

**DEPARTMENT OF CLINICAL SCIENCES,
DANDERYD HOSPITAL**

Division of Cardiovascular Medicine,
Karolinska Institutet, Stockholm, Sweden

STUDIES ON PLATELET FUNCTION

AND

MICROVESICLES IN ACUTE CORONARY SYNDROME

Sam Kafian M.D.



**Karolinska
Institutet**

Stockholm 2018

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Published by Karolinska Institutet.

Printed by Eprint AB 2018

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ISBN 978-91-7676-936-2

STUDIES ON PLATELET FUNCTION AND MICROVESICLES IN ACUTE CORONARY SYNDROME

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Sam Kafian M.D.

Principal Supervisor:

Associate Professor Bassem A. Samad
Karolinska Institutet
Department of Clinical Sciences,
Division of Cardiovascular Medicine
Danderyd Hospital

Opponent:

Associate Professor Christina Christersson
Uppsala University
Department of Medicine Sciences, Cardiology
Akademiska sjukhuset

Co-supervisor(s):

Professor Håkan Wallén
Karolinska Institutet
Department of Clinical Sciences,
Division of Cardiovascular Medicine
Danderyd Hospital

Examination Board:

Professor Tomas Lindahl
Linköping University
Department of Clinical and Experimental
Medicine

Dr. Fariborz Mobarrez
Karolinska Institutet
Department of Medicine, Solna
Karolinska University Hospital

Professor Karin Schenck-Gustafsson
Karolinska Institutet
Department of Medicine, Solna
Centre for Gender Medicine
Karolinska University Hospital

Associate Professor Mats Frick
Karolinska Institutet
Department of Clinical Sciences and Education
Division of Cardiology
Södersjukhuset

The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes.

Avicenna (980–1037, ancient Persian physician)

*To my beloved family:
Shirin, Nick and our soon-to-be-born child*

ABSTRACT

INTRODUCTION

The cornerstone of treatment of acute coronary syndrome (ACS) is percutaneous coronary intervention (PCI) and dual antiplatelet therapy (DAT) with aspirin and the ADP-receptor inhibitor clopidogrel. However, some ACS patients will suffer from recurrent cardiovascular events, and it has been shown that this may partly be due to an insufficient antiplatelet response to DAT. In this thesis, we investigated platelet function in ACS. The overarching aim was to further investigate clopidogrel non-responsiveness with established and new methods to assess platelet function.

METHODS AND RESULTS

In study I, we investigated if arterial and venous sampling give comparable results with respect to detection of poor antiplatelet responsiveness to clopidogrel and aspirin with the established method multiple electrode whole blood impedance aggregometry (MEA). Twenty-eight patients with coronary artery disease were investigated in connection to PCI. Identical number of patients with poor responsiveness to clopidogrel (7/28; i.e. 25 %) and aspirin (3/28; i.e. 11 %) were detected in arterial and venous blood samples with MEA. Thus, arterial and venous sampling can be used interchangeably in the detection of poor responsiveness to clopidogrel and aspirin when MEA is used to study platelet function.

In study II, we investigated ADP-induced platelet aggregation by MEA in 183 patients with ACS on DAT at discharge (3–5 days after admission). We also measured circulating microvesicles (MVs) and platelet derived MV (PMVs) in 154 of the ACS patients. Of note, MVs are small membrane buds released from various cells in response to activation or apoptosis. We found that around 20 % (36/183) of ACS patients were non-responders (“high on-treatment platelet reactivity”; HPR) to clopidogrel. Flow cytometry measurements showed that circulating PMV levels were significantly higher in HPR compared to those with “sufficient” clopidogrel responsiveness (“normal on-treatment platelet reactivity”; NPR). Furthermore, in patients with strong inhibition to clopidogrel (low on-treatment platelet reactivity; LPR) PMV levels were significantly lower than in NPR patients. Thus, levels of circulating PMVs reflect platelet responsiveness to clopidogrel.

Study III was performed to elucidate if MVs from ACS patients on DAT influence platelet aggregation. Thus, we added MVs from samples of patients with ACS with HPR and from patients with Non-HPR (i.e. LPR or NPR) to clopidogrel, to platelet rich plasma obtained from healthy volunteers. Results showed that MVs from HPR patients significantly enhanced spontaneous platelet aggregation as compared to MVs from patients with Non-HPR. In addition, we could also show that MVs from ACS patients with diabetes and DAT, enhanced platelet aggregation compared to MVs from ACS patients on DAT without diabetes. MVs have the potential to enhance platelet aggregation, supporting the

idea that MVs may not only be “markers” but also “makers” of physiological or pathophysiological processes.

In study IV, we investigated if circulating MVs expose a molecule of the humoral immune system pentraxin-3 (PTX3). Thus, we measured the presence of PTX3⁺-MVs in plasma in patients with acute myocardial infarction (AMI). We found that PTX3⁺-MVs were elevated at admittance in patients with acute ST-elevation myocardial infarction (STEMI; n=23) and that levels decreased after PCI. Further, the circulating PTX3⁺-MVs levels were even lower in AMI patients at discharge (3–5 days after admission; n=153), but not as low as in healthy subjects (n=15). Thus, PTX3 is exposed on circulating MVs in the acute setting of AMI, and the levels fall significantly over the days after the acute event. The PTX3⁺-MVs should be further phenotyped with our flow cytometry method to elucidate the origin of PTX3 and its possible role in acute coronary artery disease.

CONCLUSION

Clopidogrel non-responsiveness is present in every fourth to every fifth ACS patient on clopidogrel. This can be detected either in arterial or venous samples by MEA. The number of circulating MVs reflect platelet responsiveness to clopidogrel, and circulating MVs in ACS patients have the capacity to enhance platelet aggregation, indicating a possible functional role of circulating MVs in the setting of ACS. In acute AMI, MVs exposing PTX3 circulate in an elevated concentration. The role of these PTX3⁺-MVs deserves to be further investigated.

LIST OF SCIENTIFIC PAPERS

- I. **Sam Kafian**, Fariborz Mobarrez, Majid Kalani, Håkan Wallén & Bassem A. Samad

Comparison of venous and arterial blood sampling for the assessment of platelet aggregation with whole blood impedance aggregometry.
Scand J Clin Lab Invest. 2011;71(8):637–40.

- II. **Sam Kafian**, Fariborz Mobarrez, Håkan Wallén & Bassem Samad

Association between platelet reactivity and circulating platelet-derived microvesicles in patients with acute coronary syndrome.
Platelets. 2015;26(5):467–73.

- III. **Sam Kafian**, Håkan Wallén, Bassem A. Samad, Fariborz Mobarrez

Microvesicles from patients with acute coronary syndrome enhance platelet aggregation.
Manuscript.

- IV. **Sam Kafian**, Håkan Wallén, Gundars Rasmanis, Charlotte Thålin, Bassem A. Samad, Fariborz Mobarrez

Exposure of pentraxin-3 on microvesicles in patients with acute myocardial infarction.
Manuscript.

CONTENTS

| | | |
|----------|--|-----------|
| 1 | INTRODUCTION | 1 |
| 1.1 | General background..... | 1 |
| 1.2 | Acute coronary syndrome..... | 1 |
| 1.3 | Platelet function..... | 2 |
| 1.3.1 | Platelet activation..... | 2 |
| 1.3.2 | Platelet receptors..... | 4 |
| 1.3.3 | Platelet inhibitors..... | 4 |
| 1.3.3.1 | Aspirin..... | 4 |
| 1.3.3.2 | P2Y ₁₂ receptor inhibitors..... | 5 |
| I. | Ticlopidine..... | 5 |
| II. | Clopidogrel..... | 5 |
| III. | Prasugrel..... | 7 |
| IV. | Ticagrelor..... | 7 |
| V. | Cangrelor..... | 7 |
| 1.3.3.3 | Glycoprotein IIb/IIIa receptor antagonists..... | 8 |
| 1.3.4 | Measurement of platelet function..... | 8 |
| 1.3.4.1 | Light Transmission Aggregometry..... | 8 |
| 1.3.4.2 | VerifyNow..... | 8 |
| 1.3.4.3 | Multiplate..... | 9 |
| 1.3.4.4 | Flow cytometry..... | 10 |
| 1.4 | Microvesicles..... | 11 |
| 1.5 | Pentraxin-3..... | 12 |
| 2 | AIMS | 15 |
| 3 | MATERIAL AND METHODS | 16 |
| 3.1 | Study population..... | 16 |
| 3.2 | Blood collection..... | 17 |
| 3.3 | Methods..... | 19 |
| 3.3.1 | Multiple electrode aggregometry..... | 19 |
| 3.3.2 | Flow cytometric measurements of MVs and PMVs..... | 19 |
| 3.3.3 | Assessment of PTX3 by ELISA..... | 19 |
| 3.3.4 | Assessment of platelet aggregation in 96-well microplates..... | 20 |
| 3.4 | Statistical analyses..... | 21 |
| 4 | RESULTS | 22 |
| 4.1 | Study I..... | 22 |
| 4.2 | Study II..... | 23 |
| 4.3 | Study III..... | 27 |
| 4.4 | Study IV..... | 29 |

| | | |
|---|---|----|
| 5 | GENERAL DISCUSSION | 31 |
| | 5.1 Study I..... | 31 |
| | 5.2 Study II..... | 32 |
| | 5.3 Study III..... | 33 |
| | 5.4 Study IV..... | 34 |
| | 5.5 Concluding remarks and future perspectives..... | 34 |
| 6 | CONCLUSIONS | 36 |
| 7 | POPULÄRVETENSKAPLIG SAMMANFATTNING | 37 |
| 8 | ACKNOWLEDGEMENTS | 40 |
| 9 | REFERENCES | 43 |

LIST OF ABBREVIATIONS

| | |
|---------|--|
| AA | Arachnoid acid |
| ACS | Acute coronary syndrome |
| ADP | Adenosine diphosphate |
| AMI | Acute myocardial infarction |
| AUC | Area under the curve |
| CAD | Coronary artery disease |
| CD | Cluster of differentiation |
| CKD | Chronic kidney disease |
| COX-1 | Cyclooxygenase-1 |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| CYP 450 | Cytochrome P450 |
| DAT | Dual antiplatelet therapy |
| DM | Diabetes mellitus |
| EDTA | Ethylene diamine tetra acetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FCM | Flow cytometry |
| GP | Glycoprotein |
| HPR | High on-treatment platelet reactivity |
| IL | Interleukin |
| LPR | Low on-treatment platelet reactivity |
| LTA | Light transmission aggregometry |
| MACE | Major adverse cardiac events |
| MEA | Multiple electrode aggregometry |
| MVs | Microvesicles |
| NPR | Normal on-treatment platelet reactivity |
| NSTEMI | Non-ST-segment elevation myocardial infarction |
| PCI | Percutaneous coronary intervention |
| PFP | Platelet free plasma |
| PMVs | Platelet derived microvesicles |
| POC | Point of care |
| PPI | Proton pump inhibitor |

| | |
|------------------|--|
| PPP | Platelet poor plasma |
| PRP | Platelet rich plasma |
| PS | Phosphatidylserine |
| PTX3 | Pentraxin-3 |
| RT | Room temperature |
| STEMI | ST-segment elevation myocardial infarction |
| TF | Tissue factor |
| TLR | Toll-like receptor |
| TNF- α | Tumor necrosis factor-alpha |
| TRAP | Thrombin receptor activating peptide |
| TXA ₂ | Thromboxane A ₂ |
| UAP | Unstable angina pectoris |

1 INTRODUCTION

1.1 General background

Coronary artery disease, the leading cause of morbidity and mortality in Sweden ⁽¹⁾ and worldwide ⁽²⁾ is mainly caused by atherosclerosis, a progressive disease mediated by inflammation of the vessel wall causing endothelial dysfunction ^(3,4). Upon progression of atherosclerosis focal lesions are formed – so called atherosclerotic plaques – which may cause luminal narrowing of the coronary arteries. When these plaques rupture high thrombogenic components from the core of the lesion are exposed to the blood. This leads to activation of platelets and other components of the coagulation system ⁽⁵⁾ resulting in the formation of a thrombus ⁽⁶⁾. When thrombosis occurs in a coronary artery, it may have the clinical presentation of an acute coronary syndrome (ACS).

1.2 Acute coronary syndrome

ACS is the umbrella term of the clinical signs and symptoms of myocardial ischemia. Depending on the location and severity of coronary thrombus formation, the clinical presentation can be ST-segment elevation myocardial infarction (STEMI) or non-ST-segment elevation myocardial infarction (NSTEMI). Although the clinical presentation may differ, both conditions are due to insufficient blood supply causing biochemically detectable cardiac myocyte injury and necrosis. In addition, unstable angina pectoris (UAP) is also included in the definition of ACS, but in contrast to STEMI or NSTEMI, there is no biochemically detectable sign of myocardial injury in UAP.

The criteria of AMI are:

- ❖ Typical symptoms of ischemia, i.e. discomfort, pain or pressure in the chest
- ❖ New ischemia ECG changes (the occurrences of new ST-T changes or new right/left bundle branch block)
- ❖ Elevation of cardiobiomarkers (Troponin I or T), mandatory to establish the diagnosis.

Nowadays, a very common treatment in ACS is percutaneous coronary intervention (PCI) with stent implantation, in combination with dual antiplatelet therapy (DAT) with aspirin and a P2Y₁₂ inhibitor. DAT is needed both in the acute phase (to prevent acute coronary thrombus formation in connection to PCI) and as maintenance treatment to prevent recurrent thrombotic complications and death, especially to prevent so called stent thrombosis (when a thrombus is formed in stented areas of the coronaries).

However, despite DAT, there are some patients who suffer from major adverse cardiac events (MACE) including thrombotic complications within the first year ^(7, 8).

One of the contributing factors for recurrent MACE in patients with prior ACS is platelet hyperactivity, despite ongoing DAT. This seems especially to be a problem among patients treated with the P2Y₁₂ inhibitor clopidogrel ⁽⁹⁻¹¹⁾. Notably, previous studies showed wide variability in platelet inhibiting responses to clopidogrel ⁽¹²⁾, and an association between insufficient responses to clopidogrel as assessed by point of care (POC) assays and increased risk for thrombotic events ^(13, 14). POC assays have therefore been in focus to detect high risk patients with a reduced response to DAT ⁽¹⁵⁾ as this may aid in the improvement in risk stratification, and lead to a more intense medical management and follow-up.

1.3 Platelet function

Platelets, or thrombocytes, are nucleus free “cells” with a diameter of about 2–4 μm. They are derived from megakaryocytes in the bone marrow and circulate when not activated in a discoid form, with a life span of 8–10 days until cleared from the blood by liver and spleen ⁽¹⁶⁾. In a healthy person, the platelet concentration in blood is around 150–400 x 10⁹ “cells”/L.

The main physiological role of platelets is to maintain hemostasis, i.e. to prevent bleeding. However, platelets are also actively involved in other physiological or pathophysiological processes such as inflammation, angiogenesis, infection and cancer ^(17, 18). Apart from involvement in these processes, it is nowadays clear that platelets play a pivotal role in atherothrombosis and ACS.

1.3.1 Platelet activation

In a healthy person, platelet activation usually occurs in response to a vessel wall injury caused by e.g. trauma or a cut. In the ACS patient, however, the rupture of a preexisting atherosclerotic plaque is considered to be the general cause of profound platelet activation. In theory, the platelet activating factors may be similar in physiology and pathophysiology, including von Willebrand factor (vWF) and collagen exposed at the site of vessel damage. Notably, platelet activation is a multistep process involving tethering, adhesion, secretion and aggregation ⁽¹⁹⁾. In parallel, activation of the coagulation system with formation of thrombin – a very strong platelet agonist – takes place ⁽²⁰⁾.

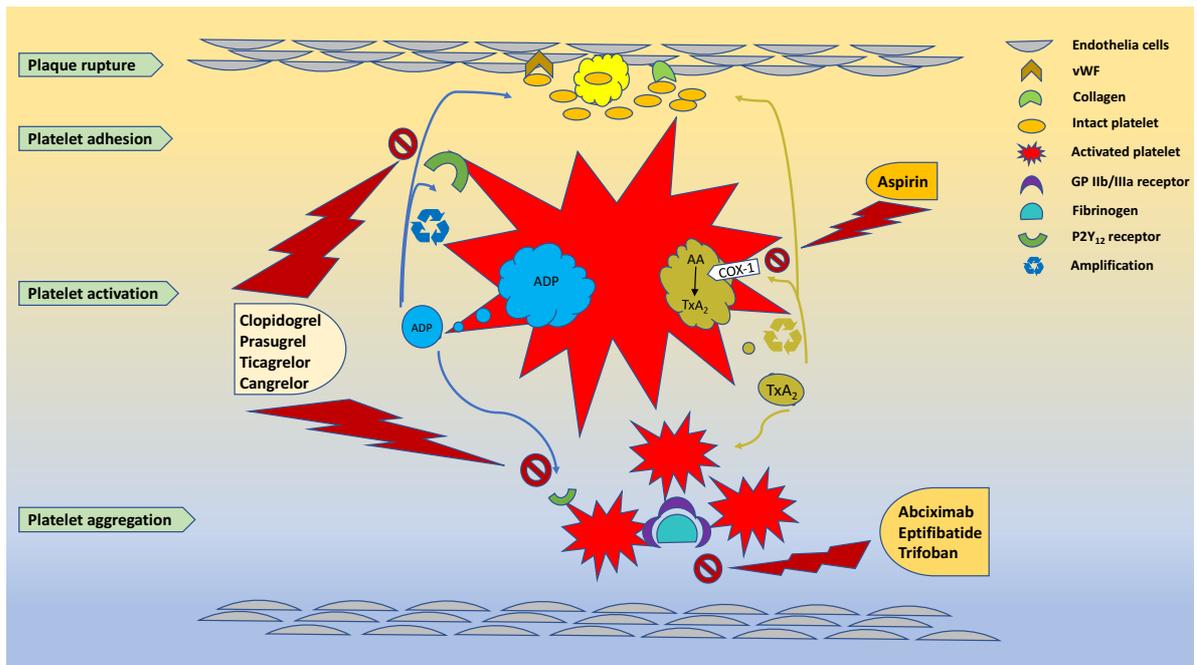


Figure 1: Schematic presentation of platelet activation and the effects of antiplatelet treatment:

Platelets interact with compounds exposed following vessel wall injury, e.g. von Willebrand factor (vWF) and collagen, which result in platelet shape change and activation, and the release of various compounds from the platelets, such as adenosine diphosphate (ADP) and thromboxane A₂ (TXA₂). Following activation, the GPIIb/IIIa receptors cluster and increase their affinity towards fibrinogen. Platelet aggregation then occurs through the binding of fibrinogen to GP IIb/IIIa receptors on platelets, as shown in the figure above. The released platelet agonists further activate already activated platelet (amplification) and also activate other non-activated platelets in the vicinity of the injury. The actions of various antiplatelet drugs are also depicted in the figure above.

The shape of platelets is changed during activation, from being discoid to irregular with protruding pseudopodia. This increases the surface of the platelets and facilitates the aggregation process. The membrane of the activated platelet also provides a surface where coagulation factors can accumulate and be activated, leading to thrombin generation and fibrin formation. Furthermore, the activated platelets release important platelet agonists such as ADP and thromboxane A₂ (TXA₂), as well as other compounds important for hemostasis, like fibrinogen and von Willebrand factor⁽²¹⁾.

In addition, activated platelets release the adhesion molecule P-selectin which is stored in α -granules and presented on the platelet membrane following degranulation. Membrane bound P-selectin may play a role both in the inflammatory and the hemostatic process, as it interacts with leukocytes, endothelial cells and also with other platelets⁽¹⁷⁾. P-selectin molecules may also be shedded from the platelet membrane into plasma as “soluble P-selectin”.

As mentioned above, platelets contribute to inflammation, as they can expose and release various pro-inflammatory molecules including CD40 ligand (CD40L). In fact, platelets have been reported to be a main source of soluble CD40L (sCD40L) in the circulation ⁽²²⁾. Another aspect of platelet activation is the release of microvesicles (MVs) into the circulation. This phenomenon is described in more detail below in section 1.4.

1.3.2 Platelet receptors

Platelets have numerous types of surface receptors including the integrins, which are important adhesion and signaling molecules. Notably, the GPIIb/IIIa receptor (see above) is an integrin and the most abundant platelet receptor, and is important in platelet aggregation. Other types of platelet receptors are the leucine rich repeat receptors of which the GPIb-IX-V-complex is the most abundant one, tetraspanins, selectins (e.g. P-selectin; see above), purinergic receptors (e.g. the P2Y₁₂ receptor), and prostaglandin receptors (including the Thromboxane receptor). For a review of platelet receptors see e.g. ^(23, 24).

1.3.3 Platelet inhibitors

1.3.3.1 Aspirin

Aspirin is without any doubt the most widely used antiplatelet agent. The “beneficial” antiplatelet effect of aspirin was described already in 1950 ⁽²⁵⁾, and the mechanism through which aspirin exerts its antiplatelet effect was shown by John Vane in 1971 ⁽²⁶⁾. Later on, numerous clinical trials have demonstrated the efficacy of aspirin in the treatment of CAD ^(27, 28).

Aspirin (acetylsalicylic acid) acetylates a serine moiety in the COX-1 enzyme. By doing so, the substrate of COX-1, i.e. arachnoid acid (AA), is unable to reach the catalytic site of COX-1 and thereby the synthesis of thromboxane A₂ is blocked, figure 1. Aspirin’s blockage of COX-1 is irreversible ⁽²⁶⁾, meaning that the thromboxane production of the platelet is inhibited for the life span of the platelet. The inhibition of COX-1 in vivo occurs already at low doses, around 30 mg daily ⁽²⁹⁾. However, present European guidelines recommend a maintenance dose of 75–100 mg daily for patients with CAD ⁽³⁰⁾.

Treatment failure seems to occur not only with clopidogrel (see below), but also with aspirin. The phenomenon “aspirin resistance” was reported by Helgason already in the 1990s ⁽³¹⁾, and there have since then been numerous reports on this phenomenon, with a wide estimated prevalence between 1–60% ^(32, 33). Indeed, aspirin resistance has been reported to be associated with an increased risk of cardiovascular complications ⁽³⁴⁾, but this observation has not been the focus of the present thesis, and is therefore not discussed in more depth here.

1.3.3.2 P2Y₁₂ receptor inhibitors

I. Ticlopidine

Ticlopidine is the first generation of the thienopyridines, i.e. a class of oral drugs which irreversibly blocks the platelet P2Y₁₂ receptor. Ticlopidine was introduced in France already around 1980 for the treatment and prevention of arterial thrombosis in coronary, cerebral and peripheral arteries. Due its severe side effects, i.e. bone marrow toxicity (leucopenia, thrombocytopenia, and pancytopenia), but also a bothersome diarrhea, newer thienopyridines have been developed^(35, 36).

II. Clopidogrel

Clopidogrel is a second generation thienopyridine, figure 1, and was introduced in the clinic after the large CAPRIE trial published in 1996⁽³⁷⁾. This drug is still one of the most used antiplatelet drugs for patients with CAD⁽³⁸⁻⁴⁰⁾ and ischemic stroke⁽⁴¹⁾.

Clopidogrel is, like the other thienopyridines, a pro-drug which as such requires hepatic metabolism to generate active metabolites. The cytochrome P450 (CYP 450) enzyme system (mainly CYP2C19) metabolizes clopidogrel in a two-step process to generate a short-lived active metabolite which irreversibly bind to and block the P2Y₁₂ receptor. Of the ingested dose only around 15% is metabolized to the active metabolite and the rest is excreted by kidneys as inactive metabolites, figure 2^(36, 42).

The maximal inhibition of platelet aggregation is obtained within 2–6 h after clopidogrel administration (loading dose of 600 mg clopidogrel), and its offset of action, which coincides with the generation of new platelets, is between 5–10 days^(38, 43).

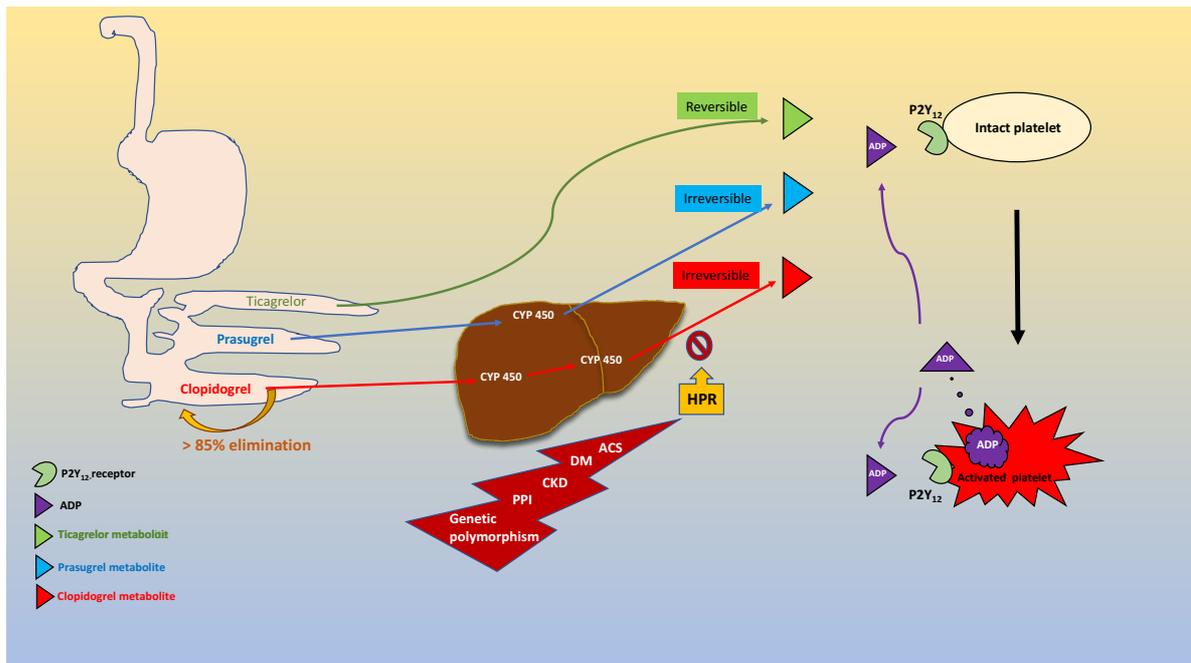


Figure 2: Schematic presentation of the pharmacology of oral P2Y₁₂ inhibitors.

Ticagrelor is a direct-acting agent with reversible antiplatelet effects. In contrast, clopidogrel and prasugrel are irreversible P2Y₁₂ inhibitors, and pro-drugs which are dependent on metabolization by the hepatic cytochrome P450 (CYP 450) system to generate active metabolites. This occurs in two steps for clopidogrel, and in one step for prasugrel (see above). The generation of active metabolites is less efficient for clopidogrel as compared to prasugrel (only 15% of the absorbed clopidogrel dose is converted to active metabolite). High-on-treatment platelet reactivity (HPR) for clopidogrel, which is considerably more common in clopidogrel than prasugrel or ticagrelor, is associated with several “clinical factors” such as acute coronary syndrome (ACS), diabetes mellitus (DM), chronic kidney disease (CKD), drug-drug interactions, and also genetic polymorphism of the hepatic CYP450 system.

The protective effects of clopidogrel to cardiovascular complications have been shown in several large prospective CAD studies^(37, 44, 45), but according to the literature approximately 20–30% of patients on clopidogrel show high on-treatment platelet reactivity (HPR)⁽⁴⁶⁾. There are several clinical factors known to be associated with clopidogrel HPR, such as genetic variation (CYP polymorphisms), drug-drug interactions (e.g. with proton pump inhibitors or statins), ACS, DM, CKD and obesity, figure⁽⁴⁷⁻⁵⁰⁾. Notably, there is a strong association between the presence of HPR and development of MACE^(13, 14, 51), and especially the risk for stent thrombosis in patients undergoing PCI^(9, 52). Therefore, new and more effective antiplatelet agents (like prasugrel and ticagrelor) have been developed.

III. Prasugrel

Prasugrel is the third generation of thienopyridines, figure 1, and was introduced in the clinic after the large TRITON study published in 2007⁽⁵³⁾. Prasugrel was shown to be more effective in reducing the risk for recurrent thrombotic events compared to clopidogrel, but at the cost of increased risk of bothersome bleeding complications⁽⁵³⁾. As depicted in figure 2, prasugrel is dependent on the CYP 450 enzyme to form an active metabolite, and this occurs in a single-step oxidation process. Importantly, prasugrel has a faster onset of action (30 min- 4 h)^(38, 43), and provide a stronger inhibition of ADP-induced platelet aggregation compared to clopidogrel, which likely explains the beneficial data on cardiovascular events observed in the TRITON study^(53, 54).

IV. Ticagrelor:

Ticagrelor is an oral non-thienopyridine reversible P2Y₁₂ antagonist, figure 1. Unlike clopidogrel and prasugrel, it is not a pro-drug and therefore it does not require metabolic activation. Ticagrelor has a fast onset of action (around 30 min-4 h), and it is important to realize that the degree of inhibition afforded by the drug is dependent on its actual plasma concentration, figure 2. The half-life of ticagrelor is 8-12 hours, and the offset of the drug is faster than for the thienopyridines (3–5 days until eliminated)⁽⁴⁶⁾. The pivotal PLATO, published in 2009, clearly showed reduced MACE in patients with ACS patients treated with ticagrelor compared to clopidogrel⁽⁵⁵⁾.

As expected (being a more potent platelet inhibitor than clopidogrel), ticagrelor treatment was associated with a higher incidence of non- coronary artery bypass grafting (CABG)-related bleedings⁽⁵⁶⁾.

V. Cangrelor

Cangrelor is a short-acting platelet P2Y₁₂ inhibitor developed for intravenous use in acute situations. Inhibition of ADP-induced platelet aggregation is observed minutes after start of drug infusion. The plasma half-life of the drug is very short (3–6 minutes), and the platelet response to ADP is completely restored in less than one hour after termination of the infusion. Because of its pharmacology, with a very rapid onset and offset, the drug is an attractive treatment in connection to PCI, when immediate inhibition of platelet activation is needed. In clinical trials the periprocedural use of cangrelor in the PCI setting was associated with improved clinical outcome⁽⁵⁷⁾.

1.3.3.3 Glycoprotein IIb/IIIa receptor antagonists

The glycoprotein IIb/IIIa (GP IIb/IIIa) receptor mediates platelet aggregation through the binding of fibrinogen, figure 1. The GP IIb/IIIa inhibitors (GPIs), abciximab, eptifibatid and tirofiban, are very potent platelet inhibiting drugs, and developed for intravenous use only⁽⁵⁸⁾. GPIs are used in some high-risk PCI patients despite rather weak available evidence, e.g. in patients with angiographic evidence of large thrombus burden, and in those with thrombo-embolic complications during PCI, e.g. slow flow and no reflow after stenting^(59, 60). Notably, the GPIs are associated with a high risk of bleeding, and combining GPIs with novel P2Y₁₂ inhibitors (prasugrel and ticagrelor) does not result in any benefit in the outcomes of patients with ACS⁽⁶¹⁾.

1.3.4 Measurement of platelet function

The interest in assessing platelet function has increased considerably during recent years. Clinical settings where measurements of platelet function are of potential value include e.g. monitoring of platelet responses to DAT, detection of bleeding disorders, and in the evaluation of platelet function pre-, per-, and postoperatively.

Some of the more commonly used platelet function methods are:

1.3.4.1 Light Transmission Aggregometry (LTA)

LTA was originally described by Gustav Born in 1962⁽⁶²⁾, and is still, by many, considered to be the gold standard platelet function test. Platelet rich plasma (PRP) is used in this test, and after adding a platelet agonist, e.g. ADP, into a cuvette with PRP, the platelets begin to aggregate and the light transmission through the plasma increases as platelets aggregate. A photocell in the aggregometer detects the change in light transmission which is proportional to platelet aggregation.

LTA has some disadvantages, such as the need for centrifugation of the blood sample (to obtain PRP) and poor standardization between laboratories. The method is not optimal as a POC test⁽⁶³⁾.

1.3.4.2 VerifyNow

VerifyNow is an automated POC test that measures platelet aggregation in anticoagulated whole blood. The test is carried out using commercially available cartridges with different agonists, like AA, ADP and TRAP. Briefly, platelets are stimulated by an agonist, and upon activation platelets bind to fibrinogen-coated beads in the cartridges which in turn leads to an increase in light transmittance. Data are registered as platelet reactivity units (PRU)⁽⁶⁴⁾. VerifyNow has been widely used to monitor platelet responses to DAT⁽⁶⁵⁾.

1.3.4.3 Multiplate

Multiplate⁽⁶⁶⁾ is the POC test used in the present thesis. It is a whole blood impedance aggregometry device⁽⁶⁷⁾, which has been commercially refined, and equipped with two pairs of electrodes per test channel; a principle by the developer called multiple electrode aggregometry⁽⁶⁶⁾. This construction provides improved quality control for each sample, figures 3 and 4. Indeed, the main weakness of the older versions of whole blood impedance aggregometers was the single pair of electrodes used (and reused) and their “between-electrode” variability.

The MEA device has five channels for parallel tests, figures 3 and 4, and can detect patients with HPR on DAT following PCI⁽⁹⁾.



Figure 3: Multiplate[®] analyzer (left), and its test cell with duplicate sensor electrodes and magnetic stirrer (right).

The test measures the change in impedance between two electrodes when activated platelets adhere and aggregate to the surface of the electrodes. The change in impedance is registered as two aggregation curves per channel on the screen of the apparatus; platelet aggregation is expressed as the area under the curve (AUC), figure 4⁽⁶⁶⁾. Adapted with permission of Roche Diagnostics Scandinavia AB.

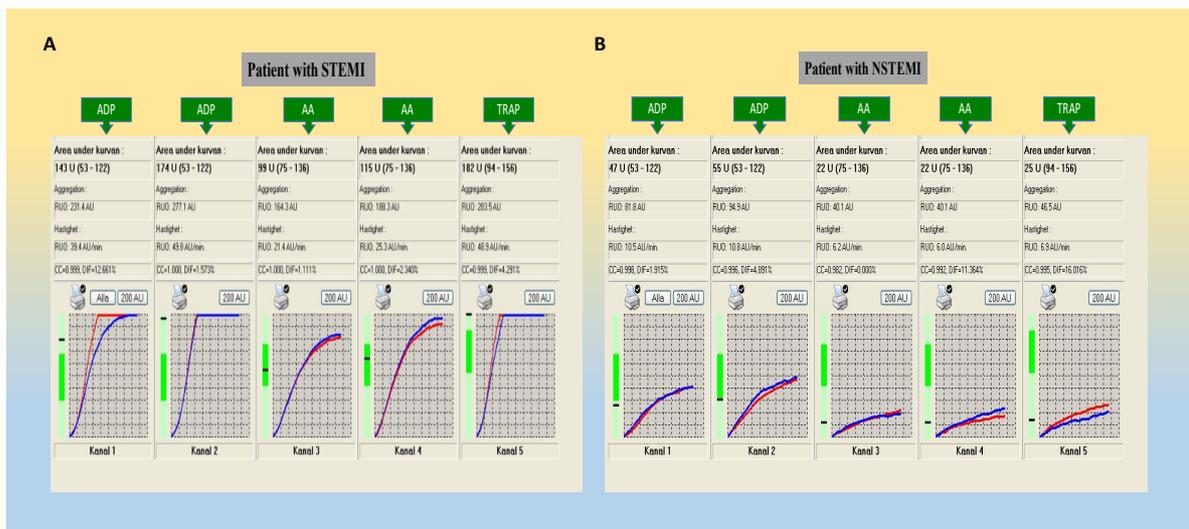


Figure 4: Platelet aggregation curves obtained by multiple electrode aggregometry (MEA). High platelet response to ADP, AA and TRAP in a patient with STEMI (panel A). Antiplatelet effects afforded by DAT in a patient with NSTEMI (panel B).

1.3.4.4 Flow cytometry

Flow cytometry (FCM) is a rapid method for assessment of platelet function. Briefly, cells or vesicles are labeled with fluorescently labeled antibodies against desired proteins or markers. In the flow cytometer, cells pass through a flow chamber with a laser beam where the size (forward scatter) and complexity or granularity (side scatter) is registered by detectors. Moreover, the emitted fluorescence (i.e. positivity for labeled antibodies) is also detected by specific fluorescence detectors.⁽⁶⁸⁾ Platelet activation measurements by FCM can be performed in whole blood, platelet rich plasma or in washed platelets⁽⁶⁹⁾.

FCM has been used for diagnosis of bleedings disorders, for assessment of platelet function⁽⁶⁹⁾, and also for monitoring of DAT⁽⁷⁰⁾. FCM is also used for measurement of MVs⁽⁷¹⁻⁷³⁾, which is described in more detail below, in section 1.4.

1.4 Microvesicles

Microvesicles (MVs) are plasma membrane vesicles, ranging from 0.1 to 1.0 μm in diameter, released from cells in response to cellular activation or apoptosis, figure 5⁽⁷⁴⁾. The phenomenon of MV formation was initially described in platelet preparations as “platelet dust” already in 1967⁽⁷⁵⁾, but later on it has become clear that many cell types can release MVs including megakaryocytes, endothelial cells, erythrocytes and leukocytes. MVs are derived from the plasma membrane which under “resting conditions” consists of two layers of phospholipids. The inner layer is negatively charged as it harbours the negatively charged phospholipid phosphatidylserine (PS), figure 5. The normal structure and integrity of the cell membrane is maintained through activities of different enzymes in the membrane (including flippase, floppase and scramblase)⁽⁷⁶⁾. However, during cell activation, intracellular calcium levels are increased, and as a consequence of complex intracellular events blebs are formed from the cell membrane. In parallel with this, the activities of flippase, floppase and scramblase are influenced, and upon “blebbing” PS is “pushed” to the outer layer of the MVs formed and released from the cell. It has, however, become clear during recent years that not all MVs expose PS, as described in several studies, figure 5⁽⁷⁷⁻⁸⁰⁾.

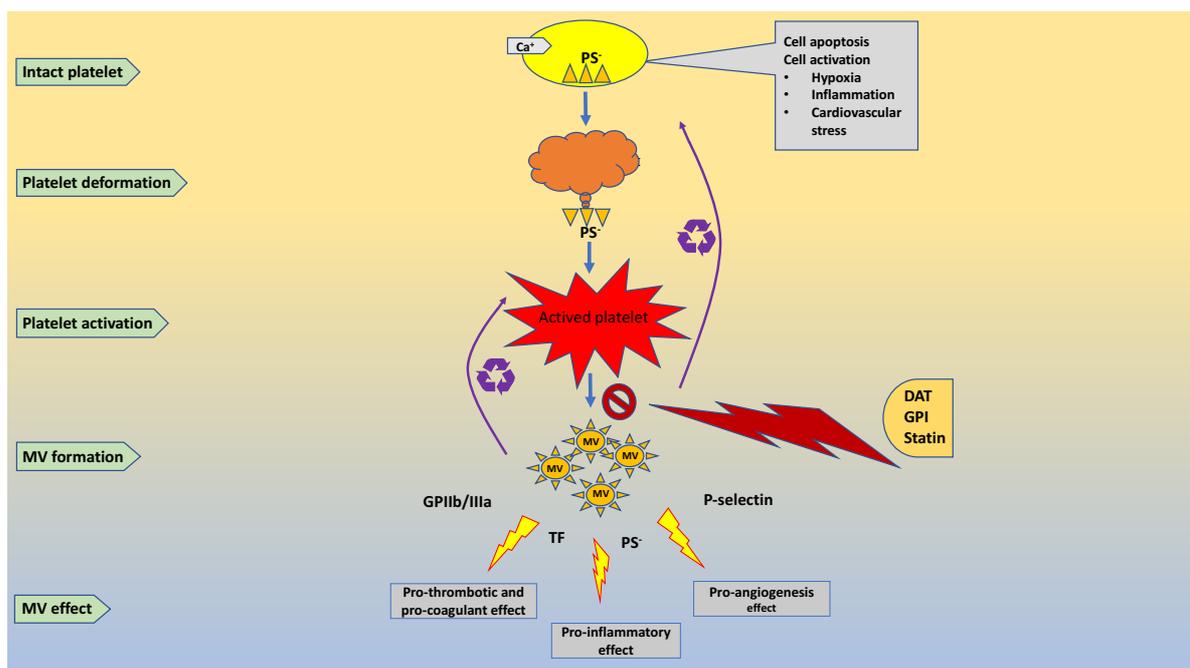


Figure 5: Schematic presentation of platelet microvesicle formation and release.

Following platelet activation intracellular levels of calcium increase, and after a series of complex intracellular events MVs are formed and released from the platelet cell membrane. Beside exposing PS, platelet MVs may also expose e.g. tissue factor, P-selectin, and platelet specific receptors like GPIIb/IIIa, glycoprotein IX (CD42a) and glycoprotein Ib (CD42b).

Platelet MVs are reported as the most abundant MVs in the circulation; around 70–90% of all MVs in healthy subjects⁽⁸¹⁾. MVs release may, according to the literature, be triggered by different conditions such as hypoxia,⁽⁸²⁾ cardiovascular stress⁽⁸³⁾, high fat consumption⁽⁸⁴⁾ and inflammation⁽⁸⁵⁾. Circulating MVs are thought to be removed from the circulation in the reticuloendothelial system (e.g. in the liver, spleen and lungs). The clearance rate in experimental studies in rodents varies between 5–50 min, and in exercising healthy humans MVs have been reported to be cleared within one hour after termination of exercise⁽⁸⁶⁾.

The exposure of PS on the membrane of MVs may enhance its elimination. Formation of MVs may be reduced by treatment with different drugs; such as P2Y₁₂ receptor antagonists, GPIIb/IIIa inhibitors (GPIs) and statins^(87, 88).

The composition of the MV membrane, including lipids and proteins, is mainly determined by the parental cells, i.e. the cell from which the MV originated. Of note, MV may pick up and carry various molecules including different cytokines, viruses, micro RNAs, DNA and mRNA⁽⁷⁴⁾. In this way, MVs have been ascribed to be important “vectors” of pathophysiological processes like infections, atherosclerosis and cancer. The role of MVs in thrombotic disorders have been in focus of several investigations, and studies have suggested that the pro-coagulant effect of MVs may be due to exposure of PS and other pro-coagulant factors including TF^(77, 89). Of note, MVs from activated platelet may be 50–100 fold more pro-coagulant than the surface of the platelets themselves⁽⁸⁸⁾.

Several studies have reported higher levels of PMVs in patients with ACS, especially in patients with STEMI, and compared to patients with stable CAD or healthy controls⁽⁸⁹⁻⁹¹⁾. Previous reports have also suggested that MVs could be used as biomarkers to aid in the detection of high-risk individuals, or to be used in the assessment of antithrombotic therapies^(92, 93).

1.5 Pentraxin-3

The view of the human immune system is that it consists of two different systems. The first one is the innate immune system, which is a quickly responding non-specific “first line” system (minutes – hours), and the second one is the adaptive immune system. This second system is antigen-specific, and responds to stimuli within days. This may lead to the development of an “immunologic memory” of the stimuli that elicited the response⁽⁹⁴⁾. Pentraxin-3 (PTX3) is a molecule belonging to a superfamily of soluble proteins – the pentraxins – and is a part of the humoral arm of the innate immune system^(95, 96).

The perhaps most “famous” of the pentraxins is C-reactive protein (CRP), an acute phase protein which has been in focus of intense research during the last two decades^(97, 98). Whereas CRP belongs to the “short pentraxins” (molecular weight around 25 kDa), PTX3 is a “long pentraxin” with a molecular weight of 45 kDa^(96, 99).

PTX3 was first identified in endothelial cells⁽¹⁰⁰⁾, but available data show that PTX3 is produced in many different cell types like neutrophils, macrophages, dendritic cells, fibroblasts and smooth muscle cells, and even cardiomyocytes seem to be able to produce PTX3^(100, 101). The production of PTX3 can be induced by different stimuli including cytokines like TNF- α , IL-1, IL-6, and the bacterial lipopolysaccharide, and perhaps also by cholesterol, figure 6⁽¹⁰²⁾. Preformed PTX3 may be stored in granules of neutrophils and released acutely in various disease states^(103, 104).

PTX3 is a multifunctional protein and when released into the circulation it can bind to different ligands such as C1q and C3 in the complement system, but also to diverse pathogens such as bacteria, viruses and fungi, and thereby promote phagocytosis^(94, 105). Notably, PTX3 may be both pro- and anti-inflammatory depending on the situation and local of action⁽⁹⁹⁾. For example, it has been shown that PTX3 can bind to “and block” the actions of P-selectin, and thereby potentially reduce the recruitment of leukocytes into areas of inflammation⁽¹⁰⁶⁾.

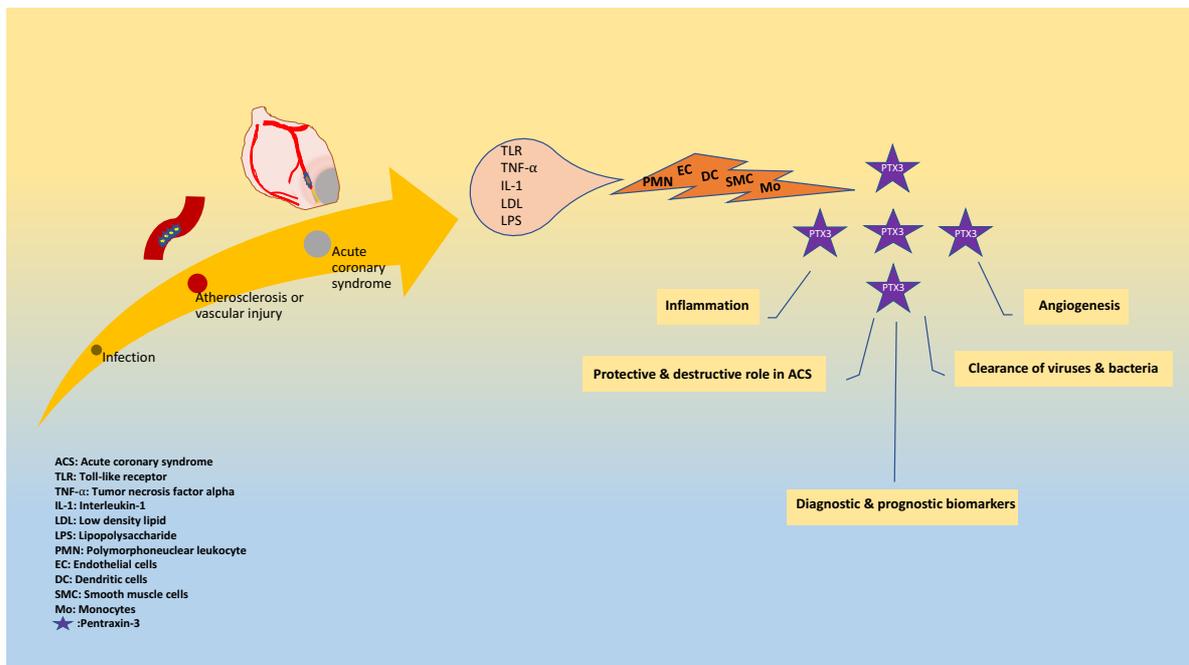


Figure 6: Schematic presentation of PTX3 production and effects.

PTX3 can be produced in and released from many cells including neutrophils, monocytes, endothelial cells, dendritic cells and smooth muscle cells in response to various stimuli. The molecule has multifunctional properties and may be involved in various processes like inflammation, atherosclerosis and angiogenesis.

PTX3 expression has been reported to be elevated in coronary plaques from patients with UAP compared to plaques from patients with stable disease^(107, 108). The plasma concentration of circulating PTX3 peaks 6–8 h from onset symptoms onset in AMI patients^(101, 109, 110) (in some studies the plasma concentration increases from 1–2 ng/mL to 200–800 ng/mL)⁽⁹⁴⁾. Interestingly, an experimental study suggested that PXT3 may have a protective role in acute myocardial infarction through the regulation of the complement cascade⁽¹¹¹⁾.

PTX3 has been suggested as a useful biomarker in the detection of ACS and has been postulated to be superior to CRP and troponin T⁽¹¹²⁾. Furthermore, PTX3 has been reported to be positively associated with Killip class and negatively associated with left ventricular ejection fraction⁽¹¹³⁾, and to be a predictor of 3-month mortality following AMI⁽¹¹⁰⁾.

2 AIMS

The overall aim of the present thesis was to assess platelet function in patients with ACS treated with clopidogrel, and its relation to markers of high risk for cardiovascular events such as inflammation and levels of circulating MVs. The specific aims were as follows:

STUDY I

To examine whether arterial and venous blood samples from patients with ACS, were comparable regarding platelet responsiveness to DAT by using MEA.

STUDY II

To investigate the possible association between platelet responsiveness to clopidogrel treatment and levels of circulating MVs and PMVs.

STUDY III

To measure the effect of circulating MVs obtained from patients with ACS on platelet aggregation, and the influence of the responsiveness to clopidogrel treatment

STUDY IV

To study the exposure of PTX3 on circulating MVs in patients with AMI.

3 MATERIAL AND METHODS

3.1 Study population

3.1.1 Paper I

We compared the arterial to the venous blood samples for assessment of platelet aggregation with whole blood impedance aggregometry in 28 patients (5 females and 23 males) with CAD. The patients were included between December 2009 and June 2010. ACS was defined as chest pain with a ST-T segment dynamic on ECG indicating an ischemic event or /and an increased level of cardiac biomarker (troponin I). The patients were pre-treated with oral loading of 500 mg aspirin and 600 mg clopidogrel unless they were on on-going continuous medication with these drugs. Coronary angiography was performed in all patients and 23 of them had PCI with stent implantation.

3.1.2 Paper II

We investigated the association between platelet reactivity and circulating MVs and PMVs in patients with ACS by including 200 consecutive patients with ACS between March 2009 and February 2011. Nine patients were excluded due to their participation in other studies after the inclusion, 2 patients died before discharge and 6 patients with 3-vessel disease were accepted for CABG. The patients were pre-treated with oral loading of DAT as mentioned in study I. Coronary angiography was performed in all patients and the majority (n=166) had PCI with stent implantation.

3.1.3 Paper III

In this study, we assessed MVs-induced platelet activation in 30 patients with ACS, who were already included in study II. All the patients were pre-treated as mentioned in study I. All patients went through coronary angiography and PCI was performed in 25 out of them. Patients were divided in two groups based on antiplatelet effect of clopidogrel measured by MEA, i.e. those with high on-treatment platelet reactivity, HPR (AUC > 46 U; n=16) and those with low or normal on-treatment platelet reactivity, Non-HPR (AUC ≤ 46; n=14). Fresh isolated platelets were obtained from healthy volunteers (n=2) without any medication.

3.1.4 Paper IV

In this study, we investigated the exposure of PTX3 on MVs in 179 patients with AMI. Patients were divided into two groups, acute STEMI group and discharge group. The STEMI group, with blood samples obtained in the acute phase, consisted of 23 patients and were included between June and December 2016. These patients were pre-treated with oral loading dose of 500 mg aspirin and 180 mg ticagrelor unless they were on on-going continuous medication with these drugs. The discharge group, with blood samples collected at discharge (3–5 days after admission), consisted of 57 STEMI patients and 99 patients with NSTEMI from the population in study II.

Plasma samples were collected from 15 healthy volunteers with no history of cardiovascular or others chronic diseases were included as a control group.

3.2 Blood collection

3.2.1 Study I

Both arterial and venous blood were obtained at the same time. For MEA analysis venous blood sample was collected in a 4.5-mL plastic vacutainer tube containing the direct thrombin inhibitor lepirudin (25 µg/mL, Recludan, Hirudin blood collection tubes), using 21-gauge needles. For arterial blood sample, after puncturing the radial (majority) or femoral artery and insertion of a 6-French arterial sheath, the arterial blood was collected through a plastic syringe and then gently filled into uncapped vacutainer tubes, as mentioned above.

3.2.2 Study II

Blood samples were obtained at discharge (3–5 days after admission), or roughly two weeks after admission in those patients receiving Abciximab (n=57) during PCI. For MEA assessment the blood samples were obtained as described in study I. Blood samples for PMV measurement were collected into 5 mL vacutainer tubes containing 0.5 mL 0.129 M sodium citrate (Becton Dickinson [BD], Plymouth, UK) using 21-gauge needles (BD Vacutainer needles). The samples were then immediately centrifuged at 2000g for 20 minutes at room temperature (RT) to obtain platelet-poor plasma (PPP). Aliquots of 500 µL PPP were later dispensed into plastic tubes and frozen at -80 °C until analysis.

3.2.3 Study III

Venous blood was obtained at discharge (3–5 days after admission) into 5 mL vacutainer tubes containing 0.5 mL 0.129 M sodium citrate. All the samples were immediately centrifuged at 2000g for 20 minutes at RT to obtain PPP. Aliquots of 500 μ L PPP were later dispensed into plastic tubes and frozen at -80 °C until analysis.

Platelet isolation:

Fresh platelets were obtained from two healthy volunteers with no history of cardiovascular disease and with no medication. Venous blood was obtained into 5 mL vacutainer tubes containing 0.5 mL 0.129 M sodium citrate. After careful mixing, the samples were centrifuged at 190g for 10 minutes in RT, to obtain PRP. Roughly 2/3 of the PRP was collected for further analysis. To obtain platelet-free plasma (PFP), the remaining plasma was centrifuged once at 2000g for 20 min, in RT. The supernatant was collected and further centrifuged at 10 000g for 30 min, in RT.

MP isolation:

PPP was thawed in a water bath for approximately 5 minutes (37 °C) and then centrifuged at 2000g for 20 min at RT, in order to remove any debris or cells that may interfere with the analysis. The supernatant was then centrifuged at 20 800g for 45 minutes in RT. After centrifugation, the supernatant was discarded and equal amount of phosphate buffer solution was added to the pellet. The sample was again centrifuged at 20 800g for 45 minutes in RT. Again, the supernatant was discarded and the remaining pellet was vortexed for roughly 30 seconds and later used in the analysis.

3.2.4 Study IV

Blood samples in the acute STEMI group were collected in the catheterization laboratory before primary PCI. After puncturing the radial artery blood was obtained from the arterial sheath inserted for angiography, and before contrast injection. Blood was drawn in a syringe and then immediately transferred into vacutainer tubes containing 0.129 M sodium citrate (Becton Dickinson [BD], Plymouth, UK) using 21-gauge needles (BD Vacutainer needles). In addition, samples were collected from an antecubital vein around 23 hours after PCI using 21-gauge needles (BD Vacutainer needles) and vacutainer tubes containing 0.129 M sodium citrate.

In the discharge group, blood samples were collected as described earlier in study II.

3.3 Methods

3.3.1 Multiple electrode aggregometry

Details of this method have been described above and previously⁽⁶⁶⁾ Briefly, after at least 30 minutes of incubation at RT, whole blood was diluted with NaCl solution (1:1) and incubated again in 37°C for 3 minutes. Platelet activation was induced by adding ADP (final conc. 6.4 µM) or AA (final conc. 0.5 mM) and aggregation was measured for 6 minutes in duplicate (i.e. two channels for each agonist). Results are expressed as area under the curve (AUC: 1U = 10 AU*min). Cut-off values for MEA data presented herein were defined as an AA value of ≥ 30 U as aspirin non-responders⁽¹¹⁴⁾ and an ADP value of more than 46 U for HPR and 46 U or less for NPR to clopidogrel, as established by ROC analyses in other studies^(115, 116). To assess the patients with a higher risk of bleeding we used cut-off values defined as an ADP value less than 19 U for low on-treatment platelet reactivity (LPR), and between 19 U to 46 U for NPR^(14, 117).

3.3.2 Flow cytometric measurement of MVs and PMVs

The measurement of MVs and PMVs was performed by flow cytometry. Briefly, PPP was thawed and later centrifuged initially at 2000g for 20 minutes and re-centrifuged at 13 000g for 2 minutes at RT. Subsequently, the supernatant, which includes MVs, were incubated for 20 minutes in the dark with specific antibodies. In study II, MVs were labelled with phalloidin-Alexa 660 (Invitrogen, Paisley, UK), lactadherin-FITC (Haematologic Technologies, Vermont, USA) and CD42a-PE (GPIX, Beckman Coulter, Brea, CA, USA). In addition, CD154-APC (abcam, Cambridge, UK) and CD62P-APC (Beckman Coulter, Brea, CA, USA) were measured in the PMV population (PS+ CD42a). In study IV, MVs were labelled with anti-PTX3-PE (Novus Biologicals, LLC, Littelton, CO, USA).

The MV gate was determined using Megamix beads (0.5, 0.9 and 3.0 µm beads, BioCytex, Marseille, France). MVs were defined as particles less than 1.0 µm in size, and positive for antibodies as described above. Conjugate isotype-matched immunoglobulin with no reactivity against human antigens was used as a negative control to define the background noise of the cytometric analysis.

3.3.3 Assessment of PTX3 concentration by ELISA

Plasma samples from 13 patients with acute STEMI and 15 healthy volunteers, were separated into 3 fractions; plasma, MV-free supernatant and MV-enriched pellet by high-speed centrifugation as described in section 3.2.3. Total concentrations of PTX3 were measured in each fraction by a commercially available ELISA kit (R&D Systems, McKinley Place NE, Minneapolis, USA) according to the manufacturer's instruction.

3.3.4 Assessment of platelet aggregation in 96-well microplate

Assessment of platelet aggregation by using a 96-well microplate is earlier described elsewhere by Armstrong et al. ⁽¹¹⁸⁾. In study III, this method was modified in order to use MVs as agonists. Briefly, 100 μ l PRP from healthy individuals, was added to the wells of a 96-well plates (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), in triplicates. MV-enriched pellet (50 μ L) from patients, was then added to the wells, in triplicates as well. As a positive control, 10 μ M of ADP (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was added in wells containing PRP (duplicates). Moreover, PRP and PFP without agonists, was used as negative control (0% aggregation) and positive control (100% aggregation). Plates were then placed in a 96-well plate reader (Tecan, Sunrise®, Zurich, Switzerland) and absorbance determined at 595nm every 15s (with shaking) for 60 min at 37°C. Changes in absorbance were converted to % aggregation by reference to the mean absorbance of PRP and PFP in both healthy volunteers. Both peak aggregation (max % aggregation) and aggregation over time (area under the curve; AUC) was assessed as seen in, figure 7.

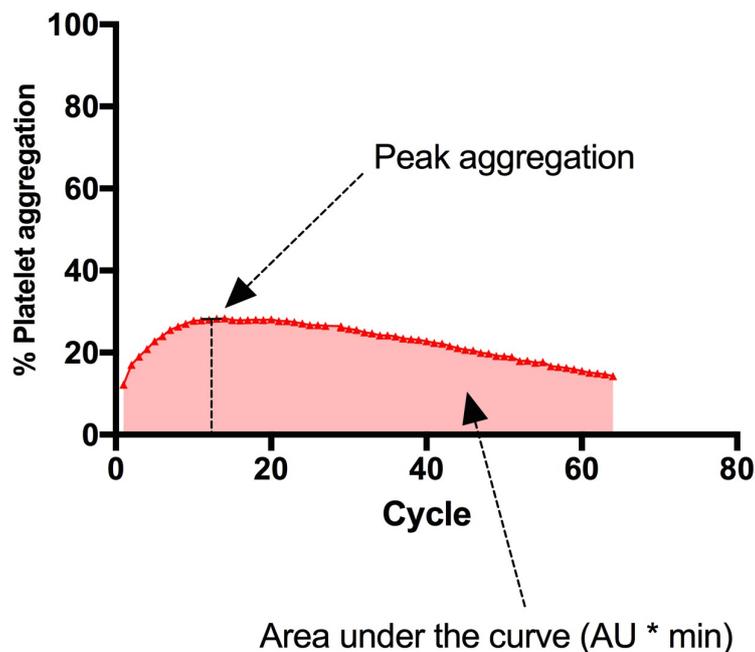


Figure 7: Microvesicle induced platelet aggregation.

Changes in absorbance were converted to percent aggregation by reference to the absorbance of platelet rich plasma (PRP) and platelet free plasma (PEP). Data presented as both peak aggregation and aggregation over time (area under the curve, AUC).

3.4 Statistical analyses

Statistical analyses were performed using SPSS software, version 22, 23 and 25 (IBM, Armonk, NY). To assess the normal distribution of the data, both visually histogram and also the Shapiro-Wilks test were used. Continuous variables are expressed as mean \pm SD for normally distributed data, and median values with lower, upper quartiles for non-normal distributed data. Comparisons of continuous variables within groups were performed by independent t-tests for normally distributed data and for non-normally distributed variables Mann-Whitney *U*-test was used. Pearson's or Spearman's correlation coefficients were used, depending on normally and non-normally distributed data, for estimation of correlations between variables. For all statistical tests and confidence intervals, two-sided tests were used and a p-value of <0.05 was considered statistically significant.

4 RESULTS

4.1 Study I

Platelet aggregation in both arterial and venous blood samples was measured by MEA and was expressed as the AUC. The result showed a significant correlation between platelet aggregation in both arterial and venous blood by use of AA and ADP-induced aggregation. The correlation coefficients (R^2) were 0.96 ($p < 0.0001$) and 0.95 ($p < 0.0001$) respectively, figure 8.

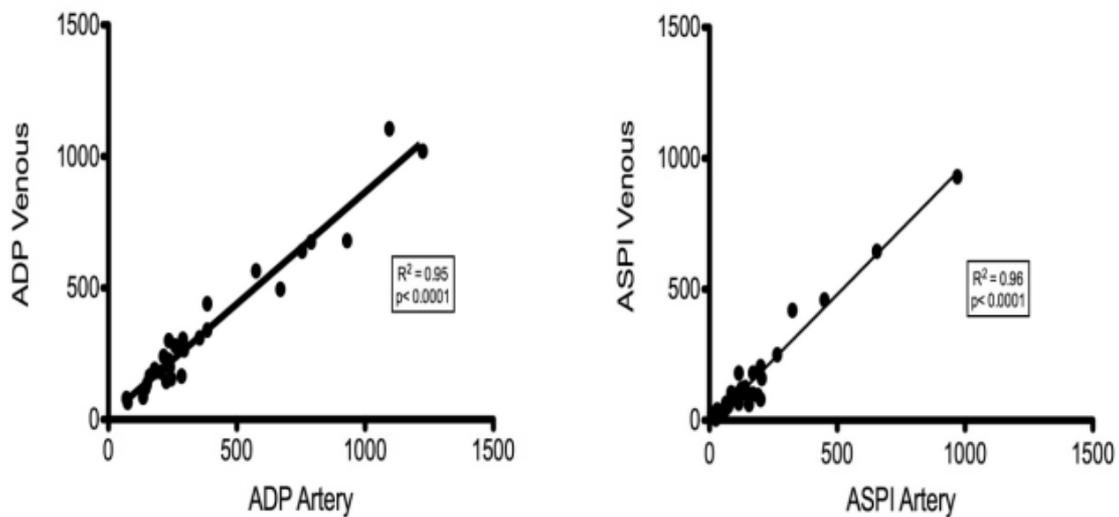


Figure 8: Correlations between platelet aggregation measured as the area under the curve (AUC) in samples collected from arterial (x-axis) and venous blood samples (y-axis).

The left panel (A) shows adenosine diphosphate (ADP) induced aggregation ($n=28$) and the right panel (B) shows arachidonic acid (AA) induced aggregation ($n=28$). Correlation coefficients (Spearman's rank correlation coefficient) and p-values are shown. Adapted with permission from Scandinavian Journal of Clinical and Laboratory Investigation.⁽¹¹⁹⁾

We also demonstrated that platelet aggregation, expressed as AUC, for both AA- and ADP-induced aggregations, were similar in arterial and venous blood. Patients were divided according to established cut-off values⁽¹¹⁷⁾ in LPR (≤ 188 AUC), NPR (189–467 AUC) and HPR (≥ 468 AUC) based on the response to clopidogrel treatment. Cut-off values for AA-response were defined as normal responders (< 300 AUC) and low responders (≥ 300 AUC) based on the response to aspirin treatment, figure 9⁽¹¹⁴⁾.

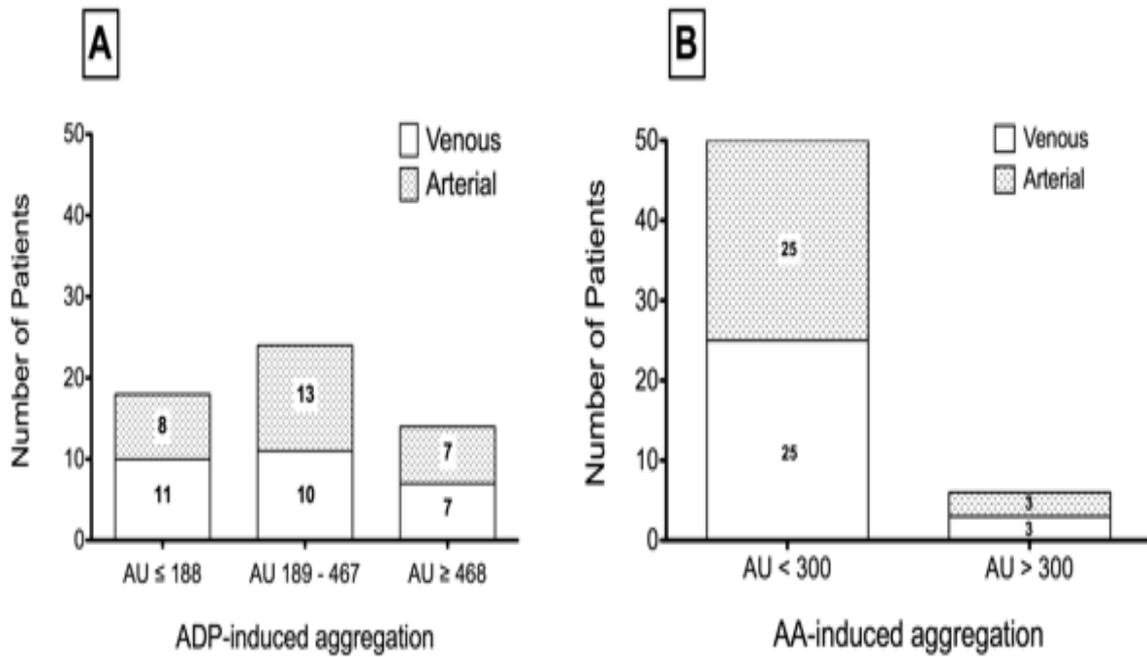


Figure 9: The left panel (A) shows the number of patients defined as low on-treatment platelet reactivity (≤ 188 AUC), (agreement 92%, kappa 0.83), normal on-treatment platelet reactivity (189-467 AUC), (agreement 92%, kappa 0.85), and high on-treatment platelet reactivity (≥ 468 AUC) to clopidogrel in arterial and venous blood samples. The right panel (B) shows the number of patients defined as normal responders (< 300 AUC) and low responders (≥ 300 AUC) to arachidonic acid (AA) induced aggregation. Adapted with permission from Scandinavian Journal of Clinical and Laboratory Investigation.⁽¹¹⁹⁾

4.2 Study II

4.2.1 Multiple electrode aggregometry

ADP-induced platelet aggregation was analyzed, by MEA, from available blood samples in 183 patients with ACS, treated with DAT (aspirin and clopidogrel). The data regarding ADP-response to clopidogrel were positively skewed. The median value of ADP-induced platelet aggregation at discharge (3–5 days after admission) was 22 U [3,5:103,5]. The results showed that 36 (20%) of these patients were with HPR, figure 10.

We also assessed the antiplatelet effect of clopidogrel treatment on MVs in 154 patients. Using the cut-off values suggested by Sibbing et al.⁽¹¹⁷⁾ 28 patients (18%) of these patients showed to be with HPR, 57 patients (37%) with NPR and 69 patients (45%) with LPR to clopidogrel.

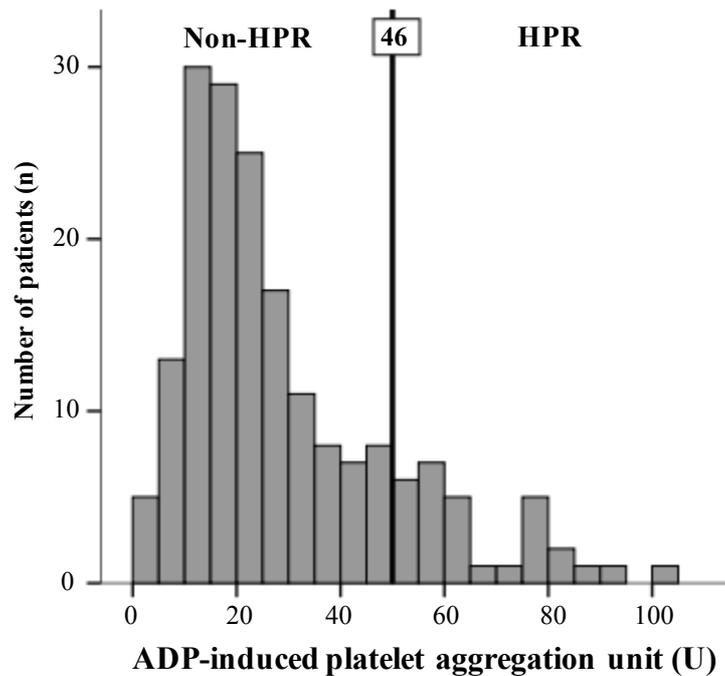


Figure 10: Adenosine diphosphate (ADP) induced platelet aggregation units (U) in 183 ACS patients on clopidogrel.

HPR: high on-treatment platelet reactivity. Adapted with permission from Platelets.⁽¹²⁰⁾

4.2.2 Microvesicles and platelet derived microvesicles

MVs (MVs exposing PS, i.e. lactadherin positive) and PMVs (MVs expressing PS together with CD42a and CD62P (P-selectin) or CD154 (CD40 ligand) were analyzed by flow cytometry as described previously. Patients with HPR to clopidogrel had significantly higher number of MVs, PMVs and PMVs exposing CD62P compared to patients without HPR to clopidogrel ($p < 0.05$ and $p < 0.01$ respectively). However, no differences were observed CD154 expression between these two groups, figure 11.

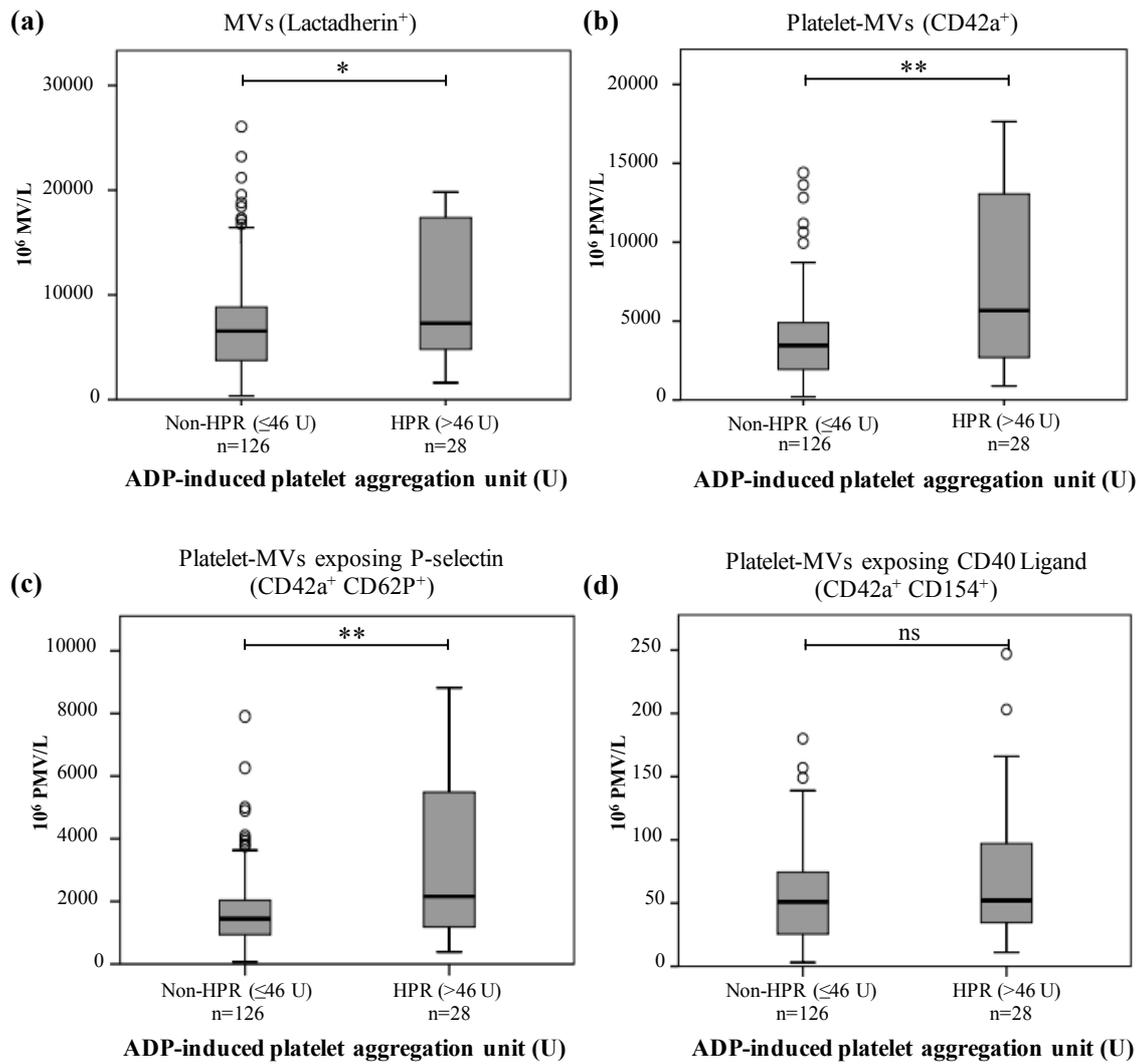


Figure 11: Effect of clopidogrel on microvesicles (MVs) and platelet-derived microvesicles (PMVs) production in ACS patients.

A value of >46 U was used to define high on-treatment platelet reactivity (HPR). *p<0.05, **p<0.01. Adapted with permission from Platelets.⁽¹²⁰⁾

Furthermore, we sub-grouped the patients using three cut-off values according to Sibbing⁽¹¹⁷⁾, i.e. LPR, NPR and HPR. Patients with LPR had significantly lower levels of PMVs compared to HPR patients to clopidogrel treatment, figure 12.

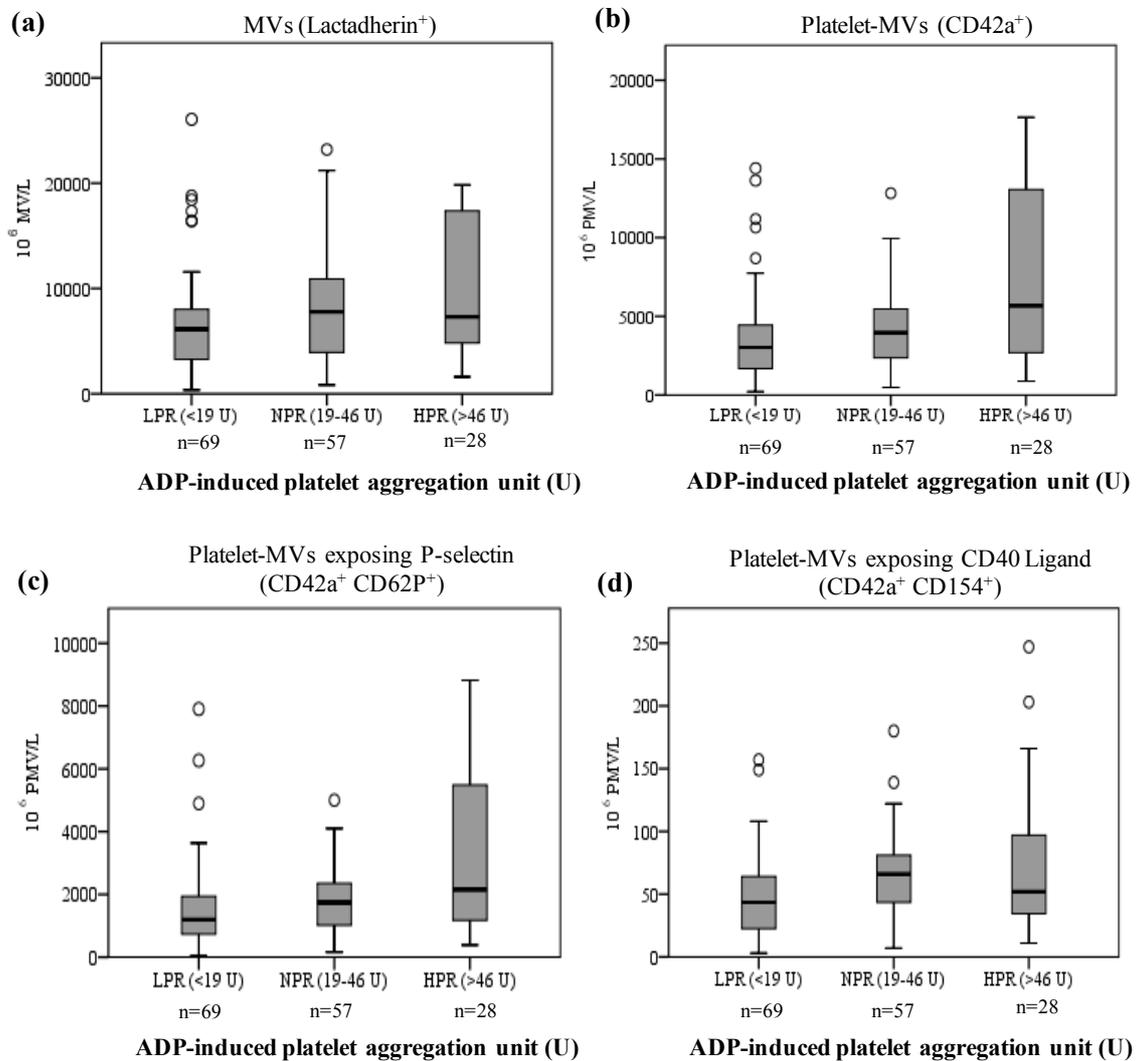


Figure 12: Effect of clopidogrel on microvesicles (MVs) and platelet-derived microvesicles (PMVs) production in ACS.

High on-treatment platelet reactivity (HPR). Normal on-treatment platelet reactivity (NPR). Low on-treatment platelet reactivity (LPR). A value of > 46 U was used to defined HPR, a value of 19–46 U as NPR and a value of < 19 as LPR. * $p < 0.05$ and ** $p < 0.01$. Adapted with permission from Platelets⁽¹²⁰⁾.

4.3 Study III

The mean age of the patients in HPR group was 66.9 ± 7.5 years and consisted of 4 women and 12 men. The median level of ADP response to clopidogrel in these patients was 77.5 [56.0:81.3] U.

The Non-HPR patients had a mean age of 69.6 ± 10.2 years and consisted of 14 men. The median level of ADP response to clopidogrel among these patients was 5.5[4.0:15.5] U.

MVs from patients with HPR to clopidogrel, induced significantly higher platelet aggregation compared to MVs from Non-HPR patients to clopidogrel as measured as aggregation over time, figure 13 (panel A) and peak aggregation, figure 13 (panel B).

Moreover, a significant correlation was found between concentrations of MVs and MV-induced platelet aggregation measured as both AUC and peak. Results showed significant correlations between MVs (PS⁺ MVs), CD42a⁺ MVs (platelet-MVs) and CD42a⁺ CD62P (platelet-derived MVs expressing P-selectin) and AUC, figure 14 (panel A-C).

In a similar way, peak aggregation in PRP was correlated to PS positive MVs ($r^2=0.32$, $p=0.023$), CD42a⁺ MVs ($r^2=0.39$, $p=0.021$) and CD42a⁺ CD62P MVs ($r^2=0.33$, $p=0.016$), but not to CD42a⁺ CD154⁺ MVs ($r^2=0.00$, $p=0.97$).

Patients with DM (n=9) had elevated platelet aggregation over time (AUC) and peak aggregation compared to patients without DM (n=21), figure 15 (panel A-B). The concentration of MVs in the DM patients did not correlate to MV-induced aggregation levels.

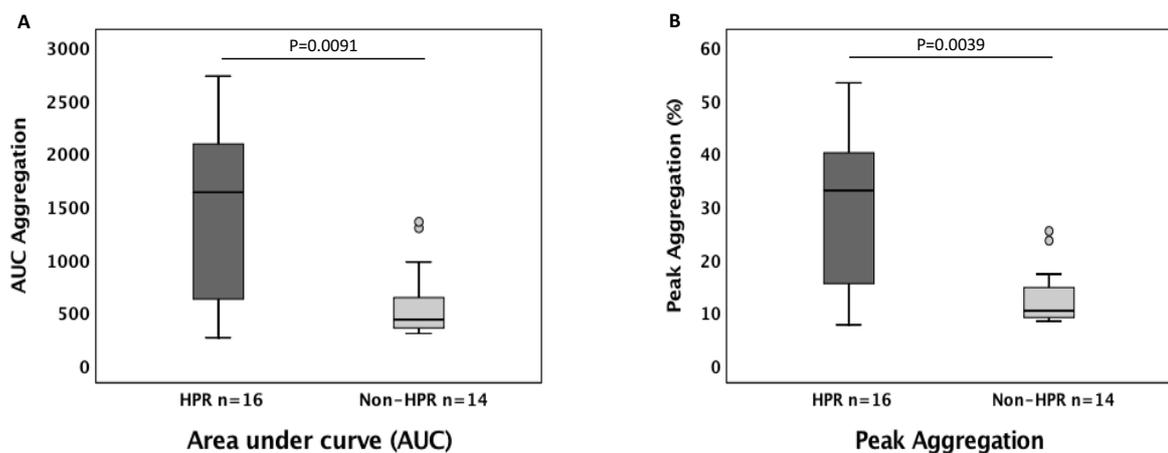


Figure 13: Microvesicle induced platelet aggregation in patient with HPR and Non-HPR to clopidogrel treatment.

The effect of MVs on platelet aggregation in HPR (n=16) and Non-HPR (n=14) patients, presented as area under curve (AUC), panel A, and peak aggregation, panel B. Data are presented as box plots (median and min-max values). Differences in AUC and peak aggregation between groups, was calculated with Mann-Whitney *U*-test.

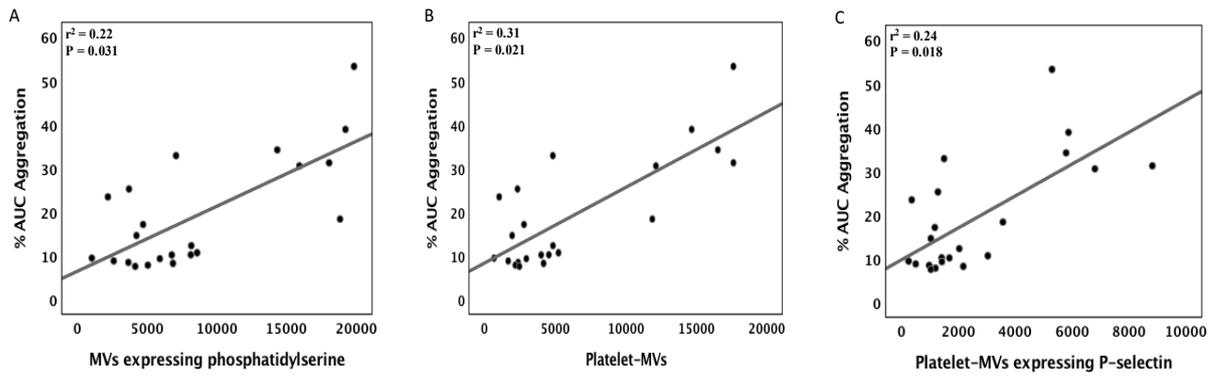


Figure 14: Correlation between MV concentration and MV activity measured as max levels of platelet aggregation over time (AUC).

MVs assessed by flow cytometry and phenotyped as phosphatidylserine expressing MVs (panel A), platelet derived MVs (panel B) and platelet derived MVs expressing CD62P (panel C). Data shows all patients (n=26), i.e. both HPR and Non-HPR. Spearman's rank correlation coefficient was used and both correlation coefficients and p-values are shown.

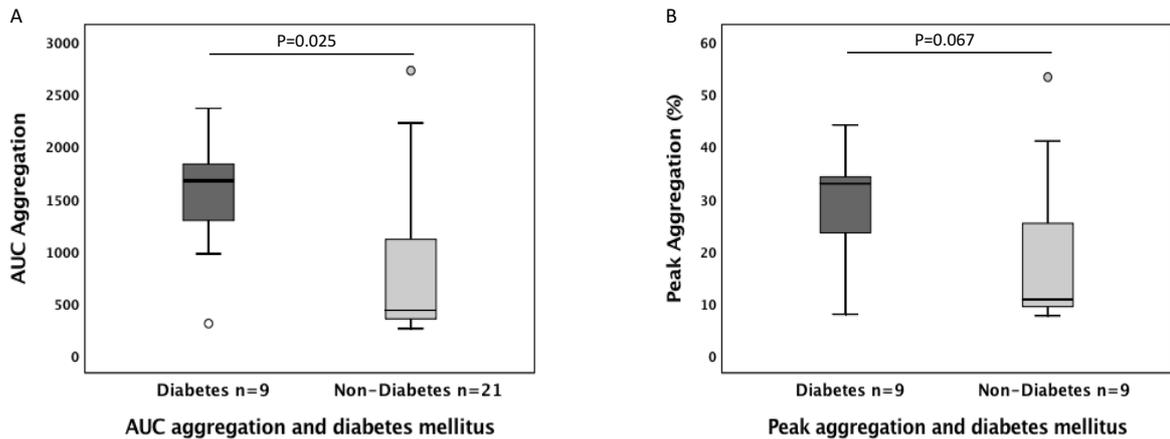


Figure 15: MV-induced platelet aggregation in all patients (n=30) with and without diabetes mellitus.

Data presented as aggregation over time (area under the curve; AUC), panel A, and peak aggregation, panel B. Data are reported as box plot (median and min-max values). Differences between groups (DM and Non-DM) were calculated with Mann-Whitney *U*-test.

4.4 Study IV

The study population baseline clinical characteristics are shown in Table II.

Table II: Baseline clinical characteristics of the study population.

| Subjects Variables | Acute STEMI (n=23) | Discharge Group (n=156) | Healthy Controls (n=15) |
|--|--------------------------|-------------------------------|-------------------------------|
| Age (years) | 67.6 ± 11.5 | 65.0 ± 11,0 | 69.0 ± 5.7 |
| Females / Males | 6 / 17 | 29 / 127 | 6 / 9 |
| Body mass index (kg/m ²) | 27 ± 4.2 | 27.3 ± 3.8 | - |
| Creatinine clearance (Cockcroft–Gault) | 67,6 ± 14,8 | 90,9 ± 29,4 | - |
| Hypertension (%) | 8 (34.8) | 92 (61,3) | - |
| Diabetes mellitus (%) | 5 (21.7) | 35 (22,4) | - |
| Active smoking (%) | 3 (13) | 36 (23,1) | - |
| Previous CAD (%) | 4 (17.4) | 32(20,5) | - |

Notes: Data are presented as mean ± standard deviation, numbers of patients and percentages.

CAD: coronary artery disease; STEMI: ST-segment elevation myocardial infarction.

4.4.1 Plasma levels of PTX3⁺-MVs assessed by flow cytometry

The median level of PTX3⁺-MVs was significantly elevated in acute STEMI patients before PCI compared to approximately 23 hours after PCI, figure 16 (panel A).

The levels of PTX3⁺-MVs in acute STEMI patients after PCI were also significantly higher compared to both patients at discharge and healthy controls, figure 16 (panel B).

4.4.2 Assessment of soluble PTX3 and MV-bound PTX3 by ELISA

To investigate the proportion of soluble and/or bound PTX3 in circulation, plasma samples were fractionated and measured with an PTX3 ELISA. 13 patients with acute STEMI with the highest PTX3⁺-MV levels measured by FCM were included, as well as 15 healthy control subjects. The results showed significantly elevated levels of PTX3 in plasma, MV-free supernatant and MV-enriched pellet in patients with acute STEMI compared to healthy subjects, figure 17. We observed a tendency to higher PTX3 levels in the MV-enriched pellet in the patients, however the differences were not significant.

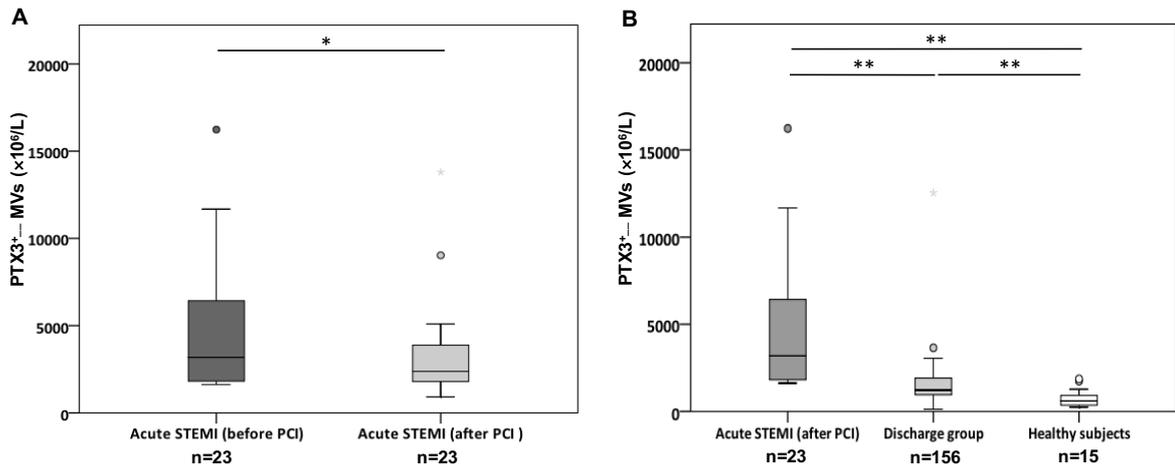


Figure 16: Flow cytometric measurement of PTX3⁺-MVs.

Levels of PTX3⁺-MVs in acute STEMI patients before PCI compared to approximately 23 hours after PCI, panel A. Levels of PTX3⁺-MVs in acute STEMI patients after PCI compared to patients at discharge (3–5 days after admission) and to healthy controls, panel B. * p<0,05 and ** p<0,001.

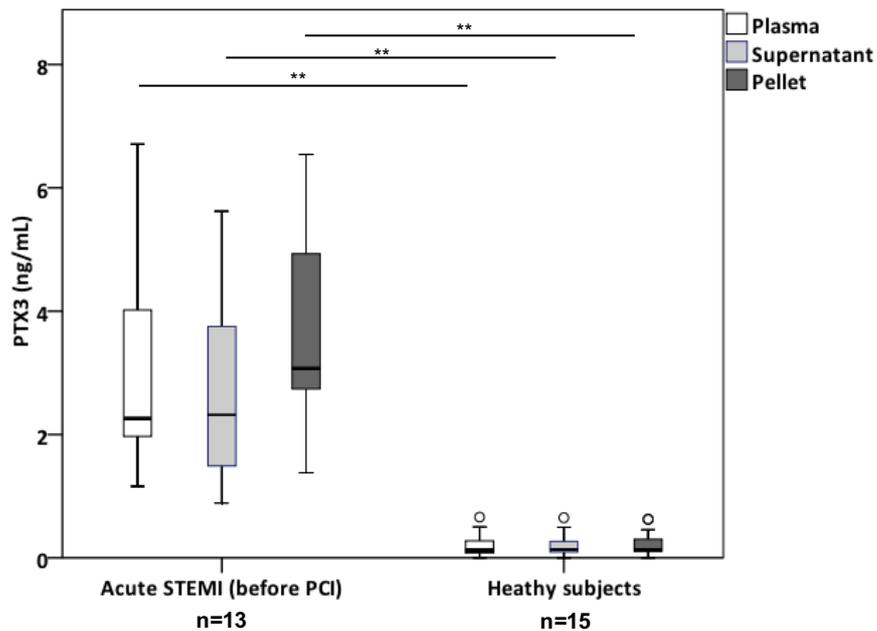


Figure 17: ELISA assessment of PTX3 in plasma, MV-free supernatant and MV-enriched pellet.

Patients with acute STEMI demonstrated elevated levels of PTX3 in both plasma, MV-free supernatant and MV-enriched pellet compared to healthy controls. ** p<0,001.

5 GENERAL DISCUSSION

The overarching aim of this thesis has been to learn more about platelet function and MVs in ACS, and to further explore the efficiency of DAT with aspirin and clopidogrel. We focused on clopidogrel since this was the first choice among P2Y₁₂ receptor inhibitors when the work with the thesis started.

5.1 Study I

When the work with this thesis started, we wanted to reduce the possibility that pre-analytical procedures influenced platelet function as assessed by MEA. More specifically, we wanted to be able to use both arterial and venous blood when assessing platelet responsiveness to DAT. Notably, blood sampled from the arterial side is easier to obtain in the acute and stressful phase of ACS in connection to PCI, as it may be obtained from the arterial sheath used for coronary angiography and PCI. On the other hand, venous blood is more easily obtained in “the everyday health-care setting” e.g. at discharge or during follow-up at the out-patient clinic.

Our results showed no significant difference between arterial blood samples and venous ones in the detection of “non-responders” to aspirin or clopidogrel, as analyzed by MEA. If data obtained are scrutinized in more detail (e.g when presented as Bland-Altman plots), measurements performed in arterial vs venous samples were not identical, but the agreement was acceptable. Some previous studies have reported, that there may be differences in test results in arterial and venous samples. Reasons for this may e.g. be a) increase in platelet aggregability in response to increased arterial shear stress; b) a higher oxygen content of arterial blood influencing platelet reactivity; c) exposure to synthetic surfaces of the long catheters used for arterial sampling⁽¹²¹⁻¹²³⁾. One study by Frumento et al suggested that it was the catheter length and lumen used to obtain blood samples rather than the oxygen content of blood that influenced test results the most.⁽¹²²⁾ However, it is important to consider that the sensitivity to pre-analytical handling may differ between different methods used to assess platelet function or other aspects of hemostasis. Thus, the study performed by Frumento used thrombelastography, which is a global hemostasis test rather than a specific platelet function test, and this method may be differently sensitive to sampling compared to other platelet function tests.

Importantly, our results in study I, showing comparable results between arterial and venous samples, were later supported by a study performed by Lancé et al. They studied ADP- and AA induced platelet aggregation with MEA⁽¹²⁴⁾. Additionally, they also used PFA-100 and studied thrombin generation (calibrated automated thrombogram). Sampling was, however, not identical with our study, as the arterial samples were obtained from the radial artery with a 20 G catheterization set, and from three different venous locations/sampling techniques (central venous line, venous line, and venipuncture by a G18 needle). Data obtained with MEA were, however, comparable with the different sampling techniques, and

as reasoned by Lancé it is likely that MEA is less sensitive to pre-analytical procedures, compared to other techniques like FCM or thromboelastography. It should also be put forward that the use of a wide bore (6F) introducer in our study may cause a comparably low shear stress and thereby likely a lower tendency to cause platelet activation. In summary, it is important to assess if pre-analytical handling influences the methods used, especially if different sampling procedures are employed in the same study, and different methods may differ in their sensitivity to pre-analytical handling.

5.2 Study II

In study II we investigated the relationship between platelet reactivity and circulating MVs and PMVs in patients with ACS at discharge, i.e 3–5 days after admission, by using MEA. The main finding was that there was a relationship between clopidogrel responsiveness and circulating MVs, and circulating PMVs; the latter defined as MVs exposing both PS and CD42a. This association was also observed when dividing patients further, i.e as being high, normal or low clopidogrel responders (i.e having low-on treatment [LPR], normal-on-treatment [NPR] and high-on-treatment platelet reactivity [HPR], respectively). Thus, we could demonstrate a concentration gradient in CD42a and CD62P positive PMVs, showing that with decreasing responsiveness to clopidogrel treatment the number of circulating PMVs increased. These findings were further supported by positive correlations between ADP-induced aggregation and PMV counts (r^2 0.26 and 0.22; $p < 0.0001$ for both).

To the best of our knowledge when published our study was the first to demonstrate an association between responsiveness to clopidogrel treatment and levels of circulating PMVs. An earlier study by Kalantzi and coworkers, showed lower PMV generation in vitro in PRP from ACS patients who were “clopidogrel responders” compared to those who were “non-responders”⁽¹²⁵⁾, thus in large supporting our findings but in an in vitro setting. Our interpretation is that in patients with a recent ACS and on aspirin, PMV formation in vivo can be reduced by clopidogrel, and our data are in agreement with a P2Y₁₂-dependent PMV formation in vivo. The data also suggest that stronger P2Y₁₂ inhibition would result in more efficient inhibition of PMV generation, but this should be studied in more detail in future studies.

In addition to study PMVs exposing CD62P, we also studied PMVs exposing CD40L (CD145⁺), which is a cytokine belonging to the TNF superfamily and secreted from and exposed on activated platelets. We have previously found that CD40L can be detected on PMVs with high sensitivity⁽¹²⁶⁾. The data obtained on CD40L positive PMVs showed a similar trend as the data observed with the other PMV phenotypes, but PMVs exposing CD40L were detected at much lower concentrations than the other PMV phenotypes investigated.

Although our data on PMVs show significant relationships to clopidogrel responsiveness and ADP-induced aggregability, indicating that ADP and inhibition of its effects are mechanistically important, the generation of PMVs in the circulation of CAD patients is likely dependent also on many other mechanisms than those related to ADP. PMVs data obtained in ACS patients should therefore – in our opinion – more be viewed upon as reflecting “global platelet activation” in vivo.

5.3 Study III

As described in the introduction of the present thesis, MVs may deliver biological effects. For example, MVs have strong pro-coagulant effects and boost thrombin generation, mainly through the exposure of PS^(88, 127). However, if MVs interact with and influence platelet aggregation had been little investigated when this thesis started, at least in the setting of ACS. After our findings made in study II, where we observed an association between MVs, and ADP induced platelet aggregability and clopidogrel responsiveness, we hypothesized that MVs could enhance platelet aggregation, and perhaps contribute to HPR to clopidogrel. In order to study the effects of MVs on platelets, we searched for a method with which we could study platelet aggregation in vitro, and that could be carried out in samples with small volumes. Notably, our simple method to enrich MVs from plasma was based on centrifugation and yielded only small volumes of MVs. The method described by Armstrong et. al⁽¹¹⁸⁾, which uses 96-well microplates to study platelet aggregation (changes in absorbance when platelets aggregate) turned out to be useful. When adding MVs from ACS patients with HPR to clopidogrel, to PRP from a healthy subject, we could demonstrate enhanced platelet aggregation compared to if MVs from patients with LPR were added to the same PRP. The MVs from patients with poor responsiveness to clopidogrel (HPR) thus had a pro-aggregatory effect which MVs from the clopidogrel responders (Non-HPR) lacked. There were significant correlations in plasma between PMV concentrations as assessed by FCM and the pro-aggregatory effects that the MV suspension caused in the wells, which further supported our idea that MVs from HPR patients may contribute to increased platelet aggregability and perhaps partly explain the phenomenon of HPR. Notably, similar data were obtained when we compared MVs from patients to diabetes mellitus – a condition associated with platelet hyperreactivity and increased levels of MVs – to MVs from patients without diabetes: MVs from diabetes patients had a significantly stronger pro-aggregatory effect irrespective of clopidogrel responsiveness.

Our experiments in study III may be viewed upon as a bit simple and incomplete, as we have not studied the enhancing effects of MVs on agonist induced platelet aggregation, e.g. on ADP, AA or TRAP induced platelet aggregation. This will require more experiments, likely with subthreshold concentrations of agonists, as enhancing effects is easier to reveal at the lower parts of the dose-response curve. But as pointed out by Bampalis et al in their methodological study of MEA and spontaneous platelet aggregation in whole blood, the tendency of spontaneous platelet aggregation leads to an increase of stimulated platelet aggregation values⁽¹²⁸⁾, so it may be that HPR as detected by MEA is partly due to the presence of pro-aggregatory MVs in the sample.

Based on our data it is tempting to speculate that generation of pro-aggregatory MVs from activated platelets may enhance platelet aggregation and increase the risk of platelet dependent thrombus-formation, e.g. at the location of an implanted coronary stent or plaque rupture. This scenario could mechanistically, at least partly, explain why HPR patients are at higher risk of a recurrent vascular complication.

5.4 Study IV

In study IV we wanted to take our FCM MV method further and assess if MVs expose an inflammatory biomarker with rapid dynamics which potentially could reflect atherosclerosis, plaque vulnerability and rupture, i.e. the long pentraxin molecule PTX3, see e.g. ⁽¹²⁹⁾. To our knowledge, we are the first to demonstrate the exposure and dynamics of PTX3 on MVs in ACS. We found substantially elevated circulating plasma levels of PTX3⁺-MV in the acute phase of STEMI before PCI and with a significant reduction after PCI. Sampling from NSTEMI patients at discharge 3–5 days after admission, showed even lower PTX3⁺-MV levels but they were not as low as in healthy subjects. We also used a conventional ELISA developed to measure plasma PTX3, and could confirm that a significant amount of the soluble PTX3 circulating in plasma is found on MVs. Although the MV bound portion may be comparatively small, it can be sensitively detected with our FCM method. In fact, this method is an attractive alternative to ELISAs, and may even be a better method to detect dynamic changes in biologically active molecules in response to various inflammatory conditions, as described by us previously for CD40L ⁽¹²⁶⁾, and perhaps also for HMBG1 ⁽¹³⁰⁾. Notably, in these two latter studies we phenotyped the MVs and could detect that they carried molecules of platelet, and monocyte origins, respectively, indicating that the MVs were released from these cell types. In this context, it is important to put forward the possibility that some of the PTX3⁺-MV that we measure may be of platelet origin, as PTX3 is known to bind to P-selectin on activated platelets ⁽¹⁰⁶⁾.

Furthermore, a future challenge will be to try to determine the cellular sources of MVs exposing PTX3. It is possible to assess if circulating PTX3⁺-MV carry antigens suggesting neutrophil origin (e.g. CD66b or Myeloperoxidase [MPO]) or vascular endothelial cell antigens such as E-selectin or CD144. Such data could add information on the source and pathophysiology of PTX3 in AMI.

5.5 Concluding remarks and future perspective

Management of thrombotic and bleeding complications is a growing challenge for health care, as the number of patients who need antithrombotic treatment is increasing. In fact, nowadays the antithrombotic treatment strategy may include drug combinations with the aim to inhibit both platelet function and coagulation. In the treatment of ACS, interventions with PCI in combination with DAT has become “standard of care” worldwide. The use of the novel P2Y₁₂ inhibitors prasugrel and ticagrelor has increased, but clopidogrel is still a commonly used P2Y₁₂ inhibitor, and perhaps even still the most used one worldwide ^(39, 40).

However, interindividual variability to antiplatelet treatment, especially clopidogrel, is a problem as poor responsiveness to clopidogrel treatment is associated with increased risk of major cardiovascular events, including stent thrombosis. The idea that platelet function testing with POC devices in this context may be of value continues to be under investigation⁽¹³¹⁾. Some large RCTs have been negative in this respect, see e.g.⁽¹³²⁻¹³⁴⁾, but a recent study with a different approach, opens up for different strategies how to use POC assay to guide antiplatelet treatment⁽¹³⁵⁾. As indicated by yet another clinical trial, it may be that we use strong platelet inhibiting treatment in a too aggressive way, and that de-escalation in antiplatelet efficiency after the first month phase following ACS can be an alternative approach⁽¹³⁶⁾. The implementation of an individualized approach according to a “therapeutic interval” regarding ADP-response to DAT as has been previously proposed^(14, 137), is in my mind a tempting approach but further studies on the matter have to be performed⁽¹³¹⁾ before such an approach can be implemented in clinical routine. The field of MVs and its role as “markers and makers” of various diseases is rapidly expanding. MVs, and especially PMVs, are more abundant in the circulation of “poor responders” to clopidogrel, according to this thesis. We could also demonstrate that MVs from “clopidogrel poor-responders” enhance platelet aggregation, and that this phenomenon also is seen with MVs from ACS patients with diabetes mellitus. However, these data were obtained in a pilot study and more studies are needed to understand this phenomenon in more detail. For example, how MVs influence platelet aggregation to various agonists have to be studied in proper experiments with full dose-response curves for the respective agonists (as alluded to above enhancing effects are likely to be more easily demonstrated at the lower part of dose-response curves). The “Armstrong method” should be a sufficient method for this purpose. Furthermore, MVs obtained from patients on ticagrelor or prasugrel should be investigated with respect to pro-aggregatory properties; these are also studies that easily can be performed.

The cellular origin of circulating MVs exposing PTX3 is another topic which should be explored in the future in patients with ACS, but this issue could also be further investigated in experimental models of inflammation, such as the endotoxin model, previously used by us^(126, 130, 138). Another aspect which deserves studying is pre-analytical handling and comparisons of arterial and venous sampling for measurement of MVs in the setting of ACS, similar to the experiments carried out in study I for MEA. Indeed, our idea to assess sample quality through the use of phalloidin exposure as described by us⁽⁷³⁾, and used by others⁽¹³⁹⁾ could be adapted in these studies.

Lastly, but most challenging, large studies to investigate the usefulness of MVs in terms of prognosis should be performed. To our knowledge, such studies are few or lacking in coronary artery disease⁽¹⁴⁰⁾.

6 CONCLUSIONS

- Both arterial and venous blood samples can be used in the detection of high on treatment platelet reactivity (HPR) to dual antiplatelet treatment with aspirin and clopidogrel, when platelet aggregation is studied with multiple electrode aggregometry (MEA).
- Patients with an acute coronary syndrome and HPR to clopidogrel, have elevated levels of circulating microvesicles (MVs) and platelet microvesicles (PMVs), which indicate ongoing platelet activation.
- Circulating MVs from ACS patients with HPR to clopidogrel are able to enhance platelet aggregation.
- Circulating pentraxin-3 (PTX3) is partially bound to MVs, and elevated in patients with acute myocardial infarction. The plasma levels of MVs exposing PTX3 decline over the first days following the infarction, likely reflecting the effects of treatment and the natural course of the disease.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund

Akuta koronara syndrom (AKS), ett samlingsnamn för akut hjärtinfarkt och svår (instabil) kärlkramp, är en mycket vanlig dödsorsak i den vuxna befolkningen. Tillståndet beror på åderförfattning (ateroskleros) i hjärtats kranskärl. Aterosklerosen skapar förträngningar vilka hindrar blodflödet ut i hjärtmuskeln med risk för syrebrist och kärlkramp. I vissa kärlområden kan aterosklerosen bilda särskilt svåra förträngningar, s.k. plack. Dessa aterosklerotiska plack kan brista och på platsen för brutna plack bildas blodproppar som riskerar helt täppa igen kranskärlen. Detta kan leda till total syrebrist och död av delar av hjärtmuskeln, en hjärtinfarkt har uppkommit. Blodproppsbildningen beror till stor del på att trombocyter (blodplättar) i blodet bildar proppar på platsen för plackrupturen.

En av hörnstenarna i behandling av AKS är medicinering med trombocythämmande läkemedel i kombination med ballongvidgning av förträngningarna (s.k. PCI). Vid PCI läggs ofta ett nät, s.k. stent, in i kärlet för att stabilisera kärlvidgningen. Denna åtgärd ökar emellertid risken för ny blodproppsbildning vilket i sin tur ökar behovet av trombocythämmande läkemedel, och för optimalt skydd mot ny propp måste två olika läkemedel ges, acetylsalicylsyra (ASA) och clopidogrel. För clopidogrel varierar den trombocythämmande effekten mellan olika individer, och det är visat att individer med mindre hämmande effekt har ökad risk att få ny blodpropp och hjärtinfarkt, särskilt på platsen för stentläggning.

Det finns idag olika metoder (blodprov) som mäter den blodproppshämmande effekten av clopidogrel, och det är visat att individer med mindre hämmande effekt av clopidogrel har ökad risk för blodpropp i kranskärlen. Det är emellertid inte vetenskapligt säkerställt att rutinmässig användning av sådana metoder, och justering av trombocythämmande medel (val av andra läkemedel) utifrån testresultaten, leder till minskat återinsjuknande i hjärtinfarkt och död.

Frågeställningar

Föreliggande avhandling har därför undersökt etablerade samt nya metoder för att mäta blodproppsbildning hos trombocyter vid AKS. Vi har undersökt en kommersiellt tillgänglig metod som mäter blodproppsbildning i ett blodprov, s.k. helblodsaggregometri (MEA), och om blodprover tagna direkt från kranskärlen i samband med ballongvidgningen ger samma testresultat som de blodprover som tas från en vanlig provtagning i en ven på armen (studie 1). Metoden MEA har jämförts med en annan metod som mäter små partiklar som knoppas av från trombocyter i blodet (s.k. mikrovesiklar [MV], studie 2), och vi har undersökt om dessa MV påverkar trombocyters benägenhet att bilda proppar (studie 3). Slutligen har vi undersökt om MV bär ett protein som visats frisättas i blodet vid hjärtinfarkt, och som kan vara av inblandat i mekanismer relevanta för hjärtinfarkt (studie 4).

Resultat och slutsatser

I studie 1 undersöktes 28 kranskärslsjuka patienter som genomgick PCI, och alla behandlades med ASA och clopidogrel. Effekten av behandlingen jämfördes i arteriellt blod (taget direkt ifrån kranskärnen vid PCI) och venöst blod taget på rutinmässigt sätt från en ven i armvecket. Blodproverna analyserades med hjälp av metoden MEA, och vi jämförde resultaten från de två provtagningstyperna.

Vi fann en god överensstämmelse mellan de två olika typerna av provtagning vad gäller att diagnosticera om patienter uppvisade god eller bristfällig hämning av clopidogrel-behandlingen. Fyndet är viktigt då det ibland är det svårt att få tillgång till det rutinmässiga venösa blodprovet i det brådskande akuta skedet. Man kan i enlighet med vår studie, sålunda använda både prover tagna från kranskärnen och blodprover tagna från en ven i armen.

I studie 2 undersöktes 183 patienter med AKS och trombocytters proppbildning (aggregation) undersöktes med hjälp av MEA. Vi fann att ca 20% av patienterna svarade bristfälligt på clopidogrel (s.k. ”non-responders”) vilket överensstämmer med tidigare studier. Vi undersökte vidare förekomsten av s.k. mikrovesiklar med en metod som utvecklats på vårt laboratorium. MV utsöndras främst från trombocyter men kan även frisättas från andra celler, t ex från vita blodkroppar eller från kärlväggens celler. Vi fann att patienter med god effekt av clopidogrel hade lägre nivåer av MV i blodet, särskilt MV från trombocyter. Dessutom fann vi att de patienter som hade mycket god trombocythämmande effekt av clopidogrel hade lägst antal trombocyt-MV i blodet. Metoden som mäter antal trombocyt-MV i blodet skulle kunna användas för att hitta patienter med risk för blodproppskomplikationer. Dessutom uppkom nu frågan om de trombocyt-MV som vi hittar i blodet hos AKS-patienter kan påverka trombocytters benägenhet till proppbildning (trombocyt-aggregation).

I studie 3 undersöktes den frågeställning som uppkommit som resultat av studie 2, dvs om MV i blodet påverkar trombocytters tendens till proppbildning (aggregation). Vi renade fram MV från patienter med god respektive bristfällig trombocythämmande effekt av clopidogrel. Dessa MV tillsattes till trombocytrik plasma från friska individer. Trombocyttaggregation undersöktes med en metod som kräver relativt små volymer prov. Vi fann att MV från patienter med bristande hämning av clopidogrel förstärkte trombocytters aggregation, talande för att hos dessa individer finns MV som kan medverka till ökad blodproppsbildning. Idén att MV i blodet påverkar trombocyttaggregation stöddes också av fynd som visade att MV från AKS patienter med diabetes, ett tillstånd med ökad trombocyttaggregation, också förstärkte trombocyttaggregation jämfört med MV från AKS patienter utan diabetes. Sammanfattningsvis finner vi i studie 3 stöd för att MV i blodet kan förstärka trombocytters tendens att bilda blodpropp (aggregera), särskilt hos patienter med bristande effekt av clopidogrel, och hos patienter med diabetes.

I studie 4 undersökte vi om MV från patienter med akut hjärtinfarkt bär på ett protein som påverkar inflammation och blodproppsbildning. Proteinet (pentraxin-3) har i andra studier visats relatera till sämre prognos efter hjärtinfarkt. Vi kunde finna att vid det akuta insjuknandet i hjärtinfarkt cirkulerar MV som bär på pentraxin-3, och nivåerna sjunker efter PCI, och är ytterligare lägre i samband med hemskrivning från hjärtinfarktavdelningen. Nivåerna når dock ej de låga nivåer i blodet som uppmäts hos friska individer. Cellursprunget av pentraxin-3 kan identifieras med vår metod i framtida studier, genom att samtidigt mäta cellspecifika molekyler på ytan av de MV som bär pentraxin-3. På detta sätt kan vi förstå var pentraxin-3 bildas och dess roll i sjukdomsprocessen vid hjärtinfarkt.

8 ACKNOWLEDGEMENTS

I owe my deepest gratitude to two groups of people, who have helped and supported me to accomplish this thesis. The first group, who were patients and voluntarily participated in clinical studies included in this work. Without you this thesis would never have been possible. The second group, is of course my dear family, friends and colleagues. In this latter group, I wish to specially express my sincere appreciation to:

Bassem Samad, my main supervisor. You have always been gentle, calm, supportive and positive during these years. I have grown both in my clinical practice and as a researcher thanks to our time together. Your clinical skills are truly outstanding. Thank you, Bassem!

Håkan Wallén, my great co-supervisor. You are not only the “king” of haemostasis with your distinct clinician skills, but also an outstanding supervisor and a true researcher, who has always ongoing ideas and interesting comments. I was always appreciative of your ready availability for needed scientific discussion and also amazed at your speed in reading and answering emails even late at night. I appreciate you enormously, Håkan!

Fariborz Mobarrez, my co-supervisor. Thanks for your support in all the lab work required, and for sharing your knowledge of microvesicles.

Majid Kalani, my co-author and former colleague at our department for your engagement and help at the beginning of my project.

Karin Malmquist, head of Department of Cardiology for being a great leader and being so enthusiastic in your support for research at the department through providing space and time for young researchers such as myself.

Ann Samnegård, my closet department head, for being always available and for your support across both my clinical and research work.

Gundars Rasmanis, my co-author and very good colleague. I have always been impressed by your clinical skills since my medical school days under your leadership in the Cardiology Department at Huddinge Hospital. Thank you again for all your professionalism and advice.

Mahbubul Alam, my mentor, for being kind and enthusiastic and always helpful.

All my colleagues at the Cardiology department, whom I deeply appreciate even without mentioning your names, for your encouragement and support over the years.

Joachim Lind, for your fantastic engagement and help for obtaining samples for the MATACS- study. I would also thank **Marie Arvidsson**, **Katherina Aguilera** and **Lena Gabrielsson** for your technical support during this time.

To the Department of Clinical Science, **Åsa Misic, Fredrik Johansson** and specially **Nina Ringart** for all your administrative engagement and always being helpful. Thank you, Nina!

Charlotte Åberg, thank you for always being professional and not the least friendly whenever I came to you in library at Danderyd Hospital.

Of course, I would also like to thank all my friends and colleagues from the broader world for being long being important parts of my life and for being patience during the last months when I have been in my own world.

My adorable / unforgettable parents, my departed mother and father for being so grateful, responsible and supportive. Hard to imagine your feelings when you let me leave you as a teenager just because you were so worried about my future at that time. I will never ever be able to compensate what you did for me. However, I know you are pleased with my achievements and I wish you were with me today. I do miss you!

Nick, my always happy son, you put the meaning in our life, and inspire me with your energy. I look forward to spending more time with you in the coming months and years.

Shirin, my best friend, companion and beloved wife. I would not be able to finish this work without your endless support and encouragement. Thank you for taking care of our small family during these years and specially in the last months, despite the demands of your pregnancy.

This work was supported by Karolinska Institutet Research Foundation, the Capio Research Foundation, the Swedish Heart & Lung Foundation, Eli Lilly Research Foundation and Stiftelsen Hjärtat.

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