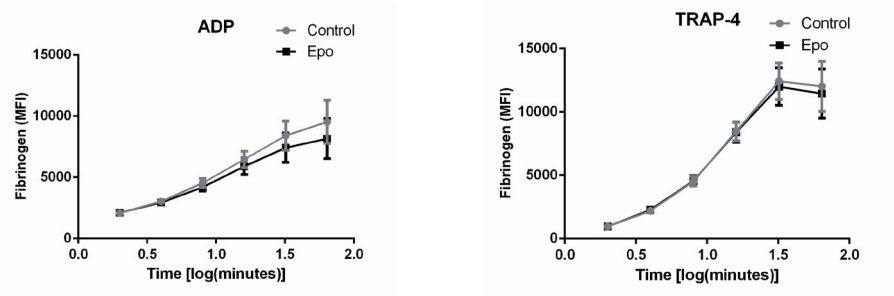


Figure 1. Epo does not affect P-selectin upregulation. Whole blood of healthy volunteers was treated with Epo (8 IU/mL) (black circles) or untreated (open diamonds) (N=10) and platelets were activated with (A) TRAP, (B) TRAP+AR, (C) TRAP4, (D) ADP, (E) CRP, (F) U46619, (G) all the agonists or (H) no agonists. Data is presented as means ± SEM. Groups were compared using unpaired t-test.

We neither observed significant differences in fibrinogen binding to platelets between Epo treated blood samples and controls (N=10) (Figure 2). We were not able to determine fibrinogen binding to platelets after 8 minutes of platelet activation with TRAP-1 in both Epo treated and untreated whole blood samples. Consequently, no data was obtained for fibrinogen binding to platelets after TRAP-1 activation of platelet in the presence of AR (data not shown).



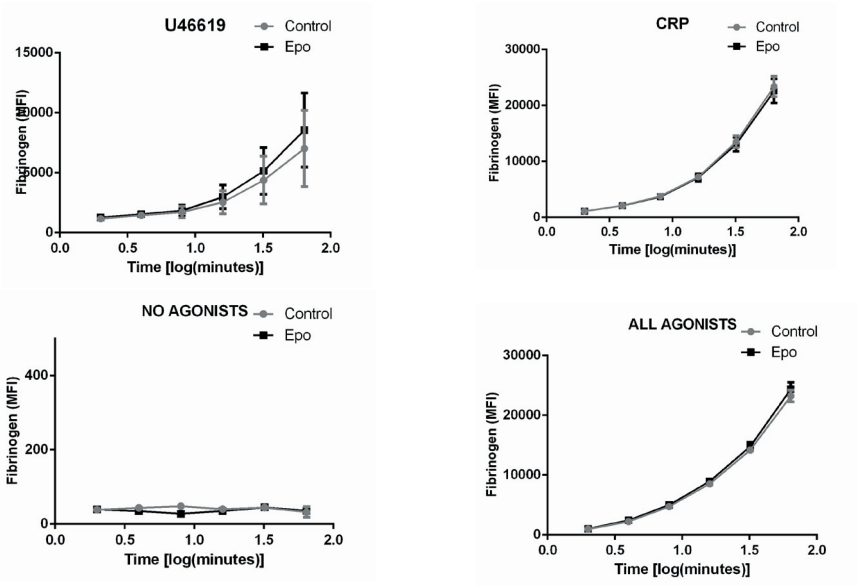
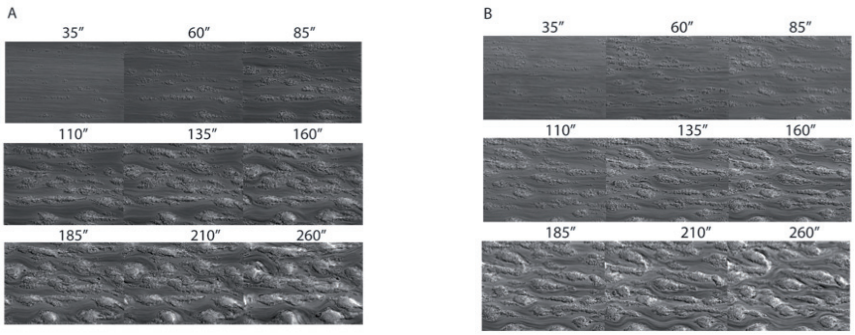


Figure 2. Epo does not affect fibrinogen binding. Whole blood of healthy volunteers was treated with Epo (8 U/mL) (black circles) or untreated (open diamonds) (N=10) and platelets were activated with TRAP-4, ADP, CRP, U46619, all agonist or no agonists. Data is presented as means ± SEM. Groups were compared using unpaired t-test.

Epo has no influence on platelet aggregation ex vivo

Real-time video microscopy was used to study whether Epo treatment affected platelet aggregation during perfusion of whole blood over a collagen coated surface. We observed no differences in platelet aggregation between Epo treated blood as compared with non-treated blood (Figure 3A and 3B). Using video quantification software, we determined that incubation of whole blood with Epo does not affect platelet aggregation under flow as compared with controls (Figure 3C) (N=5).



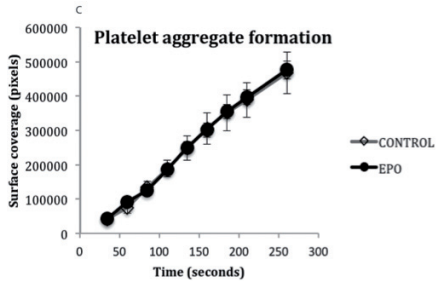


Figure 3. Epo does not affect platelet aggregation. (A) Whole blood was treated with Epo (8 IU/mL) or (B) not treated with Epo and perfused over collagen coated coverslips at a shear rate of 1600s-1(N=5). (C) Aggregate formation was determined using real-time video microscopy analysis. Data is presented as means±SEM. Groups were compared using unpaired t-test.

Discussion

Anemia and fatigue are common complications of cancer and end-stage renal disease. Severe anemia is reduced by the administration of Epo, which increases erythrocyte levels and reduces fatigue, resulting in an improvement of the quality of life of the patient[1]. However, administration of Epo is associated with venous thrombosis in patients with cancer[2-6]. Since platelet activation contributes to the development of venous thrombosis, the relationship between platelet activation and Epo has previously been studied.

These studies show that administration of Epo to healthy volunteers increases platelet activation, platelet counts and plasma levels of P-selectin and E-selectin[7, 8]. These findings demonstrate an in vivo effect of Epo on platelet activation, but it remains unclear whether Epo directly affects platelet activation or whether other factors cause platelet activation. Hence, we studied whether Epo affects P-selectin upregulation or fibrinogen binding after stimulation of the major platelet activation pathways. Furthermore, we used real-time video microscopy to study whether Epo affects platelet aggregation under flow. To study the effects of Epo on platelets, we incubated whole blood with 8 IU/mL of Epo. We based this concentration on previous reports that show optimal ex vivo effects of Epo when used at concentrations ranging between 5 to 10 IU/mL[9-14].

We found that Epo does not affect P-selectin upregulation or fibrinogen binding to platelets. Moreover, Epo treatment of whole blood did not influence thrombus formation during whole blood perfusion over a collagen coated surface. These results indicate that Epo has no direct effect on platelets, which raises the question what other factors might explain the relationship between venous thrombosis and Epo administration. Previous work shows that Epo increases the release of platelets from megakaryocytes in vivo[15], while others demonstrate that Epo increases the differentiation of megakaryocytes in vivo[16]. This suggests that Epo has an effect on megakaryocytes. It has to be noted though, that Epo receptors on megakaryocytes have not been reported. However, megakaryocytes do express thrombopoietin receptors, which share a high level of homology with Epo receptors[17]. Hence, an effect of Epo on megakaryocytes could be mediated through interactions between Epo and thrombopoietin receptors. Other indications that megakaryocytes might be affected by Epo comes from

the observation that platelet reactivity reaches a maximum 9 days after Epo infusion into healthy volunteers[7] and from the finding that circulating platelets have a lifespan of approximately 10 days[18]. This suggests that Epo increases the reactivity of newly synthesized platelets that are released from megakaryocytes. Regardless whether platelets express Epo receptors or thrombopoietin receptors, incubation of whole blood with Epo had no effect on platelet activation and aggregation in this study. Lastly, the finding of increased plasma levels of the endothelial cell activation markers thrombomodulin and von Willebrand factor may suggest a role of the endothelium in platelet activation and aggregation following Epo infusion into healthy volunteers[7].

In conclusion, our study suggests that the association between Epo and venous thrombosis[2-6] is not mediated via a direct effect of Epo on platelets.

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CHAPTER 5

Activated platelets induce IL-10 and TNF-a secretion by monocytes in a human endotoxemia model

Manuscript in preparation

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ACTIVATED PLATELETS INDUCE IL-10 AND TNF-A SECRETION BY MONOCYTES IN A HUMAN ENDOTOXEMIA MODEL

Abstract

Background The outcome after endotoxemia is associated with the intensity of cytokine production, which is partly modulated by binding of activated platelets to monocytes. We studied whether experimental human endotoxemia results in platelet activation, platelet binding to monocytes and whether platelet-monocyte complexes are associated with plasma levels of IL-10 and TNF-α. **Methods and results** Incubation of whole blood with E.coli lipopolysaccharide (LPS) O:111 resulted in the activation of platelets and binding of platelets to monocytes. The binding of activated platelets to monocytes increased IL-10 and decreased TNF-α production as compared with controls. Next, we intravenously administered E.coli LPS O:113 to healthy male volunteers (N=19) and observed platelet activation and platelet binding to monocytes. We confirmed in vitro observations of platelet activation and platelet-monocyte complex formation upon LPS administration. Moreover, we found significant correlations between platelet binding to monocytes and increased levels of IL-10 and TNF-α. **Conclusions** Induced human endotoxemia results in platelet activation and platelet binding to monocytes, which triggers the secretion of IL-10 and TNF-α. Our findings suggest that platelets are regulators of inflammatory responses and modulation of platelet binding to monocytes may improve outcome after endotoxemia.

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Introduction

Platelets circulate in large numbers in the vasculature and they are increasingly acknowledged to play key roles in inflammation and immunity[1]. Following activation, platelets increase the expression of receptors and components that are involved in atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, asthma and sepsis[2-7]. Expression of the activation marker P-selectin facilitates platelet binding to monocytes through the interaction with the constitutively expressed counter receptor P-Selectin Glycoprotein Ligand-1 (PSGL-1)[8]. The engagement of PSGL-1 activates intracellular signaling pathways that result in the expression of cytokines by monocytes[9]. Besides the induction of cytokine expression through cell-cell interaction, platelets also release a myriad of proteins that modulate the inflammatory phenotype of other cells in a paracrine way, i.e.; CXCL4, CCL5, CXCL1 and CXCL4. Moreover, both pro- inflammatory and anti-inflammatory effects of platelets on monocytes have been reported[10,11].

In this study, we investigated the effects of platelet binding to monocytes on the production of anti-inflammatory IL-10 and pro-inflammatory tumor necrosis factor α (TNF-α) by monocytes. Incubation of whole blood with LPS induced platelet activation and binding to monocytes. Also, we observed increased IL-10 and decreased TNF-α production in vitro. Using an experimental human endotoxemia model, we confirmed platelet activation and binding to monocytes after infusion of LPS. Our data suggest that modulation of platelet binding to monocytes may affect cytokine expression.

Materials and methods

Blood
Peripheral blood from healthy employees of the University Medical Center Utrecht, The Netherlands, was collected into 3.2% sodium citrate tubes. All employees were self-reported free of drugs (i.e.: acetylsalicylic acid or clopidogrel) known to affect platelet activation. Institutional ethics committee approval was obtained for this study and informed consent was obtained from all participants and all procedures were in accordance with the ethical standards of the Declaration of Helsinki.

Flow cytometry
Whole blood from healthy employees was incubated with 10 ng/mL E.coli O:111 LPS at 37^{0}C. Cells were labeled by incubation of 50 µL whole blood with 35 µL Hepes buffered saline (HBS), 5 µL CD42b-FITC, 5 µL CD14-PE and 5 µL CD62P-APC for 20 minutes at 37^{0}C. Samples were fixated by addition of 80 µL of Optilyse B (Beckman-Coulter) for 10 minutes at room temperature. Red blood cells were lysed by the addition of 855 µL demineralized water for 15 minutes. Two hundred µL sample was transferred into a 96-well ELISA plate and 10.000 designated events were counted on a FACS Canto II (BD Biosciences). In order to prevent spectral overlap, compensation controls were included and these samples were treated in the same manner as experimental samples.

Isolation of platelets and monocytes
Platelet-rich plasma was harvested from the platelet rich supernatant after whole blood centrifugation at 350 x g for 20 minutes at room temperature. Platelet count was determined using a CellDyn 1800 (Abbott, the Netherlands) and set to 200x10^3/µL. Monocytes were isolated using Ficoll gradient density separation. In short, citrated whole blood was diluted 1:1 with 10mM HBS (pH 7.3) and carefully pipetted onto 15 mL Ficoll-Pague Plus (GE Healthcare) followed by 20 minutes of centrifugation at 400 x g for 30 minutes at room temperature. Monocytes were harvested and washed twice with 10 mM HBS, resuspended in MACS buffer containing 0.5% bovine serum albumin (BSA) and 2mM ethylenediaminetetraacetic acid (EDTA) followed by incubation with CD14+ magnetic micro beads (Miltenyi Biotec, the Netherlands) for 30 minutes on ice. Monocytes were separated from remaining cells by application of a magnetic field using a miniMACS system. Monocyte counts were set at 2.5x10^4/µL using RPMI 1640 medium (Sigma-Aldrich). Counts were verified using a CellDyn1800 and a Bürker-Türk chamber.

Measurement of cytokines
Isolated monocytes (2.5x10^4/µL) were incubated with 10 ng/mL E.coli O:111 LPS or 10mM HBS in the presence or absence of platelets (50x10^4/µL) for 20 hours at 37^{0}C and 5% CO^2. Levels of IL-10 and TNF-α were determined using ELISA cytokine kits (Boster, Fremont, USA) according to manufacturer's instructions.

Experimental human endotoxemia

The study was approved by the Ethics Committee of the St. Radboud University Nijmegen Medical Center and complied with the declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all healthy male volunteers, who participated in experiments that were part of several endotoxin trials (NCT01374711, NCT01835457; www.clinicaltrials.gov). Only control subjects not submitted to additional drugs or behavioral conditions, but injected with E. Coli 113:H 10:K LPS (US reference E. coli endotoxin (lot Ec-5; Center for Biologic Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA) were included. Endotoxin was reconstituted in 5 ml glucose/saline and injected as a single intravenous bolus of 2ng/kg during 1 minute at t = 0. Blood was collected from the arterial sheath into 3.2% tri-sodium citrate tubes (Greiner Bio-One). Exclusion criteria were use of any medication, smoking, use of alcohol, caffeine, drugs within 21 days prior to study, participation in a previous LPS administration trial, surgery or trauma 3 months prior to study, history or symptoms of: cardiovascular disease, frequent vaso-vagal collapse, orthostatic hypotension, arrhythmia, hypertension or hypotension, Conduction abnormalities on the ECG consisting of a 1st degree atrioventricular block or a complex bundle branch block, renal impairment, liver function abnormality, History of asthma, obvious disease associated with immune deficiency, C-reactive protein levels above 20 mg/L, white blood cells count above $12x10^9$/L or clinically significant acute illness, including infections, within 4 weeks prior to study.

Statistical analysis

Data comparison was done using the Student t-test. Correlations between cytokine levels and levels of platelet-monocyte complexes were investigated with Spearman correlation. All statistical analysis was performed using SPSS 20.0 software (IBM Corporation) and graphs were made with Prism 6 software (Graphpad Software). A P-value below 0.05 was considered significant.

Results

LPS incubation leads to platelet activation and binding to monocytes, in vitro

To study the effect of LPS on platelet activation and platelet-monocyte complex formation, we incubated whole blood with 10 ng/mL of E.coli LPS (N=7). Using flow cytometry, we observed a significant increase in platelet binding to monocytes after 1 hour of LPS incubation (Figure 1A) as compared with controls. Also, we observed a significant increase in P-selectin expression on monocytes (Figure 1B). Unbound platelets did not show significant P-selectin upregulation after 1 hour, but were observed to be activated after 4 hours (figure 1C).

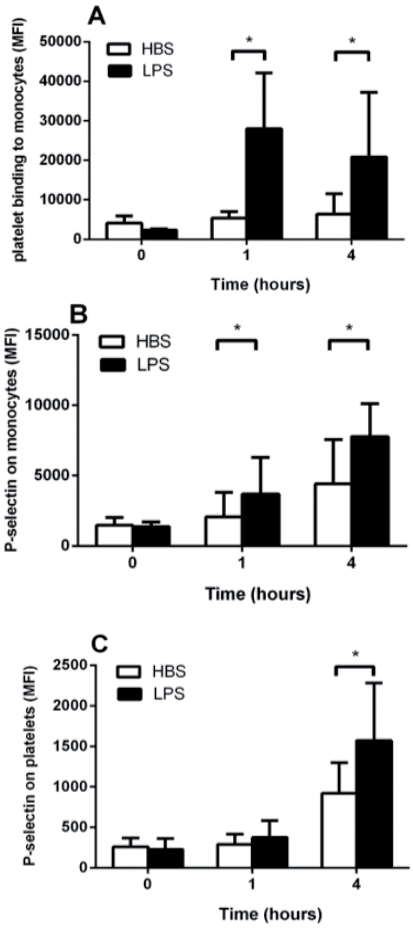

Figure 1. LPS increases platelet activation and binding to monocytes, in vitro. Incubation of whole blood with 10mM HBS or 10 ng/mL E.coli LPS (N=7) resulted in binding of platelets to monocytes (Figure 1A), presence of activated platelets on monocytes (Figure 1B) and presence of unbound activated platelets (Figure 1C). Values represent mean ± standard deviations. *P-value<0.05 using Student t-test.

Platelets increase IL-10 and decrease TNF-α production by monocytes

Monocytes from healthy donors (N=5) were incubated with HBS or E.coli LPS in the presence or absence of platelets. In agreement with previous results[11], we observed a strong increase in IL-10 (Figure 2A) and a reduction in TNF-α (Figure 2B) production when monocytes were cultured in the presence of platelets as compared without platelets. Incubation of platelets with buffer or E.coli LPS did not result in IL-10 or TNF-α production.

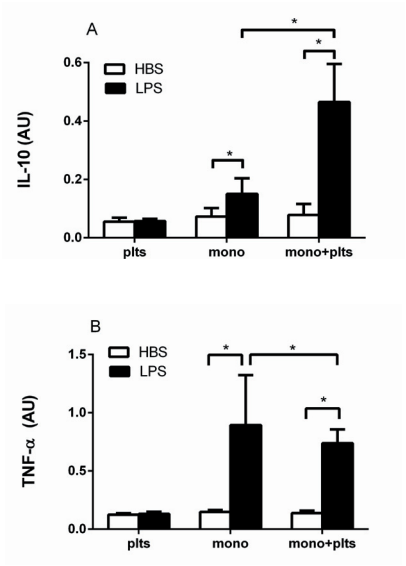

Figure 2. Platelets increase IL-10 and reduce TNF-α production, in vivo. Cells isolated from healthy volunteers (N=5) were incubated with 10mM HBS or 10 ng/mL E.coli LPS. After 20 hours levels of IL-10 (Figure 2A) or TNF-α (Figure 2B) were determined. Values represent mean ± standard deviations. *P-value<0.05 using Student t-test.

Human endotoxemia leads to platelet activation and binding to monocytes

We investigated whether LPS induces platelet activation and platelet-monocyte complex formation in vivo. Herefore, we intravenously injected 2 ng/mL E.coli

LPS into healthy male volunteers in a research intensive care unit. We found significant increased platelet binding to monocytes 1 hour after LPS challenge, and a return to baseline levels after 4 hours (Figure 3A). Monocytes were positive for the platelet marker P-selectin 1 hour after E.coli LPS challenge followed by a return to baseline levels after 4 hours, indicating that monocyte bound platelets were activated (Figure 3B). In contrast to our in vitro results, circulating platelets were only slightly activated 1 hour after E.coli LPS challenge (Figure 3C).

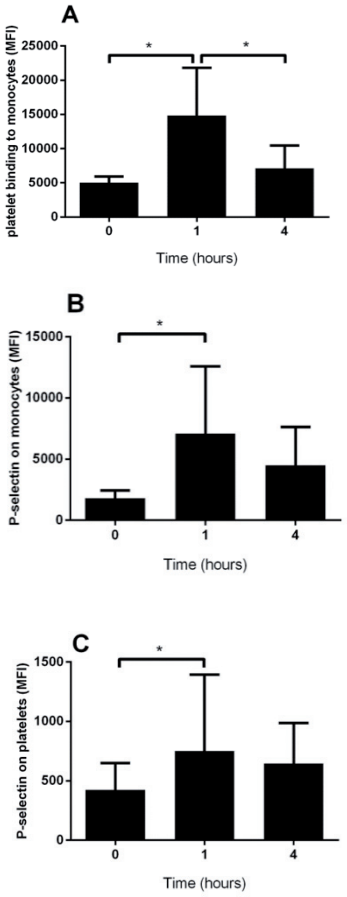

Figure 3. Human endotoxemia leads to platelet activation and binding to monocytes. Infusion of 2 ng/kg E.coli LPS (N=19) resulted in binding of platelets to monocytes (Figure 3A), presence of activated platelets on monocytes (Figure 3B) and presence of unbound circulating activated platelets (Figure 3C). Values represent mean ± standard deviations. *P-value<0.05 using Student t-test.

Kinetics of cytokine expression levels

To determine the kinetics of cytokine expression, we measured plasma levels of IL-10 and TNF-α every 30 minutes for 7 hours. We observed a similar increase in both IL-10 (Figure 4A) and TNF- α (Figure 4B) levels after 30 minutes. However, IL-10 levels peaked 3 hours after E.coli LPS challenge and TNF-α levels 90 minutes after E.coli LPS challenge. Levels of IL-10 were strongly reduced after 4 hours, while TNF-α levels gradually decreased after 1.5 hours.

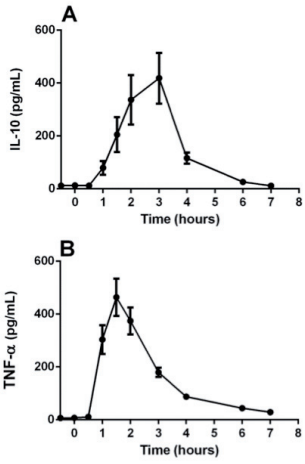

Figure 4. Kinetics of IL-10 and TNF-α during human endotoxemia. Plasma levels of IL-10 gradually increased in the first 3 hours after LPS administration, while plasma levels of TNF-α rapidly increased in the 1.5 hours. Values represent mean ± standard deviations.

IL-10 and TNF-α levels correlate with platelet binding to monocytes
Since platelet binding to monocytes modulates cytokine expression in vitro[9], we investigated whether platelet binding to monocytes correlates with plasma levels of IL-10 and TNF-α in a human endotoxemia model (N=19). Indeed, platelet binding to monocytes was strongly associated with IL-10 and TNF-α expression.

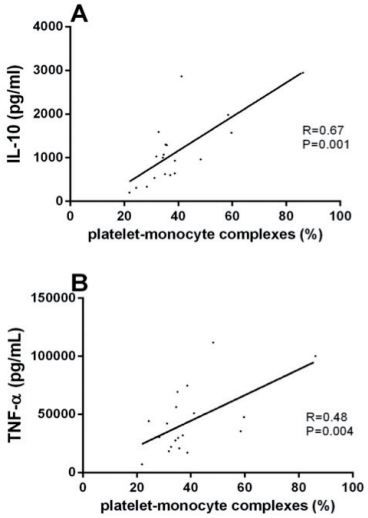

Figure 5. Platelet binding to monocytes correlates with increased IL-10 and TNF-α levels. Strong positive correlations were observed between the percentage of platelet-monocyte complexes and plasma levels of IL-10 (Figure 5A) and TNF-α (Figure 5B). The correlation was assessed using Spearman's correlation.

Discussion

Endotoxemia is a major cause of morbidity and mortality of hospitalized patients. The presence of gram negative bacteria derived LPS triggers a systemic inflammatory response that involves increased secretion of cytokines into the circulation. In vitro studies have shown that platelet P-selectin mediated binding to monocytes induces the expression of pro-inflammatory MCP-1 and IL-8[9, 12] and recent in vitro work also shows association between platelet binding to monocytes with IL-10 and TNF-α levels in a CD40 ligand dependent pathway[11]. These studies show that platelets have an eminent effect on cytokine production by monocytes in vitro. In vivo, reduced levels of circulating platelets are markedly associated with increased pro-inflammatory responses and mortality in mice and septic patients[13]. However, the effect of platelet activation and binding to monocytes on cytokine production during human endotoxemia has not yet been fully investigated. In the present study, we used a human endotoxemia model to investigate whether 2ng/kg E.coli LPS O:113 administration to healthy male volunteers induces platelet activation, platelet binding to monocytes and whether platelet-monocyte complexes are associated with levels of anti-inflammatory IL-10 and pro-inflammatory TNF-α. In vitro experiments showed increased platelet activation and binding to monocytes upon incubation of whole blood with E.coli LPS. In line with previous studies[11], we found increased levels of IL-10 and decreased levels of TNF-α upon incubation of monocytes with platelets. Hence, we investigated platelet activation and binding to monocytes in relationship to IL-10 and TNF-α production following infusion of 2 ng/mL of E.coli LPS into healthy male volunteers. We observed platelet activation and

binding to monocytes 1 hour after LPS administration. In contrast to previous and our own in vitro findings, we observed no relationship between high levels of activated platelet binding to monocytes with low levels of pro-inflammatory TNF-α. Both anti-inflammatory IL-10 as well as pro-inflammatory TNF-α levels were positively correlated with levels of platelet-monocyte complexes during human experimental endotoxemia.

Besides monocytes, numerous other cell types are major sources of TNF-α. Both endothelial cells and liver Kupffer cells are activated during the systemic inflammatory host response and have been shown to release TNF-α following LPS challenge[14,15]. These findings may suggest that platelets may reduce TNF-α production in vitro in a IL-10 dependent way, but the anti-inflammatory effects of platelets do not result in lower levels of TNF-α in vivo due to the release of TNF-α by endothelial and liver cells during endotoxemia.

A limitation of this study should be mentioned. Even though we observe strong positive correlations between levels of platelets-monocyte complexes with cytokine levels, we cannot exclude in this observational study that other factors also may contribute to increased cytokine production.

In summary, we show evidence that platelets are activated and bind to monocytes following infusion of low doses of E.coli LPS into healthy volunteers. The binding of activated platelets to monocytes was strongly associated with increased levels of circulating platelet-monocyte complexes and indicate that platelet activation is a mediator of the systemic inflammatory response during endotoxemia.

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CHAPTER 6

THE PLATELET DENSITY PER MONOCYTE IS A NOVEL PREDICTOR OF CARDIAC EVENTS IN PATIENCE AFTER PERCUTANEOUS CORONARY INTERVENTION

Manuscript submitted

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THE PLATELET DENSITY PER MONOCYTE IS A NOVEL PREDICTOR OF CARDIAC EVENTS IN PATIENTS AFTER PERCUTANEOUS CORONARY INTERVENTION

Abstract

Aims Despite advances in the treatment of patients with coronary artery disease, a large percentage of patients experience recurrent adverse cardiac events. Monocyte recruitment to the endothelium reduces the recurrence of adverse cardiac events. Since monocyte recruitment is mediated by platelets, we studied whether the platelet density per monocyte affected monocyte recruitment and was associated with the recurrence of adverse cardiac events in patients after percutaneous coronary intervention (PCI). **Methods and results** Monocytes with high and low median fluorescence intensities (MFI) of the platelet marker CD42b were isolated using cell sorting. Microscopic analysis revealed an increase in the platelet density when high platelet marker MFI monocytes were compared with low platelet marker MFI monocytes (3.4±0.7 vs. 1.4±0.1 platelets per monocyte, P=0.01). Using real-time video microscopy, we observed that a high platelet density per monocyte increased monocyte binding to activated endothelial cells as compared with monocytes with a low platelet density (mean±SD; 24±6.5 vs. 8±2.3 cells per microscopic field, P=0.01). In patients after PCI (N=263), a high platelet density per monocyte was independently associated with a 2.5-fold reduced recurrence of adverse cardiac events as compared with a low platelet density per monocyte [hazard ratio=0.4 (95% confidence interval, 0.2-0.8), P=0.01]. **Conclusions** This study shows that the platelet density per monocyte is a mechanism of monocyte recruitment to endothelial cells and a novel predictor of adverse cardiac events in patients after PCI. Our findings imply that a high platelet density per monocyte protects against recurrence of adverse cardiac events.

Introduction

In atherosclerotic patients with coronary artery disease, percutaneous coronary intervention (PCI) followed by stent implantation is the standard procedure for preventing occlusive thrombosis and recurrence of adverse cardiac events. However, this procedure also causes local vascular damage and is associated with a high risk of restenosis[1,2], while repair of the vasculature reduces the risk of restenosis and recurrence of adverse cardiac events. In recent years it has become clear that monocytes are crucial mediators of vascular repair[3]. Following vascular damage, circulating CD14+ monocytes are recruited to the vascular wall under the influence of vascular endothelial cell growth factor (VEGF) or monocyte chemoattractant protein-1 (MCP-1)[4-6]. Previous work shows that monocyte subsets with different functions are recruited after vascular damage. Initially, monocytes are recruited that are involved in phagocytosis of cellular debris, degradation of the extracellular matrix and expression of proinflammatory cytokines. In the second phase, monocytes are recruited that promote myocardial healing via myofibroblast accumulation, enhanced angiogenesis and deposition of collagen[7,8]. Besides the recruitment of monocytes that are involved in vascular healing, monocytes are also able to differentiate into myeloid endothelial progenitor cells (mEPC), that facilitate vascular repair by enhancing endothelial

re-growth and reducing restenosis[9,10]. Previously it was shown that mEPC can be cultured from monocytes in VEGF rich culture media. When these cultured cells are transplanted into mice with ischemic hind limbs they augment reperfusion by stimulating angiogenesis[11]. Lastly, a relation between monocytes and vascular repair is shown by impaired wound healing in atherosclerotic mice after depletion of monocytes[12].

Following vascular damage, platelets are exposed to the highly thrombogenic subendothelial matrix, which results in platelet activation and exocytosis of α-granules. Alpha granules are the most numerous platelet granules and they contain proteins that are involved in angiogenesis and vascular repair[13]. Moreover, the exocytosis of α-granules causes the translocation of the adhesion receptor P-selectin from the inner granule membrane to outer cell membrane, allowing interaction with the constitutively expressed monocyte receptor P-Selectin Glycoprotein Ligand 1 (PSGL-1)[14]. These interactions result in the formation of platelet-monocyte complexes that enhance the recruitment of monocytes to activated endothelial cells[15-17].

Since platelet binding to monocytes enhances the recruitment of monocytes to activated endothelial cells and both platelets and monocytes contribute to angiogenesis and vascular repair, we studied whether the platelet density per monocyte affects monocyte recruitment and whether the platelet density per monocyte is associated with the recurrence of adverse cardiac events in patients after PCI induced vascular damage. Using fluorescence-activated cell sorting and real-time video microscopy, we found that a high platelet density per monocyte increased monocyte recruitment to endothelial cells under flow as compared with a low platelet density per monocyte. In line with previous results, we found that monocytes and platelet bound monocytes increased endothelial cell re-growth. In patients after PCI, we observed that a high platelet density per monocyte was independently associated with a decreased recurrence of adverse cardiac events.

Materials and methods

Materials

For flow cytometry, we used mouse anti-human CD14 antibodies (555398) conjugated to phycoerythrin (PE) and mouse anti-human CD42b antibodies (555472) conjugated to fluorescein isothiocyanate (FITC) were purchased from BD Biosciences. For immunofluorescence microscopy, we used rabbit anti-human CD42b was purchased from Abnova (MAB0943), mouse anti-human CD14 from DAKO (M0825), goat anti-rabbit AF555 (A-21430) and goat anti-mouse AF488 (A-11034) were purchased from Invitrogen. Phorbol 12-myristate 13-acetate (PMA) and gelatin were acquired from Sigma-Aldrich. Graphs were made with Prism 5.01 software from Graphpad Software and statistical analysis were performed using SPSS 20.0 software.

Blood collection

All research in this study was approved by the ethics committees of the participating medical centers and followed the Declaration of Helsinki. All participants

provided written informed consent prior to participation. Blood from patients was drawn from the sheath catheter into citrate (3.2%) anticoagulated tubes (BD Biosciences) directly after insertion and before intervention or administration of any anticoagulants. Blood from healthy volunteers, who did not ingest platelet inhibitors for at least ten days prior to blood collection, was drawn from the median cubical vein into citrate (3.2%) anticoagulated tubes (BD Bioscienes). All samples were processed directly after collection to prevent any potential blood storage effects.

Fluorescence activated cell sorting

Whole blood was labeled with antibodies against the monocyte marker CD14 conjugated to PE and the platelet marker CD42b conjugated to FITC for 30 minutes at room temperature, followed by fixation with 4% paraformaldehyde (pH 7.4) for 20 minutes at room temperature. Monocytes, including platelet bound monocytes, were isolated using Ficoll density gradient centrifugation and the Influx flow cytometer (Becton Dickinson, Belgium) was used to separate two fractions of monocytes with high and low platelet marker MFI in PBS. Fluorescent 100nm and 200nm polystyrene beads (FluoSpheres, Invitrogen) were used for calibration of the Influx flow cytometer and samples were measured at a maximum event rate of 10.000 events per second. Monocyte counts were determined using a CellDyn 1800 (Abbott) and set to a concentration of $1.0x10^6$/mL, which was verified using a Bürker-Türk counting chamber.

Immunofluorescence microscopy

Whole blood was labeled with antibodies against the monocyte marker CD14 conjugated to PE and the platelet marker CD42b conjugated to FITC for 30 minutes at room temperature, followed by fixation with 4% paraformaldehyde (pH 7.4) for 20 minutes at room temperature. Here after, red blood cells were lysed by hypotonic shock by the addition of demineralized water. Cells were attached to glass by cytospin centrifugation and embedded overnight with DAPI prolonged gold (Invitrogen). The number of platelets bound per monocyte was determined using fluorescence and differential interference contrast (DIC) microscopy.

For obtaining representative immunofluorescence images, monocytes were isolated using were isolated using Ficoll density gradient centrifugation and suspended in PBS. Cells were blocked with 10% normal goat serum in PBS containing 0.1% bovine serum albumin (0.1% PBSA) followed by incubation with rabbit anti-human CD42b and mouse anti-human CD14 antibodies in 0.1% PBSA, for 30 minutes at room temperature. Cells were washed three times with 0.1% PBSA for 5 minutes and subsequently incubated with secondary goat anti-rabbit antibodies conjugated to AF555 and goat anti-mouse antibodies conjugated to AF444 in 0.1% PBSA. Excess antibodies were removed by washing three times with 0.1% PBSA and once with demineralized water before overnight embedment with DAPI prolonged gold (Invitrogen). Platelet bound monocytes were visualized with an inverted microscope (Carl Zeiss Axio Observer Z.1) using a 63x/1.25 oil EC-plan Neofluar objective and AxioVision

software (Release 4.6, Carl Zeiss).

Real-time video microscopy of monocyte adhesion to HUVEC under flow

Human umbilical vein endothelial cells (HUVEC) were freshly isolated from human umbilical cords by incubation with a Trypsin-EDTA solution (Invitrogen) for 15 minutes at 37^0C. HUVEC were harvested and centrifuged for 5 minutes at 350xg. The supernatant was removed and the cell pellet was resuspended in endothelial growth medium-2 (EGM-2, Lonza, USA). HUVEC were cultured until passage 3. Upon reaching full confluence, cells were trypsinized and seeded on sterile coverslips and allowed to grow until full confluence, Here after, HUVEC were activated with 100nM phorbol myristate acetate (PMA) for 60 minutes at 37^0C followed by transfer of the coverslips to polydimethylsiloxane (PDMS) perfusion chambers. Monocytes with high and low platelet densities and monocytes with no bound platelets were perfused over the HUVEC at a shear rate of 250 sec^{-1} for 10 minutes. Movies and snapshots were recorded with an inverted differential interference contrast (DIC) imaging microscope (Carl Zeiss Axio Observer Z.1) using a 10x or 40x Neofluar objective. Monocyte recruitment was quantified using blood of four different donors.

Endothelial re-growth assay

A sterile 96-wells plate was coated with 0.5% gelatin for 2 hours at 37^0C and washed two times with PBS. Cell seeding stopper (Platypus Technologies) were added to each well, followed by addition of HUVEC at a concentration of $5x10^4$ cells per well in EGM-2 medium containing 2.5% fetal bovine serum (FBS). HUVEC were allowed to attach to the bottom of the wells for 4 hours before removal of the cell seeding stoppers, except in the first well that was used as negative control. Unbound HUVEC were removed by gentle rinse and fresh EGM-2 medium containing 0.5% FBS was added. HUVEC were allowed to close the artificial wound area during 20 hours, either in the presence of $2.5x10^4$ monocytes, $5.0x10^5$ platelets or $2.5x10^4$ platelet bound monocytes that were acquired by activation of $5.0x10^5$ platelets with 625 μM TRAP-6 for 20 minutes, followed by centrifugation for 10 minutes at 2000g and suspension in PBS containing $2.5x10^4$ monocytes. After 20 hour culturing, wells were washed with PBS, fixated with 4% paraformaldehyde for 20 minutes and 100% methanol for 5 minutes, stained with May-Grünwald for 5 minutes and Giemsa for 20 minutes. Endothelial re-growth was visualized using an inverted light microscope (Carl Zeiss) with a 25x objective and ImageJ software was used to quantify the amount of HUVEC that migrated into the wound area. The well that contained the cell seeding stopper, during the 20 hours of culturing, was used as reference point. All other wells were compared to this negative endothelial re-growth area.

Study design

A detailed description of the study design has previously been published[18]. All materials used in the cohort were centrally distributed from the UMC Utrecht to ensure optimal comparability and reproducibility of measurements and prevent variation between the clinical centers. The included

study population consisted of 263 patients with coronary artery disease, who underwent PCI in four medical centers in the Netherlands: Catharina Hospital in Eindhoven, University Medical Center in Maastricht, University Medical Center in Leiden and the University Medical Center in Utrecht. Exclusion criteria were ST-segment elevation myocardial infarction (STEMI), an age below 18, suspected drug or alcohol abuse, serious comorbidities, serious infectious disease 6 weeks prior to inclusion or suspected elevated immune status. Patients were followed-up for a period of 9 months after intervention for the major adverse cardiac events (MACE) composite end point, which consisted of myocardial infarction, another PCI, coronary artery bypass grafting or cardiovascular death.

Quantification of platelet binding to monocytes in cohort samples

We diluted 50 µL of citrated blood with 40 µL PBS. Monocytes were labeled with 5 µL mouse anti-human CD14-PE and platelets were labeled with 5 µL mouse anti-human CD42b-FITC, for 30 minutes. Triplicate samples were fixated for 10 minutes with 80 µL of Optilyse B (Beckman Coulter), containing 3.4% paraformaldehyde, after which hypotonic red blood cell lysis was achieved by the addition of 825 µL of demineralized water. Monocytes were identified by scatter gating and CD14 labeling. The platelet density per monocyte was quantified by the MFI of the platelet marker CD42b-FITC on the surface of monocytes. Platelet-monocyte complexes were identified by the % of monocytes positive for CD42b. Each center was equipped with a centrally calibrated FC500 flow cytometer from Beckman Coulter and 15.000 cells were counted and subsequently analysed with the Beckman Coulter SXP SYSTEM.

Statistical analysis Platelet density and platelet-monocyte complex values were equalized among the 4 centers by the median of medians normalization method. The normalized data was grouped and transformed into natural logarithmic values so that assumptions of normality were met. Continuous values for the platelet density per monocyte and platelet-monocyte complexes were categorized into tertiles. We performed Kaplan-Meier analysis to investigate the association between the platelet density per monocyte or the percentages of platelet-monocyte complexes with the incidence of MACE after PCI. Cox proportional hazard regression models were used to calculate the hazard ratios and we adjusted for age, sex, smoking, hyperlipidemia, hypertension, diabetes mellitus, clopidogrel, aspirin and disease severity at inclusion, being: stable angina, unstable angina and non-STEMI (nSTEMI). Differences between groups were examined using paired student t-test. A P-value below 0.05 was considered statistically significant.

Results

Platelet marker MFI corresponds with platelet density per monocyte

We used fluorescence-activated cell sorting to separate monocytes with high platelet marker MFI from monocytes with low MFI (Figure 1).

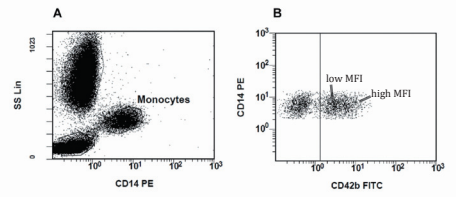
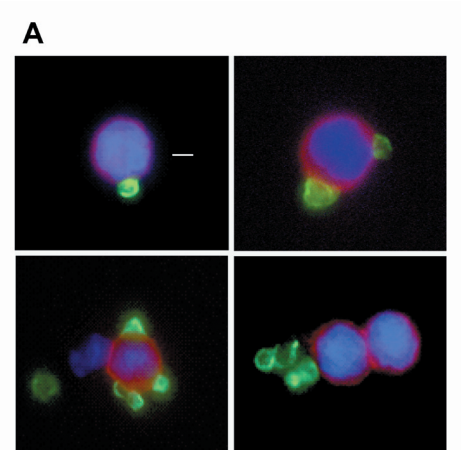


Figure 1. Fluorescence-activated sorting of monocytes. (A) Monocytes were identified based on scatter gating and CD14 labeling. (B) Monocytes with high platelet marker CD42b-FITC median fluorescent intensities (MFI) (MFI>8.0) and low platelet marker CD42b-FITC MFI (MFI<2.0) were isolated using fluorescence activated cell sorting.

Microscopic analysis revealed that monocytes with a high platelet marker MFI had more platelets bound per monocyte as compared with monocytes with a low platelet marker MFI (mean±SD; 3.4±0.7 vs. 1.4±0.1 platelets per monocyte, P=0.01) (N=4) (Figure 2).



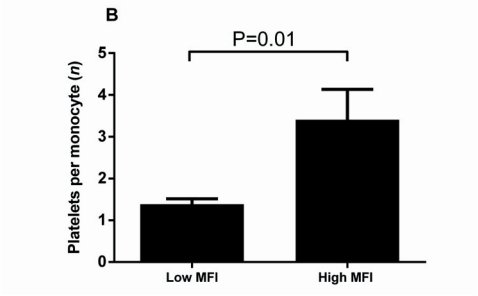
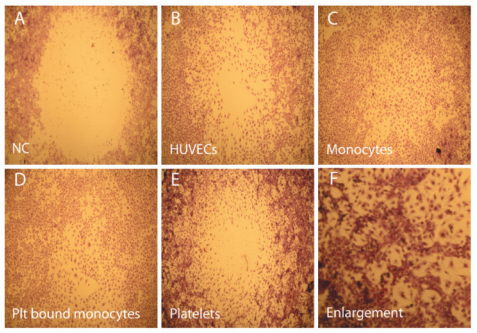


Figure 2. Platelet marker MFI per monocyte corresponded with platelet density per monocyte. (A) Representative immunofluorescence images of monocytes with high and low platelet densities. Monocyte (CD14+) in red, platelet (CD42b+) in green and nuclei (DAPI) in blue. (B) Quantification of the number of platelets bound per monocyte revealed that monocytes with high platelet marker MFI had more platelets bound per monocyte as compared with monocytes with low platelet marker MFI. Data are from 4 independent donors (mean±SD). Differences between groups were examined using paired student t-test.

Culturing of endothelial cells with monocytes increased re-growth

In line with previous studies[19-21], we observed increased endothelial re-growth when HUVEC were cultured with monocytes as compared without monocytes (mean±SD; 98.5±1.4 vs. 76.3±3.7% of re-growth, P<0.0001) (N=4) (Figure 3B-C and G). We obtained similar results when HUVEC were cultured in the presence of platelet bound monocytes as compared without monocytes (mean±SD; 95.4±4.1 vs. 76.3±3.7% of re-growth, P=0.001) (N=3) (Figure 3B-D and G), while culturing of HUVEC in the presence of platelets did not result in increased endothelial re-growth as compared without monocytes (N=4) (Figure 3B-E and G). However, HUVEC cultured in the presence of platelets displayed a phenotype that resembled in vitro angiogenesis (Figure 3F).



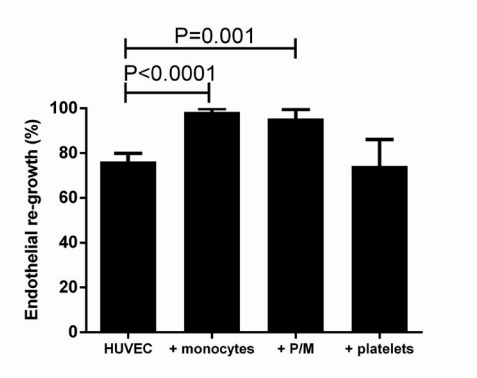


Figure 3. Monocytes and platelet bound monocytes increased endothelial re-growth. Human umbilical vein endothelial cells (HUVEC) were seeded in the presence of a cell stopper. After 2 hours , HUVEC were cultured in the presence of $2.5x10^4$ monocytes, $5.0x10^5$ platelets or $2.5x10^4$ platelet bound monocytes. May-Grunwald Giemsa staining was used to assess endothelial re-growth. (A) Negative control was set as wound area. (B) HUVEC cultured for 20 hours, (C) HUVEC cultured with monocytes, (D) HUVEC cultured with platelet bound monocytes or (E) HUVEC cultured with platelets, (F) Enlargement of the indicated area in (E). (G) Quantification showed significant increase in endothelial re-growth when HUVEC were cultured with monocytes or platelet bound monocytes. Data are from at least 3 independent donors (mean±SD). Differences between groups were examined using paired student t-test. P/M= platelet bound monocytes.

High platelet density per monocyte increased monocyte recruitment to HUVEC

To study whether a high platelet density per monocyte increases monocyte recruitment to endothelial cells, we used real-time video microscopy under flow to visualize the perfusion of monocytes with a high platelet density or a low platelet density over PMA (100nM) activated HUVEC (Figure 4A). We quantified the recruitment of monocytes and found that a high platelet density per monocyte significantly increased monocyte recruitment to activated HUVEC as compared with monocytes with a low platelet density (mean±SD; 24±6.5 vs. 8±2.3 cells per microscopic field, P=0.01) (N=4) or monocytes with no adherent platelets (mean±SD; 24±6.5 vs. 1.6±2.1 cells, P=0.01) (N=4) (Figure 4B) (video 1 and video 2). We also observed increased recruitment of monocytes with a low platelet density as compared to monocytes with no adherent platelets (mean±SD; 5.9±3.7 vs. 1.6±2.1 cells, P=0.04) (N=4).

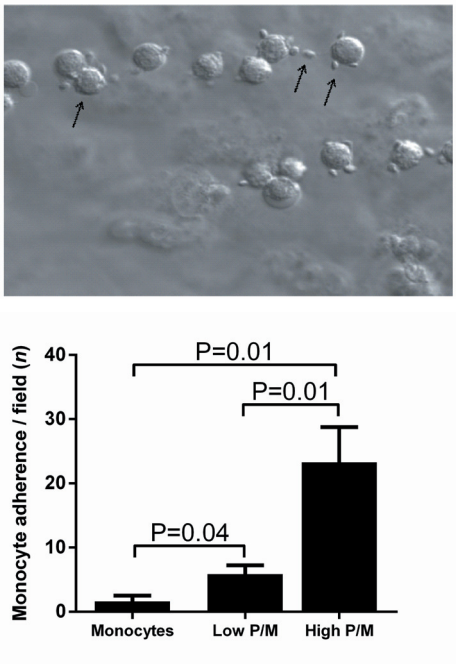


Figure 4. High platelet density per monocyte increased monocytes recruitment to endothelial cells. Human umbilical vein endothelial cells (HUVEC) were grown on cover slips till confluence. Monocytes with high and low platelet densities were isolated and perfused over PMA (100nM) activated HUVEC at 250 s-1 for 10 minutes. (A) Representative image showing binding of monocytes with high platelet densities to HUVEC under flowing conditions. Black arrowheads indicate elongated strings decorated with monocytes with high platelet densities. (B) Quantification showed that a high platelet density per monocyte increased monocyte recruitment to HUVEC. Monocyte recruitment was quantified using blood of four different donors (mean±SD). Differences between groups were examined using paired student t-test. P/M = platelet density per monocyte.

Baseline characteristics

Within the study population of 263 coronary artery disease patients, 209 patients (79%) were presenting with stable angina, 28 patients (11%) with unstable angina and 26 patients (10%) with nSTEMI. During the follow-up period of 9 months, 27 patients experienced an adverse cardiac event. Of these patients, 23 patients underwent another PCI (85%), 8 received coronary artery bypass grafting (CABG) (30%), 3 experienced myocardial infarction (11%) and there were 2 cases of death by cardiovascular disease (7%). The baseline characteristics of the cohort are depicted in supplemental table 1. Patients with nSTEMI had a more frequent history of CABG, increased numbers of diseased vessels, used less often aspirin and clopidogrel at inclusion but more aspirin after intervention as compared with patients with stable angina. Patients with unstable angina had increased numbers of diseased vessels as compared with patients with stable angina (supplemental table 1). The total cohort of patients was divided into tertiles based on the median level of platelet density per monocyte or the median level of platelet-monocyte complexes. Baseline characteristics of these subpopulations are described in supplemental table 2. We observed higher levels of hyperlipidemia in patients in the middle and high platelet density tertiles and high platelet-monocyte complex tertiles as compared with patients in the low tertiles. Also, patients in the middle platelet density tertiles used more often aspirin at inclusion, while patients in the high platelet density tertiles used more clopidogrel at inclusion as compared with patients in the low platelet density tertiles (supplemental table 2).

Platelet density per monocyte was associated with adverse cardiac events after PCI

To study whether the platelet density per monocyte was associated with adverse cardiac events, we monitored the recurrence of adverse cardiac events in patients after PCI during a follow-up period of 9 months (N=263). We found that patients with a high platelet density per monocyte had a significant reduced recurrence of adverse cardiac events as compared with patients with a low platelet density per monocyte (hazard ratio=0.3 (95%CI=0.1-0.9), P=0.03) (Figure 5A). In contrast to the platelet density per monocyte, tertiles of platelet-monocyte complexes were not associated with the recurrence of adverse cardiac events (P=0.84) (Figure 5B).

To study the influence of confounding variables, we adjusted for age, sex, smoking, hyperlipidemia, hypertension, diabetes, clopidogrel, aspirin and disease severity. We found that a high platelet density per monocyte remained significantly associated with a reduced recurrence of adverse cardiac events in patients after PCI (hazard ratio=0.4 (95%CI=0.2-0.8), P=0.01) (Table 1).

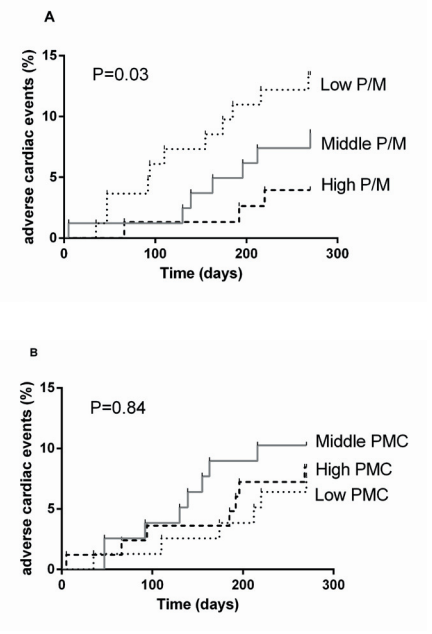


Figure 5. Patients with a high platelet density per monocyte had reduced adverse cardiac event rate. (A) Kaplan Meier analysis of adverse cardiac events in patients with a high, middle and low platelet density per monocyte. (B) Kaplan Meier analysis of adverse cardiac events in patients with high, middle and low platelet-monocyte complexes. P/M = platelet density per monocyte, PMC = platelet-monocyte complexes

Table 1 Multivariate analysis of the relationship between MACE after PCI with high platelet density per monocyte.

	Hazard-ratio (95%-CI)	P-value
high platelet density per monocyte	0.3 (0.1-0.9)	0.03
Age and sex adjusted model	0.3 (0.1-0.9)	0.03
Total adjusted model	0.4 (0.2-0.8)	0.01

Hazard ratios presented with 95% confidence intervals. The highest tertile was compared to the lowest tertile. Total model is adjusted for age, sex, smoking, hyperlipidemia, hypertension, diabetes, clopidogrel use, aspirin use and disease severity at inclusion.

Discussion

Advances in PCI have improved the treatment of atherosclerotic patients with coronary artery disease. However, a large percentage of patients require a second intervention due to restenosis[1, 2]. The repair of the vasculature after PCI induced injury plays a crucial role in the prevention of restenosis and recurrence of adverse cardiac events[22]. Monocytes and platelets enhance angiogenesis and vascular repair[3,9,10,21] and the binding of platelets to monocytes increases the recruitment of monocytes to sites of vascular injury[3, 11]. Our study shows that a high platelet density per monocyte increased the recruitment of monocytes to activated endothelial cells and was independently associated with a reduced recurrence of adverse cardiac events in patients after PCI.

Using fluorescence-activated cell sorting and microscopy, we found that a high platelet marker MFI per monocyte corresponded with a high platelet density per monocyte. Real-time video microscopy under flow showed that a high platelet density per monocyte increased the recruitment of monocytes with a high platelet density to activated endothelial cells as compared with monocytes with a low platelet density or monocytes with no adherent platelets. Previously, Bernardo and colleagues have shown that monocytes are recruited to elongated VWF-platelet strings that are released from activated endothelial cells in vitro[23]. We show that the recruitment of monocytes to endothelial cells under flow depends on the platelet density per monocyte. In vivo, the presence of VWF-platelet strings has been confirmed, although string survival time was reduced from 14 seconds in ADAMTS13 knockout mice to 4.7 seconds in to the presence of the VWF cleaving plasma protein ADAMT13[24]. Whether the in vivo recruitment of monocytes with a high platelet density to damaged endothelium occurs in a VWF dependent manner or through a rolling and firm adhesion manner remains to be elucidated.

The identification of a platelet density dependent recruitment mechanism of monocytes raised the question what role this mechanism plays in physiologic processes. Many studies focused on the role of monocyte recruitment in the progression of atherosclerosis, even though monocytes play a major role in vascular repair. To study whether a high platelet density per monocyte had an effect on vascular repair, we monitored the recurrence of adverse cardiac events during a follow up period of 9 months in patients after PCI induced vascular damage. We observed a 3-fold reduced recurrence of adverse cardiac events in patients with a high platelet density per monocyte as compared with a low platelet density per monocyte. Since the majority of recurring adverse cardiac events was due to revascularization, we investigated the influence of several established cardiovascular disease co-variables, pre- and post-interventional platelet inhibition therapy and disease severity at inclusion. We found that the association of a high platelet density with a reduced recurrence of cardiac events was independent of investigated co-factors.

A number of limitations of this study need to be considered. Even though we could exclude the influence of several confounding variables, there

may be other confounding variables that affect the relation between the platelet density per monocyte and recurrence of adverse cardiac events. Also, we present clinical data that showed an association between a high platelet density per monocyte and a reduced recurrence of adverse cardiac events in patients after PCI. This association should be confirmed in a large cohort that includes a higher number of adverse cardiac events.

In conclusion, our study shows that a high platelet density per monocyte increases monocyte recruitment to endothelial cells and is associated with a decreased recurrence of adverse cardiac events in patients after PCI. These findings show that the platelet density per monocyte is a mechanism of monocyte recruitment and a novel predictor of adverse cardiac events in patients after PCI.

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Supplemental table 1
Baseline characteristics of the total population and subpopulations

	Total population (N=263)	Stable angina (N=209)	Unstable angina (N=27)	nSTEMI (N=27)
Age in years (Median ± SD)	63 ± 10	63 ± 10	66 ± 10	65 ± 10
Men	179/263 (68%)	146/209 (70%)	17/27 (63%)	19/27 (73%)
Platelet-monocyte complexes (%)	26.0 ± 17	25.4 ± 18.1	25.3 ± 14.7	33.9 ± 3.6*
Platelet-neutrophil complexes (%)	14.2 ± 10.9	13.8 ± 10.9	13.2 ± 7.4	20.5 ± 2.8*
Previous myocardial infarction	74/263 (28%)	63/209 (30%)	10/27 (37%)	4/27 (15%)
Previous PCI	97/263 (37%)	82/209 (39%)	10/27 (37%)	5/27 (19%)
Previous CABG	22/263 (8%)	10/209 (5%)	1/27 (4%)	5/27 (19%)*
Diabetes Mellitus	49/263 (19%)	40/209 (19%)	7/27 (26%)	2/27 (8%)*
Hypertension	181/263 (69%)	148/209 (71%)	16/27 (59%)	17/27 (65%)
Hyperlipidemia	184/263 (70%)	145/209 (70%)	21/27 (77%)	18/27 (67%)
Smoking	58/263 (22%)	38/209 (18%)	9/27 (33%)	11/27 (41%)
Kidney disease	6/263 (2%)	6/209 (3%)	0/27 (0%)	0/27 (0%)
WBC count (10-3/ml)	7.2 ± 2.0	6.9 ± 1.9	8.0 ± 2.5	7.9 ± 1.6
Total cholesterol (mmol/l)	4.7 ± 1.2	4.3 ± 1.1	5.4 ± 1.9	4.7 ± 1.2
Aspirin	219/263 (83%)	179/209 (86%)	22/27 (81%)	16/27 (59%)*
Clopidogrel	146/263 (56%)	122/209 (58%)	13/27 (48%)	10/27 (37%)*
Statins	211/263 (80%)	180/209 (86%)	17/27 (63%)	14/27 (52%)
Nitrates	96/263 (37%)	86/209 (36%)	4/27 (15%)	3/27 (11%)
Calcium antagonists	88/263 (33%)	75/209 (36%)	8/27 (30%)	4/27 (15%)
Beta blockers	199/263 (76%)	177/209 (85%)	17/27 (63%)	17/27 (63%)

*P-values<0.05 and refer to comparison to the stable angina subpopulation. PCI = percutaneous coronary intervention, CABG = coronary artery bypass grafting, WBC = white blood cell count

Supplemental table 2
Baseline characteristics of patient population divided into tertiles.

	Platelet density per monocyte			Platelet-monocyte complexes		
	Low tertile MFI <3.74	Middle tertile MFI 3.75-6.15	High tertile MFI >6.16	Low tertile <14.4%	Middle tertile 14.5-30.4%	High tertile >30.4%
Age, years (Median ± SD)	65 ± 9	62 ± 10	62 ± 10	63 ± 9	64 ± 10	63 ± 10
Men	62/87 (71%)	70/88 (80%)	56/88 (64%)	62/87 (71%)	70/88 (80%)	58/88 (66%)
Previous MI	25/87 (29%)	25/88 (28%)	27/88 (31%)	23/87 (26%)	30/88 (34%)	23/88 (26%)
Previous PCI	23/87 (26%)	34/88 (39%)	31/88 (35%)	37/87 (43%)	33/88 (38%)	31/88 (35%)
Previous CABG	7/87 (8%)	9/88 (10%)	8/88 (10%)	5/87 (6%)	11/88 (13%)	9/88 (10%)
Diabetes Mellitus	19/87 (22%)	14/88 (16%)	14/88 (16%)	12/87 (14%)	18/88 (20%)	23/88 (26%)
Hypertension	54/87 (62%)	70/88 (80%)	71/88 (81%)	54/87 (62%)	67/88 (76%)	61/88 (69%)
Hyperlipidemia	48/87 (55%)	72/88 (82%)*	72/88 (82%)*	48/87 (55%)	67/88 (76%)*	65/88 (74%)
Smoking	19/87 (22%)	19/88 (22%)	19/88 (22%)	21/87 (24%)	16/88 (18%)	24/88 (27%)
Kidney disease	1/87 (1%)	2/88 (2%)	2/88 (2%)	10/87 (11%)	3/88 (3%)	3/88 (3%)
WBC count (10-3/ml)	6.9 ± 1.9	7.1 ± 1.8	7.4 ± 2.2	7.1 ± 2.0	7.2 ± 2.0	7.2 ± 2.0
Total cholesterol (mmol/l)	4.3 ± 1.1	4.6 ± 1.3	4.3 ± 1.0	4.6 ± 1.3	4.2 ± 1.0	4.3 ± 1.0
Aspirin before intervention	66/87 (76%)	78/88 (89%)*	73/88(83%)	72/87 (79%)	75/88 (85%)	70/88 (80%)
Aspirin after intervention	73/87 (84%)	75/88 (80%)	79/88 (80%)	77/87 (89%)	75/88 (85%)	75/88 (85%)
Clopidogrel before intervention	42/87 (51%)	47/88 (53%)	56/88 (64%)*	52/87 (60%)	53/88 (60%)	47/88 (53%)
Clopidogrel after intervention	65/87 (75%)	61/88 (69%)	72/88 (80%)	70/87 (80%)	65/88 (74%)	63/88 (72%)
Statins	68/87 (85%)	71/88 (81%)	74/88 (84%)	73/87 (84%)	69/88 (78%)	71/88 (81%)
Nitrates	28/87 (36%)	32/88 (36%)	33/88 (38%)	24/87 (28%)	32/88 (36%)	34/88 (40%)
Calcium antagonists	29/87 (37%)	28/88 (32%)	30/88(34%)	25/87 (29%)	32/88 (36%)	34/88 (40%)
Clopidogrel	42/87 (51%)	47/88 (53%)	56/88 (64%)*	52/87 (60%)	53/88 (60%)	47/88 (53%)
Beta blockers	72/87 (85%)	67/88 (76%)	65/88 (74%)	70/87 (80%)	72/88 (82%)	62/88 (70%)

*P-values<0.05 are shown in bold and refer to comparison to the lowest tertile.
MI = myocardial infarction, PCI = percutaneous coronary intervention, CABG = coronary artery bypass grafting, WBC = white blood cell count

CHAPTER 7

Plasma levels of active VWF are increased in patients with first ST-segment elevation myocardial infarction: a multicenter and multiethnic study

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PLASMA LEVELS OF ACTIVE VWF ARE INCREASED IN PATIENTS WITH FIRST ST-SEGMENT ELEVATION MYOCARDIAL INFARCTION: A MULTICENTER AND MULTIETHNIC STUDY

Abstract

Aims

Von Willebrand factor (VWF), a key player in hemostasis and thrombosis, is released from endothelial cells during inflammation. Upon release, VWF is processed by ADAMTS13 into an inactive conformation. The aim of our study was to investigate whether plasma levels of active VWF, total VWF, ADAMTS13 and osteoprotegerin (OPG) are risk factors for first ST-segment elevation myocardial infarction (STEMI).

Methods and results

We assessed 1026 patients with confirmed first STEMI and 652 control subjects from China, Italy and Scotland, within 6 hours after cardiovascular event. Median plasma levels of total VWF, active VWF and OPG were increased, while plasma levels of ADAMTS13 were decreased in patients compared to controls. The odds ratio (OR) of STEMI in patients with high plasma levels of active VWF was 2.3 (interquartile range (IQR): 1.8-2.9), total VWF was 1.8 (1.4-2.3), ADAMTS13 was 0.6 (05-0.8) and OPG was 1.6 (1.2-2.0). The OR for total VWF and active VWF remained significant after adjustment for established risk factors, medical treatment, C-reactive protein, total VWF, ADAMTS13 and OPG. When we adjusted for levels of active VWF, the significance of the OR for VWF disappeared while the OR for active VWF remained significant

Conclusions

We found evidence that plasma levels of active VWF are an independent risk factor for first STEMI in patients from 3 different ethnic groups. Our findings confirm the presence of VWF abnormalities in patients with STEMI and may be used to develop new therapeutic approaches

Introduction

VWF is a multimeric protein that plays a crucial role in hemostasis by supporting platelet adhesion and thrombus formation after vascular damage[1,2]. This function of VWF is evident from the bleeding disorder von Willebrand disease, which is characterized by quantitative or qualitative deficiencies in VWF[3]. VWF is present in plasma, platelets and endothelial cells. In endothelial cells, it is stored as ultra large VWF (UL-VWF) in Weibel-Palade bodies, where it is in association with OPG[4]. UL-VWF and OPG are released from endothelial cells upon stimulation with inflammatory agonists[5,6] and recent studies have implicated OPG in endothelial dysfunction[7,8]. UL-VWF is highly thrombogenic, as it exposes a multitude of platelet glycoprotein (GP)Ib binding domains[9]. To prevent the release of this highly thrombogenic moiety into the circulation, UL-VWF is cleaved by the plasma protein ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13) at the Tyr1605-Met1606 peptide bond[10]. It is suggested that cleavage results in a conformational change in which the active GPIb binding domain A1 is covered by either the A2 or the D'D3 domains, that leads to the shielding of the GPIb binding domain[11,12]. Hence, cleavage by ADAMTS13 allows VWF to circulate in plasma in an inactive form, thereby preventing

spontaneous thrombus formation. The importance of ADAMTS13 in the regulation of VWF activity is evidenced by the high prevalence of spontaneous microthrombi in the vasculature of patients with thrombotic thrombocytopenic purpura, who have markedly decreased plasma levels of ADAMTS13. Besides the role of ADAMTS13 in the inactivation of VWF during its release, ADAMTS13 also attenuates thrombogenesis through cleavage of VWF following vascular damage[13]. Previous research has shown that the distal carboxyl-terminal domains of ADAMTS13 are required for the regulation of thrombus formation[14]. Increased plasma levels of inactive VWF have been reported in various cardiovascular diseases[15-19] and efforts are being made to develop inhibitors of the interaction of VWF with platelets[20]. Inactive VWF becomes activated upon shear induced conformational changes, that may be caused by the high shear stress present in atherosclerotic arteries or by binding to exposed subendothelial collagen after vascular damage. Activated VWF presents its active A1 domain, resulting in the capture of platelets through interaction with the GPIb receptor[21]. The interaction between GPIb and VWF leads to activation-induced conformational changes in the platelet GPIIb/IIIa receptor complex, which consequently exposes binding sites for fibrinogen[22]. Fibrinogen then acts as a bridging protein in the formation of a thrombotic plug, which is the leading cause of coronary artery occlusion and subsequent STEMI. Previous work, from the laboratory of Clinical Chemistry and Haematology at the University Medical Center in Utrecht, resulted in the development of a llama derived single-domain antibody that specifically recognizes the active form of VWF[23]. Utilizing this antibody, higher levels of active VWF were shown in patients with thrombotic thrombocytopenic purpura, HELLP, malaria, dengue and in a small cohort of young premenopausal women with AMI[24-26]. Despite the potentially significant role of active VWF in various diseases, no information is available on the relation between active VWF and first STEMI. We hypothesized that VWF, active VWF, ADAMTS13 and OPG are biomarkers of endothelial dysfunction. Hence, we investigated the relationship between first STEMI and plasma levels of active VWF, total VWF, ADAMTS13 and OPG in a large-scale study including 3 different ethnic groups.

Methods

Study population

The First Acute Myocardial Infarction (FAMI) study was a multiethnic and multicenter study that has been reported in more detail elsewhere[27]. We included 1026 patients with confirmed first STEMI and 652 controls from 32 centers in urban areas in China, Italy and Scotland between October 2002 and April 2007. Subjects and healthy controls were included from these countries based on data from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) study, which reported 4-fold differences in the incidence of cardiovascular disease between subjects from these countries, with the lowest incidence in China and highest in Scotland, with Italy in an intermediate position[28]. Control subjects

were matched for gender, age, ethnicity, environment (enrolled from the same city), drug use and comorbidities. Inclusion criteria were electrocardiography evidence of STEMI, blood sampling within 6 hours of symptoms and no history of previous coronary artery disease. The FAMI study was approved by all local medical and ethical committees and followed the Declaration of Helsinki. All participants provided written informed consent before inclusion into the study.

Blood collection

Arterial blood was collected from patients into 3.2% sodium citrate tubes (Becton Dickinson, Plymouth, UK) directly before coronary recanalization and before administration of any anticoagulant. Blood from fasting case controls was collected and processed similarly. All samples were centrifuged at 2000g for 15 minutes at 4°C and aliquots were immediately frozen at -80°C. Subsequently, all samples were transported by air and road couriers in dry ice to the Universita Vita-Salute San Raffaele Core laboratory in Milan, Italy, where the plasma was stored at -80°C until use (Polar 530V Ahsi Angelantoni freezer, Milan, Italy).

Measurement of total

VWF, active VWF, ADAMTS13 and OPG An in-house developed llama derived single-domain antibody directed against the A1 domain of VWF, which is only exposed upon activation of VWF, was used to quantify plasma levels of active VWF(23). Plasma levels of total VWF include both the inactive and active form of VWF, although the majority of total VWF is in the inactive conformation. Plasma levels of total VWF, active VWF, ADAMTS13 and OPG were measured at the University Medical Center Utrecht, the Netherlands, using a semi-automated ELISA robot (Freedom EVO, Tecan, Switzerland). The intra-assay variation coefficient was 11% for active VWF, 2.9% for VWF, below 10% for ADAMTS13 and 4.7% for OPG. All inter-assay variation coefficients were below 10%, except for active VWF which was below 15%. The lower limit of detection was above 100 ng/mL for active VWF, above 1 µg/mL for VWF, above 10% for ADAMTS13 and above 0.01 ng/mL for OPG. ADAMTS13 levels were compared to normal pooled plasma of healthy donors, which was set at 100%. Wells were coated overnight at 4°C with 0.0775 µg/mL anti total VWF IgG (DAKO, A0082), 5 µg/mL anti active VWF VHH (UMC-Utrecht, clone: AU/VWF-a11), 0.5 g/ml anti ADAMTS13 IgG (Sanquin, D053) or 1 µg/mL anti OPG IgG (R&D, MAB8051). Hereafter, wells were washed with phosphate-buffered saline (PBS) containing 0.5% Tween-20, and samples were incubated for 2 hours at room temperature. Next, wells were washed and incubated with 0.55 µg/mL of polyclonal rabbit anti human VWF, active VWF, ADAMTS13 or OPG antibodies conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. Subsequently, the wells were washed and incubated with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Substrate development was stopped by the addition of 50 uL 0.3 M H2SO4 and measured on a Spectramax reader (MDS Analytical Technologies) at 450 nm.

Statistical analysis

Comparison of the levels of total VWF, active VWF, ADAMTS13 and OPG was done between patients and controls using the Student t-test. Levels of total VWF, active VWF, ADAMTS13 and OPG were divided into quartiles based on the distribution in the control group. The relationship between levels of total VWF, active VWF, ADAMTS13 and OPG and the occurrence of first STEMI was assessed using multivariate logistic regression. Patients in the highest quartile were compared with patients the lowest quartile. We adjusted for age, gender, smoking, diabetes mellitus, hypertension, hyperlipidemia, statins, angiotensin-converting-enzyme (ACE) inhibitors, clopidogrel, acetylsalicylic acid, high sensitivity C-reactive protein (hsCRP) and levels of total VWF, active VWF, ADAMTS13 and OPG. Correlations between the levels of total VWF, active VWF, ADAMTS13 and OPG were investigated with Spearman correlation. All statistical analyses were performed using SPSS 20.0 software (IBM Corporation) and graphs were made with Prism 6 software (Graphpad Software).

Results

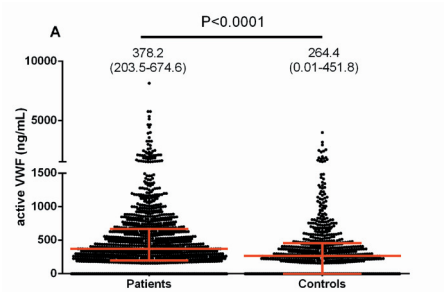
Study population

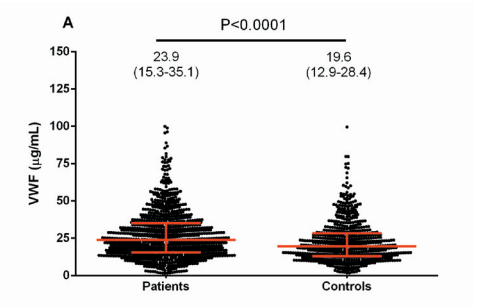
A total number of 1026 patients with first STEMI and 652 controls were included from China, Italy and Scotland (Table 1). Of the total population, 76% of patients were men, with a 9 year lower mean age compared to the mean age of women (mean ± SD; 67 ± 11.4 vs. 59 ± 11.5 years). STEMI was diagnosed by the electrocardiography evidence of ST-segment elevation at arrival in the hospital and by the creatine phosphokinase peak: (median, [IQR]; 1,647 [708-3063] U/L). Of the 1026 included patients, 897 patients (87.4%) received coronary reperfusion therapy. The baseline characteristics are depicted in supplemental table 1. We observed significant differences in the prevalence of diabetes mellitus (P<0.0001), hypertension (P<0.0001), dyslipidemia (P<0.05) and smoking (P<0.0001) between subjects from China, Italy and Scotland. Expectedly, due to exclusion of patients with a history of previous coronary artery disease, there was a very low use of drugs against cardiovascular diseases. Scottish patients were administered statins more frequently and Chinese patients received calcium antagonists more frequently (supplemental table 1).

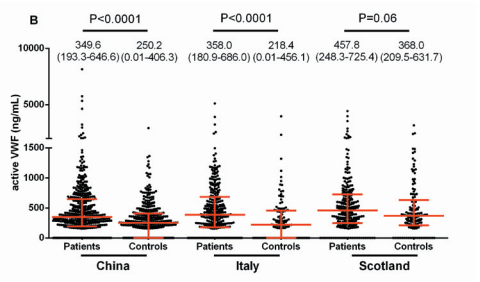
Table 1. Inclusion numbers, age and gender distribution of the cohort.

	Patients (n=1026)		Controls (n=652)	
	Men	Woman	Men	Woman
TOTAL (n=1678) Men=76%	59 ± 11	68 ± 12	58 ± 11	66 ± 11
CHINA (n=850) Men=76%	58 ± 12	68 ± 12	57 ± 12	67 ± 10
ITALY (n=435) Men=77%	60 ± 10	67 ± 12	61 ± 11	63 ± 11
SCOTLAND (n=393) Men=74%	59 ± 12	68 ± 12	58 ± 12	65 ± 12

Values are expressed as mean ± SD.







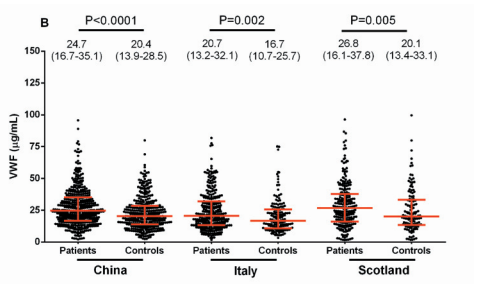


Figure 1. Plasma levels of active VWF are increased in patients. (A) Levels of active VWF in patients versus controls in total cohort. (B) Levels of activate VWF in patients versus controls per country. The bars and numbers below the P-value represent medians with interquartile ranges.

Figure 2. Plasma levels of total VWF are increased in patients. (A) Levels of total VWF in patients versus controls in total cohort. (B) Levels of VWF in patients versus controls per country. The bars and numbers below the P-value represent medians with interquartile ranges.

Levels of active VWF were 1.5-fold higher in patients than controls

Median levels of active VWF were nearly 1.5-fold higher in patients as compared with healthy controls (median, (IQR); 378.2 ng/mL (203.5-674.6) vs. 264.4 ng/mL (0.01-451.8), P<0.0001) (Figure 1A). Levels of active VWF were increased in Italian patients (385.0 ng/mL (180.9-686.0) vs. 218.4 ng/mL (0.01-456.1), P<0.0001) and Chinese patients (349.6 ng/mL (193.3-646.6) vs. 250.2 ng/mL (0.01-406.3), P<0.0001) as compared with controls, while a strong trend was observed in Scottish patients (457.8 ng/mL (248.3-725.4) vs. 368.0 ng/mL (209.5-631.7), P=0.06) (Figure 1B) as compared with controls.

Levels of total VWF were higher in patients than controls

Median levels of total VWF were significantly increased in patients as compared with healthy controls (median, (IQR); 23.9 µg/mL (15.3-35.1) vs. 19.6 µg/mL (12.9-28.4), P<0.0001) (Figure 2A). Levels of total VWF were increased in Italian patients (20.7 µg/mL (13.2-32.1) vs. 16.7 µg/mL(10.7-25.7), P=0.002), Scottish patients (26.8 µg/mL (16.1-37.8) vs. 20.1 µg/mL (13.4-33.1), P=0.005) and Chinese patients (24.7 µg/mL (16.7-35.1) vs. 20.4 µg/mL (13.9-28.5), P<0.0001) (Figure 2B) as compared with controls.

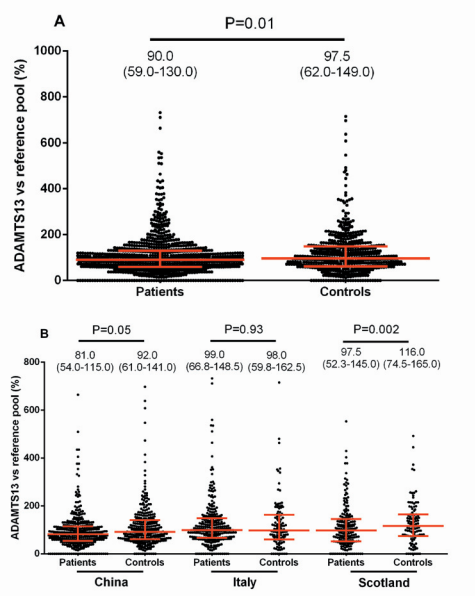


Figure 3. Plasma levels of ADAMTS13 are decreased in patients. (A) Levels of ADAMTS13 in patients versus controls in total cohort. (B) Levels of ADAMTS13 in patients versus controls per country. The bars and numbers below the P-value represent medians with interquartile ranges.

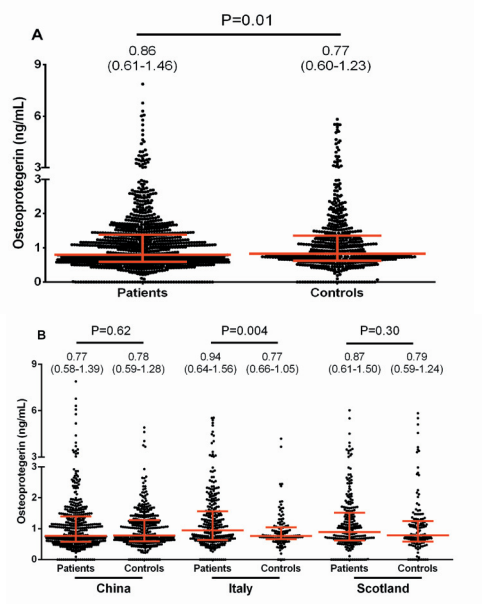


Figure 4. Plasma levels of OPG are increased in patients. (A) Levels of OPG in patients versus controls in total cohort. (B) Levels of osteoprotegerin in patients versus controls per country. The red bars and numbers below the P-value represent medians with interquartile ranges.

Levels of ADAMTS13 were lower in patients than controls

Median levels of ADAMTS13 were significantly lower in patients as compared with healthy controls (median, (IQR), 90% (59.0-130.0) vs. 97.0% (62.0-149.0), P=0.01) (Figure 3A). When subdivided per country, ADAMTS13 levels were significantly lower in Scottish patients (97.5% (52.3-145.0) vs. 116.0% (74.5-165.0), P=0.002) and Chinese patients (81.0% (5.0-115.0) vs. 92.0% (61.0-141.0), P=0.05), but not Italian patients (99.0% (66.8-148.5) vs. 98.0% (59.8-162.5), P=0.93) (Figure 3B) as compared with controls.

Levels of OPG were higher in patients than controls

OPG levels were significantly increased in patients as compared with the healthy controls (median, (IQR); 0.86 ng/mL (0.61-1.46) vs. 0.77 ng/mL (0.60-1.23), P=0.01) (Figure 4A) and in Italian patients as compared with controls (0.94 ng/mL (0.64-1.56) vs. 0.77 ng/mL (0.66-1.05), P=0.004) (Figure 4B). In contrary, OPG levels were not significantly increased in Scottish (0.87 ng/mL (0.61-1.50) vs. 0.79 (0.59-1.24), P=0.03) and Chinese patients (0.77 ng/mL (0.58-1.39) vs. 0.78 ng/mL (0.59-1.28), P=0.62) as compared with controls (Figure 4B).

Adjustment for established risk factors.
We investigated correlations between levels of total VWF, active VWF, ADAMTS13 and OPG. We found strong positive correlations between active VWF and total VWF (R=0.58, P<0.0001). Furthermore, we observed weak correlations between active VWF and OPG (R=0.23, P<0.0001), and between total VWF and OPG (R=0.26, P<0.0001). ADAMTS13 levels did not correlate with total VWF, active VWF or OPG (Table 2).

Table 2. Correlations between total VWF, active VWF, OPG and ADAMTS13.

	Active VWF		ADAMTS13		OPG	
	R	P	R	P	R	P
Total VWF	0.58	<0.0001	0.02	0.54	0.26	<0.0001
Active VWF			0.02	0.61	0.23	<0.0001
ADAMTS13					0.02	0.40

Univariate logistic regression analysis showed that the OR of STEMI in patients with high levels of total VWF, active VWF and OPG was significantly increased (>1.0), while the OR of STEMI in patients with high levels of ADAMTS13 was significantly decreased (<1.0) in the total cohort of patients but not in each country (Table 3).

Table 3. Relation between STEMI and unadjusted levels of total VWF, active VWF, ADAMTS13 and OPG.

	China	Italy	Scotland	Total
	OR	OR	OR	OR
Total VWF	1.9* (1.3-2.6)	2.2* (1.2-3.9)	1.6* (1.0-2.6)	1.8* (1.4-2.3)
active VWF	3.0* (2.1-4.4)	2.3* (1.4-4.0)	1.3 (0.9-2.1)	2.3* (1.8-2.9)
ADAMTS13	0.4* (0.3-0.7)	0.8 (0.5-1.3)	0.6 (0.3-1.0)	0.6* (0.5-0.8)
OPG	1.2 (0.8-1.7)	3.1* (1.8-5.6)	1.8* (1.1-2.9)	1.6* (1.2-2.0)

Odds ratios (OR) are presented with 95% confidence intervals.

The hightest quartile was compared with the lower quartiles. *P-value<0.05

The significance of the OR for ADAMTS13 and OPG disappeared after adjustment for age, gender, smoking, hypertension, diabetes mellitus, dyslipidemia, statins, ACE inhibitors, clopidogrel, acetylsalicylic acid, hsCRP, total VWF, ADAMTS13 and OPG, while the increased OR for total VWF and active VWF remained significant after adjustment. When active VWF was incorporated into the multivariate model the significance of the OR for total VWF disappeared (Table 4). This may suggest that plasma levels of active VWF are an independent risk factor for first STEMI in patients from three different ethnic groups.

Table 4. Relation between STEMI and adjusted levels of total VWF, active VWF, ADAMTS13 and OPG

	Total population			
	Unadjusted OR	OR1	OR2	OR3
Total VWF	1.8* (1.4-2.3)	1.8* (1.5-2.3)	1.6# (1.2-2.2)	1.3 (0.9-1.8)
active VWF	2.3* (1.8-2.9)	2.3* (1.8-3.0)	2.0* (1.4-2.8)	2.0* (1.4-2.8)
ADAMTS13	0.6* (0.5-0.8)	0.7* (0.5-0.9)	0.8 (0.6-1.0)	0.8 (0.6-1.0)
OPG	1.6* (1.2-2.0)	1.6* (1.3-2.1)	1.3 (1.0-1.8)	1.2 (0.9-1.7)

Odds ratios (OR) are presented with 95% confidence intervals. The highest quartile was compared with the lowest quartile. #P-value<0.01, *P-value<0.0001. OR1 is adjusted for age and gender, OR2 is adjusted as OR1 and additionally for smoking, diabetes mellitus, hypertension, hyperlipidemia, statins, ACE inhibitors, clopidogrel, acetylsalicylic acid, hsCRP, total VWF, ADAMTS13, ratio VWF/ADAMTS13 and OPG, OR3 is adjusted as OR2 and additionally for active VWF .

Discussion

This study found increased plasma levels of total VWF, active VWF and OPG in patients with first STEMI as compared with controls. It also demonstrated that plasma levels of ADAMTS13 were lower in patients as compared with controls. The association of first STEMI with plasma levels of total VWF and active VWF was independent of established risk factors, medical treatment, C-reactive protein, total VWF, ADAMTS13 and OPG, while the association of first STEMI with ADAMTS13 and OPG was dependent on these co-variables. The relation between first STEMI and plasma levels of active VWF was independent of total VWF, active VWF, ADAMTS13 and OPG, while the relation between first STEMI and total VWF disappeared after adjustment for active VWF. These findings suggest that the active component of VWF explains the relationship between first STEMI and levels of total VWF.

Highly thrombogenic UL-VWF is processed by ADAMTS13 into an inactive conformation upon release from endothelial cells. Following vascular damage, shear stress induces conformational changes in inactive VWF that lead to the formation of active VWF. Hence, increased levels of active VWF may indicate increased vascular damage and, consequently, an increased risk of STEMI. Therefore, we investigated if levels of active VWF were increased in patients with first STEMI. We found an approximate 1.5-fold increased level of active VWF in patients with first STEMI as compared with controls. In a small number of patients we observed very high levels of active VWF. Comorbidities, such as renal impairment or inflammatory disease, may be responsible for the observed increase in levels of active VWF. Importantly, these cases did not skew our data distribution. Because ADAMTS13 is one of the components that controls the level of active VWF, through cleavage of UL-VWF following release from endothelial cells and during the formation of a thrombus, we

investigated if levels of ADAMTS13 were associated with levels of active VWF. We found no association between levels of ADAMTS13 and active VWF. Moreover, adjustment for levels of ADAMTS13 did not affect the relationship between active VWF and STEMI, suggesting that the variation in ADAMTS13 levels is not a major determinant of active VWF levels. ADAMTS13 is not the only protein that is involved in the conversion of active VWF into the inactive plasma conformation, as granzyme B and Thrombospondin are also able to inactivate VWF through cleavage[29,30]. Although ADAMTS13 levels had no relationship with active VWF levels, we did find a reduced OR of STEMI for subjects with high ADAMTS13 levels. Since all evidence for a relationship between ADAMTS13 and STEMI is obtained from observational studies, it is currently impossible to distinguish whether low plasma levels of ADAMTS13 are the cause of the high risk of STEMI or the result of ADAMTS13 binding to VWF present in thrombi that are formed during STEMI. Previously, an inverse relationship between levels of ADAMTS13 and VWF has been reported[31]. We did not observe such an inverse relationship, which is in line with other studies that also show the absence of an inverse relationship between ADAMTS13 and VWF[32,33].

Recent studies indicate that plasma levels of OPG are associated with endothelial dysfunction and predict the incidence of mortality in patients with acute coronary artery disease[7,34]. Hence, we investigated whether plasma levels of OPG were associated with first STEMI. We found that levels of OPG were increased in patients with first STEMI as compared with healthy controls. However, the association between OPG and first STEMI disappeared after adjustment for co-variables. These findings suggest that OPG is not an independent risk factor for first STEMI.

We observed several differences in plasma levels of measured markers when patients from the 3 ethnic groups were compared. We found lower levels of VWF in Italian patients as compared with Scottish and Chinese patients, higher levels of active VWF in Scottish patients as compared with Italian and Chinese patients and lower levels of ADAMTS13 in Chinese patients as compared with Italian and Scottish patients. Differences in plasma levels of VWF due to genetic variations have previously been reported[35,36], but the mechanisms behind the observed differences in levels of active VWF and ADAMTS13 between the 3 different ethnic groups remain to be elucidated.

A number of limitations apply to this study. We observed inconsistencies when results between patients and controls from the three countries were compared. Firstly, we did not observe lower levels of ADAMTS13 in Italian patients compared to controls, while we observed higher levels of total VWF in Italian patients compared to controls. Besides genetic or environmental factors, the explanation that levels of ADAMTS13 antigen do not fully reflect levels of ADAMTS13 activity might also be considered here. Nevertheless, levels of ADAMTS13 appear to correlate with the incidence of STEMI per country, with the lowest incidence of AMI in China, the highest incidence in Scotland and Italy in an intermediate position, as previously observed in the MONICA study[28].

Secondly, levels of OPG were increased in Italian and Scottish patients versus controls, but not in Chinese patients compared to controls. Finally, from our cross-sectional study we cannot conclude whether increased levels of active VWF are the cause or consequence of first STEMI. Future prospective studies are necessary in order to determine causality.

In conclusion, our data show that plasma levels of active VWF are an independent risk factor for first STEMI in patients from 3 different ethnic groups. Plasma levels of total VWF are also associated with first STEMI, but this relationship disappears after adjustment for levels of active VWF. These findings provide new insight in the occurrence of VWF abnormalities in patients with STEMI and supply additional rationale for the development of drugs targeting VWF.

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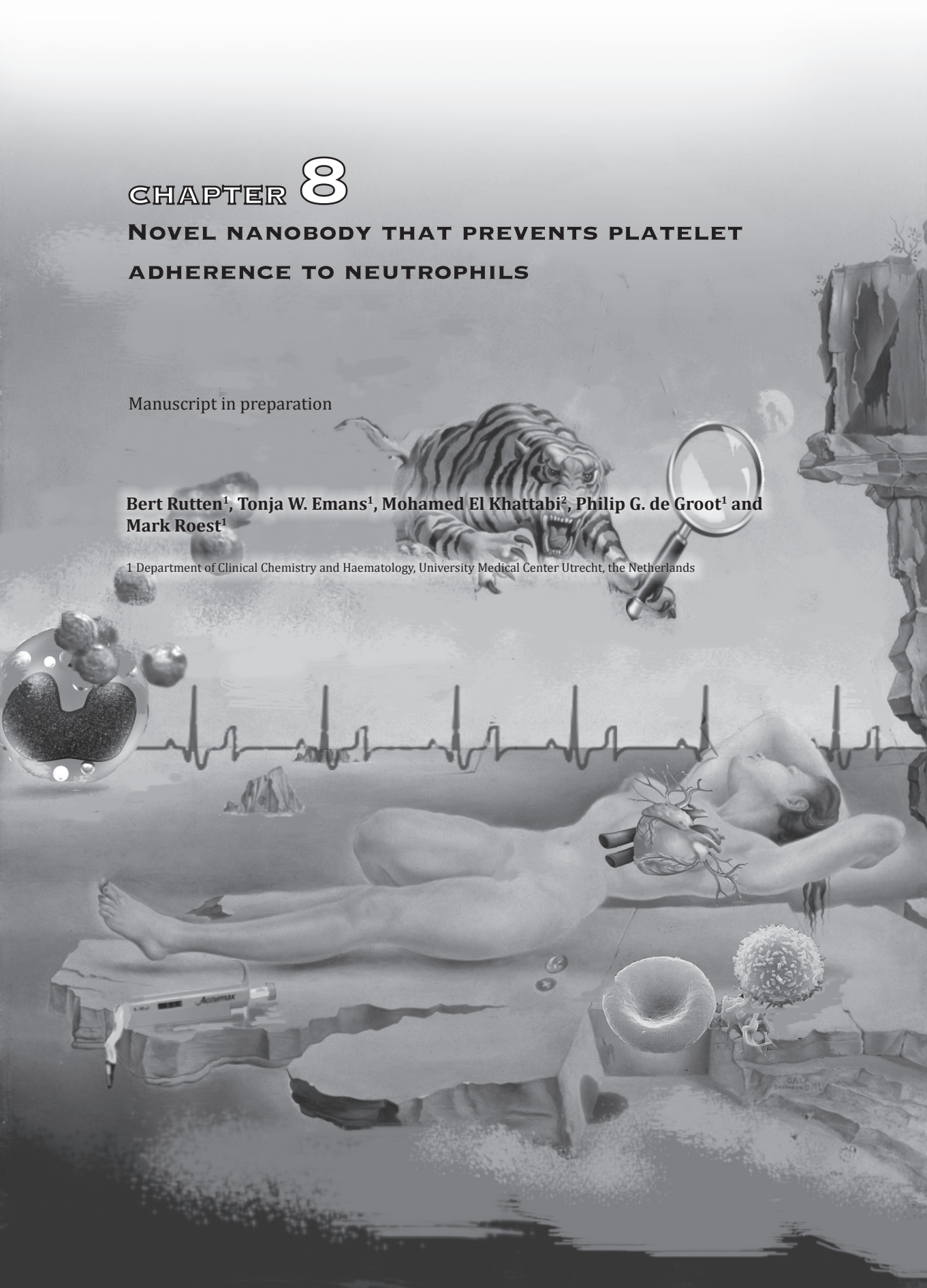
Supplemental table 1. Baseline characteristics of patient cohort

	China	Italy	Scotland	Total	P
Time to blood sampling (min)	222 ± 115	192 ± 137	167 ± 101	200 ± 121	<0.0001
Diabetes Mellitus	114 (13.5%)	41 (9.4%)	27 (6.9%)	182 (10.9%)	<0.0001
Hypertension	462 (54.8%)	188 (43.3%)	130 (33.2%)	780 (35.3%)	<0.0001
Dyslipidemia	243 (29%)	166 (38.2%)	104 (26.5%)	513 (30.8%)	<0.05
Smoking	398 (47%)	160 (36.7%)	136 (34.7%)	694 (41.4%)	<0.0001
Aspirin	150 (17.6%)	94 (21.5%)	75 (19.1%)	319 (18.6%)	NS
Beta blockers	59 (6.9%)	48 (11%)	47 (12%)	154 (9%)	NS
Statins	37 (4.4%)	24 (5.5%)	55 (14%)	116 (6.8%)	<0.0001
Calcium antagonists	120 (14.1%)	18 (4.1%)	19 (4.8%)	157 (9.1%)	<0.0001
Clopidogrel	6 (0.7%)	4 (0.9%)	3 (0.8%)	13 (0.8%)	NS
ACE inhibitors	82 (9.6%)	52 (11.9%)	45 (11.5%)	179 (10.4%)	NS
hsCRP (mg/L)	7.6 ± 19.1	8.6 ± 18.6	5.1 ± 9.9	7.2 ± 16.9	<0.05
Creatine (mg/L)	8.4 ± 6.1	8.9 ± 3.4	10.5 ± 8.2	9.3 ± 6.4	<0.0001
HDL (mg/L)	407 ± 96	425 ± 116	457 ± 136	423 ± 114	<0.0001
LDL (mg/L)	1354 ± 444	1809 ± 585	1515 ± 552	1503 ± 542	<0.0001
Heart rate (beats per minute)	77.5 ± 19.9	76.5 ± 15.5	76.8 ± 17.4	77.0 ± 17.1	NS
Systolic blood pressure	133.4 ± 28.5	136.0 ± 25.0	139.8 ± 29.6	135.8 ± 27.8	NS
Diastolic blood pressure	80.3 ± 20.7	80.1 ± 15.2	81.7 ± 18.8	80.6 ± 18.7	NS

Values are expressed as mean ± SD or N (%). P-value refers to comparison of total population vs. Chinese, Italians and Scottish population. NS = not significant

CHAPTER 8

Novel nanobody that prevents platelet adherence to neutrophils



Manuscript in preparation

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8

Abstract

Background The binding of platelet P-selectin to PSGL-1 on neutrophils plays a major role during inflammatory processes. Our aim was to develop a nanobody that prevents platelet binding to neutrophils.
Methods and results We immunized Llamas with recombinant human P-selectin and used phage display selection to isolate nanobodies with high affinity for P-selectin. Using ELISA and flow cytometry, we obtained several nanobodies that bound to P-selectin expressed on activated platelets. Next, we artificially created platelet-neutrophil complexes by incubating blood with 100 ng/mL E.coli lipopolysaccharide (LPS). Using flow cytometry and real-time video microscopy, we identified several nanobodies that prevented the binding of platelets to neutrophils.
Conclusion We developed a novel nanobody directed against human P-selectin that prevented the interaction between platelets with neutrophils. This nanobody can be used to block interactions between platelets and neutrophils during conditions that are unfavorable for conventional antibodies.

Introduction

Platelets contribute to the progression of inflammatory diseases. Upon activation and adherence to inflamed endothelium, they create a surface that promotes leukocyte recruitment[1]. The recruitment of leukocytes is a central event in the pathogenesis of atherosclerosis. Neutrophils, which are the most populous of the leukocytes, are involved in defense against microbial invaders[2], but they are also play a role in early stages of atherosclerotic plaque progression[3]. The binding of platelets to neutrophils is mediated by the engagement of platelet P-selectin with the constitutively expressed neutrophil counter receptor P-Selectin Glycoprotein Ligand-1 (PSGL-1)[4,5]. However, engagement of PSGL-1 triggers intracellular activation pathways that lead to cytokine production[6,7]. Hence, inhibition of platelet-neutrophil interactions by PSGL-1 antagonism may lead to unwanted leukocyte activation. Previous work shows that monoclonal antibodies directed against P-selectin reduce pancreatitis[8], cutaneous inflammation[9], deep vein thrombosis[10,11] and sickle cell diseases in murine models[12]. We aimed to develop an nanobody that prevents the interaction between platelets and leukocytes through P-selectin antagonism. Nanobodies have several advantages over conventional antibodies. For example, nanobodies have a convex shape that allows deep penetration into antigen pockets, thereby reaching epitopes that are not accessible to conventional antibodies[13], nanobodies lack a Fc receptor and thereby they do not initiate Fc receptor mediated processes, nanobodies are able to resist high temperatures and low pH as compared with IgG[14, 15] and, lastly, they have been shown to be very effective diagnostic tools evidenced by the specific recognition of unstable atherosclerotic plaques in vivo[16].

Methods

Isolation of nanobodies from a phage-display library

A phage display library was created from RNA isolated from peripheral blood lymphocytes of a llama immunized with recombinant human P-selectin. RNA was reverse-transcribed into cDNA using random hexamers (SuperScript III, Invitrogen) and cleaned using the QIAquick PCR purification kit (Qiagen). Next, the cDNA was restricted using BstEII and SfiI and purified by gel electrophoresis, followed by ligation into a phagemid vector for display on bacteriophages. E.coli TG1 competent cells were electro transformed and stored at -80°C as phage-display libraries. We coated 96-wells maxisorp plates (Nunc) with rabbit anti-human Fc immunoglobulin G (IgG) overnight at 4°C. Next, wells were washed with PBS and blocked with 4% marvel-PBS (mPBS) for 1 hour at room temperature (RT). Blocked wells were incubated with 2 µg/mL recombinant human Psel/Fc chimera (R&D Systems) for 1 hour at RT. Simultaneously, phages were precipitated from the phage library using 20% polyethylene glycol (PEG) in 2.5 M NaCl during 15 minutes on ice. Phages were spun at 11000 x g for 5 minutes, resuspended in PBS, and incubated with recombinant human Psel/Fc chimera (R&D Systems) for 2 hours at room temperature. After incubation, wells were washed five times with PBS-Tween (0.05% v/v) followed by PBS. Bound phages were eluted using 0,1M triethylamine (TEA) and the high pH of the TEA buffer was neutralized by addition of Tris (1M, pH=7.5). Selections were carried out for two rounds as following; phages were amplification using E. coli TG1 infection during 30 minutes at 37°C and 5% CO^2 while gently shaking. Ten-fold serial dilutions were made and 5 µL of each dilution was spotted on 2xTryptane Yeast (2xTY) agar plates supplemented with 0.1mg/ml ampicillin and 2% glucose. Plates were incubated overnight at 37°C and 5% CO^2 and remaining samples were incubated in 5mL 2xTY supplemented with 0.1mg/ml ampicillin and 2% glucose overnight at 37°C and 5% CO^2. The following day, individual colonies were picked and diluted 100 times into a 5 mL TY medium containing 0.1mg/ml ampicillin and 2% glucose. After 2 hours of incubation at 37°C and 5% CO^2, 2µl of helper phage was added for 30 minutes at 37°C and 5% CO^2. Samples were spun for 10 minutes at 4000 x g and the pellet was resuspended in 50 ml 2xTY containing 0.1mg/ml ampicillin and 0,1mg/ml kanamycin. After overnight incubation at 37°C and 5% CO^2, phages were precipitated with PEG and incubated in a 2µg/ml recombinant human Psel/Fc chimera coated maxisorp plate as described above. Next, individual colonies were transferred into an ELISA plate well containing 100 µL 2xTY medium supplemented with 0.1mg/ml ampicillin and 2% glucose, and incubated for 4 hours 4 hours at 37°C and 2% glucose, followed by addition of 100 µL glycerol and storage at -80°C. E.coli contain phages directed P-selectin, were grown by inoculation of 2xTY medium, supplemented with 0.1 mg/mL ampicillin and 2 % glucose, until reaching an optical density (OD) of 600 (OD600). Next, 10 µL of 250-fold diluted helper phage was added to the culture for 30 minutes at 37°C and 5% CO^2. Bacteria were spun at 700 x g for 10 minutes

and the pellet was resuspended in 600 µL of 2x TY medium containing 0.1mg/ml ampicillin and 0.1mg/ml kanamycin, followed by overnight incubation at 37°C and 5% CO^2. Here after, phages were harvested from the supernatant and incubated with 2 µg/mL P-selectin coated maxisorp for 2 hours at RT. Next, wells were washed five times with PBS-Tween and an anti-M13 antibody conjugated to horse radish peroxidase (HRP) was allowed to bind phages during 30 minutes of incubation, followed by the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate and stoppage of the reaction with 1M H2SO4. Analysis was performed on the Spectra Max L reader at 450 nm.

Polymerase chain reaction (PCR) and sequencing

DNA was amplified using Dream Taq buffer (Fermentas), containing 0.04 mM dNTP (Fermentas), 0.5 µM M13 reverse primer and 0.5 µM MPE25WB forward primer, demineralized water and Taq DNA polymerase (Fermentas). The PCR product was digested with the restriction enzyme; Hinfl (Fermentas) during 2 hours at 37°C. Samples were mixed with 5x loading buffer supplemented with etidium bromide and transferred into the wells of a 2% agarose gel, using a 1Kb DNA ladder (Thermo Scientific) to estimate DNA size. After electrophoresis, samples were analyzed on an Odyssey Fc Imager (Li-Cor Bioscience). Next, plasmids from samples containing the desired insert were isolated with The NucleoSpin® Plasmid- Kit (Macherey-Nagel) and sequenced using a sequencing buffer (AB), M13 reverse primer (Sigma-Aldrich) and BigDye Terminator V3.1 (AB). The PCR product was transferred into eppendorf's containing 64 µL 95% ethanol and 16 µL demineralized water. After thoroughly mixing, samples were stored in the dark and on ice for 20 minutes. Then, samples were spun at 20.000 x g and the pellet was resuspended in 250 µL 70% ethanol. Again, samples were spun at 20.000 x g and the pellet was air dried. Pellet was mixed with 25 µL highly deionized formamide and incubated for 2 minutes at 95°C, followed by DNA sequencing. All data were analyzed using Clone-manager suite 6.

Nanobody production

Nanobody DNA was digested from the plasmid and transformed into a pMek219 vector. Plasmids and vectors were digested using SFIl (fermentas) and Eco91l (Fermentas) restriction enzymes using Tango buffer (Fermentas). The pMek219 vector was transformed into Top Ten competent E. coli bacteria (Invitrogen) using heat-shock at 42°C for 30 seconds, followed by incubation on ice for 2 minutes. Here after, 400 mL of 2xTY medium, supplemented with 0.1mg/mL ampicillin and 1% glucose, was inoculated with 4 mL overnight E coli culture for 4 hours until OD600. Nanobody expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 24 hours at 28°C and 5% CO^2. Next, pellets were spun at 4000 x g. resuspended in 5 mL PBS and stored at -80°C for 2.5 hours. Nanobodies were purified by immobilized metal affinity chromatography using Talon metal affinity resin (ClonTech). In short, beads were washed with high salt buffer containing 300 mM NaCl and 50mM NaH2PO4 and loaded onto a gravity-flow column (ClonTech).

Nanobodies were eluted from the column using 150 mM imidazole dissolved in 300mM NaCl and 50mM NaH2PO4 and dialyzed overnight against PBS at 4°C.

ELISA detection of Nanobodies directed against P-selectin

We coated 2 μg/mL recombinant human P-selectin/FC chimera (R&D Systems) overnight on white maxisorp 96 wells plate (Nunc). After blocking with 4% mPBS, 200 μL supernatant of overnight E.coli culture, containing secreted nanobodies, was incubated with the coated antigen for 1 hour. Bound nanobodies were detected using 10 μg/mL sheep anti-myc 9E10 antibody (Millipore). The primary anti-myc antibody was detected using a rabbit anti-mouse IgG antibody conjugated to HRP (Dako, Denmark). We included a biotinylated sheep anti-human P-selectin as positive control. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was added for 30 minutes, after which the reaction was stopped with 1 M HCl. Analysis was performed on the Spectra Max L reader at 450 nm.

Affinity ELISA using nanobodies directed against P-selectin

An ELISA was carried out to determine which nanobodies were able to bind with high affinity to P-selectin. White 96-well maxisorp plate (Nunc) were coated with 2 μg/mL recombinant Psel/Fc chimera (R&D Systems) and incubated overnight at 4°C. After blocking with 4% mPBS, wells were incubated with 4-fold serial dilutions of nanobodies (starting concentration 50 μg/mL) or negative control nanobodies for 1 hour. Bound nanobodies were detected with 10 ug/mL sheep anti-myc 9E10 antibodies and 0.5 μg/mL rabbit anti-sheep conjugated to streptavidin-HRP antibodies (Dako, Denmark). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was added for 30 minutes and the reaction was stopped with 1 M HCl. Analysis was performed on the Spectra Max L reader at 450 nm.

Blood collection

Blood from healthy employees of the University Medical Center Utrecht, The Netherlands, was collected into 3.2% sodium citrate tubes. All employees were self-reported free of platelet function inhibiting drugs (i.e.: acetylsalicylic acid or clopidogrel), informed consent was obtained from all participants and all procedures followed the Declaration of Helsinki.

Flow cytometry detection of nanobodies directed against P-selectin

Whole blood was incubated for 20 minutes with 1 μg/mL or 5 μg/mL nanobody clones in the presence or absence of 0.625 mM thrombin receptor activating peptide-1 (TRAP), followed by fixation with 0.2% formaldehyde. Fixated samples were transferred into wells of ELISA plates and diluted 3-fold with 150 μL 0.2% formaldehyde. An anti-myc IgG conjugated to FITC (Invitrogen) was used to detect platelet bound nanobodies on a FACS Canto II flow cytometer (BD Bioscience). Ten thousand platelets were counted by positive labeling with CD42b antibody conjugated to PE and scatter plots profiles. A commercially available anti-human P-selectin antibody conjugated to FITC (BD Pharmingen) was used as positive control.

8

Prevention of platelet interactions with leukocytes using flow cytometry

We used the following commercially available IgGs that block platelet-leukocyte interactions; anti-human P-selectin (R&D, BBA30), anti-human PSGL-1, (BD Biosciences, PL-1), anti-human MAC-1 blocker (R&D, 5845NF) and anti-human GP1b blocker (Sanquin, M1539). In order to create platelet-leukocyte complexes, we incubated whole blood with 100 ng/mL E.coli LPS:O111 for 1.5 hours, in the presence or absence of 1 µg/mL anti-Pselectin nanobody clones. Here after, platelets were labeled with CD41a conjugated to PE (Sanquin PeliCluster) and samples were fixated with Optilyse B (Beckman Coulter). Red blood cells were lysed by addition of 800 µL demineralized water and 200 µL of each sample was analyzed on a FC Canto II flow cytometer (BD Biosciences).

Platelet isolation

Whole blood was centrifuged at 160 x g for 15 minutes, and platelet-rich plasma was harvested from the supernatant. Next, platelets were centrifuged 340 x g for 15 minutes after addition of 1:10 volume of acid citrate dextrose (ACD) containing 2,5% trisodium citrate, 1,5% citric acid and 2% D-Glucose. Platelets were resuspended in Hepes-Tyrode's (HT) buffer (pH 6,5) and spun at 340 x g for 15 minutes after addition of 10 ng/mL prostaglandin analogue (PGI2). Platelets were carefully resuspended in HT buffer (pH 7,3), and left undisturbed for at least 30 minutes at room temperature.

Neutrophil isolation

Whole blood (35 mL) was transferred into 50 mL falcon tubes containing 15 mL Ficoll-Paque (GE Healthcare). Tubes were centrifuged at 890 x g during 20 minutes at 20°C. Supernatant was removed and neutrophils were harvested and transferred into a new falcon tube. Red blood cells were lysed by addition of 45 mL of red blood cell lysis buffer (Qiagen), followed by centrifugation at 600 x g for 5 minutes. Here after, pellet was resuspended in HT buffer (pH 7.3).

Real-time video microscopy perfusion

Sterile coverslips were coated with 100 mg/mL fibrinogen and blocked with 1% human serum albumin. Washed platelets at a concentration of 3.0x1011 were perfused over the coverslips at a shear rate of 250 sec^{-1} using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Subsequently, neutrophils at a concentration of 5x1010 in a PBS buffer containing 1mM calcium chloride (CaCl) were perfused at a shear rate of 250 sec-1 over the activated and adherent platelets. The rolling velocity of neutrophils was analyzed in the presence of absence of 16 µg/mL nanobody directed against P-selectin during analysis of movies that were recorded with an inverted differential interference contrast (DIC) imaging microscope (Carl Zeiss Axio Observer Z.1) with a 10 x or 40 x Neofluar objectives.

Statistical analysis

Data were presented as means ± standard deviations. Differences between groups were determined using the Student t-test. A P-value <0.05 was considered statistically significant.

Results

Nanobodies recognizes recombinant human P-selectin

We used an ELISA assay to investigate binding of selected nanobodies to recombinant human P-selectin. Nanobodies bound to wells coated with recombinant human P-selectin and not to IgG or PBS (Figure 1). A commercially available anti-human P-selectin IgG was used as positive control. The selected nanobodies bound with similar intensities to P-selectin as commercially available IgGs directed against P-selectin. Additionally, a PBS coated well showed absence of aspecific binding after by incubation with the commercial IgG directed against P-selectin.

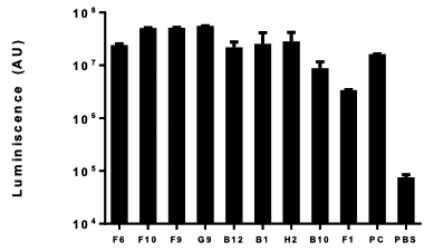

Figure 1. Nanobodies bound to recombinant human P-selectin in an ELISA assay. Nanobodies (1 µg/mL) were incubated with recombinant human P-selectin, IgG or not coated (PBS) wells for 1 hour followed by detection at 527 nm. A commercially available anti-human P-selectin IgG was used as positive control.

Nanobodies binds P-selectin expressed on activated platelets

We used flow cytmetry to study the binding of nanobodies to P-selectin expressed on activated platelets, we incubated blood with the platelet activator TRAP-1. We selected nanobodies that were highly expressed in our bacterial expression system, and observed binding of these nanobodies to activated platelets and not to platelets that were not activated (Figure 2). The commercially available anti-human P-selectin antibody (PC) confirmed platelet P-selectin expression after TRAP-1 activation.

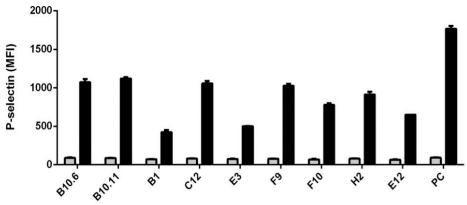

Figure 2. Nanobodies bound P-selectin expressed on activated platelets, in a flow cytometry assay. Nanobodies (1 µg/mL) were incubated with TRAP-1 activated blood (black bars) or not activated blood (grey bars). A commercially available anti-human P-selectin IgG was used as positive control (PC).

Nanobodies prevent platelet binding to leukocytes

Incubation of whole blood with 1µg/mL E.coli O:111 LPS resulted in the formation of platelet-neutrophil complexes (PNC) and platelet-monocyte complexes (PMC), but not platelet-lymphocyte complexes (PLC) (Figure 3). Next, we studied whether incubation of whole blood with E.coli LPS in the presence of nanobodies or commercially available IgG antibodies, which were reported to block platelet binding to leukocytes, prevented the formation of artificial complexes. We observed that blocking neutrophil integrin Mac-1 or platelet glycoprotein (GP) 1b did not prevent the formation of platelet-neutrophil or platelet-monocyte complexes, respectively (Figure 3). However, blocking P-selectin and PSGL-1 with commercially

available antibodies did prevent the formation of platelet-neutrophil or platelet-monocyte complexes. Next, we investigated the blocking potential of two nanobody (B10.6 and C12) and found that clone C12 prevented the formation of platelet-neutrophil and platelet-monocyte complexes.

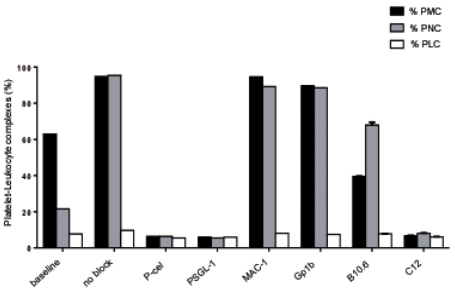

Figure 3. Nanobody C12 prevents the binding of platelets to monocytes and neutrophils. Platelet-monocytes complexes and platelet-neutrophil complexes were induced by incubation of whole blood with 1µg/mL E.coli LPS in the presence of commercial antibodies against P-selectin, PSGL-1, MAC-1, GP-1b or nanobodies against P-selectin. Nanobody C12 was found to prevent platelet adherence to monocytes and neutrophils. PMC = Platelet-monocyte complexes, PNC = platelet-neutrophil complexes, PLC =platelet-lymphocyte complexes.

Nanobodies prevent binding of neutrophils to activated platelets under flow

Using perfusion experiments, we studied the rolling and adhesion of neutrophils over activated platelets that were perfused over fibrinogen coated coverslips, in the presence or absence of nanobody clone C12 at a flow rate of 250 s^{-1}. Using real-time video microscopy at a speed of 4 frames per second, we observed an increase in the rolling velocity of neutrophils over activated platelets in the presence of nanobody C12 (Figure 4).

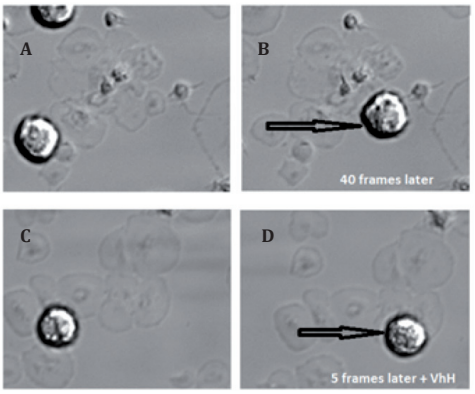

Figure 4. Nanobody C12 (vhH) reduces neutrophil rolling velocity under flow. Neutrophils were perfused over platelets bound to fibrinogen coated coverslips in the absence (A+B) or presence of nanobody C12 (C+D) . The rolling velocity was determined by counting the number of frames from start (A+C) to finish (B+D) of video analysis. In the absence of nanobody C12 neutrophils had a higher rolling velocity (B) as compared with the presence of nanobody C12 (D).

Discussion

Interactions between P-selectin and PSGL-1 facilitate the formation of platelet-leukocyte complexes, which are involved in the progression of atherosclerosis[17]. The aim of this study was to develop a nanobody that blocks the formation of platelet-leukocyte complexes. We selected two nanobodies that were expressed at high levels in our bacterial expression system and that recognized P-selectin in ELISA and flowcytometry experiments (clone B10.6 + C12). In contrast to clone B10.6, clone C12 prevented the binding of platelets to neutrophils.

During flow cytometry experiments, we observed decreased nanobody binding to activated platelets as compared with the binding of a commercially available antibody to activated platelets. The creation of a bivalent nanobody, which is characterized by two antigen recognition domains and created by the introduction of two copies of the nanobody gene into the expression plasmid, has been reported to increase the affinity of nanobodies[18]. Our flow cytometry results showed that clone C12 prevented the binding of platelets to monocytes and neutrophils during incubation with E.coli LPS. Surprisingly, the levels of platelet-monocyte complexes and platelet-neutrophil complexes after incubation with clone C12 were lower than baseline levels. This may suggest that nanobody C12 has a competitive advantage over leukocyte PSGL-1 in the binding to platelet P-selectin. In addition, clone C12 reduced the rolling velocity of purified neutrophils over activated platelets under flowing conditions. Since neutrophil recruitment is involved in the early progression of cardiovascular diseases, we suggest to study the effect of clone C12 in a murine ApoE knock out model. Besides the role of P-selectin and PSGL-1 interaction in the progression of atherosclerosis, tumor cells also express PSGL-1[19]. Hence, it should be studied whether nanobody C12 prevents the binding of platelets to tumor cells.

In conclusion, we developed a novel nanobody that prevents the binding of platelets to neutrophils.

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CHAPTER 9

Discussion
Nederlandse samenvatting
Publieks samenvatting
Dankwoord
List of publications
Curriculum vitae

Discussion

We studied the role of platelet adherence to leukocytes during inflammatory processes. Since platelet adherence to leukocyte attributes to the progression of atherosclerosis[1, 2], alters the function of leukocytes[3-5] and is associated with adverse cardiac events[6-8], the findings of this thesis contribute to our understanding why inflammatory processes are the greatest medical challenge to humanity since ancient times[9-13]. The major finding of this thesis is that the platelet density per monocyte is a novel predictor of adverse cardiac events in atherosclerotic patients after percutaneous coronary intervention. Furthermore, we give insight into the role of platelet adherence to monocytes during atherosclerosis and endotoxemia. Lastly, we show that levels of active VWF are a more accurate marker of first myocardial infarction then levels of total VWF.

Platelet adherence to leukocytes contributes to chronic inflammatory diseases

Platelet activation, which leads to the formation of platelet–leukocyte complexes[14, 15], is a critical step in the progression of atherosclerosis[16]. Hence, we studied in **chapter 2** whether severe flow-limiting stenoses in atherosclerotic patients are associated with altered platelet activation and levels of platelet-monocyte and platelet-neutrophil complexes. Since platelet-monocyte complexes are markers of platelet activation[8, 17, 18], we hypothesized that platelet activation and levels of platelet-leukocyte complexes would be increased in patients with severe stenosis. In contrast to our hypothesis, we found that severe stenosis was associated with reduced levels of platelet-leukocyte complexes and no association was found with platelet activation. Previous groups found that increased luminal obstruction was associated with increased platelet activation[19, 20]. How then to explain these differences? While other groups investigated ex vivo platelet aggregation after mental and physical stimulation, we investigated ex vivo platelet P-selectin expression after stimulation with several agonists. The observed association between platelet aggregation and severe stenosis might be explain by exercise induced factors that sensitize platelets in vivo. Using flow cytometry, Milovanovic et al. measured fibrinogen binding to platelets after ex vivo activation of platelets with TRAP or ADP. They found a decrease in the percentage of platelets that bound fibrinogen in patients with severe stenosis as compared with mild stenosis[21]. We measured P-selectin expression after ex vivo activation of platelets with TRAP, ADP and CRP and observed no differences in the expression of P-selectin between patients with severe stenosis as compared with mild stenosis. It is important to note that plasma levels of fibrinogen are associated with disease severity and this may influence the percentage of circulating platelets that bound fibrinogen[22]. Since P-selectin expressing platelets rapidly adhere to leukocytes[1, 23], we anticipated that absence of differences in levels of P-selectin expression between patients with severe as compared with mild stenosis could occur. However, we did not expect to observe decreased levels of platelet-leukocyte complexes in patients with severe stenosis as compared with mild stenosis. A study on clearance of activated (P-selectin expressing) platelets

and platelet-leukocyte complexes could help to explain these findings. Recently, a mechanism of platelet clearance by macrophages was reported in patients with von Willebrand disease[24]. Interestingly, the authors found co-localization of platelets with macrophages in the spleen, which may suggest that activated platelets and platelet-leukocyte complexes are withdrawn from the circulation by retention in the spleen. Hence, future work that studies altered platelet reactivity in patients with severe stenosis should focus on mechanisms that are involved in the removal of hyper reactive platelet from the circulation. Besides retention in the spleen, reduced levels of circulating platelet-leukocyte complexes may be due to transmigration into the atherosclerotic plaques[25]. In **chapter 3**, we report of an observation that may supports this suggestion. Our results show that platelet activation after stimulation with ADP is associated with platelet-monocyte complex formation and monocyte infiltration into atherosclerotic plaques. The role of platelet activation in platelet-monocyte complex formation, monocyte adherence and atherogenesis has been shown in various other studies[2, 7, 26]. In these studies, P-selectin expression was shown to be crucial for the adherence of monocytes to the endothelium and subsequent exacerbation of atherosclerosis. However, none of these studies investigated the association between P-selectin upregulation and platelet stimulation with increasing agonist concentrations. Since the reactivity of platelets towards agonists determines the upregulation of P-selectin, we provide additional insight into the association of platelet reactivity with P-selectin expression, platelet-monocyte complex formation and monocyte infiltration. Moreover, due to the use of increasing concentrations of agonist, we obtained dose-response curves for platelet reactivity. During careful analysis, we observed that neither the half maximal effective concentration (EC50), nor the slope of the reaction or the maximum activation value correctly represented the reactivity of platelets towards ADP. Hence, we converted the dose-response curves into 'area under the curve'-values. These values gave improved representations of the dose-response curves as compared with other parameters. Future investigation should study whether chronic diseases reduce the time that platelets spend in circulation and whether there are longitudinal changes in the reactivity of circulating platelets or in the levels of platelet-leukocyte complexes. Longitudinal changes may also explain why recent clinical trials have found a high risk of venous thrombosis and morbidity in patients who are treated with epoetins. Since platelet aggregation and reactivity are major determinants of thrombosis, we investigated whether epoetin has a direct effect on platelet aggregation under flowing conditions and platelet activation after stimulation with various platelet agonists during an approximate time course of 1 hour. Besides the high risk of venous thrombosis and morbidity, previous studies also report increased platelet reactivity and plasma levels of the platelet activation marker P-selectin after administration of epoetin to healthy volunteers[27]. Our findings, described in chapter 4, show

that platelet aggregation and reactivity is not affected by incubation of whole blood with epoetin ex vivo. This shows that neither direct platelet activation nor changes in platelet aggregation by epoetin are the cause of the high risk of venous thrombosis after epoetin treatment. We investigated a direct effect of Epo, i.e.; approximately 60 minutes, on platelet reactivity towards agonists. However, this does not exclude possible effects of Epo on platelet reactivity at later time points. However, our finding that also platelet aggregation, which is a major determinant of thrombus formation, was not affected by epoetin suggests that other factors cause the association with thrombosis and morbidity. It is suggest that changes in platelet reactivity in vivo are caused by phenotypic alteration of megakaryocytes. A study that investigates the effect of epoetin on bone marrow residing megakaryocytes, or newly formed platelets derived from megakaryocytes, may provide these insights. To achieve this aim, novel approaches to determine reactivity of megakaryocytes need to be established.

Does quantity or quality matter during monocyte recruitment?

A major novelty of this thesis is the quantification of the number of platelets per monocyte and the use of this parameter for the prediction of adverse cardiac events after vascular damage. While percentages of platelet-monocyte complexes are qualitative markers of platelet activation, we suggest that the platelet density per monocyte is a quantitative marker of platelet activation. In **chapter 6**, we show that quantity matters during the recruitment of monocytes to endothelial cells and the recurrence of adverse cardiac events after vascular damage. Monocytes are important mediators of vascular repair and platelet binding to monocytes increases monocyte recruitment to the endothelium. Hence, we hypothesized that a high number of platelets per monocyte, also referred to as the platelet density per monocyte, increases the recruitment of monocytes to endothelial cells and is associated with a reduced incidence of adverse cardiac events after PCI induced vascular damage. Commonly, platelet adherence to leukocytes is determined by measuring the percentage of leukocytes that are positive for a platelet specific marker. However, this approach gives limited insight into the intensity of platelet adherence to leukocytes, despite the importance of quantitative factors. For example, when a cardiologist investigates the intensity of atherosclerosis in patients, the number of diseased vessels (1-vessel, 2-vessel, 3-vessel, etc.) is taken into account before the intensity of atherosclerosis is diagnosed. This concept should also be applied to measurements of platelet adherence to leukocytes. Not only should we determine whether a leukocyte is bound by a platelet, we should also quantify how many platelets are bound per leukocyte. In the early nineties, when flow cytometry was still in its infancy, Rinder et al. determined the platelet density on monocytes and postulated in the first sentence of the discussion: "Binding of activated platelets to leukocytes is likely of physiologic importance not only for the targeting of both cell types to appropriate inflammatory and/or hemostatic sites but also for functional alterations in these cells"[34]. Indeed, in chapter 5 we

showed that adherence of activated platelets to monocytes alters the function of monocytes. The idea that binding of activated platelets to leukocytes affects the targeting of leukocytes prompted us to investigate whether the quantity of platelet binding per monocyte (i.e.; platelet density per monocyte) affects the recruitment of monocytes to endothelial cells. We show that the platelet density per monocyte is crucial to the recruitment of monocytes to activated endothelial cells under flow. Previously it was reported that platelet adherence to monocytes increases monocyte recruitment to endothelial cells under flow[35-37]. However, the finding that the platelet density per monocyte is a key factor in the recruitment of monocytes has not been reported before. While platelet adherence to monocytes is well-known to play a detrimental role in the progression of atherosclerosis, we are the first to suggest that platelet adherence to monocytes plays a beneficial role after cardiac intervention. In line with our suggestions, previous reports show that platelet binding to monocytes can alter the phenotype of the monocyte and cause a shift towards a pro-angiogenic state[5,38,39]. Hence, we suggest that increased platelet adherence to monocytes results in increased recruitment of monocytes, an increased pro-angiogenic state increased vascular repair. This implies that enhancing platelet binding to monocytes during periods of vascular repair, improves strategies to reduce future adverse cardiac events.

In summary, measurements of platelet reactivity and platelet-leukocyte complexes are associated with atherosclerosis, but additional factors, such as disease severity and clearance mechanisms of platelets and platelet-leukocyte complexes, influence the relationship between atherosclerosis, platelet reactivity and platelet adherence to leukocytes, which affects the applicability of these parameters as reliable markers for atherosclerotic disease progression.

Does platelet adherence alter the function of leukocytes?

In **chapter 5**, we studied the effects of platelet adherence to monocytes during induced human endotoxemia. Our preliminary results suggest that endotoxemia triggers platelet activation and adherence to monocytes, which in turn affects the production of cytokines by monocytes. Several other groups have obtained similar results in vitro[28,29]. While we can conclude that platelet adherence to leukocytes alters the function of leukocytes, a key question remains unanswered: what induces the expression of P-selectin on platelets in whole blood and in vivo? Since E.coli LPS does not induce P-selectin upregulation on platelets in plasma or buffer[30], while we observe P-selectin upregulation in vivo and in whole blood flow cytometry assay, we suggest that a leukocyte derived factor triggers platelet activation. The identification of this factor will contribute to our understanding why septic patients have a high incidence of deep vein thrombosis and thrombocytopenia[31]. Future studies, for instance using purified leukocyte fractions, mass spectrometry, high affinity pull downs, knock out animal models and large scale cohort studies, could identify this factor. Due to its platelet activation capacity, secretion by leukocytes and high levels in patients

with sepsis[32,33], platelet activating factor might be a possible candidate.

Is activity the key to a longer life?

During the formation of an occlusive thrombus, the aggregation of platelets is associated with levels of the bridging molecule VWF and the VWF cleaving factor ADAMTS13. Since the conformation of VWF determines its capacity to bind platelets and initiate thrombus formation, we investigated, in **chapter 7**, whether active VWF is a marker of first myocardial infarction. Previous groups found an association between VWF, and ADAMTS13. Hence, we included these factors into our study. We also investigated association between first myocardial infarction and OPG, since VWF is bound to OPG in the Weibel-Palade bodies of endothelial cells[40]. The study consisted of a large cohort of patients from China, Italy and Scotland, which were compared with matched healthy controls. Using a novel single domain antibody directed against the active form of VWF, we found that active VWF was an independent marker for myocardial infarction. The association between VWF and first myocardial infarction disappeared after adjustment for levels of active VWF, while the association between first myocardial infarction with ADAMTS13 and OPG disappeared after adjustment for the inflammatory marker C-reactive protein (CRP). This finding suggests that levels of active VWF are responsible for the association between VWF and first myocardial infarction, while ADAMTS13 and OPG are associated with myocardial infarction due to inflammatory responses. However, others have reported that association between ADAMTS13 and myocardial infarction was not affected by adjustment for CRP[41]. These different findings highlight that cross-sectional studies are not suitable to conclude whether increased levels of markers are the cause or the consequence of myocardial infarction. Future prospective studies are necessary to determine causality. Nevertheless, our findings show that the active form of VWF explains the relationship between myocardial infarction and levels of total VWF. This implies that drugs targeting the interaction between platelets and the active A1 domain of VWF benefit therapies to reduce myocardial infarction.

Can we solve big problems with small solutions?

Based on our findings, we propose that platelet activation and adherence to leukocytes has different causes and consequences that dependent on the stage of the disease. Firstly, platelets are activated and adhere to monocytes during chronic inflammatory processes such as stable angina. The adherence of platelets to monocytes may increase the clearance of platelet-monocyte complexes from the circulation into atherosclerotic plaques or to sites such as the spleen. Hence, we observed reduced levels of circulatory platelet-leukocyte complexes in patients with stable angina. Secondly, platelet adherence to leukocytes during acute thrombotic events is a consequence of platelet interactions with the exposed pro-thrombotic subendothelial matrix. Hence, increased levels of platelet-monocyte complexes are observed in patients with nSTEMI as compared with stable angina[42].

Lastly, complexes formed after plaque ruptures are suggested to contribute to monocyte recruitment to the endothelium in order to augment vascular repair.

These suggestions encouraged us to develop single domain antibodies (nanobodies, VhH), which have beneficial physical properties and easy low-cost manufacturing platforms as compared with conventional antibodies. In **chapter 8**, we describe the development of nanobodies that prevented platelet adherence to leukocytes, that are detrimental during early stages of atherosclerotic disease progression. However, despite that we were able to acquire a nanobody that prevented platelet binding to neutrophils under flowing conditions, much work remains to be done. We need to investigate whether our novel nanobody also prevents binding of platelets to monocytes and endothelial cells under flow. Next, we should address whether the nanobody prevents platelet binding to leukocytes in vivo and we need to develop a solutions for the high in vivo clearance rate of nanobodies[43]. Before we can embark on such a voyage, more urgent steps need to be taken. First, the cDNA of our nanobody needs to be transferred into a eukaryotic vector that allows high expression without causing protein aggregation, which is commonly observed during antibody production[44]. If we are able to develop a nanobody that fulfils these requirements and we continue to study the mechanisms that cause cardiovascular disease, future scientists might observe a significant decrease of atherosclerosis in patients from the early 21st century.

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Nederlandse samenvatting

Het aantal bloedplaatjes per monocyte voorspelt toekomstige hart- en vaatziekten.

Slagaderverkalking is een wereldwijde epidemie die jaarlijks ongeveer 17 miljoen slachtoffers eist. Het dotteren van patiënten en het plaatsen van een stent is de meest voorkomende behandeling van slagaderverkalking. Ondanks vooruitgang in de behandeling van patiënten met slagaderverkalking, zijn er veel patiënten die een tweede dotterbehandeling moeten ondergaan. Het is niet geheel duidelijk waarom sommige patiënten een tweede dotterbehandeling nodig hebben. We weten dat slagaderverkalking een chronische beschadiging van de slagaderen is, waarbij de interactie tussen bloedplaatjes en monocyten een belangrijke rol speelt. Bloedplaatjes zijn cellen die gespecialiseerd zijn in het dichten van wondjes middels binding aan beschadigde vaatwand cellen. Aangezien bloedplaatjes gespecialiseerd zijn in de binding aan beschadigde vaatwand cellen, vroegen wij ons af of bloedplaatjes ook aan andere cellen kunnen binden. Door middel van microscopie laten wij zien dat bloedplaatjes inderdaad ook aan andere cellen binden; namelijk aan monocyten. Het is belangrijk om te weten dat monocyten cellen zijn, die verantwoordelijk zijn voor het herstellen van de vaatwand na beschadiging. Om hun werk goed te kunnen uitvoeren, is het cruciaal dat de monocyten binden aan beschadigde vaatwand cellen. Aangezien bloedplaatjes gespecialiseerd zijn in het binden aan beschadigde vaatwand cellen, hebben wij onderzocht of monocyten met veel bloedplaatjes beter instaat waren om te binden aan beschadigde vaatwand cellen dan monocyten met weinig bloedplaatjes. Met behulp van een microscoop en een videocamera hebben wij deze binding kunnen visualiseren. Dankzij deze techniek hebben wij ontdekt dat monocyten met veel bloedplaatjes beter binden aan beschadigde vaatwand cellen dan monocyten met weinig bloedplaatjes. Vervolgens wilden we onderzoeken of patiënten met veel bloedplaatjes per monocyte een beter herstel van de vaatwand hadden. We weten dat het dotteren van patiënten met slagaderverkalking leidt tot een beschadiging van de vaatwand. Daarnaast is wel bekend dat een slecht herstel van de vaatwand ertoe leidt dat patiënten een tweede dotterbehandeling nodig hebben. Aangezien monocyten zorgen voor het herstel van de vaatwand, hebben wij onderzocht of patiënten met veel bloedplaatjes per monocyte minder vaak een tweede dotterbehandeling hoefden te ondergaan dan patiënten met weinig bloedplaatjes per monocyte. Wij vonden dat patiënten met veel bloedplaatjes per monocyte 3 keer minder vaak een tweede dotterbehandeling nodig hadden dan patiënten met weinig bloedplaatjes per monocyte. Aangezien dit verschil veroorzaakt kon worden doordat de patiënten met veel bloedplaatjes per monocyte andere karakteristieken hadden, zoals meer of minder medicijn gebruik, hebben we onderzocht of andere factoren verantwoordelijk waren voor de afname van tweede dotterbehandelingen in patiënten met veel bloedplaatjes per monocyte. Wij laten met behulp van statistische methodieken zien dat zowel de ernst van slagaderverkalking, het gebruik van medicijnen, de leeftijd, het geslacht en het roken van sigaretten niet verantwoordelijk was voor de verminderding in het aantal benodigde

dotterbehandelingen. Wij hopen deze bevindingen in de toekomst te kunnen gebruiken om het risico op een tweede dotterbehandeling te kunnen voorspellen. Daarnaast impliceren onze resultaten dat het hebben van veel bloedplaatjes per monocyte beschermt tegen toekomstige hart- en vaatziekten. Zodoende hopen wij in de toekomst een therapie te kunnen ontwikkelen waarbij wij hart- en vaatziekten kunnen verminderen door het aantal bloedplaatjes per monocyte te vergroten.

Publieks samenvatting

Het aantal bloedplaatjes per witte bloedcel voorspelt toekomstige hart- en vaatziekten.

Bert Rutten heeft ondekt dat patiënten met veel bloedplaatjes per witte bloedcel 3 keer minder vaak een 2e dotterbehandeling nodig hebben.

Slagaderverkalking is een wereldwijde epidemie die jaarlijks 17 miljoen slachtoffers eist. Het is een chronische beschadiging van de slagaderen waarin de interactie tussen bloedplaatjes en witte bloedcellen een belangrijke rol speelt. Bloedplaatjes zijn cellen die gespecialiseerd zijn in het binden aan beschadigde vaatwand cellen. Rutten laat zien dat bloedplaatjes ook aan witte bloedcellen kunnen binden. Dit is van groot belang, omdat witte bloedcellen zorgen voor het herstel van de vaatwand. Met behulp van microscopie heeft Rutten ontdekt dat witte bloedcellen met veel bloedplaatjes beter binden aan beschadigde vaatwand cellen dan witte bloedcellen met weinig bloedplaatjes. Vervolgens heeft Rutten onderzocht of patiënten met veel bloedplaatjes per witte bloedcel minder vaak een 2e dotterbehandeling nodig hadden. Uit het onderzoek bleek dat deze patiënten 3 keer minder vaak een 2e dotterbehandeling nodig hadden, dan patiënten met weinig bloedplaatjes per witte bloedcel. Daarnaast constateerde hij dat zowel de ernst van slagaderverkalking, het gebruik van medicijnen, de leeftijd, het geslacht en zelfs het roken van sigaretten niet de reden waren voor deze uitkomst. Rutten hoopt met zijn bevindingen in de toekomst een therapie te kunnen ontwikkelen waarbij het aantal 2e dotterbehandelingen wordt verminderd door op het juiste moment het aantal bloedplaatjes per witte bloedcel te vergroten.

Dankwoord

Some departments have the brightest minds in the field and other departments have a great atmosphere. I was fortunate to do my PhD in a department that has both. Therefore, I would like to thank the following people for their contribution.

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Leontien "Crazy Ti-Ta-Tinus" Korteweg! Telkens als ik met jou afspreek wordt het een dolle Saturday Night Disco Fever bedoeling! Dansen tot de broek ervan afzakt! Ik ben ontzettend blij dat je samen met Michiel "ik heb geen ADHD, ik heb ADSL" zo'n fantastisch mooi gezin hebt! Dikke kus aan Jaap (aka Wesley).

Breggieeeeeeee! Halve gare! Is het niet in de hoek van mijn tuin, dan is het wel van de top van een steiger. Wat moet ik altijd lachen om die acties van jou. Nu samen met die mafkees van een Jankees weet ik zeker dat je nog heel veel mooie avonturen gaat beleven!

Roelie boy, je bent er echt op vooruit gegaan bij je verhuizing van Utrecht naar Leidsche Rijn. Daarnaast heb je 2 schatten van kids en 'n super aardige vrouw. Doe je gaat jonguh! Gaan we snel weer een keer voetbal of K1 kijken?!

Henkie! Ouwe gangsta rappert! Een van de weinige mensen die ik absoluut niet begrijp en daardoor volledig volg. Gaaf dat je nu in Chicago zit en ik hoop dat je er je geluk vind!

Shane, endless questions about biology you had during our high speed bus trips through El Salvador and during extreme volcano hikes in Nicaragua. The simplest questions are usually the hardest to answer! Thanks for being one of my best friends for all these years! I am extremely proud to be the Godfather of your adorable son! Lisa, forgive me, but at a certain point I have to kidnap your husband because there is a highway from Canada to Chili waiting for us!

Lieber Franz von Paola, vielen dank für Paulaner Bier.

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My dear Romain; I wanted a sigarette, you needed a beer and that is how we became friends. We went on a great roadtrip through Europe and I am shure that is not the last adventure that we will have together. We had great times at de Poort en Der Derrick, although you never asked me whether I wanted to join you to Der Derrick ;-) I am extremely proud that I was your paranimf, and it is a great honour that you are mine! Merci voor alles!

Maui, ik ben ontzettend blij dat je zo gelukkig bent met Romain! Ik zou zeggen niks meer aan doen! Heel succes met het afronden van je PhD.

Mallikieee! I have never met a person who is so persistently willing to help other people. You are one of those few people who would first help to extinguish a fire at the neighbors before turning your attention to your own house. I hope that one day we will enjoy a beer together on the beaches of Algeria!

Mister Coen Maas. De Sun Tzu van de wetenschap! Tot op het bot gemotiveerd voor het bedrijven van wetenschap en het drinken van bier. Van salsa bij Stairway tot drum n base bij Tivoli, we hadden altijd de grootste lol (en de dag erna keihard proeven doen in het lab alsof er niks was gebeurd). Je gaat altijd tot het naadje en je weet nooit van ophouden. De perfecte combinatie voor vriendschap en wetenschap!

Beste Dapne, slim en daardoor een perfecte combinatie met Coen. Jullie gaan een mooie tijd tegenmoet. Ik verheug me erop om bij jullie op bezoek te komen als de kleine er eenmaal is!

Remo "Je luisterd niet naar me" Diego. Wat een feest is het altijd met jou. Ik weet niet wat het is, maar ik associeer jou met dronken zijn en dat bevalt me prima! De 1e keer dat ik jou zag was op de stoep bij Stairway to Heaven. Het duurde drie tellen voordat we besloten elkaar niet op de bek te slaan maar dikke maten te worden. Ik hou ook van jou!

Adam, you are a silly Pom! Good to see that are still scientists who also don't take themselves too serious! Lovely! Give my greetings to Francesca!

Tobias,"Why? What do you mean?". Er komt een dag that I will destroy you before I destroy myself! Super geil dat we zulke goeie vrienden zijn!

Vale "de baracuda" de Angelis. I wish we didn't had to live in different cities as I extremely enjoy your company. You always cheered me up when I felt sad, you always invited me when I felt lonely and you always knew what advice to give when I was in doubt. You are really my brother from another mother!

True Bee, thanks for the muay thai trainings in the jungle of Thailand and for teaching me that being knocked down doesn't mean that you are knocked out.

Nacho, my crazy Spanish Mexican friend who speaks with a Scottish accent. It's great to have you as a friend and I hope that we get to do many more rooftop-parties.

Rene Holtackers, ik heb je leren kennen op de MLO en nu 15 jaar later zijn we nog steeds goeie vrienden. Ik hoop nog vaak bij je op bezoek te kunnen komen in Zurich.

Bartje Boonen, wanneer pakken we weer de auto om met 180 km/u over der Autobahn naar Rene toe te scheuren? Lekker ouwehoeren en Rammstein luisteren!

Beste Chani, Anja, Wong, Bak or however that we may call you. I am looking forward to see you cycle through London after a whole night of parting. Hope you can stay on your bike this time!

Tonja, behalve een goed ontwikkeld gevoel voor humor (Kont-ent) heb je ook een uitstekend gevoel voor experimenten en bijbehorende controles. Ik ben ervan overtuigd dat je het heel goed gaat doen als PhD kandidaat! Heel veel succes!

Elena, from the 1st day as my student (when you fell of your bike in the morning and busted your knee, while playing Twister, in the evening) I knew we would get along very well. You did excellent work on the Epo project and I wish you all the best with your PhD!

Beste Ax, bedankt voor het uitstekende gezelschap en het prettige samenwonen op de Asselijnstraat. Heel veel succes met het afronden van je PhD!

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Beste paranimf, beste Danny, dank voor al die keren dat je geïnformeerd hebt naar het wel en wee van mijn promotietraject en voor al die keren dat we samen de bloemetjes buiten hebben gezet! We zijn al jaren beste matties en dat zullen we ook nog jaren blijven! Groeten aan Ilona en een dikke knuffel aan je schat van een dochter Demi!

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Ronald (en Hennie) Prak. Al jaren staan we vooraan in de pit en ik hoop er nog jaren naast je te staan!

Lieve Pim, ik ben je onzettend dankbaar voor je onmisbare steun toen ik weer eens aan een nieuwe studie begon. Zonder jouw steun toen, was ik nu niet hier geweest!

Jolique, als een vakantie naar Israël net zo lachen is als een weekendje London met jou…dan hoop ik dat het er snel van komt!

Casper, altijd in voor een lolletje en een feestje. Keep up the good work!

Rene Scriwanek, altijd leuk om effe bij je langs te lopen en te ouwehoeren over Timboektoe! Wanneer is er weer een barbecue bij je thuis?

Daphne, wanneer gaan we weer naar Der Derrick? Wat een lol was het met jou tijdens Koningsdag!

Stephin, mede-limburger. Het was super gezellig in Cambridge met jou!

Edo, het was heerlijk samen plaatjes draaien met jou op vrijdagmiddag in het van der Sluijs lab. Eindelijk iemand die NOFX weet te waarderen.

Diane, de eerste en enige keer dat ik uit cafe de Poort ben gegooid was met jou.. Wat een lol was dat zeg! Wij zijn niet gemaakt om samen naar het smartlappen festival te gaan. Dat we door de velg heen zakten van die 'gevonden' fiets op weg naar het centraal station is nog steeds hilarisch! Doe de groeten aan je stoere trucker (Pistolen Paultje)!

Mathijs, dat was wat he…toen je telefoon de oude gracht in vloog. Mooie tijden waren het met jou in het van der Sluis lab.

Bianka, Ow's it going mate? Good to see that you are enjoying yourself so much in Australia! I am very jealous of that!

Timo, het jonkie toen, het jonkie nu en toch kom je d'r wel! Goed bezig!

Joost en Janine, bedankt voor de uitstekende begeleiding tijdens mijn stage bij GenMab. Het was een groot plezier om met jullie samen te mogen werken. Joost, wanneer ga je weer optreden met je bandje? Kom ik de pit afbreken!

Luus, bedankt dat je me het email adres van Mark gaf op die bewuste zondagmiddag in cafe Weerbericht.

Lieve Bram; ik ken niemand die werkelijk elk telefoonnummer en alle teksen van Doe Maar uit zijn hoofd kent. Je hebt een groot talent, gebruik het wijselijk.

Lieve Ankie; ondanks dat je 4 jaar ouder bent was je vroeger altijd mijn kleine zusje. Als ik nu zie wat je allemaal hebt bereikt in je werk en je dagelijks leven, dan kan ik met trots zeggen dat je nu mijn grote zus bent! Daarnaast ben ik je eeuwig dankbaar voor je gigantische inzet bij de tot standkoming van dit proefschrift. Ik had me geen betere zus kunnen wensen!

Maarten, je stelde bij verre de beste vraag tijdens de Science & Supper lezing. Ik heb het even nagekeken, maar bloedgroep heeft geen invloed op plaatjes binding aan monocyten.

Lieve Kaat, ik verheug me erop om je later alles te vertellen wat jouw moeder en ik allemaal uitgevreten hebben!

Lieve pap, als leraar, en later als directeur, wil jij kinderen helpen door de dingen net iets anders te doen dan anderen. Toen ik vroeger les van jou kreeg botste dat soms omdat ik ook de dingen net iets anders wilde doen dan anderen. Nu weet ik van wie ik dat heb. Ik wordt nu doctor, pap, en ik weet zeker dat ik dat niet voor elkaar gekregen had zonder de wijze lessen die ik van jou heb geleerd. Bedankt daarvoor!

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Mi dulce Emma, what happens when the moon hits my eyes like a big pizza pie? Well, then the world seems to shine like I had too much wine. I want to thank you from the bottom of my heart for all those times that you supported and inspired me. You accept me for who I am and you stimulate me to become who I want to be. You make me Mister Fahrenheit and I could not have done this without you! Ik ben jouw halve sinaasappel y tu eres mi media naranja!

List of publications

Plasma levels of active VWF are increased in patients with first ST-segment elevation myocardial infarction: a multicenter and multiethnic study.
B. Rutten, A. Maseri, D. Cianflone, A. Laricchia, N.A. Cristell, A. Durante, M. Spartera, F. Ancona, L. Limite, D. Hu, H. Li, N.G. Uren, P.G. de Groot, P.M. Mannucci and M. Roest
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The relationship between fractional flow reserve, platelet responsiveness and platelet leukocyte complexes in stable coronary artery disease.
B. Rutten*, J.W. Sels*, T.C. van Holten*, M.A.K. Hillaert, J. Waltenberger, N.H.J. Pijls, G. Pasterkamp, P.G. de Groot and M. Roest. *Equal contributor
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Manuscript submitted for publication

Recombinant erythropoietin has no direct effect on platelet reactivity
B. Rutten, E. Chouri, J. Aragonés, I. Ott, D. Neumann and M. Roest
Manuscript submitted for publication

The platelet density per monocyte is a novel predictor of cardiovascular events in patients after percutaneous coronary intervention
B. Rutten, M. Roest, E.A. McClellan, J.W. Sels, A. Stubbs, J.W. Jukema, P.A. Doevendans, J. Waltenberger, A.J. van Zonneveld, G. Pasterkamp, P.G. de Groot* and I.E. Hoefer*. *Equal contributor
Manuscript submitted for publication

Curriculum Vitae

Bert Rutten was born on the 8th of August 1978 in Beringe, the Netherlands. After completing the MAVO in 1994, he studied medical microbiology at the MLO in Blerick. During this study, he performed an intership at the Plant Research Institute in Wageningen and an internship at MSD Merck in Boxmeer. After graduation, Bert went to travel around the world for 2.5 years. Hereafter, he decided to study 'Molecular Life Sciences' at the HLO in Utrecht, which he finished with a Honours degree from the University of Brisbane in Australia. Upon his return to the Netherlands in 2006, Bert worked in the pharmaceutical industry and the healthcare sector, where he became acquainted with the methodologies of both fields. As he was interested in the molecular machinery that carries out complex biological processes, he enrolled in 2008 in the Master program 'Cellular Life Sciences' at the University of Utrecht. In 2010, he accepted a PhD-candidate position to study the role of platelet adherence to immune cells during inflammatory diseases. The results of his research are described in this thesis. Bert presented his work at numerous scientific meetings, including the International Society of Thrombosis and Hemostasis Congress in 2013, but also at scientific outreach events such as the FameLab Competition of the University of Cambridge in 2014. Throughout his PhD, Bert was actively involved in improving the training and supervision of PhD candidates as a representative of the Cardiovascular Research Track and as a member of the PhD Council. He was also the first PhD candidate to be elected as a full-member of the Faculty Board of Medicine of the University Medical Center in Utrecht. This gave him to opportunity to influence decision related to the education of students within the Faculty of Medicine. In 2014, Bert founded his own company, The PhD Cooperation, which aims to unite PhDs in order to reduce the financial costs of thesis printing. Currently, he works at the department of Clinical Chemistry and Heamatology where he combines his research with science communication and public engagement.