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PLATELET FUNCTION AND THROMBIN GENERATION IN ISCHEMIC STROKE – CLINICAL CORRELATES AND PROGNOSTIC IMPORTANCE

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Platelet function and thrombin generation in ischemic stroke – clinical correlates and prognostic importance
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Frida and Jakob: stay curious!

"*Not everything that can be counted counts,*
and not everything that counts can be counted."
William Bruce Cameron

1 Cor 13:1-2

ABSTRACT

Platelet function is central to atherothombotic diseases and antiplatelet agents prevent non-cardioembolic ischemic stroke (IS). Individual differences in platelet reactivity may limit the response to antiplatelet treatment, leading to so-called high on-treatment platelet reactivity (HPR). HPR is associated with increased risk of cardiovascular events. Platelet activation leads to the release of platelet microvesicles (PMV), and circulating PMV are considered a measure of in vivo platelet function. Activated platelets and PMV enhance thrombin generation and thrombin in turn is a strong platelet agonist.

The overall aim of this thesis was to study the importance of platelet function, circulating PMV, thrombin generation and associations with clinical characteristics and prognosis in patients with IS or transient ischemic attack (TIA).

The prevalence of HPR to clopidogrel was assessed by whole blood aggregation in a cross-sectional study of 72 patients treated with clopidogrel one month after IS or TIA. Associations between HPR and clinical variables were determined, focusing on glucose metabolism, IS subtype and arterial vessel changes (study 1 and 2). Circulating PMV, expressing activation markers P-selectin or tissue factor (TF), and thrombin generation variables were measured in the acute and convalescent phase of IS/TIA in a cohort study of 211 patients (study 3 and 4). Associations with prognosis were evaluated after a 5 to 7 year follow-up. The primary outcome was a composite of recurrent IS, myocardial infarction and ischemic cardiovascular death; recurrent IS was a secondary outcome.

HPR to clopidogrel was found in 16/72 IS/TIA patients (22%) and was associated impaired glucose tolerance/diabetes, insulin resistance, hypertension and radiological white matter changes (WMC), indicating cerebral small vessel disease (CSVD). There were no associations between HPR and carotid atherosclerosis, other manifestations of large vessel atherosclerosis or IS stroke subtype.
PMV populations expressing P-selectin or TF were substantially elevated in the acute phase of IS/TIA, and remained elevated in the convalescent phase. Only PMV expressing TF and lacking phosphatidylserine (PS) were associated with poor outcome, with a hazard ratio (HR) of 1.86 [1.04-3.31] p=0.036 after adjustment for cardiovascular risk factors. Unexpectedly, high levels of several PMV populations appeared inversely associated with poor outcome. Despite being elevated in patients, endogenous thrombin generation potential (ETP) and peak thrombin measured in the acute phase were associated with *reduced risks* of primary outcome and recurrent IS after adjustment for cardiovascular risk factors, HR 0.40-0.53, $p < 0.05$ for all. MV-induced thrombin generation potential and MV peak thrombin were associated with increased risk of recurrent IS in univariate analysis. F1+2 was lower in patients than healthy controls, but not associated with outcome. There were no correlations between thrombin generation variables and PMV populations.

In summary, HPR to clopidogrel in patients with IS or TIA was common and the risk of HPR increased in the pre-diabetic phase. HPR was associated with CSVD but not with large artery

atherosclerosis. Circulating levels of PMV populations after an episode of IS/TIA had different associations with outcome; PMV expressing TF but lacking PS merit further investigation as they were associated poor prognosis. High levels of ETP and peak thrombin in the acute phase were associated with *reduced risk* of recurrence, while MV-induced thrombin generation was associated with *increased risk* of recurrent IS.

LIST OF SCIENTIFIC PAPERS

I. **Lundström A**, Laska A-C, Von Arbin M, Jörneskog G, Wallén H: Glucose intolerance and insulin resistance as predictors of low platelet response to clopidogrel in patients with minor ischemic stroke or TIA. *Platelets 2014; 25(2):102-110*

II. **Lundström A**, Wallén H, Von Arbin M, Jörneskog G, Gigante B, Höeg Dembrower K, Laurencikas E, Laska A C: Clopidogrel resistance after minor ischemic stroke or transient ischemic attack is associated with radiological cerebral small-vessel disease. *J Stroke Cerebrovasc Dis 2015; 24(10):2348-2357*

III. **Lundström A**, Mobarrez F, Rooth E, Thålin C, Von Arbin M, Henriksson P, Gigante B, Laska A C, Wallén H: Platelet microvesicles are elevated after ischemic stroke or TIA – specific subpopulations have different associations to prognosis. *Manuscript.*

IV. **Lundström A**, Rooth E, Mobarrez F, Thålin C, Gigante B, Laska A C, Wallén H: High thrombin generation in the acute phase of ischemic stroke or TIA is associated with reduced risk of recurrent ischemic event – a cohort study. *Manuscript.*

OTHER PUBLICATIONS

Publications not discussed in this thesis:

I. Thålin C, Blomgren B, Mobarrez F, **Lundström A**, Laska A-C, Von Arbin M, Von Heijne A, Rooth E, Wallén H, Aspberg S: Trousseau's syndrome, a previously unrecognized condition in acute ischemic stroke associated with myocardial injury. *J Investig Med High Impact Case Rep 2014 Jun 24; 2(2)*

II. Thålin C, Lundström S, Seignez C, Daleskog M, **Lundström A**, Henriksson P, Helleday T, Phillipson M, Wallén H, Demers M: Citrullinated histone H3 as a novel prognostic blood marker in patients with advanced cancer. *PLoS One 2018 Jan 11; 13(1)*

III. Paues Göranson S, Thålin C, **Lundström A**, Hållström L, Lasselin J, Wallén H, Soop A, Mobarrez F: Circulating H3Cit is elevated in a human model of endotoxemia and can be detected on circulating microvesicles. *Submitted to Sci Rep 2018-03-08, under revision.*

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACS	Acute coronary syndrome
AF	Atrial fibrillation
AGE	Advanced glycation end product
AMI	Acute myocardial infarction
APC	Activated protein C
CAA	Cerebral amyloid angiopathy
CAD	Coronary artery disease
CAT	Calibrated automated thrombogram
CSVD	Cerebral small vessel disease
COX-1	Cyclo-oxygenase 1
CVD	Cardiovascular disease
eGFR	Estimated glomerular filtration rate
ETP	Endogenous thrombin potential
HOMA-IR	Homeostasis model of assessment insulin resistance
HPR	High on-treatment platelet reactivity
ICH	Intracerebral hemorrhage
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IR	Insulin resistance
IS	Ischemic stroke
LTA	Light transmission aggregometry
MEA	Multiple electrode aggregometry
MRI	Magnetic resonance imaging
MV	Microvesicles
MV-PT	Microvesicle-induced peak thrombin
MV-TGP	Microvesicle-induced thrombin generation potential
NO	Nitric oxide
NR	Non-responder to clopidogrel
OGTT	Oral glucose tolerance test

Ox-LDL	Oxidized low-density lipoprotein
PAR	Protease-activated receptor
PCI	Percutaneous coronary intervention
PGI_2	Prostacyclin
PMV	Platelet microvesicles
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein 1
R	Responder to clopidogrel
TIA	Transient ischemic attack
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TRAP	Trombin receptor agonist peptide
VSMC	Vascular smooth muscle cell
VWF	Von Willebrand factor
WBA	Whole blood aggregometry
WMC	White matter changes

1 INTRODUCTION

1.1 GENERAL BACKGROUND

Stroke is the second most common cardiovascular disease after coronary artery disease (CAD) and a major cause of death and disability in Sweden and globally. The majority of strokes are ischemic (85%), caused by a thrombus or embolus occluding a cerebral artery. Ischemic stroke (IS) is a heterogeneous condition with different underlying pathologies. The three major IS subtypes are large artery atherosclerosis, small vessel disease and cardioembolism, which is often caused by atrial fibrillation (AF).

In the absence of cardioembolism, antiplatelet treatment is given as secondary prophylaxis after IS or transient ischemic attack (TIA). Platelets have a direct role in arterial thrombus formation following rupture of an atherosclerotic plaque. Mounting evidence suggests that platelets also have a role in the atherosclerotic process (1-3). While intensified platelet inhibition has resulted in net clinical benefits for patients with acute coronary syndrome (ACS), this has not always been the case for patients with IS as several studies have shown a marginal improvement which has often been offset by an increased risk of major bleeding (4, 5).

The view of platelet function in hemostasis and thrombosis has evolved substantially over the last 10-20 years. It has been shown that there are individual differences in the response to antiplatelet treatment, in particular to clopidogrel but also to aspirin, so-called high on-treatment platelet reactivity (HPR) (6, 7). Platelets have been found to have an important role in inflammation, interacting with the endothelium and leukocytes (8). Upon activation platelets release microvesicles (MV) into the circulation which stimulate coagulation (9); further activated platelets bind MV in the thrombus which promote fibrin formation (10). Most of the clinical studies on platelet function in cardiovascular disease (CVD) have been performed in patients with CAD. However, patients with IS differ from those with CAD in risk factor profile, underlying vessel pathology, acute treatment and bleeding risk. Therefore the aim of this thesis was to investigate platelet function in patients with IS or TIA, focusing on HPR and MV.

1.2 CLINICAL ASPECTS OF STROKE AND STROKE SUBTYPES

1.2.1 Stroke epidemiology and risk factors

The updated stroke definition recognizes stroke as one manifestation of cerebrovascular diseases (11). Stroke is defined as acute, focal neurological symptoms persisting for more than 24 hours, or typical symptoms of shorter duration with radiological evidence of an acute lesion in an appropriate neuroanatomical location (11). If the symptom duration less than 24 hours and no corresponding lesion is found, the patient is diagnosed with TIA. Patients with TIA have a substantial risk of developing IS in the following weeks-months.

The stroke incidence in Sweden is presently about 25 000 per year (12). In addition there are about 10 000 cases of TIA per year. About one quarter of strokes are recurrent events. The incidence of stroke in Sweden has decreased by approximately 2000 cases per year in the last decade (12). This is likely due to improved risk factor control in the population with improved treatment of hypertension and hyperlipidemia, increased anticoagulant treatment of AF and reduced rates of smoking. The risk of recurrent IS remains relatively high despite secondary prevention: 3-7% in the first three months after IS/TIA and thereafter 2-3%/year in modern intervention studies (13, 14).

The main risk factors for stroke are the established cardiovascular risk factors, but their relative importance differs from those of other CVD. Hypertension is by far the most important risk factor, giving an odds ratio (OR) of about 3 and globally representing about half of the population attributable risk (15). Age is a strong, non-modifiable risk factor, and the average age is higher for stroke patients than for patients with acute myocardial infarction (AMI) (16). Male sex is not a clear risk factor for stroke overall (16). Smoking is a risk factor for stroke with an OR 1.7 and dose-dependent increase of risk for current smokers (15, 16). Diabetes increases the relative risk of stroke (16), but epidemiologic studies show a limited independent contribution to the population attributable risk (15). Other aspects of the metabolic syndrome such as waist-to-hip ratio or BMI may be more important for stroke (15, 17). Hyperlipidemia is a risk factor for stroke, but statin treatment reduces the risk of recurrence less after IS than after AMI (18, 19). Overall smoking, diabetes and hyperlipidemia are all weaker risk factors for stroke than for AMI (15, 20). High alcohol consumption increases the risk for stroke with an OR of about 2 (15), while for AMI moderate alcohol consumption overall is protective (20). Stress, low level of exercise and a diet low in fruit and vegetables are important modifiable risk factors (15). AF and carotid stenosis are etiological risk factors, as discussed below.

Hemorrhagic stroke or intracerebral hemorrhages (ICH) comprise about 15% of strokes. The main risk factors for ICH are hypertension, alcohol and stress (15). The most common forms of ICH are hypertensive ICH and subarachnoid hemorrhage, where the latter is often caused by a ruptured cerebral aneurysm. Rare causes include cerebral amyloid angiopathy (CAA), vessel malformations and tumors.

IS has many different causes. According to a recent large meta-analysis, cardioembolism causes 22% of IS, large artery atherosclerosis 23% and small vessel disease 22% (21). In 26% of cases the cause was undetermined, which includes the cases where no cause can be determined, so-called cryptogenic stroke. Other determined causes contributed 3% of IS, including conditions such as dissection of cerebral vessels, venous sinous thrombosis, migraine, vasculitis and prothrombotic/procoagulant conditions. The distribution of IS subtypes varies depending on ethnic background and socioeconomic conditions (21).

1.2.2 Ischemic stroke subtypes

1.2.2.1 Cardioembolism

Cardioembolism involves thrombus formation in the heart, often due to disturbed flow conditions. The thrombus, or part of it, may release, follow the blood stream and cause occlusion of cerebral arteries. AF is by far the most common cause, with thrombi often forming in the left atrial appendage. Other underlying conditions include left ventricular mural thrombus due to AMI or pronounced heart failure, valvular heart disease (in particular mitral stenosis), thrombus formation on mechanic valve prostheses (often due to inadequate anticoagulation), endocarditis, cardiac tumors and patent foramen ovale with paradoxical embolism from the venous system.

AF is common with an estimated prevalence of 3% in adults and higher in the elderly (22). In AF, the electrical conduction system of the heart is disturbed by micro-reentry circuits in the atria, causing fast and irregular atrial contractions. The ventricular heart rate is also irregular as only random electrical impulses pass the atrial-ventricular node. Factors that increase the risk for AF include structural heart disease, hypertension and possibly diabetes (22). The pathology of AF is characterized by structural remodeling with atrial distension, cardiac fibrosis and endocardial damage. These factors both increase the risk of AF and are aggravated by AF, creating a viscous circle (23). Overall, AF increases the risk for IS by a factor 2-5, where the individual risk depends on comorbidities/cardiovascular risk factors. These are often summed up in scores, and the CHA_2DS_2-VASc score (0-9 points) is presently the one recommended (22). The yearly risk of IS due to AF in Sweden varies between 0 and 17% from the lowest to the highest CHA_2DS_2-VASc score (24). Other scores are investigated to improve the prediction of which AF patients are at risk of IS/thromboembolism (25).

Factors that contribute to thrombus formation in AF are stasis of blood flow, activated coagulation and endocardial damage/activation (23). Certain plasma platelet activation markers have also been found elevated, which may however be secondary to underlying cardiovascular disease or comorbidities (23). Anticoagulant treatment is given to prevent IS caused by AF or other forms of cardioembolism. Further discussion cardioembolic IS will be limited in this thesis, which concerns primarily non-cardioembolic IS.

1.2.2.2 Large artery atherosclerosis

Atherosclerosis with stenosis of the carotid bifurcation or proximal internal carotid artery is estimated to cause 10% of IS. IS can also be caused by large artery atherosclerosis of the intracranial vessels, vertebral or basilar arteries or more proximal vessels including the aortic arch. IS due to large artery atherosclerosis has the strongest commonality to AMI. However, in contrast to AMI, where plaque rupture often leads artery occlusion in direct proximity to the plaque, large artery IS often involves artery-to-artery embolization.

The development of atherosclerotic plaque is a complex process stretching over many years, often decades. Only some key events in plaque development will be mentioned here (26). Plaque typically form at artery branch points or areas of turbulent blood flow. The initial lesion, the fatty streak, results from accumulation of lipoproteins in the intima layer of the artery wall, where they bind to proteoglycans. Once resident in the vessel wall, lipoproteins are prone to oxidization which generates pro-inflammatory breakdown products. These cause endothelial dysfunction and recruitment of leukocytes to the lesion. Amongst others monocytes transmigrate into the intima, and differentiate into macrophages which phagocytize oxidized low-density lipoprotein (ox-LDL) and transform into foam cells. Foam cells may undergo apoptosis, enhancing lipid accumulation. The inflammatory reaction leads to recruitment of vascular smooth muscle cells (VSCM) from the arterial wall media. In response to inflammatory cytokines, in particular platelet-derived growth factor and transforming growth factor β, VSCM proliferate and produce extracellular matrix, resulting in progression to a fibrofatty lesion. Activation of platelets and the coagulation system occur in association to the lesion, resulting in microthrombi, which contribute to plaque progression, hence the term atherothrombosis. As plaques become larger and more complicated they may develop a necrotic core, neovascularization and a thin fibrous cap, all of which contribute to plaque instability and risk of rupture. Plaque rupture exposes collagen and tissue factor (TF) which activate platelets and the coagulation system leading to thrombus formation.

1.2.2.3 Cerebral small vessel disease

Cerebral small vessel disease (CSVD) is the most common cause of so-called lacunar infarcts, which are often central infarcts with a size less than 20 mm in the acute phase. CSVD is a collective term for different pathological changes in penetrating arteries and arterioles less than 0.5 mm in diameter; cerebral venules and capillaries may also be affected (27, 28). The general characteristic of CSVD is a spread but segmental breakdown of the vessel wall architecture. CSVD may manifest as different cerebral lesions detectable with magnetic resonance imaging (MRI) (29):

- Small subcortical infarcts
- Lacunes of presumed vascular origin
- White matter changes (WMC)
- Expanded perivascular spaces
- Cerebral microbleeds
- Large ICH (deep or lobar)
- Brain atrophy

Sporadic CSVD is considered to have two major causes: hypertensive arteriolosclerosis and CAA (27). These manifest in different locations with hypertensive changes typically occurring in deep, central structures and CAA in subcortical, cortical and leptomeningeal arteries. The two conditions may co-exist.

Increasingly, CSVD is considered a syndrome affecting the whole brain (30). In the absence of stroke, CSVD may cause cognitive decline, gait disturbances, mood disorders and urinary incontinence. A meta-analysis of population-based studies of WMC, considered to be the earliest and most common CSVD lesion, showed that WMC increase the risks of stroke (hazard ratio, HR 3.3), dementia (HR 1.9) and death (HR 2.0) (31). While the short-term prognosis of lacunar infarcts is generally good, the medium to long term prognosis is worse, with an increased risk of all the above-mentioned complications.

The etiology of CSVD has remained elusive, and there is limited knowledge as to which patients develop clinical symptoms from CSVD, which progress radiologically/clinically and why patients develop certain CSVD manifestations and not others. In population-based studies, cardiovascular risk factors are associated with WMC and lacunes, in particular hypertension and age. However, cardiovascular risk factors are not substantially more common in patients with clinical lacunar infarcts than in patients with IS of other subtypes (32, 33). Also, a recent study showed that cardiovascular risk factors explained a very limited part of the variance in WMC in both a population-based and a stroke cohort (34). The authors concluded that CSVD has primarily a 'non-atheromatous' etiology. Hereditary factors are important (35, 36) and genome-wide association studies have identified certain candidate genes for IS caused by CSVD (37).

The pathology of lacunar infarcts was first characterized in ground-breaking work by Fisher 1960's (38). In the majority of cases, the artery supplying a lacune was occluded. Characteristic vessel changes included segmental lipohyalinosis with arterial wall thickening, fibrinoid necrosis and loss of VSMC. Similar changes are seen in CAA, where vessel deposits consist of beta-amyloid peptides. Chronic hypoperfusion with local hypoxia has been assumed to contribute to observed changes (39). Also increased permeability of the blood-brain barrier has been implicated (40), supported by the fact that lipohyalinosis contains fibrin and other plasma proteins. However, it has recently been questioned whether reduced cerebral blood flow is a cause or an effect of CSVD changes, as a longitudinal study found no association between baseline cerebral blood flow and WMC progression (41). Also, CSVD has been found to be a dynamic process, where lacunes, microbleeds and WMC may disappear in long-term follow-up (42). This has sparked speculation of underlying dysfunction in other cerebral structures or processes, such as the newly discovered glymphatic system for cerebral fluid transport (43) or the oligodendrocyte network (44).

Studies on the role of platelets and CSVD are scarce and partly contradictory. Most studies suggest that platelet activation is more important in large vessel IS than small vessel IS (45). Oberheiden et al showed enhanced platelet activation in patients with CSVD as compared to healthy controls (46).

1.3 HEMOSTASIS AND THROMBOSIS – A PLATELET-CENTERED PERSPECTIVE

Platelets are 2-5 µm anucleate cell fragments released into the circulation from megakaryocytes in the bone marrow. The normal platelet concentration in blood is approximately 150-400 x 10^9/l and the life-time in circulation 8-10 days. The primary function of platelets, together with the vessel wall and coagulation system, is to limit blood loss after vessel damage. A breach of the endothelial lining causes rapid and dramatic changes in platelet morphology and function. Aspects of platelet functions which are central to hemostasis and thrombosis are discussed below. The first two sections introduce platelet function according to the traditional model of hemostasis with primary and secondary hemostasis. Section 1.3.3 discusses the role of MV in atherothrombosis, section 1.3.4 addresses the advances in understanding the dynamic role of platelets in thrombosis and section 1.3.5 gives an overview of platelet function and cardiovascular risk factors.

1.3.1 Platelets in primary hemostasis

Under normal condition platelets circulate in an inactive, discoid shape. Healthy endothelial cells contribute to maintaining platelets inactive by releasing the platelet inhibitors nitric oxide (NO) and prostacyclin (PGI_2) and expressing ectoADPase (CD39), which breaks down adenosine diphosphate (ADP), one of the main platelet agonists (see below). Endothelial damage leads to exposure of sub-endothelial proteins which bind platelet receptors (generally termed glycoprotein receptors, GP), causing platelet adhesion and activation. Under the flow conditions in arteries and arterioles, sub-endothelial collagen together with von Willebrand factor (VWF) are central for platelet adhesion (47). Exposed collagen binds circulating VWF, which subsequently binds the platelet receptor GPIbα of the GPIb-IX-V complex, see figure 1. This leads to platelet tethering and translocation; firm adhesion involves binding of platelet GPVI and integrin $\alpha_2\beta_1$ to collagen. Binding of these receptors causes platelet activation with stimulation of intracellular signal pathways and increased calcium concentrations. This leads to platelet cytoskeletal rearrangements with spreading and shape change, activation of the platelet receptor GPIIbIIIa through inside-out signaling, generation of thromboxane A2 (TxA2) and degranulation of platelet granulae, see figure 1.

GPIIbIIIa is the most abundant platelet receptor. Once activated, GPIIbIIIa can bind to several ligands. It stabilizes platelet adhesion to collagen via VWF. Further it supports platelet-platelet bonds via molecular bridges, primarily of soluble fibrinogen, but also fibrin, VWF and fibronectin can function as bridges (48). GPIIbIIIa is thus the central receptor for platelet aggregation. Binding of GPIIbIIIa to its ligands activates the receptor further, increasing platelet activation by outside-in signaling.

Platelet activation causes degranulation, which contributes to the recruitment of platelets to the forming thrombus (49). Platelets contain two types of granules, α-granules and dense granules, where the former are most numerous.

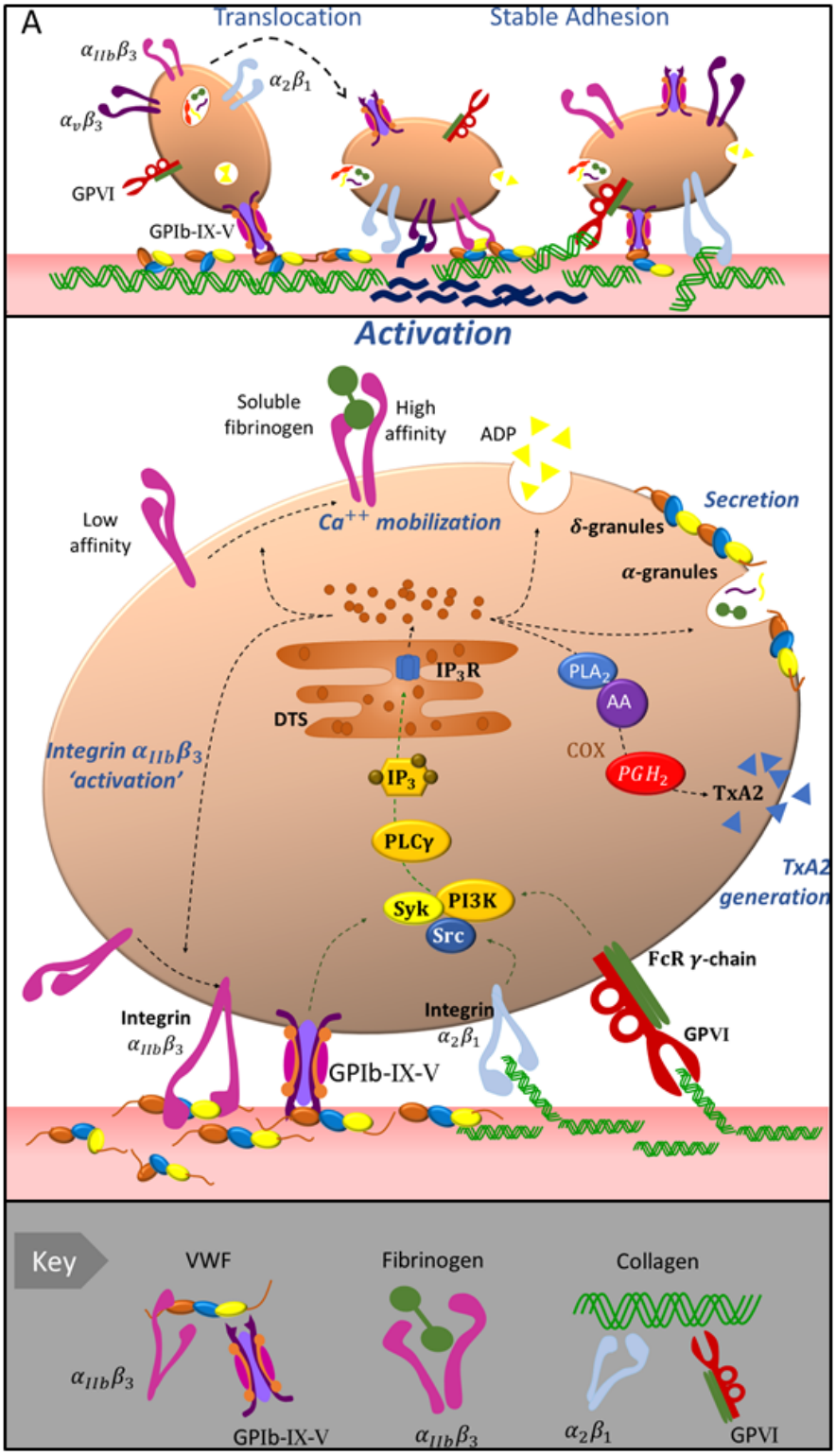

Figure 1: Platelet adhesion and activation, simplified version based on (47). Platelet receptors with their main ligands: GPIb-IX-V binding VWF immobilized on collagen, integrin $\alpha_{IIb}\beta_3$ (GPIIbIIIa) binding fibrinogen and VWF, GPVI and integrin $\alpha_2\beta_1$ binding collagen. Receptors signal via the kinases Src, Syk and PI 3 kinase (PI3K), which amongst other activates phospholipase Cγ (PLCγ) generating the inositol-1,4,5-triphosphate (IP3) which stimulates calcium release from the dense tubular system (DTS). Laminin and its receptor integrin $\alpha_6\beta_1$ and the fibronectin receptor $\alpha_5\beta_1$ have been omitted to limit complexity. Image by Jakob Svensson.

α-granules contain a large number of proteins (more than 300), many related to adhesion, coagulation, anti-coagulation, fibrinolysis and wound healing. Importantly α-granules release VWF and fibrinogen, supporting platelet aggregation via GPIIbIIIa. Dense granules contain mainly non-protein small molecules, in particular ADP, adenosine tri-phosphate (ATP), calcium, magnesium and serotonin. In addition they contain polyphosphates. Both α-granules and dense granules carry membrane-bound platelet receptors such as GPIb-IX-V, GPVI and GPIIbIIIa, which are exposed on the platelet surface after degranulation, enhancing platelet adhesive properties. In addition, α-granules contain membrane bound P-selectin, which is only exposed on the platelet surface after α-granules release. P-selectin is thus a marker of platelet activation. P-selectin is important for platelet interactions with other cells, in particular leukocytes.

Once the first layer of platelets has deposited over the sub-endothelium, platelet recruitment to the growing thrombus depends largely on the availability of soluble platelet agonists. The main soluble platelet agonists in vivo are thromboxane A2 (TxA2), ADP and thrombin (from the coagulation system). All of these signal via G-coupled receptors on the platelet (50). TxA2 is formed in activated platelets by several enzymes, see figure 1. Activated phospholipase A2 cleaves off arachidonic acid (AA) from the plasma membrane; AA is then converted to TxA2 by cyclooxygenase 1 (COX-1) and thromboxane synthetase. TxA2 diffuses out of the platelet and activates thromboxane receptors on near-by platelets, signaling in an auto- and paracrine manner. TxA2 is a moderately strong platelet agonist, but has a short half-life (about 30 seconds) (50). ADP is released from dense bodies and may also be released from injured cells in the vessel wall. It activates the platelet purinerg receptors $P2Y_{12}$ and $P2Y_1$ in an auto- and paracrine manner. ADP has an important role in amplifying the effect of other platelet agonists (50). Via $P2Y_{12}$ ADP regulates overall platelet reactivity through inhibition of the enzyme adenylyl cyclase which generates cyclic AMP (cAMP). PGI_2 from endothelial cells activates adenylyl cyclase, increasing cAMP levels, which acts as a general brake and suppresses platelet response to agonists. ADP stimulation of $P2Y_{12}$ counteracts the platelet inhibitory effect of PGI_2, enhancing the response to other agonists. Thrombin is generated by the coagulation system and stimulates protease-activated receptors (PAR) on platelets, in humans PAR-1 and PAR-4. Thrombin also binds to GPIbα, which enhances platelet activation. Thrombin is a strong platelet agonist and higher concentrations cause a sustained increase of platelet calcium levels. In addition to the above agonists, platelets can be activated by circulating catecholamines, notably norepinephrine (51) but also epinephrine (50). Both signal primarily via the adrenerg α_{2A} receptor on platelets (52), reducing cAMP levels, but likely also having other effects. On their own catecholamines are considered to be relatively weak agonists, but they potentiate the response to other agonists (50). Serotonin from dense granules is a relatively weak platelet agonist (53), which also potentiates the response to other agonists. It signals via the G-coupled receptor $5\text{-}HT_{2A}$ in platelets and also has a vasoconstrictive effect.

In addition to the above platelet receptors and agonists, platelet activation and aggregation depend on local flow conditions. In healthy arteries flow is laminar, with maximum flow velocity in the center and flow decreasing to zero at the vessel wall. The differences in flow velocities between neighboring fluid layers near the wall causes shear stress which exerts a pressure on flowing and adhering cells and molecules. Shear is generally measured as shear rate in the unit s^{-1}. In veins and large arteries shear rate is generally low, about 500-1000 s^{-1}, whereas in arterioles it can be up to 5000 s^{-1} and in stenotic arteries can reach levels ten times higher than in normal blood vessels (3, 47). Platelet aggregation under high shear rate depends critically on VWF binding to GPIb and GPIIbIIIa, which becomes more efficient as shear rate increases, likely due to increased exposure of VWF binding sites (54). Turbulent flow with rapidly varying shear can also cause platelet aggregation, independent of TxA2 and ADP signaling (54). While laminar flow will tend to wash out soluble platelet agonists, disturbed flow with eddies and vortices downstream of an arterial stenosis or thrombus can cause accumulation of soluble platelet agonists, maintaining platelet activation (47).

Under the flow conditions in arteries and arterioles, the primary platelet plug cannot maintain stability for longer times and requires stabilization by fibrin fibers from the coagulation system, so-called secondary hemostasis.

1.3.2 Platelets and secondary hemostasis

The main component of secondary hemostasis is the coagulation system, consisting of a number of serine proteases and their co-factors which circulate in inactive form as zymogens. At vessel damage these are activated to produce **thrombin** which cleaves **fibrinogen** to **fibrin**. Fibrin molecules associate into fibrils which crosslink to form the fibrin network of blood clots. Coagulation is a powerful process which if activated systemically can have fatal consequences to the host. The coagulation system is therefore tightly regulated, amongst others by anticoagulant and fibrinolytic proteins.

One of the primary functions of platelets in secondary hemostasis is to localize the coagulation process to the thrombus by anchoring coagulation factors to the surface of activated platelets, the so-called cell-based model of coagulation (55). On the activated platelet surface, coagulation factors assemble into coagulation complexes. These complexes generally consist of an enzyme, its co-factor(s) and the substrate being cleaved (56). Binding of coagulation complexes to the activated platelet surface increases their enzymatic efficiency by up to three orders of magnitude or more, substantially enhancing the amount of thrombin formed (56).

The main coagulation complexes relevant to hemostasis and thrombosis are illustrated in figure 2 (56); coagulation factors are denoted f, the roman number and ‘a’ added for the activated form. At vessel damage, membrane bound TF is exposed on sub-endothelial cells and binds fVIIa, which is present in the circulation in low amounts. The resulting **extrinsic tenase** complex generates small amounts of fIXa and fXa which initiate coagulation. FXa

associates with fVa on activated platelets, forming the **prothrombinase** complex which cleaves prothrombin to thrombin (fII to fIIa). The small amount of thrombin generated during initiation activates fVIII and fXI; the latter in turn activates fIX. FIXa associates with its co-factor fVIIIa to form the **intrinsic tenase** complex. The intrinsic tenase complex generates larger amounts of fXa and amplifies thrombin generation by the prothrombinase complex, so-called propagation.

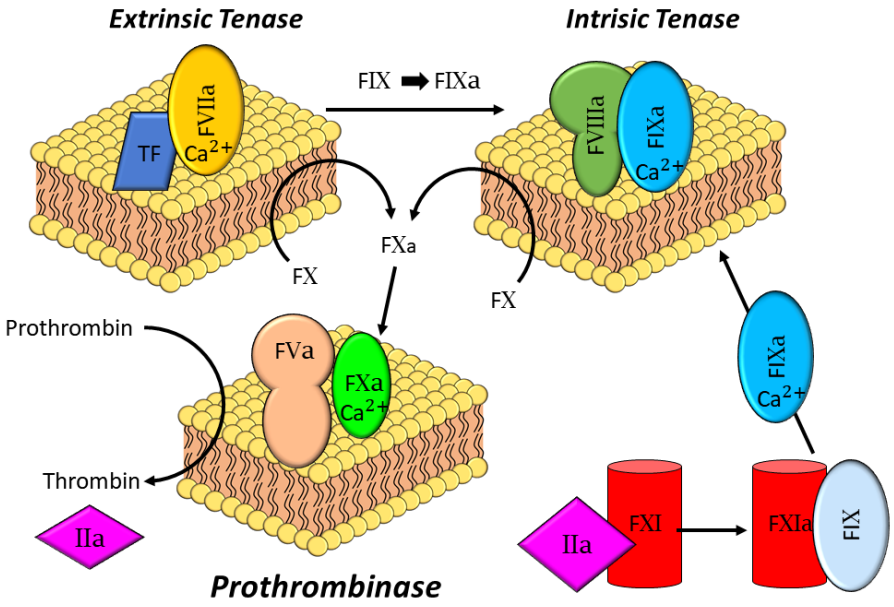

Figure 2: The main coagulation complexes extrinsic tenase, intrinsic tenase and prothrombinase; also shown is the activation of fXI by thrombin (fIIa) and the subsequent activation of fIX by fXIa.. FIIa = thrombin. Image by Jakob Svensson.

Thrombin is essential to the coagulation process not only as the final serine protease generating fibrin. It also activates several other coagulation factors: fV and fVIII, the necessary co-factors in the prothrombinase and tenase complexes, and fXI, which generates fIXa. In addition it activates fVII. Thrombin thus amplifies its own generation. Further, thrombin activates fXIII, which crosslinks fibrin fibers, forming the fibrin network. As mentioned above thrombin is also a strong platelet agonist. The prothrombotic roles of thrombin are illustrated in figure 3.

In addition to the above complexes, coagulation can be initiated via the contact pathway, resulting in activation of fXII which activates fXI. As deficiency of contact pathway proteases in humans does not cause bleeding, the contact system is considered to have limited importance for hemostasis, but may be important in thrombosis, see section 1.3.4.5.

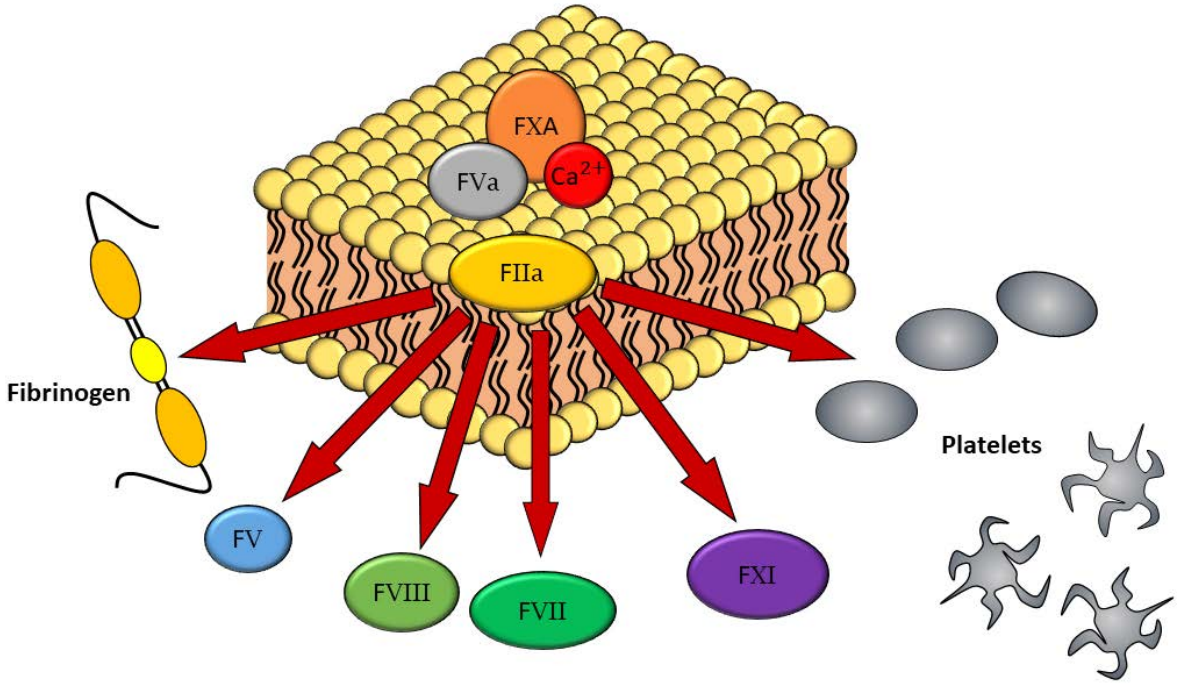

Figure 3: The different prothrombotic functions of thrombin (fIIa). Image by Jakob Svensson.

Anticoagulant proteins restrict the coagulation process in time and space. Tissue factor pathway inhibitor (TFPI) inhibits the extrinsic tenase complex quickly, limiting its function to initiation of coagulation (56). Circulating antithrombin inactivates thrombin, fIXa, fXa, fXIa, fXIIa and possibly also fVIIa in the extrinsic tenase complex. Thrombin together with thrombomodulin activates protein C bound to endothelial protein C receptor on the surface of neighboring endothelial cells. Activated protein C (APC) then dissociates from the endothelial surface and associates with cofactors (among them protein S) on the activated platelet surface to cleave fVa and fVIIIa, inhibiting the intrinsic tenase and prothrombinase complexes (56). Thrombin thus has a self-limiting function.

Activated platelets bind coagulation factors by different mechanisms. The vitamin K-dependent coagulation factors (prothrombin, fVII, fIX and X) and anticoagulants (protein C and protein S) contain a gamma-carboxyglutamic domain (Gla domain) which after activation and in the presence of calcium ions develops a hydrophobic patch, that allows incorporation into plasma membranes expressing negatively charged phospholipids. In resting platelets, as in other cells, an asymmetrical distribution of phospholipids is maintained, where the outer plasma membrane consists of neutral phosphatidylcholine and sphingomyelin and negatively charged phospholipids including phosphatidylserine (PS), phosphatidyl-etanolamine and phosphatidyl-inositol are found on the inner plasma membrane (57). This distribution is maintained by a balance of ATP-dependent aminophospholipid translocases, so-called flippase and floppase. Platelet activation, with elevated calcium concentration, inhibits flippase and activates floppase and scramblase, where the latter causes random transport of phospholipids across the membrane. This results in PS exposure on the

outer platelet membrane surface. (The platelet scramblase has recently been identified as TMEM16F (58).) PS binds the Gla domain of vitamin K-dependent coagulation factors and is thus a procoagulant factor of activated platelets. FV and fVIII contain different domains which allow binding to phospholipids of the plasma membrane, notably the C-domain. In addition, activated platelets have been shown to have specific binding sites for the coagulation factors of the intrinsic tenase and prothrombinase complex and fXI, in particular in their activated form (57). Both the binding affinity and enzyme complex efficiency can be higher after binding to activated platelets than after non-specific binding to PS on synthetic phospholipid vesicles (56, 57). Platelet expression of specific binding sites depend on the type and strength of platelet agonist stimulation, with thrombin combined with collagen having the strongest effect.

In addition to anchoring the coagulation complexes, platelets contribute to and regulate coagulation in different ways. α-granules contain fibrinogen, fV and fXIII, and the presence of fVIII, fIX and fXI has also been reported (56). Degranulation increases the local concentration of these factors in the thrombus. Several granule proteins, including fibrinogen and fV are not synthesized by megakaryocytes, but taken up selectively from plasma by receptor-mediated, clathrin-dependent endocytosis in both megakaryocytes and platelets. FV is then 're-tailored' creating platelet-derived fV which is distinct from plasma fV physically and functionally (56). Notably, platelet-derived fV is more readily activated, has higher co-factor activity and is a poorer substrate for APC inactivation than its plasma counterpart (56). Furthermore, platelet fV in α-granules is partially activated at release, contributing to the initiation of coagulation as discussed above. Platelets also contain anticoagulants, among them antithrombin, TFPI and protein S as discussed above, but also others, such as protease nexin 1 and 2, where the former is a potent membrane-bound inhibitor of thrombin and the latter inactivates fXIa (57). In addition α-granules contain several proteins of the fibrinolytic systems, with both activating and inhibiting effects (49). Platelet activation can thus both promote and limit coagulation and fibrinolysis, and the regulation of these effects is unclear. Differential granule release may occur by different intracellular signaling pathways (59). α-granules are heterogeneous in content, and it has been proposed that their release may be regulated differentially in time and space for an appropriate effect (60).

1.3.3 Microvesicles in hemostasis and thrombosis

1.3.3.1 History of microvesicles, general introduction

In addition to platelets and coagulations factors, Chargaff and West in the 1940's discovered a subcellular, sedimentable factor in plasma which could be removed by ultracentrifugation and supported thrombin generation (61). Wolf showed in 1967 that this factor consisted mainly of minute particles budding from platelets, which he termed 'platelet dust' (62). These particles were later called microparticles and the presently recommended term is microvesicles (MV) (63). MV have a diameter of 0.1-1.0 μm and are released from the plasma membrane at activation. Following Wolfs discovery it was found that MV can be

released from the plasma membrane of virtually all cell types at activation or apoptosis. MV form part of the larger group extracellular vesicles (EV) which also comprise the smaller exosomes (40-100 nm in diameter), released from intracellular multi-vesicular bodies, and the generally larger apoptotic bodies (64). The majority of circulating MV express platelet/megakaryocyte surface markers (65, 66). Erythrocytes, endothelial cells and leukocytes also release MV in the circulation, which can be identified by their respective specific surface markers (66).

1.3.3.2 Platelet-derived microvesicles

The majority of circulating MV carry platelet/megakaryocyte markers, and are termed platelet-derived MV (PMV). PMV are of importance for hemostasis and thrombosis in different ways. The procoagulant activity which led to their discovery is thought to be related partly to the expression of PS, which can bind coagulation factors as discussed above. MV expression of PS is considered connected to the mechanisms resulting in MV release (63) and PS has been shown to contribute substantially to MV capacity for thrombin generation (67). MV of non-platelet origin also express PS and can thereby contribute to coagulation. Notably, half or more of PMV in healthy volunteers do not express PS as identified by the PS marker annexin V (68, 69). In vitro generated PS-positive PMV bind coagulation factors fV, fVIII, fIX and fX with high affinity (70-73). In the latter study, PMV generated by calcium ionophore stimulation were estimated to be almost two orders of magnitude more procoagulant per surface area than activated platelets (73), indicating that coagulation factor binding sites can be enriched on PMV. Interestingly, in vitro generated PMV have also been shown to have anticoagulant effects (74).

In addition to their effects on coagulation, PMV are of interest as markers of intravascular platelet activation. The half-life of MV in the circulation is limited and was found to be about five hours in humans (75). In particular PS-positive MV (PS^+MV) bind different opsonins and are cleared by macrophages (76) or taken up by endothelial cells (77, 78). Despite this, PMV circulate in healthy at substantial concentrations (69), suggesting that MV expressing platelet surface markers are constitutively released. Flaumenhaft et al demonstrated that megakaryocytes can release PS^+MV constitutively, which likely contribute to the circulating pool of MV identified as PMV (79). It has also been suggested that PMV may be released by aging platelets. However, even in healthy volunteers a subset of PMV express P-selectin indicating that they are released from activated platelets (79, 80). PMV levels have been found elevated in various pathological conditions related to platelet activation, including IS/TIA and AMI (63, 67, 80-82). It has been suggested that PMV expressing P-selectin could be more specific markers of platelet activation than overall PMV concentrations (83).

The heterogeneity of PMV is increasingly recognized and depends on the circumstances leading to their release (76, 84). PMV can be generated by different types of stimulation: platelet agonists, inflammatory signals (e g lipopolysaccharide, immune complexes) or physical conditions (shear stress, hypoxia, storage) (76). The type and strength of stimulation

influence not only the amount of PMV generated but also their surface markers and cytoplasmic content (63). Once generated PMV can be taken up by various target cells, for instance endothelial cells, but also cells of the immune system (85). The effect of PMV on target cells also depends on their content and how they were generated.

1.3.3.3 Tissue factor positive microvesicles

As discussed above, TF is the main initiator of coagulation. In the cell-based model of hemostasis, TF was considered external to the circulation, coming into contact with blood only at vessel injury (55). In the '90's several authors reported low concentrations of circulating TF in plasma and other body fluids (86, 87), but the results were questioned (88). However, in 1999, Giesen et al demonstrated that TF from human blood accumulated in experimental thrombi on pig arterial media and collagen-coated glass slides (89). Immuno-electronmicroscopy identified TF-positive MV (TF^+MV) in clusters near platelets. TF was shown to be functional, as blocking anti-TF-antibodies reduced the size of thrombi and fibrin formation substantially. TF^+MV expressed the leukocyte marker CD18, and subsets of TF-positive neutrophils and monocytes adhering to the thrombus were identified (89). TF^+MV were estimated to be present in blood in sub-picomolar concentrations and TF was proposed to circulate 'encrypted', to be activated only in the microenvironment of the thrombus.

Since their discovery, TF^+MV have spurned research and debate (90-92). In line with the above study, Falati et al showed TF^+MV accumulation in an in vivo mouse model of laser-induced thrombosis, which lacked endothelial denudation (10). TF^+MV bound to the thrombus via P-selectin glycoprotein 1 (PSGL-1) on MV and P-selectin on activated platelets. In the same study, TF^+MV isolated from human blood expressed CD14, indicating monocyte lineage. Animal experiments suggested that also the generation of TF^+MV depended on P-selectin stimulation of PSGL-1 on monocytes (93, 94). In contrast to the study by Falati, mouse models of vascular injury thrombosis with endothelial denudation showed no significant contribution from blood-borne TF or TF^+MV to thrombus formation (95). The source of TF in circulating TF^+MV has been debated, and neutrophils, platelets and endothelial cells have been proposed as alternative TF sources to monocytes (91). Monocyte-derived TF^+MV have been shown to bind to and fuse with activated platelets (96), which may explain the finding of circulating TF^+MV expressing platelet markers (80, 97). Notably, the specificity of TF antibodies to identify TF on MV has been questioned (98). In addition to circulating TF^+MV, atherosclerotic plaque contain TF^+MV with active TF, which are highly procoagulant and thought to be derived from macrophages and VSMC (99).

The mechanisms resulting in activation or 'decryption' of TF on TF^+MV within a thrombus have not been unequivocally determined but may include PS exposure on platelets, modifications of disulfide bonds and/or conformational changes (100, 101). The clinical importance of circulating TF^+MV in human thrombosis has not been fully clarified and may vary depending on pathophysiological conditions. However, TF^+MV, in particular if combined with PS, may represent a circulating pool of important procoagulant potential.

1.3.4 Advances in the understanding of platelet function in thrombosis

As illustrated by the discussion above, thrombus formation is a complex process, requiring dynamic coordination in time and space of the different elements of hemostasis with those of the injured vessel wall. In the last decades, improved molecular probes, microfluidic flow chambers and in vivo thrombosis models have clarified some of these interactions, sometimes with surprising results. Some important findings are summarized in this chapter.

1.3.4.1 Dynamics of thrombus formation, alternative pathway for thrombus initiation

The cell-based model of hemostasis was developed largely based on how isolated components of hemostasis behaved in vitro, viewing primary and secondary hemostasis as separate and partially sequential events. Ground-breaking work by the Furie group, using an in vivo mouse model with laser-induced thrombus formation in cremaster arterioles, illustrated how intimately platelets and coagulation interact to establish the thrombus, already from the earliest phase of thrombus initiation, summarized in (102). As this model did not include exposure of sub-endothelial structures, it suggested that alternative pathways for thrombus initiation were possible, including endothelial activation. Blood-borne TF^{+}MV was shown to contribute to initiation of thrombin generation and thereby platelet activation as discussed above (10). TF accumulation and fibrin formation was shown start immediately after laser stimulation and increased over several minutes, while platelet accumulation occurred in two waves: a small one peaking at about 15-20 s and a larger one peaking after 1-2 minutes. Platelet P-selectin started to increase only 2 minutes after thrombus initiation (103), and leucocyte adherence to the thrombus started after 2-3 minutes and peaked at 8 minutes (104). Protein disulfide isomerase (PDI), a chaperone protein, was shown to be released from activated endothelial cells and platelets, and was found to be critical for thrombus formation in this model (10, 105). Recently circulating vitronectin has been identified as a PDI substrate; breaking of disulfide bridges in vitronectin allows binding to endothelial $\alpha_v\beta_3$, which is one of the earliest events in the laser-induced thrombosis model (106).

1.3.4.2 Different platelet subpopulations

Platelet activation in thrombosis was initially considered digital, with platelets being either activated or not. Research has shown that this is a simplified view and that platelets respond differently to different types of platelet activation and can adopt different ‘phenotypes’. The first evidence of this was found by Behnke in the ‘90’s. He identified two different platelet populations positive respectively negative for the enzyme phospho-tyrosine phosphatase (107). Platelets lacking the enzyme responded faster to thrombin and collagen stimulation and were the first to adhere after vessel injury (108). Heemskerk et al identified platelet populations positive respectively negative for PS, where the prevalence of PS exposure was enhanced by collagen and thrombin stimulation (109). The Dale-group subsequently identified a subpopulation of ‘coated’ platelets after platelet stimulation with thrombin and collagen, which expressed PS, bound fV and retained α-granule proteins on the platelet

surface (110, 111). Despite strong stimulation, only a proportion of platelets in healthy, on average 30%, adopted this phenotype. While healthy donors varied in the propensity to form coated platelets, this propensity was fairly stable over time in the same individual. Kempton et al using thrombin and convulxin also identified two platelet populations by flow cytometry, which differed in their ability to bind coagulation factors fV, fIX and fX (112).

Combining flow cytometry, microfluidic flow cells and in vivo mouse models, Munnix et al could show that different platelet subpopulations formed also under physiological conditions (113). Two major platelet populations were identified: aggregating, PS-negative platelets, with only weak binding of coagulation factors fVa, fXa and prothrombin, and non-aggregating, PS-positive platelets with strong binding of the same coagulation factors. (Coated platelets were a small minority of PS-positive platelets in these experiments.) The two populations separated in microdomains in thrombi, with PS-positive platelets developing a 'ballooning' morphology. Ex vivo experiments by Agbani et al also showed a population of PS-positive, ballooning, procoagulant platelets which were prone to microvesiculation, in contrast to spreading/aggregating PS-negative platelets (114). (They also observed two other platelet populations ex vivo.) In a follow-up study it was shown that platelet ballooning and PS-exposure were synchronized and could spread between platelets in platelet aggregates (115). Yakimenko in a mouse thrombosis model could show that PS-positive platelets were found close to the endothelium and in the thrombus core (116). While different authors have proposed other terms for PS-positive, procoagulant platelets, platelet procoagulance appears to be mediated largely by a subpopulation of platelets in thrombi (117).

1.3.4.3 Discoid platelets in thrombus formation

In addition to the above platelet phenotypes it has been found that discoid platelets associate reversibly to the outer surface of a forming thrombus (102). Shear-induced platelet aggregation can occur without irreversible platelet activation (118). Maxwell et al showed that adhesion of discoid platelets in vitro occurred via platelet membrane tethers (distinct from platelet filipodia) in a shear-dependent process (119). Tethering and platelet adhesions depended on GPIb and GPIIbIIIa binding to VWF. Reversible aggregates of discoid platelets formed under shear where discoid platelets would cover an activated, spread platelet. The process was independent of ADP, but ADP was central for converting unstable aggregates of discoid platelets to stable aggregates of activated platelets. It was proposed that an outer layer of aggregated discoid platelets could serve to protect the inner microenvironment of the thrombus from the effects of flow in the initial phases of thrombus formation. This would allow accumulation of soluble platelet agonists, enhancing platelet activation. It could also allow assembly of coagulation complex on activated platelets in the inner part of the thrombus, while restricting exposure of procoagulant surfaces to the bulk of the blood flow.

1.3.4.4 Platelet interactions with other cells, neutrophil extracellular traps

The extent of platelet interactions with other cells and the role of platelets in inflammation and atherosclerosis are increasingly recognized (8, 99). Activated platelets can interact with

leukocytes, in particular monocytes, neutrophils and lymphocytes, by platelet P-selectin binding to PSGL-1 on leukocytes or platelet CD40 ligand (CD40L) binding to leukocyte CD40. Platelets also express PSGL-1, whereby they can tether and roll on activated endothelial cells expressing P-selectin (after endothelial release of Weibel-Palade bodies). Subsequent cell adhesion involves additional binding of different adhesion molecules, including integrins, which activate both interacting cell types further. In these processes, platelets may release a number of proinflammatory chemokines, for instance PF4, NAP-2, MCP-1 and RANTES (8). Platelets can also be induced to synthesize IL-1β, amongst others by thrombin stimulation (120). In animal models of atherosclerosis, platelets and platelet-leukocyte aggregates contributed to leukocyte recruitment to the endothelium, leukocyte activation and leukocyte transmigration (1, 2). Notably, several of the inflammatory functions of platelets can be mediated also by PMV, which can commute inflammatory signals remotely (85, 121). In humans it has not been verified that antiplatelet treatment reduces atherosclerosis, but circulating leukocyte-aggregates are increased in cardiovascular disease, suggesting that the above interactions have a role also in humans.

In 2004, Brinkmann et al discovered a new form of neutrophil activation: Neutrophil Extracellular Traps (NETs) (122). On encountering pathogens, neutrophils decondensed their nuclear chromatin which mixed with granular contents. Chromatin strands decorated with antimicrobial enzymes and proteins were then released extracellularly. In infection, NETs were interpreted to limit dissemination of circulating pathogens. Since their discovery NETs have been found to play a role in various diseases and pathologies, including thrombosis (123). In sepsis, platelets stimulate NETosis via activation of the platelet TLR-4 receptor, expression of P-selectin and High Mobility Group protein B1 (124). If released in the circulation NETs are highly thrombogenic. They activate the contact pathway via factor XII, generating thrombin which leads to platelet activation (125). NETs also bind VWF and fibrin which contribute to platelet activation. Further, NETs may express TF (126), and NETs associated neutrophil elastase cleaves the anticoagulant TFPI (125). The importance of NETs for venous thrombosis is established (123), but NETs have also been found in arterial thrombosis, including acute IS (126-128).

1.3.4.5 Platelet polyphosphates

In 2004 it was discovered that phosphate was stored in dense granules in the form of polyphosphate chains of on average 70-75 phosphate groups (129). Dense bodies were proposed to be similar to acidocalcisomes of bacteria, which contain longer polyphosphates known to be procoagulant. Muller et al therefore studied platelet polyphosphates and found them to be procoagulant, which was claimed to be due to activation of fXII and the contact pathway (130). The latter claim was questioned and could not be reproduced in vitro as platelet sized polyphosphates induced only a weak activation of factor XII (131). Alternative procoagulant effects of platelet polyphosphates have been proposed, including enhanced activation of fV and fXI, and inhibition of TFPI (132). Recently, retention of polyphosphates

on activated platelet surface in form of nanoparticles were shown in vitro, which supported fXII activation (133). It is thus possible that the contact pathway contributes to thrombosis via platelet polyphosphates. In animal models of arterial and venous thrombosis, treatment with phosphatases was protective without increasing the risk of bleeding (134), supporting a role for platelet polyphosphates in thrombosis and suggesting that targeting polyphosphates could be a future treatment strategy.

1.3.5 Cardiovascular risk factors and platelet function

Certain cardiovascular risk factors may mediate risk in part by their effect on platelet function, with diabetes being the most notable example. The balance of platelet reactivity is governed by specific signal pathways, where in particular high cAMP and cGMP levels inhibit platelet activation, as illustrated in figure 4. Cardiovascular risk factors may disturb the balance in different ways; some of the mechanisms involved are discussed below.

1.3.5.1 Diabetes, prediabetes and platelet function

DM is known to increase platelet reactivity and a number of mechanisms are thought to be involved (135). Both chronic and acute hyperglycemia increase platelet activation. Platelets from patients with DM have higher expression of platelet activation markers and surface receptors than healthy controls (135). TxA2 synthesis is enhanced in type 2 DM (136). Several of the above changes are associated with HbA_{1c} and respond positively to improved metabolic control. Acute hyperglycemia in type 2 DM enhances shear-induced platelet activation, with parallel increases in circulating VWF (54). Acute hyperglycemia also increases platelet sensitivity to agonists by different mechanisms: activation of protein kinase C, reduction of platelet NO synthesis and increased mobilization of calcium from intracellular stores, see figure 4 (135). Chronic hyperglycemia causes protein glycation with formation of advanced glycemic end products, AGEs. Glycation of platelet membrane proteins can influence protein function and decreases platelet membrane fluidity, which enhances platelet response to agonists.

Type 2 DM and the metabolic syndrome are characterized by insulin resistance and high insulin levels in the early phase of disease. The effect of insulin on platelets is controversial. Platelets have insulin receptors, which inhibit platelets by increased cAMP levels, increased numbers of PGI_2 receptors and influx of magnesium ions (135). Certain studies on healthy subjects found an inhibitory effect of insulin on platelet aggregation (137, 138). However, there are several studies showing the opposite effect, with insulin causing increased platelet aggregation, in particular for patients with obesity or diabetes (139-142). It has been proposed that platelets develop insulin resistance in a similar manner as other tissues (135). Interestingly, Vaidyula et al found that exposing healthy controls to simultaneously high glucose and high insulin levels by clamps increased platelet activation, monocyte TF expression and TF activity which resulted in enhanced basal thrombin generation (143, 144), suggesting a possible synergy of high glucose and high insulin levels on platelet function and hemostasis.

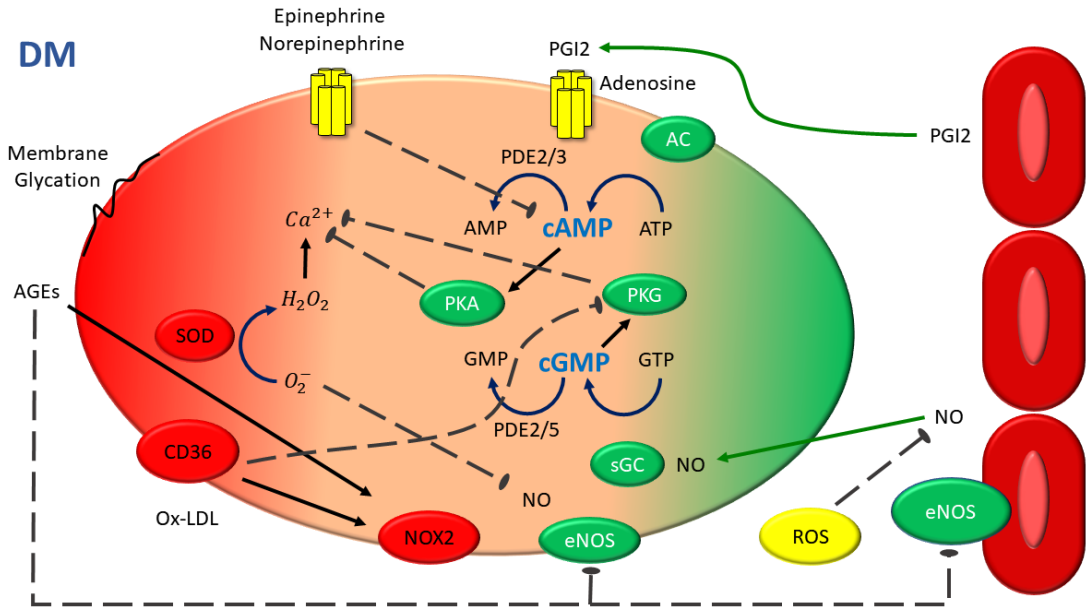

Figure 4: Schematic figure of factors that increase (red) and decrease (green) platelet reactivity. (Certain intracellular signaling pathways have been omitted to limit complexity). cAMP and cGMP are general inhibitors of platelet activation. cAMP signals primarily through protein kinase A (PKA) and cGMP via protein kinase G (PKG). These enzymes have various platelet inhibiting effects, amongst others limiting calcium mobilization (145). The healthy endothelium on the right releases prostacyclin (PGI2) which activates adenylyl cyclase (AC), increasing cAMP levels. It also releases nitric oxide (NO) which activates soluble guanylyl cyclase (sGC) forming cGMP. Endothelial dysfunction due to DM, hypertension or inflammation reduces endothelial generation of PGI2 and NO, increasing platelet reactivity. Oxidative stress inactivates NO. The catecolamines epinephrine and norepinephrine amongst others inhibit cAMP generation. Platelet inflammatory reactions can activate NAPDH oxidase 2 (NOX 2) (146), generating superoxide (O_2^-) which inactivates NO. Superdioxide dismutase (SOD) converts O_2^- to hydrogen peroxide (H_2O_2) which enhances calcium mobilization, increasing platelet reactivity (146). Ox-LDL binds the scavenger receptor CD36, which activates NOX2 and inactivates PKG (147). PDE: phosphodiesterases, enzymes that breakdown cAMP and cGMP. → : activating/stimulating effect; - -● : blocking/inhibiting effect. Image by Jakob Svensson.

In addition to the above effects, patients with DM compared to healthy controls have higher platelet turnover, with higher proportion of recently formed, reticular platelets, known to be more reactive (135) . AGEs binding to the receptor for AGE (RAGE) on endothelial cells cause endothelial dysfunction with reduced release of NO and PGI_2, which contribute to platelet hyper-reactivity (135), see figure 4. DM is also associated with dyslipidemia and inflammation which affect platelet reactivity (135), as discussed below.

1.3.5.2 Dyslipidemia and platelet function

Dyslipidemia is often seen in diabetes patients, with high triglycerides and low high-density lipoprotein (HDL). This attracted attention to how dyslipidemia may affect platelet function. Platelets express several lipoprotein receptors. Pedreno et al showed binding of LDL, VLDL and IDL to the platelet surface; in vitro LDL stimulated platelet aggregation to a similar degree as common platelet agonists (148). The platelet scavenger receptor CD36 binds

modified/oxidized lipids including glycated LDL and oxLDL, which causes platelet activation and aggregation, see figure 4 (147, 149). The lectin-like ox-LDL receptor 1 (LOX-1) has similar effects (150). Notably, platelets can contribute to LDL oxidization via NAPDH oxidase 2 (150). High HDL levels reduce platelet aggregation, partly by cholesterol efflux mechanism in both platelets and endothelial cells, which cause platelet inhibition (151). Statin treatment also reduces platelet aggregation and PMV levels (67, 150).

1.3.5.3 Inflammation, oxidative stress and platelet function

As discussed in section 1.3.4.4, activated platelets can interact with leukocytes and endothelial cells and release proinflammatory substances. In the last decades, it has become clear that platelets should be considered inflammatory cells in their own right, linking hemostasis and immunity in both directions, and having the capacity to regulate inflammatory reactions (152). Platelets have a number of immunologic receptors including Toll-like receptors, complement receptors, cytokine receptors and receptors for the Fc domain of IgG and IgE (153). They have a repertoire of antimicrobial host defense mechanisms, including the ability to phagocytize smaller pathogens and can release antimicrobial peptides. Further, platelets can generate reactive oxygen species by NAPDH oxidase 2 (153), which is activated during platelet aggregation (146). Platelet thus contribute to oxidative stress and in turn superoxide enhances platelet aggregation response (154). Platelets interact with monocytes and neutrophils in inflammation, but notably also with different types of lymphocytes and dendritic cells, indicating that they can modulate also the adaptive immune response (155). Notably, these interactions may stimulate either pro- or anti-inflammatory responses. Platelets also regulate endothelial permeability under normal conditions and in inflammation (156). While the above platelet responses are adequate in for instance wound healing and infections, they likely contribute to enhanced platelet reactivity in chronic inflammatory conditions such as diabetes and systemic diseases.

1.3.5.4 Hypertension and platelet function

Hypertension is not considered to have direct influence on platelet function but involves endothelial dysfunction, which may lead to less efficient endothelial inhibition of platelets and enhanced platelet-endothelial interactions. Also, increased vessel stiffness could negatively influence shear and turbulence, which may contribute to platelet activation. Certain studies have found increased platelet sensitivity to agonists, altered NO availability and altered calcium metabolism in hypertensive patients, summarized in (157).

1.4 MEASUREMENT OF PLATELET FUNCTION

While hemostasis and thrombosis depend on interactions between circulating elements and the vessel wall, establishing the contributions of different components may be important in assessing the risk of thrombosis or bleeding. Early methods to assess platelet function included platelet counts and bleeding time; technological advances have led the development

of a number of methods that are more reliable and specific. A brief overview of some of the available techniques is given below.

1.4.1 Light transmission aggregometry

In light transmission aggregometry (LTA) or Born aggregometry, a platelet agonist is added to illuminated platelet-rich plasma (PRP) under stirring. As platelets aggregate, the light transmission through plasma increases, which is a measure of aggregation (158). The original light transmission is set to 0% and platelet-poor plasma (PPP) is set to 100% transmission; the measurement is thereby by definition individual and relative. LTA allows testing of different agonist and agonist concentrations, and it also gives a time profile for the platelet response. However, the method also has disadvantages. Testing has to be performed in close connection to blood sampling, and requires relatively large blood volumes if multiple agonists are to be tested. Testing is performed in the absence of other blood cells, centrifugation may result in loss of larger, more reactive platelets and the result depends platelet count. LTA requires expert knowledge and is relatively work intensive. Pre-analytical and analytical conditions are not standardized, making inter-laboratory comparisons difficult.

LTA is by many considered the golden standard of platelet aggregation testing, and results correlate with risk of bleeding risk and thrombosis (158). Due to the disadvantages above, LTA is mainly used for investigations of specific platelet defects and in research.

1.4.2 Flow cytometry methods

In flow cytometry, cells or particles in suspension are guided through a narrow flow cell illuminated by lasers. When passing the interrogation aperture, cells scatter the light, allowing them to be counted individually. Scattering properties depend on cell size and internal granularity, allowing separation of different cell types. Labelling cells with fluorescent antibodies allows evaluation of specific surface markers. Flow cytometry of platelets can be performed in whole-blood, PRP or on washed platelets in suspension, all of which require analysis in relatively close connection to blood sampling. Gating strategies allows detailed investigation of specific subpopulations. Flow cytometry requires advanced laboratory equipment and specialist competence but is a powerful and versatile method. A wide range of antibodies allows the investigation of many aspects of platelet function; often simultaneous labelling with two or more antibodies is used. Some examples of platelet function aspects that can be studied with flow cytometry are discussed below.

1.4.2.1 Platelet activation and reactivity

Labelling of platelet surface markers expressed at activation allows evaluation of the degree of platelet activation under different circumstances or in response to platelet agonists. Common examples of activation markers include P-selectin, CD40L, activated GPIIbIIIa (labelled by the antibody PAC1), CD63 or surface-bound coagulation factors (159). Expression of platelet PS can be assessed by fluorescently labelled annexin V (and calcium)

or lactadherin (160). The output is often reported as percentage of platelets positive for a particular marker.

1.4.2.2 Platelet-leukocyte aggregates

A combination of scattering properties and expression of leukocyte-specific markers such as CD14 allows identification of specific leukocyte populations in blood, such as monocytes and neutrophils (159). The proportion of respective leukocyte population positive for platelet markers for instance GPIIb (CD41) can then be assessed.

1.4.2.3 VASP-phosphorylation

In addition to labelling platelet surface markers, platelets can be permeabilized in order to label intracellular antigens. This has led to the development of a specific application to assess the phosphorylation state of a protein down-stream of the $P2Y_{12}$ receptor: vasodilator-stimulated phosphoprotein (VASP). The degree of phosphorylation is a measure of $P2Y_{12}$ inhibition (159). A commercial kit of reagents and antibodies has been developed to simplify and standardize the measurement (BioCytex, Marseille, France).

1.4.2.4 Microvesicles

All the above measurements are performed on functional platelets/cells, which have to be analyzed in close connection to blood sampling. MV are present in plasma and can be analyzed by flow cytometry after one freeze-thaw cycle, allowing storage of samples. While sample handling for larger patient groups is easier, MV enumeration with flow cytometry is technically challenging (63). One aspect is the small size of PMV (median 200-300 nm) combined with a detection limit of flow cytometers, which is in the range half the wavelength of the exiting laser or more for scattering. Generally flow cytometers thus detect a minority of PMV, the so-called ‘tip-of-the-iceberg’ effect (161). Platelet activation may occur during or after blood-sampling, leading to spurious PMV release, which must be avoided or minimized (161). Other errors may occur, for instance swarm effects of smaller particles such as exosomes, protein aggregates, lipoproteins or aggregates of labelling antibodies (161).

1.4.3 Whole blood aggregometry

Whole-blood aggregometry (WBA) is an electric measurement, in which two electrodes are inserted into diluted whole-blood under stirring. After stimulation with an agonist, platelets adhere to and aggregate on the electrodes (158). This causes a change in the impedance between electrodes, which can be monitored. In the original Chrono-log instrument, cuvettes and electrodes were washed between measurements and re-used, limiting reproducibility. In multiple electrode aggregometry (MEA), disposable cuvettes with two electrode pairs per cuvette are used, resulting in a more reliable measurement (158). In MEA, impedance is tracked for six minutes and the area under the curve in arbitrary units is used as a measure of aggregation.

WBA by MEA has the advantage that it uses unprocessed, anticoagulated whole-blood diluted 1:1 with sodium-chloride solution. There is thus no influence of centrifugation, and all blood components are present. Different agonists can be tested, and the blood volume per cuvette is limited (300 µl). Limitations of the method include dependence on platelet count, hematocrit and temperature; further the results are influenced by the delay between blood-sampling and analysis (158). While partly automatized, MEA requires pipetting and a certain amount of training. MEA is mainly used to evaluate response to antiplatelet treatment and bleeding risk.

1.4.4 VerifyNow

VerifyNow is a point-of-care optical method for whole-blood, which was developed primarily to evaluate response to antiplatelet treatment. Blood is inserted into cartridges containing fibrinogen-coated polystyrene beads and a platelet agonist; once activated platelets bind to and agglutinate the beads via GPIIbIIIa binding of fibrinogen (158). The increase in optical transmission due to agglutination depends the number of activated GPIIbIIIa receptors and platelet aggregation. Three types of cartridges with different agonists are available: one with AA, one with a combination of ADP and prostaglandin E_1, and one with thrombin receptor-activating peptide (TRAP). The output for the first is reported as aspirin response units (ARU), for the second as $P2Y_{12}$ units (PRU) and for the third as platelet aggregation units (PAU). Also the percent platelet inhibition of the first two cartridges relative to PAU is reported. (The TRAP cartridge is designed to give maximum platelet aggregation).

VerifyNow has the advantage of being a true point-of-care method which is easily operated. It is standardized, allows direct comparison of results and is the most commonly used method to assess clopidogrel response. Limitations include a dependency on platelet count, hematocrit, triglyceride and fibrinogen levels.

1.4.5 Other methods to assess platelet function

The below methods are at present less common in large clinical research studies. Unless otherwise stated the information refers to (158).

Plateletworks is a cell-counting technique which compares platelet counts in anticoagulated, mixed whole blood with and without the addition of a platelet agonist. It is easy to use and requires only limited instrumentation.

Thromboelastography is based on the principle of increasing viscosity as blood clots. Anticoagulated whole blood is placed in a sample cup heated to 37^{o}C which has a suspended rotating pin. After addition of a platelet agonist, blood starts to clot and the pin encounters increased movement inertia which can be measured. Several variables can be deduced from the output curve. The method has limited sensitivity for platelet defects and the effect of antiplatelet drugs.

The Platelet Function Analyzer 100/200 was developed to assess platelet aggregation under shear. Blood is aspirated through a tube 150 µm in diameter which contains an aperture membrane coated with collagen and epinephrine or collagen and ADP. The time to occlusion is a measure of shear-induced activation. The method has limited sensitivity and specificity, which has led to a decline in use.

T-TAS is a new instrument for the study of platelet aggregation in whole blood in microfluidic capillaries where shear can be adjusted. The output is time to occlusion, and the evolution of the thrombus is documented by microscope (162). The capillary is coated with collagen or collagen and TF.

P-selectin and CD40L are cleaved off from the platelet surface after activation and soluble P-selectin and CD40L as measured by ELISA are sometimes used as markers of platelet activation.

TxA2 is quickly metabolized in blood but the TxB2 metabolites are more stable and can be measured in blood or urine as an index of platelet TxA2 generation. Non-platelet generation of TxA2 limits the sensitivity.

1.5 ANTIPLATELET TREATMENT AND RESPONSE

1.5.1 Mechanisms of antiplatelet treatment

1.5.1.1 Aspirin

Aspirin inhibits COX-1 irreversibly by acetylation of the catalytic site, inhibiting platelet generation of TxA2 and thereby limiting platelet amplification and aggregation. Platelets do not synthesis of COX-1, and the enzyme therefore remains inhibited for the platelet life-time. Synthesis of prostacyclin in endothelial cells occurs both via COX-1 and COX-2. Low-dose aspirin treatment only marginally affects endothelial prostacyclin synthesis, resulting in a clear net platelet inhibiting effect. High-dose aspirin inhibits also COX-2, but endothelial cells can resynthesize the enzyme. Selective COX-2 inhibitors can shift the TxA2/prostacyclin balance, which may be one explanation for the increased risk of thrombosis seen for selective COX-2 inhibitors.

1.5.1.2 $P2Y_{12}$ receptor inhibitors

The first $P2Y_{12}$ receptor inhibitors ticlopidine and clopidogrel are structurally related thienopyridines. Both are prodrugs, which are metabolized into their respective active metabolites in the liver. Ticlopidine has largely been abandoned due to unacceptable side effects. Clopidogrel is for the most part hydrolyzed by hepatic carboxylases into an inactive metabolite (85-90%). The remaining 10-15% are metabolized by cytochrome P450 enzymes in two steps to form the instable, short-lived active metabolite R-130964 (163). R-130964 encounters platelets in the hepatic circulation and inhibits the $P2Y_{12}$ receptor irreversibly. Under maintenance treatment with 75 mg/day, platelet inhibition increases incrementally

reaching a plateau after 4-5 days (164). After a loading dose of 300-600 mg clopidogrel, platelet inhibition can be demonstrated after 2-5 hours.

Both ticlopidine and clopidogrel have been reported to have 'off target effects', which include a reduction of fibrinogen levels, reduced erythrocyte aggregation, enhanced NO production and reduced TF expression in endothelial cells which may contribute to their antithrombotic effect (164).

The relatively long response time and large individual variations in platelet response to clopidogrel (see section 1.5.2.2) have prompted the development of more effective $P2Y_{12}$ receptor inhibitors for ACS. Prasugrel is a thienopyridine metabolized more effectively than clopidogrel as metabolization starts in the intestine. The time of onset is therefore quicker and platelet inhibition more uniform than for clopidogrel (164). Cangrelor is an ATP-analogue which is not broken down by circulating ectonucleotidases. It is administered intravenously, has a short half-life and reversibly inhibits the $P2Y_{12}$ receptor (164). Ticagrelor is an oral reversible inhibitor of the $P2Y_{12}$ receptor, with a binding site different from the ADP binding site. Ticagrelor also inhibits the equilibrative nucleoside transporter 1 (ENT-1), increasing adenosine levels (165). In clinical studies on ACS, prasugrel and ticagrelor compared to clopidogrel have enhanced platelet inhibition and improved clinical outcome at the cost of increased bleeding risk (166, 167).

1.5.1.3 Dipyridamole, cilastozol

Dipyridamole was developed as a vasodilator in the 1960's, and its antithrombotic effect has emerged during clinical use. Its working mechanisms are diverse and are not restricted to platelets but involve different cells in the circulation. Dipyridamole inhibits the adenosine transporter, primarily in erythrocytes, resulting in increased adenosine concentrations and longer adenosine half-life in the circulation (168). Adenosine binding to platelet adenosine receptors stimulates adenylyl cyclase, which increases platelet cAMP levels and cause platelet inhibition. Dipyridamole also inhibits the breakdown of platelet inhibitory cAMP and cGMP by inhibiting platelet phosphodiesterases (PDE), in particular PDE5, which can potentiate the platelet inhibiting effect of endothelial NO and PGI_2. Further, dipyridamole stimulates endothelial PGI_2 production (168). Dipyridamole in combination with aspirin reduces the number of PAR-1 receptors on platelets, suggesting that it can modulate platelet response to thrombin. It has antioxidant and anti-inflammatory effect including reduced superdioxide generation by neutrophils, reduced lipid peroxidation and reduced oxidation of LDL (168). It inhibits VSMC proliferation after vessel injury and reduces thrombus formation in in vivo models. Finally, dipyridamole appears to improve microvascular circulation and resistance to ischemia. It does not increase bleeding in large studies, however, it has a number of side effects including headache.

Cilostazol is a selective, reversible PDE3 inhibitor which limits the break-down of platelet cAMP, resulting in a general platelet inhibiting effect (169). In addition it has been found to

reduce triglyceride levels and increase HDL. In Sweden it is only approved for treatment of peripheral artery disease, but it is used for stroke prevention in Asia (170). It has a number of side effect, including increased mortality in patients with heart failure, but does not appear to increase bleeding.

1.5.1.4 GPIIbIIIa inhibitors

As GPIIbIIIa is the central receptor for platelet aggregation, it is an attractive target for antiplatelet treatment. Three intravenous treatments have been developed and are mainly used for short-term treatment in connection with percutaneous coronary intervention (PCI): the antibody abciximab and the low molecular weight molecules tirofiban and eptifibatide. Abciximab binds and inhibits also integrin $\alpha_v\beta_3$ on endothelial cells, VSMC and platelets as well as $\alpha_M\beta_2$ on leukocytes, thereby inhibiting platelet interactions with the endothelium and leukocytes (171).

Oral GPIIbIIIa inhibitors were developed and tested in large scale studies but were found to increase bleeding and mortality (172).

1.5.1.5 Other platelet inhibitors

The platelet TxA2 receptor inhibitor terutroban was tested versus aspirin for patients with IS or TIA, but the result was neutral (173).

Vorapaxar is an antagonist of the thrombin receptor PAR-1. Vorapaxar in addition to clopidogrel for patients with ACS gave borderline significance for improved effect but also increased bleeding rates, in particular intracranial bleeding (174).

1.5.2 High on-treatment platelet reactivity

It is well-known that there are individual variations in both baseline platelet function and platelet aggregation response to antiplatelet treatment. Seminal work by Helgason et al in the early nineties showed that incomplete inhibition of platelet aggregation by aspirin as measured by LTA was not uncommon in patients with acute or previous IS (175, 176). The authors proposed that certain patients should be considered biologically ‘aspirin resistant’. Similarly, Järemo et al in 2002 showed large inter-individual variations in the response to loading dose of clopidogrel in patients with stable angina pectoris the day after PCI (177). These findings were confirmed in a larger study by Gurbel et al, who found marked individual variation in clopidogrel response also after 30 days of treatment, with 15% of patients remaining ‘clopidogrel resistant’ (178). These pioneering studies have led to the development of the term ‘high on-treatment platelet reactivity’ (HPR): high residual platelet aggregation in vitro despite antiplatelet treatment.

While HPR has been a fruitful scientific concept to discuss platelet response to treatment, it has limitations. Firstly, HPR should not be interpreted as lack of therapeutic effect. Atherothrombosis is a highly complex process, and targeting the platelet, which is only one component, cannot be expected to eliminate the risk of recurrent ischemic events. Overall,

single antiplatelet therapy reduces the risk of a new vascular event in patients with CVD by 22% (179), i e recurrences can be expected without HPR. Secondly, HPR concerns in vitro platelet aggregation, which is an artificial event. As discussed above platelet function can be measured by different techniques, with different agonists, and different cut-offs have been used to define 'high residual platelet aggregation'. Considering the varying measurement conditions of the above methods (whole-blood versus PRP, centrifugation or not, flow conditions, anticoagulants, contact surfaces et cetera) it is not surprising that there is limited correlation between methods in identifying patients with HPR (180, 181). To limit these effects it has been agreed to evaluate HPR relative to the platelet signal pathway inhibited, i e stimulated by AA for the effect of aspirin and by ADP for the effect of $P2Y_{12}$ receptor inhibitors (182).

Despite the above limitations, meta-analyses indicate that HPR, as established by different measurement methods, is a clinically important phenomenon. Krasopoulos et al found HPR to aspirin to be associated with increased OR of CVD event (OR 3.85), ACS (OR 4.06) and death (OR 5.99) in 2 930 patients from 20 studies, 28% of which were classified as 'aspirin resistant' (183). Similarly Aradi found patients with HPR under clopidogrel treatment to have increased risk of AMI (OR 3.00), CVD event (OR 4.95) and cardiovascular death (OR 3.35) in a meta-analysis of 9 187 patients, where 33% had HPR (184). These OR's are remarkably high considering an expected maximum treatment effect of 30%; not taking the drug at all would statistically result in a relative risk (RR) of at most 1.5 if patients were otherwise comparable (185). The existence of a generally 'hypo-responsive' or globally hyper-reactive platelet phenotype has been proposed to explain the high OR's (182, 186), but selection and publication bias cannot be excluded.

As the pharmacodynamic and pharmacokinetic aspects of HPR to aspirin and $P2Y_{12}$ receptor inhibitors vary, they will be discussed separately.

1.5.2.1 HPR to aspirin

In healthy persons, aspirin results in a near complete inhibition of COX-1, and HPR to aspirin, interpreted as inability of aspirin to inhibit its target COX-1 is rare. However, platelet TxA2 synthesis under aspirin treatment may occur by various mechanisms. Full TxA2 suppression requires 97% inhibition of COX-1 (185) and a residual COX-1 activity of 10% is sufficient to generate TxA2-induced platelet aggregation (163). Platelets released 12-24 hours after aspirin intake may have COX-1 activity and they also express some COX-2 which can generate TxA2 (163). High platelet turnover, as is seen for instance in DM, with a higher than normal proportion of newly formed platelets increases the risk of HPR to aspirin (187). Drug interactions can also influence aspirin response. PPI reduces aspirin bioavailability and NSAID such as ibuprofen and naproxen competitively but reversibly bind COX-1, limiting the effect of aspirin (187). In inflammatory conditions, COX-2 in endothelial cells and monocytes may generate prostaglandin H_2 which can be transferred to platelets via cell-cell interactions and there be converted into TxA2 by thromboxane synthase independently of

COX-1 (163). Shear-induced platelet activation is known to be insensitive to ASA treatment (188). Finally, compliance is always a possible explanation for HPR. In one study comparing platelet aggregation under self-reported maintenance treatment with low dose aspirin with aggregation two hours after witnessed intake of 325 mg aspirin, the prevalence of HPR dropped from 9% to less than 1% (one patient out of 190) (189).

Increasing the aspirin dose can reduce the proportion of patients with HPR to aspirin but has so far not been found to improve outcome and increases the risk of bleeding. As true HPR to aspirin is rare, platelet function tests are presently not recommended (7). It is possible that HPR to aspirin should be considered a non-modifiable risk marker. Changing to dosage to twice daily may both reduce the prevalence of HPR and improve outcome in patients with high platelet turnover such as diabetes, but has so far not been implemented in the clinic.

1.5.2.2 HPR to ADP

Most of the insights on HPR to ADP are based on studies on clopidogrel in patients with ACS and/or patients treated with PCI. The recommended measurement techniques and their respective cut-off limits have been agreed in a consensus document (182).

Given the complex metabolism of clopidogrel, platelet aggregation response variability has been considered to depend largely on variable and insufficient generation of the active metabolite (182). A significant effort has been devoted to clarify clopidogrel absorption, hepatic metabolism, drug interactions et cetera. This has led to the discovery genetic polymorphisms, in particular for cytochrome P450 (CYP) enzymes. The CYP2C19 loss of function allele CYP2C19*2 has attracted particular interest (163). CYP2C19 is involved in both steps of clopidogrel activation and is associated with HPR to ADP (190). Despite a high heritability of HPR to ADP under clopidogrel treatment, the CYP2C19*2 allele was found to explain on 12% of the variation in ADP-induced aggregation (191). Notably, in the CHARISMA trial, CYP2C19 variants resulting in poor metabolization of clopidogrel were not associated with higher risk of primary outcome in the treatment arm but overall poor metabolizers had higher risk, independent of clopidogrel treatment (192).

Several CYP enzymes involved in clopidogrel metabolism are susceptible to drug interactions or enzyme induction by other substances. Thus proton pump inhibitors, lipophilic statins and calcium channel blockers compete with clopidogrel for metabolization by CYP2C19 and CYP3A4, and ex vivo tests have shown a reduced platelet response to clopidogrel under co-medication (182). However, these interactions appear to have a limited or no effect on clinical outcome (193). St John’s wort, smoking and rifampicin stimulate enzyme activity of CYP1A3 and CYP3A4, enhancing clopidogrel metabolism. Clinical variables have also been associated with HPR to ADP under clopidogrel treatment, most consistently age, DM, high BMI, chronic kidney disease, ACS and PCI (163).

The improved clinical effects of prasugrel and ticagrelor compared to clopidogrel for ACS patients suggest that HPR to ADP and its associated risk can be overcome (166, 167).

However, studies on platelet function guided adjustment of antiplatelet therapy in patients with HPR have so far been negative (7, 194, 195). In addition, certain patients have a strongly suppressed platelet aggregation response to $P2Y_{12}$ inhibitors, so-called low on-treatment platelet reactivity (LPR), which is associated with increased risk of bleeding (7, 196). It has been proposed that there is a 'therapeutic window' of platelet response to ADP where the risk of both ischemic events and bleeding are low, so-called optimal platelet reactivity (OPR) (197).

1.6 PLATELET FUNCTION AND ANTIPLATELET TREATMENT IN ISCHEMIC STROKE

1.6.1 Platelet function in ischemic stroke

There is ample evidence for a role of platelet activation in IS. Elevated levels of platelet activation markers PF4 and β-thromboglobulin have been found in several studies on patients with IS, summarized in (198). Platelet activation as measured by flow cytometry has also been found elevated in several studies. Grau et al found higher proportion of platelets expressing P-selectin and CD63 in patients with acute IS as compared to healthy controls; levels remained elevated also in the chronic phase (199). Zeller et al found elevated levels of platelets expressing P-selectin, CD63 and thrombospondin in acute IS caused by atherosclerosis, but not in cardioembolic IS (200). Meiklejohn et al found increased platelet expression of P-selectin and platelet fibrinogen binding in patients with acute non-cardioembolic IS, and levels remained elevated three months after symptoms (201). Garlichs et al found elevated platelet expression of P-selectin and CD40L and soluble CD40L in the acute phase of IS or TIA compared to matched controls, as well as higher levels of platelet-monocyte aggregates (202). Levels remained elevated three months after symptoms, and notably differences were small between patients with IS and TIA, and also between different IS subtypes.

In animal models, the extent of cerebral infarction depends on secondary platelet activation in ischemic cerebral microvessels (198). In a model of photo-induced IS, infarct size was reduced by blocking platelet GPIb and GPVI, but not GPIIbIIIa, where inhibition instead caused infarct hemorrhage (203). Microvascular occlusions after IS contain platelets, fibrin and typically neutrophils (198). Early platelet adhesion and activation in the ischemic microvasculature are thought to contribute to 'no reflow' and reperfusion injury after IS (204). In humans, a phase III trial of abciximab for acute stroke was abandoned due to lack of efficacy and increased risk of bleeding (205).

While the role of platelet activation and the preventive effect of antiplatelet treatment are established in IS, maintained platelet function reduces the risk of hemorrhagic transformation of IS, which is a common and sometimes symptomatic complication (198).

1.6.2 High on-treatment platelet reactivity in ischemic stroke

HPR, in particular to ADP, has been less studied for IS than for ACS. In addition, there is less agreement in the stroke community on HPR definitions, cut-off levels, optimal timing of measurement and so forth. Lim et al reviewed platelet function studies in stroke and reported varying prevalence of HPR in patients with IS/TIA, largely depending on differences in measurement techniques and HPR definitions (206). A recent meta-analysis of 8 364 patients with IS/TIA in 52 studies evaluated the prevalence of HPR to aspirin respectively to ADP under clopidogrel treatment, and found HPR in 23% of patients treated with aspirin and in 27% of clopidogrel treated patients (207). A minority was treated with clopidogrel (1 783 patients) and there was significant heterogeneity between studies. Outcome was analyzed in 18 of the 52 studies and showed HPR to be associated with a significantly increased risk of recurrent IS/TIA, RR 1.81, $p < 0.001$, again with important heterogeneity. Numerically the relative risk was somewhat higher in clopidogrel treated patients, but the difference was not significant. In a large meta-analysis by Taglieri et al, HPR to ADP under clopidogrel and aspirin treatment in patients treated with PCI was associated with increased risk of stroke during follow-up, RR 1.84 (208), supporting the notion that HPR is clinically important for stroke.

There are no randomized studies on platelet function guided adjustment of antiplatelet therapy in patients with IS/TIA, but certain findings should be mentioned. Depta et al investigated outcome in a retrospective, observational study of IS/TIA patients referred to LTA measurement by their responsible physician (209). The majority of patients were treated for a recurrent event and several had dual antiplatelet treatment at the time of testing. HPR to aspirin was seen in 43% of patients on aspirin treatment, HPR to clopidogrel in 35% and HPR to both aspirin and clopidogrel in 19% of patients on dual anti-platelet treatment. Based on the results, anti-platelet treatment was intensified in 23% of patients, either by dose increase, adding an additional antiplatelet agent or switching to a more potent anti-platelet agent as decided by the responsible physician. Patients with intensified treatment had higher risk of the composite endpoint death, new ischemic event or bleeding when analyzing the data with propensity score matching (HR 2.24, $p=0.02$). In univariate analysis intensified treatment resulted in both higher rates of ‘any bleeding’ and ‘recurrent IS/TIA’. While there are likely selection effects, the study illustrates the complexity of intensified antiplatelet treatment in patients with IS/TIA. In another observational study, Maruyama et al studied prevalence of HPR to ADP as measured by VerifyNow in IS patients treated with clopidogrel, cilostazol or a combination of the two. The combination cilostazol and clopidogrel had low HPR prevalence, less than 3% as compared to 25% for clopidogrel (210). Outcome was not assessed.

In summary, the prevalence of HPR in patients with IS/TIA appears to be similar to that found in patients with ACS and confers an increased risk of recurrent IS/TIA. Whether intensified platelet inhibition can reduce the risk without increasing bleeding remains to be demonstrated.

1.6.3 Antiplatelet treatment for ischemic stroke and TIA

The protective effect of antiplatelet therapy against CVD is established since the 1990's. In the updated meta-analysis from the Antithrombotic Trialists' Collaboration, antiplatelet therapy in patients with previous IS or TIA reduced the risk of a new cardiovascular event by 22% compared to placebo (179). The risk reduction for short term treatment after acute IS was 11%. Subsequent studies have compared antiplatelet treatment with each other rather than placebo.

A summary of the large antiplatelet trials comparting antiplatelet treatments for IS is given in table 1. Most early studies had a postponed randomization in relation to the index event to avoid hemorrhagic transformation/ICH. In spite of this, dual antiplatelet therapy with aspirin and clopidogrel compared with aspirin gave negative results in the MATCH trial, with no significant protective effect and increased risk of life-threatening bleeding (4). The SPS3 study on patients with lacunar infarcts, (primarily due to CSVD) gave similar results, showing that clopidogrel in addition to aspirin increased both bleeding and mortality (5). CAPRIE compared aspirin to clopidogrel, finding no significant difference in patients with IS (211). The combination of aspirin with dipyridamole compared to aspirin alone was studied in ESPS-2 and ESPRIT, showing a clear added protective effect without increased bleeding as compared to aspirin (212, 213). However, the PROFESS study could show no advantage of combined aspirin/dipyridamole compared to clopidogrel in monotherapy (13), and in fact the bleeding rate was marginally higher in the aspirin/dipyridamole arm. Importantly, the discontinuation rate was higher for aspirin/dipyridamole than clopidogrel in PROFESS, which was also noted in ESPRIT (213).

More recent studies have focused on reducing recurrent IS in the first months after IS/TIA, when the relative risk per time unit is the highest. The CHANCE trial found that early dual antiplatelet treatment with aspirin and clopidogrel in the first three weeks after IS/TIA could improve outcome without increased risk of bleeding (214). The cohort was Chinese, relatively young (average 62 years) and had high rates of smoking (> 40%), which gave rise to questions of external validity. The SOCRATES trial did not show a significant benefit of ticagrelor over aspirin in the first three months after IS/TIA, but also no increase in bleeding (215). The recent TARDIS trial compared triple therapy (aspirin, clopidogrel and dipyridamole) with guideline recommended treatment (clopidogrel monotherapy or aspirin/dipyridamole) (216). The study was terminated prematurely due to excess bleeding in the triple therapy arm, emphasizing the risks of intensified antiplatelet treatment in the acute/subacute phase of IS/TIA.

Considering the high risk of recurrence in the first weeks and months after IS/TIA, Rothwell et al in a meta-analysis analyzed the effects of early aspirin treatment and the added effect of dipyridamole from previous placebo-controlled studies (217). They could show that aspirin strongly reduced both the incidence (HR 0.42) and severity of recurrent IS in the first six weeks of treatment but also between six and twelve weeks. Notably, there was no significant

effect of aspirin versus control after twelve weeks. Dipyridamole did not have an additive effect in the first twelve weeks but did reduce risk after twelve weeks (OR 0.72). Notably, in ESPRIT the added benefit of dipyridamole to aspirin was apparent only after about two years (213). The optimal timing of antiplatelet treatment as well as the delay until treatment effect may differ between antiplatelet agents.

Study	Intervention	Nr patients	Rx timepoint	Follow-up time	Outcome	RRR	AR diff bleeding
ESPS-2 1996	Asp vs placebo Dp vs placebo Asp+Dp vs placebo	6 602	< 3 mo	24 mo	Recurrent stroke	-21% -19% -41%	+1.7% ns +1.9%
CAPRIE* 1996	Clo vs Asp	6 431	'Recent'	1.9 y	Recurrent IS, AMI, vasc death	NS (-7%)	NS
MATCH 2004	Asp+clo vs clo	7 599	< 3 mo	18 mo	Recurrent ischemic event	NS (-6%)	+1.3%
ESPRIT 2006	Asp+Dp vs Asp	2 739	< 6 mo	3.5 y	Recurrent stroke, AMI, CVD death, major bleed	-20%	-1.3%
PROFESS 2008	Asp+DP vs clo	20 332	< 4 mo	2.5 y	Recurrent IS	NS	+0.5%
SPS3 2012	Asp+clo vs Asp	3 020	< 6 mo > 2 we	3.4 y	Recurrent stroke	NS (-8%)	+1%/y
CHANCE 2013	Acute Clo+Asp vs Asp	5 170	< 24 h	3 mo	Recurrent stroke	-32%	NS
SOCRATES 2016	Ticagr vs Asp	13 199	< 24 h	3 mo	Recurrent stroke, AMI, death	NS (-11%)	NS
TARDIS 2018**	Asp+Clo+Dp vs Asp+Dp or C	3 096	< 48 h	1 mo	Recurrent stroke or TIA	NS (-10%)	2%

Table 1 Summary of large randomized controlled trials comparing antiplatelet treatment for patients with ischemic stroke (IS) or TIA. Nr patients: number of patients in trial; Rx timepoint: timepoint of randomization relative to index event; RRR: relative risk reduction for primary outcome; AR diff bleeding: absolute risk difference for major or fatal bleeding; Asp: aspirin; Dp: dipyridamole; clo: clopidogrel; Ticagr: ticagrelor. *) Stroke subgroup of CAPRIE study, **) TARDIS was interrupted due to bleeding complications. (4, 5, 13, 212-216)

The effect of antiplatelet treatment may also depend on IS subtype, and in particular patients with large vessel disease can be expected to benefit from intensive antiplatelet treatment. A subgroup analysis of the SOCRATES trial investigating patients with ipsilateral extra- or intracranial stenosis showed significant reduction of stroke, AMI or death with ticagrelor as compared aspirin, HR 0.68 (218).

2 AIMS

The overall aim of this thesis was to investigate platelet function and response to antiplatelet treatment in patients with ischemic stroke (IS) or TIA. This included in particular study of High on-treatment Platelet Reactivity (HPR) to clopidogrel and microvesicles (MV) in IS/TIA cohorts. A further aim was to assess thrombin generation variables and their relation to platelet function in patients with IS/TIA, since thrombin 1) is a strong platelet agonist 2) links platelets with coagulation and 3) is a measure of coagulant activity/potential. Thus, the specific aims of the studies in the thesis were:

- To determine the prevalence of HPR under clopidogrel treatment in patients with IS or TIA (Study 1 and 2).
- To investigate the association between HPR to clopidogrel and clinical variables in patients with IS or TIA, with particular emphasis on:
    - Disturbances in glucose metabolism (study 1).
    - Ischemic stroke subtype and presence of large artery atherosclerosis respectively cerebral small vessel disease (CSVD) (study 2).
- To investigate platelet microvesicles (PMV), thrombin generation variables and microvesicle-induced thrombin generation in patients with IS or TIA, and the association between these variables and future cardiovascular complications (study 3 and 4).

3 METHODS

3.1 STUDY POPULATIONS AND STUDY DESIGN

3.1.1 Study 1 and 2

Study 1 and 2 were cross-sectional studies, investigating prevalence of HPR under clopidogrel treatment one month after IS/TIA, and associations between HPR and clinical variables. Patients were selected from the StrokeDiabetes study at Danderyd Hospital (Clinical Trials NTC 01648985), which recruited 144 patients with minor IS or TIA between 2010 and 2012 for the study of glucose metabolism, platelet function and life-style factors. Patients of study 1 and 2 were those receiving clopidogrel as secondary prevention and were still on this treatment at the one month control, in total 66 patients for study 1 (recruited until end of 2011) and 72 patients for study 2 (after completed recruitment). Inclusion criteria were acute minor IS/TIA and susceptibility to life-style changes. Exclusion criteria were inability to perform oral glucose tolerance test (OGTT) and estimated life expectancy less than one year. Between study 1 and study 2 five patients were excluded (two due to dual antiplatelet treatment with aspirin and clopidogrel, two due to platelet aggregation tests only being available from the subacute phase and one due to final diagnosis other than IS or TIA); 61 patients were thus common to study 1 and 2. Patients were recruited median 2 days after symptoms (range 0-19 days) and were evaluated at the one month control median 34 days after symptoms (range 20-51 days).

3.1.2 Study 3 and 4

Study 3 and 4 were cohort studies. The cohort was recruited 2007-2009 at two stroke centers in Stockholm (Danderyd Hospital and Södersjukhuset) within the Proppstopp study. The original study had two objectives: 1) to screen patients with IS/TIA for occult atrial fibrillation (AF, completed (219)) and 2) measuring MV concentrations and hemostatic variables in the acute and convalescent phase of IS/TIA (220). Inclusion criteria were IS/TIA within 14 days of recruitment and age above 45/65 years (the age limit was raised during inclusion to obtain a representative cohort). Exclusion criteria were known AF or AF on admission ECG and inability to operate a handheld ECG device. In total 249 patients were recruited. For the analysis of MV and hemostatic variables 38 patients were excluded due to patient request, incorrect diagnosis or complete lack of blood samples, resulting in a cohort of 211 patients. Fifty-three age and sex-matched healthy controls without antithrombotic, anti-hypertensive, lipid-lowering, corticosteroid or SSRI medication were recruited for comparison.

The exposing factors were MV concentrations in study 3 and thrombin generation variables in study 4, as measured in the acute and convalescent phase of IS/TIA. Patients were followed until end of 2014 with respect to recurrent ischemic events and all-cause mortality.

3.2 BLOOD SAMPLING AND PLASMA PREPARATION

Blood samples were generally taken in the morning, after an over-night fast and after rest for at least 10 minutes in semi-reclining position, with no or minimal stasis. (For a minority of patients in study 3 and 4, acute phase blood samples were taken just before lunch, due to short time between patient recruitment and hospital discharge.) In study 1 and 2 blood samples for platelet aggregation tests were drawn in hirudin tubes (Refludan Dynabyte), after blood had been collected for routine blood chemistry and metabolic tests (to avoid procedure-related platelet activation). In study 3 and 4, blood for preparation of PPP was drawn in citrate collection tubes (0.129 mM, filled 1:9) and centrifuged immediately at 2000 g for 20 minutes in room temperature (RT). PPP was then aliquoted and frozen at -80^{o}C until analysis.

3.3 MEASUREMENT OF PLATELET FUNCTION, MICROVESICLES AND THROMBIN GENERATION

3.3.1 Platelet aggregation tests

Blood from hirudin test tubes was analyzed by WBA MEA in the Multiplate^{TM} instrument (Roche Diagnostics, Basel, Switzerland) within 30-179 minutes of collection, according to the manufacturer's instructions. The method has been described in detail previously (221). In brief, 300 µl of whole-blood is diluted 1:1 with 0.9% sodium-chloride solution, and pre-heated to 37^{o}C in dedicated cuvettes for 3 minutes under stirring. A platelet agonist is thereafter added, causing platelets to aggregate on two pairs of silver-coated copper electrodes. Platelet aggregation causes an increase in electrical impedance between electrodes, which is recorded for 6 minutes. The instrument output is the average area under the curve for the two electrode pairs, in arbitrary units (AU). For study 1 and 2 the following platelet agonists were used:

- AA for assessment of the TxA2 pathway (MEA AA). AA was added to a final concentration of 0.5 mM.
- ADP for assessment of the $P2Y_{12}$ and $P2Y_1$ pathways and effect of clopidogrel treatment (MEA ADP). ADP was added to a final concentration of 6.4 µM.
- TRAP for evaluation of the thrombin receptor PAR-1 pathway. TRAP was added to reach 10% of the recommended final concentration, 3.2 µM, to asses sub-maximal PAR-1 response (MEA TRAP).

All reagents and materials were provided by Dynabyte Medical, Munich, Germany. For each patient five cuvettes were used: one with AA and two each with ADP respectively TRAP as the agonist. In the analysis, the mean of two measurements was used for MEA ADP and MEA TRAP. Patients were classified as responders (R) or non-responders (NR) to clopidogrel based on a MEA ADP value below respectively above 468 AU, as established by receiver-operating characteristics analysis in a previous study and confirmed by consensus

(182, 222). (After study 1 and 2, the MEA ADP cutoff was shifted one order of magnitude and is now 46 AU.) No cut-off was used for MEA AA or MEA TRAP.

3.3.2 Measurement of microvesicle concentrations

MV concentrations in plasma were enumerated by multi-colour flow cytometry. The methods for MV isolation and labelling have been described in detail previously (80, 223). In brief, one aliquot of PPP (≈ 500µl) was thawed in water-bath at 37ºC for 5 minutes and then centrifuged in RT for 20 minutes at 2000 g. Thereafter, the supernatant (SN) was centrifuged at 13 000 g for 2 minutes. From the resulting MV-rich SN, 20 µl was incubated in the dark with 5 µl each of following fluorescent surface markers in suitable combinations (maximum four markers per flow cytometer sample):

- Phalloidin-Alexa660 (Invitrogen, Paisley,UK), binding actin to distinguish cell fragments from MV (223).
- Lactadherin-FITC (Haematologic Technologies, VT, USA) in a concentration of 8 µg/ml, binding phosphatidylserine (160).
- Mouse anti-human antibodies to CD41-PC7 (BD, NJ, USA), binding GPIIb of the platelet/megakaryocyte receptor GPIIbIIIa.
- Mouse anti-human antibodies to CD62P-APC (BD, NJ, USA), binding P-selectin.
- Mouse anti-human antibodies to CD142-PE, (BD, NJ, USA), binding TF.

After 20 minutes incubation the samples were fixed with low concentration formaldehyde (BD Cellfix, Becton Dickinson, CA, USA). For size determination and establishment of the MV gate, Megamix beads (0.5, 0.9 and 3.0 µm) were run on separate samples; beads were also used for calculation of MV concentrations. Non-specific isotype mouse antibodies were as used as negative controls. Samples were analyzed in a Beckman Coulter Gallios^{TM} flow cytometer. Gating for MV events was set to size less than 1 µm and negative for phalloidin.

PMV populations and TF^{+}MV populations not expressing the platelet/megakaryocyte marker GPIIb were analyzed according to the hierarchy of figure 5; the subpopulations at lower levels are subsets of the population immediately above when connected with a line.

3.3.3 In vitro thrombin generation by Calibrated Automated Thrombogram

The in vitro thrombin generation potential was assessed by the Calibrated Automated Thrombogram (CAT) assay developed by Hemker et al (224), using the commercial instrument from Thrombinoscope (Thrombinoscope BV, Maastricht, Netherlands). In brief, thrombin generation in PPP is initiated by the addition of TF, phospholipids and calcium in the presence of an excess of a fluorogenic thrombin substrate. As coagulation starts, thrombin cleaves the substrate which generates a fluorescent signal. By comparison with a second well containing a thrombin calibrator of known concentration, the generated thrombin concentration in test plasma as a function of time can be calculated. An example of a CAT output curve is shown in figure 6. From this curve, four output variables evaluated in study 4

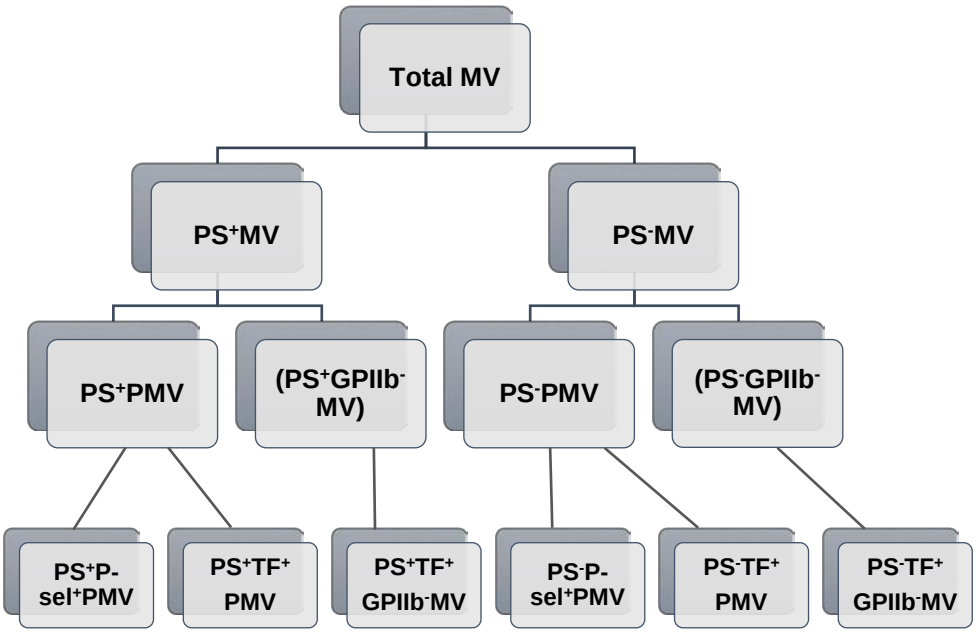

Figure 5: Hierarchy of analyzed MV. All MV populations are a subset of the MV population one level above. P-sel: P-selectin. GPIIb: marker of the GPIIb moiety of the platelet receptor GPIIbIIIa. MV populations in parenthesis were not enumerated.

are obtained as shown in figure 6: lag time (time to start of thrombin generation), peak thrombin concentration, time to peak thrombin concentration and endogenous thrombin potential (ETP, area under the curve).

As previously described (220), CAT tests were performed with 80 µl PPP sample per well. Reagents were from Thrombinoscope. TF was added to a final concentration of 5 pM and phospholipids to a final concentration of 4 µM. Fluorescence was measured every 30 seconds for 60 minutes by a Fluoroskan Ascent microwell fluorometer (Fisher Scientific, Vantaa, Finland). Tests were performed in triplicate. The four CAT variables were calculated by the commercial software of the instrument (Thrombinoscope version 2007).

3.3.4 In vivo thrombin generation: prothrombin fragment F1+2

Prothrombin fragment F1+2 is cleaved off when prothrombin is activated to thrombin and thus reflects in vivo thrombin generation. F1+2 concentration was measured by a commercial ELISA (at the time of testing Dade Behring, now Siemens Healthcare Diagnostics, Marburg, Germany).

3.3.5 Microvesicle-induced thrombin generation potential

The CAT assay above was modified to assess the thrombin generation capacity of the MV pellet, as originally described by Mobarrez et al (67). In brief, one PPP aliquot (≈ 500 µl) was thawed in water-bath as above and centrifuged 2000 g for 20 minutes in RT. The MV-rich SN was centrifuged 20 800 g for 45 minutes in RT, after which the MV pellet was re-suspended in phosphate-buffered saline and again centrifuged 20 800 g for 45 minutes. The

washed MV pellet was then re-suspended in normal pooled plasma (centrifuged 10 000 g for 45 minutes in RT). The CAT assay was performed with 80 µl MV-rich plasma, without addition of TF or phospholipids. These tests were performed in duplicate for a subset of patients in study 4 (96 patients in the acute phase and 98 patients in the convalescent phase).

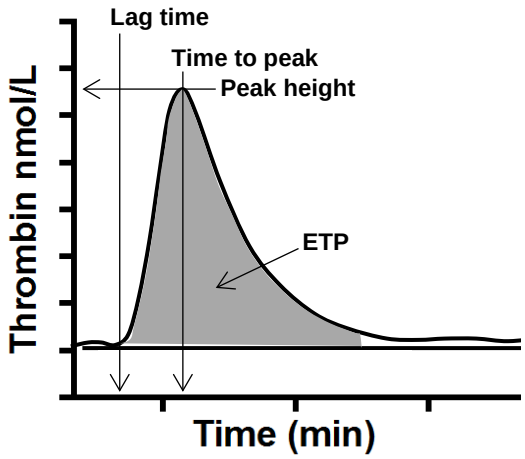

Figure 6: Schematic view of a thrombin concentration curve of the CAT assay, showing lag time (time to start of clotting), peak thrombin (peak height), time to peak thrombin and endogenous thrombin potential (ETP).

3.4 METHODS FOR CLINICAL CHARACTERIZATION

3.4.1 Glucose metabolism and insulin resistance

Patients in study 1 and 2 were characterized with respect to glucose metabolism and insulin resistance. Fasting plasma glucose (fP-glucose) and glycated hemoglobin (HbA_{1c}) were measured for all patients. P-glucose was analyzed by glucose oxidase method in Beckman Synchron LX 20 (Beckman Coulter Inc, CA, USA). HbA_{1c}, was measured with high performance liquid chromatography in a Variant II analyzer (Bio-Rad, Hercules, CA, USA).

Patients without known diabetes were further characterized by oral glucose tolerance test (OGTT) and fasting serum insulin. OGTT was performed according to the guidelines of WHO (225), with measurement of p-glucose before respectively 2 hours after ingestion of 75 g glucose dissolved in water after an overnight fast. According to WHO limits, patients were characterized as having:

- Normal glucose tolerance (NGT): fP-glucose $\leq$ 6.0 mM and 2h-P-glucose $\leq$ 7.8 mM.
- Impaired fasting glucose (IFG): fP-glucose: 6.1-6.9 mM and 2h-P-glucose $\leq$ 7.8 mM.
- Impaired glucose tolerance (IGT): fP-glucose < 7.0 mM and 2h-P-glucose 7.9-11.0 mM.
- DM : fP-glucose $\geq$ 7.0 mM or 2h-P-glucose $\geq$ 11.1 mM.

Patients were divided into three groups for the statistical analysis: NGT, IFG/IGT and DM (previously known or according to OGTT at the one month control).

Fasting serum insulin (fS-insulin) was measured in non-diabetic patients by ElectroChemiLuminescence Immunoassay (Elecsys Insulin Modular E, Roche Diagnostics, Rotkreuz, Switzerland). The degree of insulin resistance (IR) was estimated by the Homeostasis Model of Assessment (HOMA-IR), as calculated by the formula: fP-glucose (mM) x fS-insulin (mU/l)/22.5 (226).

3.4.2 Stroke suptype, vessel pathology

Patients in all four studies were categorized with respect to most likely underlying cause of IS/TIA as determined by TOAST classification (227), which specifies the categories:

- Large-artery atherosclerosis
- Cardioembolism
- Small vessel occlusion
- Other determined cause of stroke
- Undetermined cause of stroke

The classification was blinded to all tests of platelet function and thrombin generation, by at least two stroke physicians in consensus.

In study 2, large vessel pathology in carotid arteries was categorized based on carotid ultrasound investigations in the acute phase, or, for two patients by CT/MR angiography. (Two out of 72 patients were not assessed; one due to symptoms from the vertebro-basilary circulation and one where operation of a possible carotid stenosis was considered excluded.) The evaluation was performed by the clinical physiology or X-ray departments respectively, blinded to all other test results. The degree of atherosclerosis in the internal carotid artery was categorized as:

- 0 p: normal vessel wall
- 1 p: atherosclerosis/atherosclerotic plaques < 50% of lumen diameter
- 2 p: carotid stenosis 51-74%
- 3 p: carotid stenosis 75-99%
- 4 p: carotid occlusion

The score reported was the average between the right and left internal carotid arteries, with atherosclerosis considered to be present if the score was $\geq$ 0.5 p, i e atherosclerosis in at least one of the internal carotid arteries.

In study 2, presence of cerebral small vessel disease was assessed using Fazekas scale for WMC (228) based on acute CT scans of the brain. Fazekas scale was developed to grade periventricular hyperintensities (PVH) and deep white matter hyperintensities (DWMH) on MRI and has also been used on CT (229). PVH are graded as absent (0 p), periventricular

'caps' or pencil-thin lining (1 p), 'smooth halo' (2 p) or irregular PVC extending to deep white matter (3 p). DWMH are graded as absent (0 p), punctate foci (1 p), beginning confluence (2 p) or confluent (3 p). The total Fazekas score is thus 0-6 p. (In the published article the total was erroneously 0-9 p, see section 6.1.3.) The correct score was used to denote overall WMC as 'none' (total 0 p), 'mild' (1-2 p), 'moderate' (3-4 p) or 'extensive' (5-6 p); patients were then divided into the categories none-mild WMC versus moderate-extensive WMC.

3.4.3 Routine laboratory analyses and evaluation of kidney function

Routine laboratory analyses were performed at the Clinical Chemistry Laboratory, Karolinska University Hospital, Solna, Sweden. Estimated glomerular filtration rate (eGFR) was calculated from the plasma creatinine value by the Modification of Diet in Renal Disease (MDRD) formula.

3.5 EVALUATION OF OUTCOME

For studies 3 and 4, outcome was evaluated until 2014-12-31. Primary outcome was a composite of recurrent ischemic events: recurrent IS, AMI or ischemic CVD death. Secondary outcomes were fatal/non-fatal recurrent IS or all-cause mortality respectively. The following ICD codes were used for recurrent ischemic events:

- IS: ICD I63.0-6 and I63.8-9.
- AMI: I21.0-4, I21.9, I22.0-1, I22.8-9, I24.8-9.
- Ischemic CVD death: I46.0-1, I46.9 or death with any of the above as underlying cause.

Events and date of events were obtained from the Swedish register for in-patient care, the Swedish register for cause of death, and where possible confirmed by hospital records. Patients were censored after a first outcome event, at the time of death or at the end of follow-up 2014-12-31.

3.6 STATISTICAL METHODS

In studies 1 and 2, R and NR to clopidogrel treatment, as defined by the established cut-off above, were compared as independent groups. Group differences of continuous variables were analyzed by Student t-test for normally distributed variables and by Mann-Whitney U-test for non-normally distributed variables. Differences in categorical variables were analyzed by χ^2 test. In study 1, correlations between MEA values and metabolic variables were evaluated by Pearson's correlation coefficient.

In study 3 and 4, the methods above were used to compare patients with healthy controls. Correlations between independent variables were analyzed by Pearson or Spearman correlation coefficient as appropriate. Changes and correlations between acute and convalescent phase values were analyzed by paired Student t-test or Wilcoxon signed rank

test as appropriate. The statistical analyses of exposing factors versus outcome were performed in steps. In a first step, exposing factors were screened by comparing patients with and without outcome and analyzing differences by Student t-test or Mann-Whitney U-test as appropriate. Variables with $p < 0.2$ for difference were retained. In a second step, Kaplan-Meier survival analysis based on median split was performed, and the p-value for difference was calculated by log-rank test. Variables with $p < 0.1$ in the second step were retained for Cox regression and calculation of hazard ratios (HR). Multivariate Cox regression was performed in a forward, stepwise manner, adjusting for cardiovascular risk factors in order of importance in the patient cohort. The number of variables included in the multivariate model was limited to have 8-10 patients with events per variable, in order not to overfit the model.

P-values < 0.05 were considered significant with the exception of variable screening.

4 RESULTS

4.1 STUDY 1

HPR to clopidogrel treatment is associated with pathological glucose tolerance and insulin resistance.

Out of 66 clopidogrel-treated patients, 14 (21%) had MEA ADP value above the cutoff 468 AU and were considered NR. In terms of clinical characteristics, NR had higher prevalence of known diabetes than R, 36% versus 12%, see table 3. They also had higher frequency of previous IS, 43% versus 6%, p= 0.0012. NR had higher leukocyte and platelet count than R, but there were no differences in CRP, blood lipids or eGFR. NR also had higher levels of MEA AA (840 vs 500 AU, $p < 0.0001$), suggesting an underlying general platelet hyper-reactivity.

Eleven patients in the cohort had known diabetes at inclusion. Based on OGTT at the one month control another ten patients were classified as having DM, two had IFG and 15 had IGT. Fasting plasma glucose (fP-glucose) was marginally higher in NR than R, but there was no significant difference in HbA_{1c}. Pathological glucose tolerance, defined as DM (known or according to OGTT) or IFG/IGT, was more common for NR (93%, all but one patient) than R (48%), p=0.001, see table 2. Conversely, IFG/IGT appeared to confer a similar risk for NR as DM. In non-diabetic patients, HOMA-IR was higher for NR than R, 4.5 versus 2.1, p=0.001. Notably, the one NR with NGT had very high HOMA-IR (12.9), suggesting that a pre-diabetic/diabetic metabolism is a 'near pre-requisite' for HPR to clopidogrel, while NGT with normal HOMA-IR appeared to largely preclude HPR.

Metabolic variable	R (n =52)	NR (n =14)	P-value
DM 2 at incl, n (%)	6 (12%)	5 (36%)	0.045
fP-glucose, mmol/l, median (IQR)	5.3 (5.0-6.0)	6.0 (5.5-6.7)	0.023
HbA_{1c}, mmol/mol, median (IQR)	40 (37-44)	42 (34-46)	0.26
Pathological OGTT/DM, n (%)	25 (48%)	13 (93%)	0.001

Table 2: Variables of glucose metabolism for responders (R) and non-responders (NR) to clopidogrel. Incl: inclusion, fP-glucose: fasting plasma glucose, IQR: interquartile range.

4.2 STUDY 2

HPR is associated with WMC, indicating CSVD, but not carotid artery sclerosis.

After completed recruitment, there were 72 patients on clopidogrel treatment at the one month control (61 of which were analyzed in study 1, see section 3.1.1 for details). Sixteen patients were NR (22%). The above differences between NR and R with regard to clinical and laboratory characteristics and variables of glucose metabolism were confirmed in this somewhat larger patient cohort. The concept of a general platelet hyper-reactivity for NR was further supported by significantly higher MEA TRAP for NR compared to R, both at inclusion and the one month control (310 versus 160 AU and 245 versus 160 AU respectively, $p < 0.01$ for both).

Concerning IS subtype, there was no statistical difference in the distribution of TOAST categories between NR and R. To assess the importance of existing vessel changes, patients were evaluated with respect to carotid atherosclerosis, reflecting large artery atherosclerosis disease, and WMC on CT scans of the brain, indicating presence of CSVD. Overall, 51% of patients had carotid atherosclerosis. Contrary to our original hypothesis, there was no difference in the prevalence or severity of carotid atherosclerosis between NR and R, see figure 7. Neither was there any difference between NR and R in the prevalence of other atherosclerotic manifestations such as history of ischemic heart disease or peripheral artery disease. Moderate-extensive WMC were found in 23 patients (32%) and was significantly more common in NR (56%) than R (25%), OR 3.9 (CI 1.2-12, p=0.03), see figure 7. A subgroup analysis based on the categories ‘no vessel changes’ (20 patients), ‘isolated carotid atherosclerosis’ (28 patients), ‘isolated WMC’ (14 patients) and ‘both carotid atherosclerosis and WMC’ (8 patients), suggested that only WMC was associated with clopidogrel non-response; the prevalence of NR in patients with isolated carotid atherosclerosis was similar to that in patients without vessel changes.

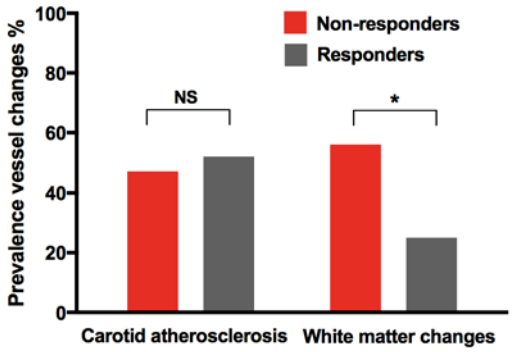

Figure 7: Prevalence of vessel changes in non-responders (NR) and responders (R) to clopidogrel; carotid atherosclerosis (55 R, 15 NR) and white matter changes (56 R, 15 NR). *) $p < 0.05$.

Based on the above result, patients were characterized in more detail, in particular with respect to hypertension. While there was no significant difference in the prevalence of hypertension at inclusion, all NR had hypertension (diagnosed or treated) at the one month control, versus 64% of R. NR also had more intensive anti-hypertensive treatment than R, but still had higher systolic blood pressure at the one month control, median 145 versus 135 mm Hg, p=0.02. Also the pulse pressure was higher for NR than R, which may suggest increased arterial vessel stiffness. NR thus had both higher prevalence of pre-diabetic metabolic changes and more advanced hypertension than R.

The study cohort was not large enough to perform a multivariate Cox regression analysis of which clinical variables were independently associated with HPR as there were only 16 patients with HPR. A multivariate linear regression model was performed to investigate which variables were predictive of the MEA ADP value. The traditional CVD risk factors age, sex, smoking, hypertension and diabetes together gave an R^2 value of 0.13 (adjusted 0.064); thus explaining only 13% of the variance in MEA ADP. In contrast a model with age, pathological glucose tolerance/DM, BMI, platelet count and leukocyte count resulted in R^2 of 0.47 (adjusted 0.43), thus explaining almost half of the variance in MEA ADP. In this model, hypertension and WMC were not found to give a further independent contribution to MEA ADP. These results were presented as poster at the International Society on Thrombosis and Haemostasis conference in Toronto 2015.

4.3 STUDY 3

PMV, TF^+MV and their subpopulations are enhanced in both the acute and convalescent phase of IS/TIA. The associations with outcome differ between MV populations and depend on the time of measurement.

Blood samples for MV enumeration were available for 199 out of 211 patients in the acute phase and 189 patients in the convalescent phase. The follow-up time for patients with MV data was 1100 patient years. The primary outcome recurrent ischemic event occurred in 54 patients in the cohort. Secondary outcome recurrent ischemic stroke occurred in 43 patients, and 31 patients died.

4.3.1 Cross-sectional evaluation of circulating MV in the acute and convalescent phase compared to matched controls

Compared to healthy controls, patients had higher levels of all enumerated MV populations in the acute phase ($p < 0.0001$ for all), see figure 8. Differences were particularly pronounced for PMV expressing the activation markers P-selectin and TF, both for populations positive and negative for PS, which were median 7-77 times higher in patients than controls. MV levels generally remained high in the convalescent phase. MV populations had different temporal profiles between the acute and convalescent phase. MV expressing PS and/or TF decreased, while PS^-MV, PS^-PMV and PS^-PMV expressing P-selectin increased between the acute and convalescent phase. PS^+MV and PS^-MV populations with otherwise identical

surface markers were not correlated, neither in healthy controls nor in patients at either measurement time.

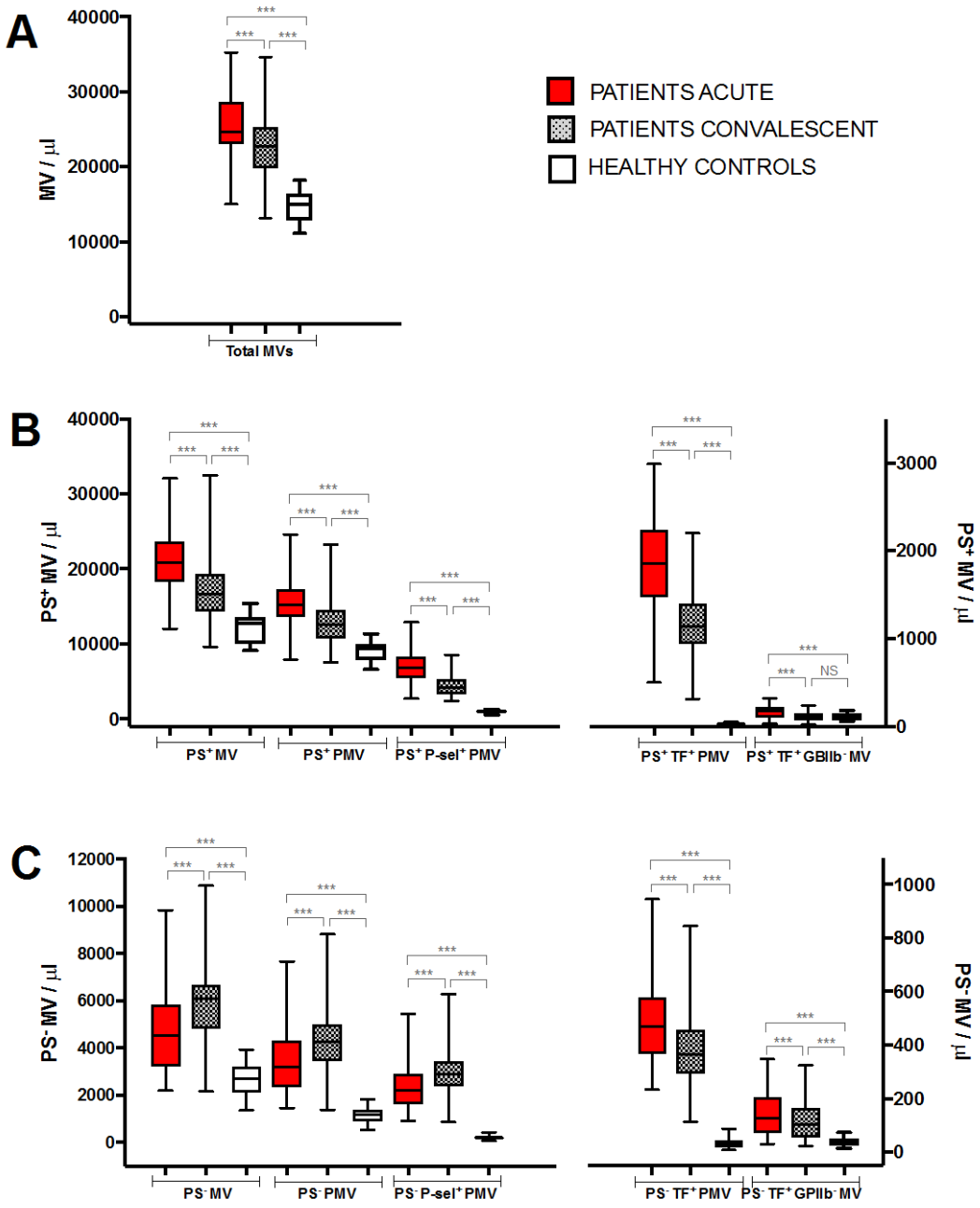

Figure 8: Concentrations of total MV (A), PS^+MV populations (B) and PS^-MV populations (C). *** $p<0.0001$, NS: non-significant.

4.3.2 Associations between MV populations and outcome

The screening of MV populations versus outcome gave negative results for several types of MV expected to be pro-thrombotic. Thus, comparing patients with and without primary outcome there were no significant differences in the levels of PS^+TF^+PMV or PS^+TF^+MV lacking the platelet marker GPIIb (TF^+MV of non-platelet origin) neither in the acute nor convalescent phase. Furthermore, levels of PS^+PMV expressing P-selectin, generally viewed

upon as reflecting platelet activation, were similar in patients with and with primary outcome. Notably, the only MV population with a positive association to primary outcome in variable screening lacked pro-coagulant PS. Thus $PS^{-}TF^{+}$PMV above median in the acute phase gave an unadjusted HR 1.72 for primary outcome, see figure 9B. Adjustment for cardiovascular risk factors diabetes, eGFR, age and male sex resulted in a significant HR 1.86 (CI 1.04-3.31, p=0.036) with p=0.001 for the model. In contrast, PS^{+}PMV above median in the acute phase conferred a reduced risk for primary outcome with crude HR 0.59, see figure 9A. This association was not independent of cardiovascular risk factors. Interestingly, there was moderate negative correlation between $PS^{-}TF^{+}$PMV and PS^{+}PMV measured in the acute phase, r=-0.35, p < 0.0001.

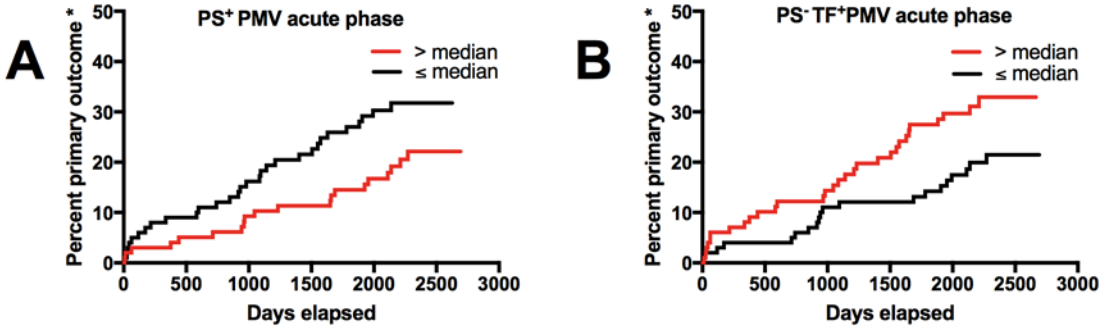

Figure 9: Univariate Kaplan-Meier survival curves for PS^{+}PMV and $PS^{+}TF^{-}$PMV in the acute phase versus primary outcome. Red curves: concentrations above median, black curves: concentrations at and below median.

In the convalescent phase, variable screening showed PS^{-}P-sel^{+}PMV to be inversely associated with primary outcome, figure 10A, and $PS^{+}TF^{+}$MV of 'non-platelet origin' to be inversely associated with the secondary outcome recurrent IS, figure 10B. These MV populations thus appeared to confer reduced risks, but associations were not significant after adjustment for cardiovascular risk factors.

None of the enumerated MV populations at either measurement point were associated with the secondary outcome all-cause mortality.

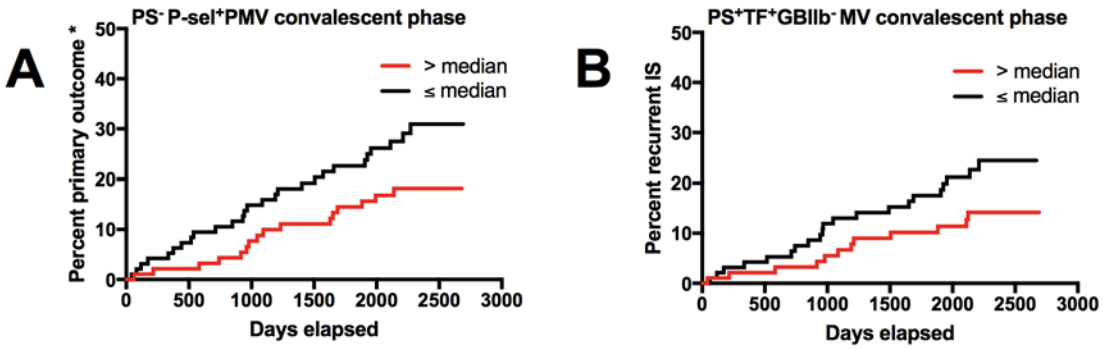

Figure 10: Univariate Kaplan-Meier survival curves for convalescent concentrations of PS^{-}P-sel^{+}PMV versus primary outcome and $PS^{+}TF^{-}GPIIb^{-}$MV versus secondary outcome recurrent IS. Red curves: concentrations above median, black curves: concentrations at and below median.

4.4 STUDY 4

High peak thrombin and ETP measured in the acute phase of IS/TIA is associated with reduced risk of recurrent ischemic events. High MV-induced thrombin generation in the acute phase is associated with increased risk of recurrent IS.

The relationships between thrombin generation variables and outcome were evaluated in the same patient cohort as above. Excluding patients with anticoagulant treatment there were 190 patients in the cohort; of these 180 patients in the acute phase and 181 patients in the convalescent phase had data on thrombin generation. The follow-up period with respect to primary outcome was 986 patient years. In this cohort, primary outcome occurred in 46 patients and the secondary outcome recurrent IS in 36 patients; 25 patients died.

4.4.1 Cross-sectional evaluation of thrombin generation variables in the acute and convalescent phase as compared to healthy controls.

Baseline CAT data were reported previously, showing higher peak thrombin in patients in the acute and convalescent phase, and higher ETP in the acute phase as compared to healthy controls (220). Median F1+2 was lower in patients than in healthy controls, both in the acute and convalescent phase (170 and 207 versus 243 pmol/l, $p < 0.001$ and $p < 0.05$ respectively). In the subset of patients tested for MV-induced thrombin generation, MV peak thrombin (MV-PT) and MV thrombin generation potential (MV-TGP) were higher in patients than healthy controls, both in the acute and convalescent phase (MV-PT 117 and 115 vs 87 nM, $p < 0.001$ for both). MV lag time and MV time to peak were also marginally shorter.

4.4.2 Association between thrombin generation variables and outcome

Screening of CAT variables with respect to outcome unexpectedly showed lower acute phase values of peak thrombin and ETP in patients with events as compared to those without. The Kaplan-Meier survival curves based on median split confirmed a reduced risk in patients with peak thrombin or ETP above median both with respect primary outcome, see figure 11A) and B), and secondary outcome recurrent IS (data not shown). Hazard ratios for both outcomes remained significant after adjustment for cardiovascular risk factors, HR 0.40-0.53, $p < 0.05$ for all associations.

Variable screening showed no significant association between F1+2 and outcome.

In the subset of patients tested for MV-induced thrombin generation potential, MV-PT and MV-TFP measured in the acute phase were positively associated with the secondary outcome recurrent IS in an unadjusted analysis. The Kaplan-Meier curve for MV-PT is shown in figure 11C). The number of events in this group was too small to allow adjustment for cardiovascular risk factors.

There were no significant associations between thrombin generation variables and all-cause mortality.

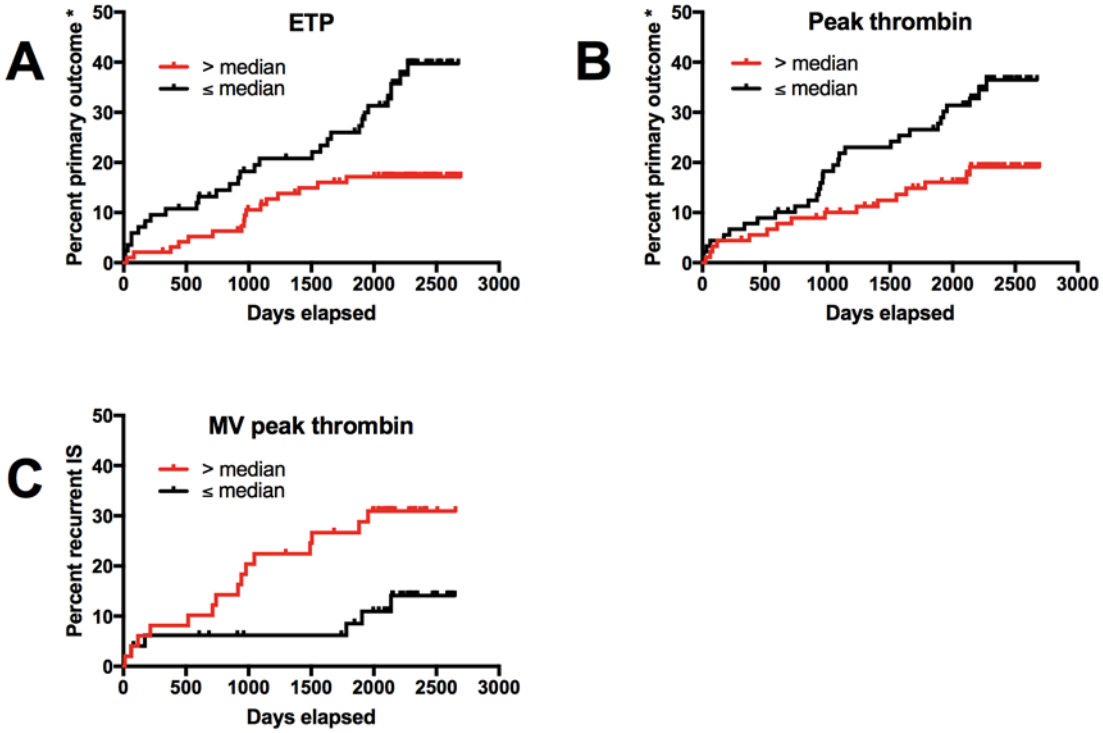

Figure 11: Univariate Kaplan-Meier survival curves based on median split. Red curves: values above median; black curves: values at median and below. A) ETP measured in the acute phase versus primary outcome B) peak thrombin measured in the acute phase versus primary outcome and C) MV-PT (peak thrombin) in the acute phase versus secondary outcome recurrent IS (a subset of patients).

5 DISCUSSION

5.1 STUDY 1 AND 2

In study 1 and 2, several clinical variables were found to be associated with HPR under clopidogrel treatment by univariate analysis 1) pathological OGTT/DM, 2) insulin resistance, 3) hypertension and 4) WMC indicative of CSVD. In addition, patients with NR had significantly higher platelet aggregation response to AA and submaximal TRAP stimulation than R, as well as higher platelet and leukocyte counts. Together these results suggest that HPR to clopidogrel to an important extent reflects a general platelet hyperreactivity, as has been proposed by others for patients with CAD (182, 186).

5.1.1 High on-treatment platelet reactivity and impaired glucose tolerance

At the time of study 1, the elevated risk of HPR in patients with DM was well-known in CAD patients (182, 230), but the association between HPR and IFG/IGT had not been investigated to our knowledge. The importance of DM for clopidogrel response for IS patients was confirmed in a study by Meves et al in 2014. They investigated HPR by MEA in 159 clopidogrel-treated patients shortly after acute IS (231). The prevalence of HPR was 44% after on average two days of clopidogrel treatment. (In a subset of HPR patients retested after one week only 43% retained HPR status, suggesting a prevalence under maintenance treatment similar to the one we found.) In a multivariate analysis only DM and platelet count were independently associated with HPR; glucose tolerance was not investigated.

In 2015, Ueno et al evaluated OGTT, insulin resistance and platelet aggregation measured by LTA in 65 non-diabetic CAD patients on dual antiplatelet treatment with aspirin and clopidogrel, tested in the stable phase after PCI (232). IGT was found in 30 patients and NGT in 35. Patients with IGT had clearly higher rates of HPR than those with NGT, and IGT was the only independent predictor of HPR in a multivariate analysis (OR 7.5), supporting our results. More than 55% of patients with IGT had HPR, while HPR was found in less than 15% of patients with NGT. In comparison we found HPR in only 2/32 patients (6%) with NGT, one of whom had insulin resistance. Together the two studies suggest the possibility to use OGTT as a tool to risk stratify non-diabetic patients with respect to HPR, which merits further investigation.

5.1.2 High on-treatment platelet reactivity and insulin resistance

We found HOMA-IR to be about twice as high for NR as R to clopidogrel. Insulin resistance was also evaluated in the above study by Ueno et al; 13/65 patients (20%) had insulin resistance, defined as HOMA-IR > 2.5; eight of these also had IGT (232). Patients with insulin resistance had higher platelet aggregation than those without, but insulin resistance was not an independent predictor of HPR in the multivariate analysis. However, the platelet aggregation response during OGTT, measured at baseline, and at 1 h and 2 h, deviated for patients with IR as compared to those without, in that it did not decrease over time. Together

our results and the Ueno study suggest an association between insulin resistance and HPR to clopidogrel. Larger studies are needed to determine if insulin resistance is associated to HPR independently of IGT.

With respect to mechanisms that could be involved in the association between insulin resistance and HPR, the enzyme/chaperone protein PDI is highly interesting. PDI was released from activated endothelium and platelets in the laser-induced thrombosis model and was found necessary for thrombus formation (105). PDI inactivates insulin in vitro by reducing disulfide bonds resulting in separation of the insulin A and B chains (233). PDI is expressed on the platelet surface of platelets even before they are activated (234). Circulating PMV from healthy controls expressed PDI with enzymatic capacity to reduce insulin, and PDI on PMV substantially contributed to platelet aggregation (233). Further, these PDI-positive PMV were found elevated in patients with DM type 2. The role of PDI as a mediator of both platelet hyper-reactivity/HPR and insulin resistance merits further investigation, see section 1.3.4.1. Of note, targeting PDI has recently been proposed as a novel antiplatelet treatment (235).

5.1.3 High on-treatment platelet reactivity and hypertension

In study 2 several findings supported an association between HPR and hypertension. At the one month control, patients with HPR had higher prevalence of hypertension, higher systolic blood pressure, higher pulse pressure and more intensive antihypertensive treatment than those with normal clopidogrel response. Hypertension has been proposed to increase platelet reactivity (see section 1.3.5.4) but an association to HPR was not established at the time of study 2. Supporting our finding, Kukula et al recently found hypertension to be a associated with HPR as measured by MEA in CAD patients (236). Further, in a study on ACS patients Verdoia et al found that hypertension influenced the MEA ADP response under ticagrelor treatment (237). Whether there is an independent association remains to be established.

5.1.4 High on-treatement platelet reactivity and ischemic stroke subtype, white matter changes and carotid atherosclerosis

Concerning IS subtype, there was no difference in the distributions of TOAST classifications between patients with and without HPR. The study was small and had only 16 patients with HPR, likely making it underpowered in this respect. Furthermore, TOAST is a relatively blunt classification tool, with overrepresentation in the ‘undetermined’ category, which may have contributed to the lack of difference. We also investigated associations between HPR and existing vessel changes. Contrary to our original hypothesis there was no association between HPR and carotid atherosclerosis or other manifestations of large artery atherosclerosis. As most patients had minor IS or TIA, the overall atherosclerosis burden in this cohort may have been too limited to reveal an association and there were also methodological limitations, see section 6.1.3. However, it is notable that patients with carotid atherosclerosis had a similar HPR prevalence to those without any discernable vessel changes.

In contrast to the result for atherosclerosis, HPR was associated with moderate to extensive WMC, indicative of CSVD. One possible explanation could be a confounding effect of hypertension and impaired glucose tolerance; these factors may contribute to both increased platelet reactivity and WMC without a causal relationship between the two. However, considering the recent finding that cardiovascular risk factors explain only a small part of the variance in WMC (34), this explanation is not entirely convincing. In a small study by Oberheiden et al, patients with mild, moderate or extensive WMC according Fazekas scale had higher platelet expression of CD40L and P-selectin in flow cytometry than to healthy controls (46). They also had higher number of platelet-monocyte aggregates and higher monocyte TF expression. The authors proposed that platelet activation may be a pathological factor in CSVD. Other studies for patients with IS have often found higher platelet activation in large vessel disease as compares to lacunar IS, in particular in the acute phase (45).

One factor that may differ between large vessel atherosclerosis and CSVD is shear-induced platelet activation. As the carotid arteries are among the larger in the body, they have low shear under normal conditions and significant stenosis is required for shear-induced platelet activation in these vessels. In addition, carotid atherosclerosis/stenosis occurs over a limited distance, typically a few centimeters, representing on a limited part of the platelet 'time-of-flight' in the circulation. Holmes et al showed that shear rates of about 10 000 s^{-1}, corresponding to more than 80% stenosis in coronary vessels, were required to achieve shear-induced platelet activation ex vivo; the time of shear exposure and geometry of stenosis were also important (238). CSVD on the other hand, affects the small arteries and arterioles, where shear is high also under healthy conditions and microstenoses can potentially cause extreme shear values. Further, CSVD involves diffuse changes throughout the cerebral vascular tree, where platelets can be exposed to shear-induced activation for longer times. Similar arguments could be applied also to platelet interactions with the dysfunctional endothelium in CSVD, where there is likely more time and larger surface area for interactions than for large vessel lesions. Thus we propose that shear-induced platelet aggregation and/or cerebral endothelial dysfunction in CSVD could be contributing factors to the association between HPR and WMC. As study 2 was small, the association between HPR and CSVD should be confirmed in larger studies using MRI, which has the added advantage that it can quantify the different manifestations of CSVD and establish their individual associations with HPR.

5.1.5 Multivariate analysis

An important question with respect to the above clinical variables is which of them are independently associated with HPR. The total number of NR was not sufficient to perform a multivariate Cox regression, and instead a multivariate linear regression model was used to investigate which variables predicted MEA ADP. In this model pathological glucose tolerance/DM, age, BMI, platelet count and leukocyte count were predictors of MEA ADP. Hypertension, insulin resistance and CSVD did not give a further independent contribution, likely partly due to covariance with the other variables. The variables of the model explained

almost half of the MEA ADP variance. Factors explaining the remaining half could include variations in clopidogrel absorption and metabolism, drug interactions and other genetic aspects of platelet function.

5.1.6 Conclusions

In summary, HPR appears to be at least as common in patients with IS/TIA as in patients with ACS (239), despite the fact that the former are not exposed to thrombogenic stents. The recent meta-analysis by Fiolaki et al suggests that the clinical consequences of HPR in terms of risk for recurrence are also similar for IS/TIA as for ACS (207). While it is clear that HPR is a risk marker, it is less clear how it should be handled clinically. For patients with CAD the present recommendation is that platelet function testing may be considered in selected, high-risk patients on dual antiplatelet therapy with aspirin and clopidogrel (240, 241). However, for patients with IS/TIA, clopidogrel is given as monotherapy and often indefinitely. Screening of IS/TIA patients with high risk of HPR could therefore be warranted. Studies 1 and 2 identified pre-diabetic metabolic changes, advanced hypertension and presence of WMC as important factors for HPR. The importance of these factors should be confirmed in larger cohorts, but together they provide an indication of which patient groups may benefit the most from targeted platelet function tests.

With respect to interventions, our results suggest that HPR may respond to life-style changes and more aggressive risk factor management. As the risk of HPR is increased already in the pre-diabetic phase, life-style changes such as exercise, weight-loss and improved diet could conceivably reduce the prevalence of HPR in pre-diabetic patients. It would also be highly interesting to investigate whether medication that improves plasma glucose and insulin levels could reduce HPR in non-diabetic patients. In this respect metformin is of particular interest, as it reduces plasma glucose and improves insulin sensitivity without causing hypoglycemia.

It would intuitively seem appropriate to change clopidogrel to other antiplatelet treatment in IS/TIA patients with HPR. Lending some support to this concept, the PROFESS subgroup analysis suggested that aspirin with dipyridamole may be superior to clopidogrel for patients with diabetes, small vessel disease and patients with previous IS/TIA (13). However, if the association between HPR and CSVD is confirmed, IS/TIA patients with HPR may also be at increased risk of bleeding (5), which makes the selection of antiplatelet treatment more complicated. Since one effect of HPR is insufficient cAMP levels in platelets, addition of dipyridamole or cilostazol to clopidogrel would be interesting, in particular as these substances are not expected to increase bleeding risk substantially.

5.2 STUDY 3

Study 3 investigated PMV and TF^{+}MV subpopulations and their association with prognosis, and generated a number of results. Several were unexpected and/or contradicted our original hypotheses. While the majority of detected MV expressed PS, a PS-negative population was

the only one with a positive association with prognosis. PS^+MV populations displayed no association or unexpected protective effects.

As expected PMV populations expressing activation markers P-selectin or TF showed more pronounced elevations relative to healthy controls than overall PMV levels but the individual associations with recurrent events and relative changes between the acute and convalescent phase differed between PMV populations.

5.2.1 Platelet microvesicles and P-selectin-positive platelet microvesicles

Overall PS^-PMV and PS^-P-sel^+PMV showed stronger elevations relative to healthy controls than their PS^+ counterparts. Also the proportions of PMV positive for P-selectin were higher for PS^-PMV than for PS^+PMV, 68% versus 46% in the acute phase and 68% and 33% in the convalescent phase. These results may partly be due to 'diluting' effect of PS-positive, megakaryocyte-derived MV which would be counted as PS^+PMV (79). In support of this hypothesis, the median concentrations of PS^+PMV lacking P-selectin were similar for patients in the acute and convalescent phase and for healthy controls (8 570, 8 563 and 8 338 /µl respectively). These MV could thus comprise constitutively released megakaryocyte MV; however, this hypothesis would have to be confirmed by evaluating megakaryocyte-specific MV markers (79). In comparison with PS^+PMV, P-selectin positive PMV would appear to be more appropriate markers of platelet activation, as suggested by others (80, 83). However, there were notable differences between PS^+P-sel^+PMV and PS^-P-sel^+PMV, both in dynamic behavior and association with recurrence. PS^+P-sel^+PMV decreased between the acute and convalescent phase, as would be expected after a thrombotic event. In contrast PS^-P-sel^+PMV increased between the acute and convalescent event. A discussion of possible explanations for this result by necessity becomes speculative, as the PS^-PMV generation mechanisms and binding/clearance mechanisms are presently unknown. Shear-induced platelet activation has been shown to result in PMV release ex vivo (238); it was not specified to what extent these expressed PS. Considering that shear-induced platelet activation may be reversible and does not require platelet PS-exposure (119), it is tempting to speculate that shear-induced platelet activation could generate PS^-PMV. Another interesting hypothesis is that PS^+PMV and PS^-PMV may, in the setting of thrombosis, be released from PS^+ and PS^- platelet subpopulations respectively. Munnix et al found aggregating plaletets to be PS^- and while procoagulant platelets were PS^+ (113). While in vitro experiments indicate that PS^+ procoagulant platelet are particularly prone to MV release, some MV appeared to be released also from aggregating platelets (115). It would be interesting to investigate if PS^+P-sel^+PMV correlate with the propensity to form PS^+ platelets in vitro. As to binding and/or clearance, platelets adhere to cerebral ischemic microvesseles in IS (198) and similar effects may occur with PMV, which would decrease the concentration of circulating PMV. In this respect it would be interesting to subtype PS^+P-sel^+PMV and PS^-P-sel^+PMV further with respect to adhesion molecules that mediate interactions with the endothelium. Similarly, IS leads to leukocyte activation, in particular monocytes and neutrophils, and hypothetically leukocyte binding of

PS^+P-sel$^+$PMV and PS^-P-sel$^+$PMV could differ, which could influence their circulating concentrations.

Concerning associations with prognosis, PS^+PMV overall in the acute phase and PS^-P-sel$^+$PMV in the convalescent phase appeared to be inversely associated with prognosis, although the associations were not significant after adjusting for cardiovascular risk factors. Other PMV populations did not have any association with outcome. The reasons for these unexpected results are unknown. It is possible that platelets in high-risk patients are chronically activated and 'exhausted', leading to a suppressed platelet response in connection with a thrombotic event. It has also been reported that PMV can have an anticoagulant effect which may explain a protective effect (74). Also the timing for PMV measurement should be considered: PMV responses in the acute and early convalescent phase of IS/TIA may not be associated with long-term risk. It cannot be excluded that measurement in a more stable phase (3-6 months) would reveal other associations.

5.2.2 Tissue factor positive microvesicles and platelet microvesicles

Several findings are notable for TF$^+$MV populations after IS/TIA. Firstly, TF$^+$PMV were drastically elevated levels in patients as compared to healthy controls: median PS^+TF^+PMV was 77 times higher for patients in the acute phase and 49 times higher in the convalescent phase. For PS^-TF^+PMV the corresponding figures were 15 and 11 times. Further, the absolute levels were substantially higher than those previously found by our group for patients with ACS by the same method (80). While results are not directly comparable due to minor differences in flow cytometry settings, the difference was about one order of magnitude, while concentrations for healthy controls were comparable. Further, for patients a clear majority of TF$^+$MV expressed the platelet marker GPIIb, almost 90% both in the acute an convalescent phase, while for healthy controls the corresponding figure was only about 30%. Finally, only PS^-TF^+PMV measured in the acute phase were positively associated with recurrent events; PS^+TF^+PMV were neutral and PS^+TF^+MV of non-platelet origin measured in the convalescent phase appeared protective.

As discussed in section 1.3.3.3, TF$^+$MV, their source and clinical importance are controversial. Most studies have identified monocytes as the most likely source of TF$^+$MV. TF$^+$PMV have been detected in several studies (80, 242), but after del Conde et al showed that activated platelet could take up monocyte-derived TF$^+$MV (96), the original TF source has still largely been assumed to be monocytes. We found concentrations of TF$^+$PMV to be much higher than concentrations of TF$^+$MV of non-platelet origin (about one order of magnitude), putting this concept into question. There are reports both demonstrating and disproving the presence of TF in resting and activated platelets, recently excellently summarized in (101). Our results suggest that platelets are strongly involved in the generation of TF$^+$MV after IS/TIA, however it cannot be excluded that TF$^+$PMV are hybrid MV generated by interactions with leukocytes. Interestingly, Leon et al found platelet expression

of TF, where TF was inactive in its native form, but could be activated by platelet-neutrophil interactions in vitro in an ADP-dependent manner (242).

The difference in TF^+PMV concentrations of patients with IS/TIA as compared to patients with ACS is notable, and suggests differences in TF^+PMV formation in cerebral as opposed to coronary vessels. It is possible that increased permeability of the blood-brain barrier is involved, as the brain is particularly rich in TF which may leak into the circulation and be taken up by platelets. Such effects have been demonstrated in traumatic ICH (243). However, cerebral TF is would be expected to be active, of which we have no evidence. In fact, the TF-positive population with the highest concentration, PS^+TF^+PMV, was not associated with prognosis, which suggests that TF is inactive on these PMV. Also, none of the TF^+MV subpopulations in patients correlated with any of the thrombin generation variables in study 4. It would be interesting to investigate if PS^+TF^+PMV and PS^-TF^+PMV differ in their expression of PSGL-1, which could influence their incorporation into thrombi.

5.2.3 Conclusions

Overall PMV and TF^+MV levels are not associated with recurrence in patients with minor IS/TIA, but specific populations measured in the acute and convalescent phase display associations with either increased risk (PS^-TF^+MV in the acute phase) or decreased risk (PS^+PMV in the acute phase, PS^-P-sel^+PMV and $PS^+TF^+GPIIb^-$MV in the convalescent phase). A more detailed subtyping to these MV populations may provide insights into platelet activation mechanisms and possibly cell interactions underlying an increased respectively decreased risk of recurrent events in patients with IS/TIA. The study also emphasizes the importance of the timing of MV measurement in relation to symptoms, as both MV levels and their association with outcome change between the acute phase and one month after symptoms.

5.3 STUDY 4

As in study 3, the results in study 4 were unexpected and partly contradicted the original hypotheses. Despite the fact that acute phase values of both ETP and peak thrombin were elevated in patients as compared to healthy controls, high levels of ETP and peak thrombin were associated with a reduced risk of recurrent ischemic events. In contrast, high MV-induced peak thrombin and MV thrombin generation potential in the acute phase increased the risk of recurrent IS in the subset of patients tested. F1+2 was lower in patients than healthy controls and tended to be lower in patients with recurrent events than those without but was not significantly associated with outcome.

One previous population-based study on IS and one small study on ACS patients have found low ETP to be positively associated with outcome (244, 245). In both studies it was proposed that high risk patients may have elevated thrombin generation in vivo which would result in decreased in vitro thrombin generation, explaining the associations. We measured F1+2 as marker of in vivo thrombin generation and found that it was lower in patients than healthy

controls and also tended to be lower in patients with primary outcome than those without (at least in the convalescent phase). In addition, there was no indication of larger infarction in patients with recurrent events as stroke severity was the same in patients with and without primary outcome, both in the acute phase and at the one month control. It is thus difficult to reconcile our results with a pure consumption effect. The modified MV-induced CAT assay uses normal pooled plasma and is not dependent on patient concentrations of coagulation/anticoagulation factors. The fact that this assay had a positive association with the outcome recurrent IS suggests that coagulation/anticoagulation may be 'rebalanced' after IS/TIA in high risk patients. Possibly MV populations may be involved in this 'rebalancing' but we found no clear correlations between the MV-populations measured in study 3 and peak thrombin or ETP. With respect to 'rebalancing' of coagulation/anticoagulation it would be interesting to evaluate TFPI, which has often been found increased in CVD (246).

The associations to outcome differed somewhat between ETP and peak thrombin. A somewhat stronger association was found for ETP, in particular with respect to recurrent IS. Also, the Kaplan-Meier curves suggested that high ETP was particularly protective against early recurrences while the for peak thrombin the curves separated later (1-2 years). The study was not large enough to test these findings for statistical significance. It would be interesting to test the predictive potential of the combination of low ETP and high MV-TP in a larger study.

5.4 OVERALL DISCUSSION OF STUDY 3 AND 4

Together study 3 and 4 suggest that in patients with high risk of recurrent ischemic event, the response of the hemostatic system to a thrombotic event is disturbed in the acute phase. This disturbance is characterized by increased $PS^{-}TF^{+}$PMV, possibly decreases in other PMV subpopulations, reduced levels of ETP and peak thrombin and, for recurrent IS, increased levels of MV-induced thrombin generation and MV-induced peak thrombin. Some of these variables appear to have a particular importance for early recurrences when the relative risk per time interval is highest, notably ETP. If the underlying mechanisms could be uncovered, this may lead to the discovery of treatment targets to reduce early recurrence.

6 METHODOLOGICAL LIMITATIONS

6.1 STUDY 1 AND 2

6.1.1 Definition of high on-treatment platelet reactivity

We used the MEA ADP cut-off for HPR agreed by cardiological consensus (182). This value comes from a medium-sized cohort (1608 patients) going through planned PCI due to ACS or stable angina after a loading dose of 600 mg of clopidogrel (222). The cut-off was established by receiver-operating characteristics analysis of AMI in the first month comprising 10 cases, most of which occurred in the first week after PCI. The conditions of our study differ from the above in almost all aspects. As was noted in the consensus document, the appropriate cut-off value may be different in patient cohorts with CVD other than ACS/PCI (182). Ideally a larger study should be performed to establish the optimal HPR cut-off for IS/TIA patients.

6.1.2 Insulin resistance

Insulin resistance was assessed by HOMA-IR, which is a surrogate index; definite diagnosis of insulin resistance requires testing with hyperinsulinemic, euglycemic clamp. The correlation between the two methods is good for patients with a limited loss of β-cell (226). As the latter condition was likely met for the majority of patients tested, the error is likely limited, but hyperinsulinemic, euglycemic clamp may be required to establish an independent association between HPR and insulin resistance.

6.1.3 Diagnosis of vessel changes

The degree of carotid atherosclerosis was evaluated based on clinical ultrasound investigations aimed at identifying carotid stenosis with indication for surgery. A research-based protocol including measurement of carotid-intima thickness would have been more appropriate. We can thus not exclude an association between large vessel and HPR.

The degree of WMC was evaluated on CT scans of the brain, while Fazekas scale was developed for MRI. Wattjes et al show good correlation of Fazekas scale on modern CT instruments compared to MRI (229), but we cannot exclude some misclassification where lacunes may have been interpreted as WMC. Both lacunes and WMC are manifestations of CSVD and they are correlated in studies, but differences in detailed pathophysiology cannot be excluded. Ideally a larger study should be performed relating HPR to CSVD manifestations on MRI. Also, by a misunderstanding of the Fazekas scale, DWMC were counted twice, both for characteristic appearance and number of DWMC. This resulted in a total Fazekas score of 0-9 points instead of 0-6 points. As the main result was obtained after dichotomizing the ordinal scale, we do not think this error influences our conclusions.

6.1.4 Statistical methods

In study 1, correlations between MEA ADP and variables of glucose metabolism were calculated by Pearson's correlation coefficient. As the variables were non-normally distributed, Spearman's correlation coefficient should have been used.

6.2 STUDY 3

PPP was prepared by a single centrifugation step, while presently double centrifugation to obtain platelet-free plasma is recommended to avoid in vitro generation of PMV or platelet fragments being counted as PMV (247). MV enumeration was performed before the guidelines were established. As we have used phalloidin to avoid counting platelet fragments (223), and data indicated low number of these events, the error introduced by single step centrifugation should be minimal.

6.3 STUDY 4

The modified CAT assay for determining MV-induced thrombin generation in normal pooled plasma was performed without addition of corn trypsin inhibitor and thus includes the contact pathway contribution to thrombin generation. Notably, it was recently reported that no measurement of MV-induced thrombin generation could not be obtained when adding corn trypsin inhibitor to the assay (248).

7 CONCLUSIONS

- High on-treatment platelet reactivity (HPR) under clopidogrel treatment in the stable phase after ischemic stroke (IS) or TIA was common and observed in approximately one of five patients.
- Patients with HPR to clopidogrel tended to have stronger platelet responses to arachidonic acid and thrombin receptor activating peptide compared to those who responded normally to clopidogrel.
- HPR to clopidogrel was associated with disturbed glucose metabolism, insulin resistance, advanced hypertension and radiological signs of cerebral small vessel disease (CSVD).
- HPR to clopidogrel was not associated with carotid atherosclerosis or other manifestations of large artery atherosclerosis.
- Platelet microvesicles (PMV) expressing activation markers P-selectin or tissue factor (TF) were strongly elevated in the acute phase of IS/TIA and remained elevated in the convalescent phase one month after symptoms.
- Several PMV subpopulations expected to be prothrombotic were not associated with outcome, neither in the acute nor the convalescent phase.
- PMV expressing TF but lacking phosphatidylserine measured in the acute phase were positively and independently associated with poor outcome.
- Several MV subpopulations appeared to be inversely associated with poor outcome, suggesting a protective effect.
- Chronic activation with ‘platelet exhaustion’ in high risk patients may explain the seemingly protective effect of certain PMV populations.
- Further research is required to determine if different PMV populations reflect different types of platelet activation.
- In vitro thrombin generation in plasma, assessed as endogenous thrombin potential and peak thrombin measured in the acute phase, were associated with *reduced risks* of recurrent ischemic events.
- In addition, thrombin generation in vivo was lower in patients than healthy controls, and not associated with outcome.
- MV-induced thrombin generation and peak thrombin measured in the acute phase, were associated with an increased risk of recurrent ischemic stroke, in a subset of patients.
- We found no relationships between thrombin generation variables and circulating PMV concentrations.

8 FUTURE PERSPECTIVES

8.1 HIGH ON-TREATMEANT PLATELET REACTIVITY TO CLOPIDOGREL

It would be of high priority to confirm the association between HPR and CSVD in a larger study. Such a study should use MRI to assess the not only WMC but all the different CSVD manifestations and their respective associations with HPR. In particular, the association between HPR/platelet function and cerebral microbleeds in patients with IS/TIA is of interest, to determine whether patients with HPR and CSVD also have a risk of bleeding. Such a study could be designed as a case-control study with HPR patients as the cases.

As HPR has a high prevalence in patients with IS/TIA, it should be investigated whether HPR status can be modified by interventions. Pilot crossover studies could be performed to investigate if metformin or the addition of dipyridamole or cilostazol to clopidogrel can reduce HPR prevalence. It would also be of interest to study the effect of life-style changes and more aggressive risk factor management in patients with HPR. Comparing the clinical effect of clopidogrel treatment versus aspirin combined with dipyridamole in IS/TIA patients with HPR would require a large, multicenter study.

8.2 MICROVESICLES

Study 3 illustrates both the potential and the complexity of subtyping MV to a greater level of detail than just cell of origin. In particular two aspects deserve further study. Firstly, the one population positively and independently associated with outcome, $PS^{-}TF^{+}$PMV, should be characterized further, and secondly the differences between PMV expressing respectively not expressing PS should be clarified.

A more detailed characterization of $PS^{-}TF^{+}$PMV could include the investigation of other surface markers on these PMV, for instance PSGL-1 and neutrophil- and monocyte-specific markers. Also functional tests of this subpopulation are of interest. Possibly $PS^{-}TF^{+}$PMV could be isolated by lactadherin columns and/or magnetic beads with anti-TF antibodies, which could allow a more detailed characterization.

The differences in dynamic behavior and association with outcome between PS^{+}P-sel^{+}PMV and PS^{-}P-sel^{+}PMV suggest that they may reflect different aspects of platelet activation. Also the negative correlation between PS^{+}PMV and PS^{-}P-sel^{+}PMV merits further study. More detailed characterization of these PMV populations may provide clues to the mechanisms behind PS^{-}PMV release, which are presently unknown. In particular it would be interesting to determine whether circulating levels of PS^{+}P-sel^{+}PMV correlate with the propensity to form PS^{+} procoagulant platelets in vitro and vice versa for PS^{-}P-sel^{+}PMV and PS^{-} aggregating platelets.

8.3 THROMBIN GENERATION VARIABLES

It is possible that the contradictory results of study 4 could be clarified by investigating CAT response in PRP which would include platelet contribution to thrombin generation.

8.4 PLATELET MICROVESICLES, THROMBIN GENERATION AND VESSEL CHANGES

Given the association between HPR and CSVD found in study 2, the associations between PMV respectively thrombin generation variables and existing vessel changes in the cohort of study 3 and 4 should be investigated. The data has been collected but not yet analyzed. If CSVD respectively large artery atherosclerosis have different ‘profiles’ of PMV populations and thrombin generation variables respectively, this may provide clues both to the underlying pathological mechanisms and possibly new treatment targets.

9 SVENSK SAMMANFATTNING

Trombocyter (blodplättar) är centrala för kardiovaskulära sjukdomar som stroke. Vid stroke aktiveras trombocyter av en kärlskada och klumpar ihop sig (aggregerar) till en propp som blockerar blodförsörjningen till en del av hjärnan. Detta ger syrebrist, förlust av hjärnceller och funktionsbortfall. Stroke på grund av propp behandlas med läkemedel som hämmar trombocyternas förmåga att aggregera. Forskning visar att individer skiljer sig åt både vad gäller trombocyternas benägenhet att aggregera och hur väl trombocyter hämmas av behandling. Hos vissa patienter med hjärtkärlsjukdom bibehåller trombocyterna sin förmåga att aggregera trots behandling med trombocythämmande läkemedel. Detta gäller framför allt läkemedlet clopidogrel. De patienter som inte svarar på behandling kan betraktas som ’clopidogrel-resistenta.’ Clopidogrel-resistens innebär en ökad risk för återinsjuknande.

När trombocyter aktiveras kan de frisätta små blåsor från cellmembranet, så kallade mikrovesikler. Koncentrationen av trombocytmikrovesikler kan mätas i blod och speglar graden av trombocytaktivering. Aktiverade trombocyter och trombocytmikrovesikler kan i sin tur aktivera koagulationssystemet (blodlevringssystemet) som bildar enzymet trombin. Trombin bidrar till proppbildningen genom att bilda ett nätverk av fibrin; vidare stimulerar trombin trombocytaggregationen. Forskning tyder på att trombocyter och koagulation, utöver att bilda blodproppar, också bidrar till ateroskleros (åderförfettning), vilket är en underliggande orsak till både stroke och hjärtinfarkt.

Det första målet med denna avhandling var undersöka förekomsten av clopidogrel-resistens hos patienter med stroke eller transient ischemisk attack (TIA), samt vilka faktorer som är kopplade till clopidogrel-resistens (studie 1 och 2). Vi fann clopidogrelresistens hos 22% av de 72 patienter vi undersökte, vilket är lika mycket eller något högre än vad man tidigare har funnit hos patienter med kranskärlssjukdom. Clopidogrel-resistenta patienter hade oftare diabetes och nedsatt glukostolerans än de med normalt svar på clopidogrel; omvänt var clopidogrel-resistens mycket ovanligt hos individer utan tecken på diabetes eller nedsatt glukostolerans. Clopidogrel-resistenta hade även högre insulinnivåer och högre blodtryck. Förekomsten av åderförfettning i halskärlen var inte högre hos clopidogrel-resistenta. Däremot var kärlsjukdom i hjärnans små kärl betydligt vanligare hos clopidogrel-resistenta patienter.

Det andra målet var att undersöka trombocytmikrovesikler och trombinbildning efter stroke eller TIA och deras betydelse för återinsjuknande. Vi undersökte totalt 211 patienter och jämförde med 53 ålders- och könsmatchade friska kontroller. Patienterna hade betydligt högre koncentrationer i blod av trombocytmicrovesikler som bar på aktiveringsmarkörerna P-selectin och tromboplastin (studie 3). Bara en typ av trombocytmikrovesikler var kopplad till återinsjuknande: höga nivåer trombocytmikrovesikler som bar på tromboplastin men inte fettmolekylen fosfatidylserin nästan dubblerade risken för återinsjuknande. Något oväntat verkade höga nivåer i blodet av flera andra typer av mikrovesikler ha en skyddande effekt.

Trombinbildningsförmågan i blodet var högre hos strokepatienter än hos friska individer. Trots detta visade studie 4 att hög trombinbildningsförmåga i den akuta fasen av stroke var förenat med en *minskad* risk för återfall. Däremot var mikrovesiklernas funktionella förmåga att bilda trombin kopplad till en ökad risk för återfall i stroke.

Sammanfattningsvis är clopidogrel-resistens vanligt efter stroke/TIA och kan i vissa grupper finnas hos upp mot 40% av patienterna. Risken för clopidogrel-resistens ökar redan under förstadiet till diabetes. Förbättrad livsstil och mer intensiv behandling av riskfaktorer kan tänkas minska återfallsrisken för dessa patienter. Större studier behövs emellertid för att undersöka om andra trombocythämmande läkemedel än clopidogrel kan förbättra prognosen för strokepatienter med clopidogrelresistens. Studie 3 visar att en detaljerad kartläggning av trombocytmikropartiklar kan ge information om trombocytaktiveringen vid stroke/TIA. Vissa typer av trombocytmikrovesikler var kopplade till ökad respektive minskad risk för återfall. De bakomliggande mekanismerna är okända och bör kartläggas närmare, framför all vad gäller trombocytmikrovesikler som bär på tromboplastin men saknar fosfatidylserin, då dessa vesikler var kopplade till ökad risk för återfall. Hos patienter med hög risk för återinsjuknande är trombinbildningen störd i akutfasen av stroke/TIA, med tecken på nedsatt trombinbildningsförmåga.

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