From DEPARTMENT OF MOLECULAR MEDICINE AND SURGERY
Karolinska Institutet, Stockholm, Sweden

POINT-OF-CARE TESTING OF PLATELET P2Y12-INHIBITION IN PATIENTS WITH ACUTE CORONARY SYNDROME

by

Manne Holm

Karolinska Institutet

Stockholm 2018

Published by Karolinska Institutet.

ISBN 978-91-7831-029-6
Printed by Eprint AB 2018

ABSTRACT

Background: The point-of-care platelet function test Plateletworks® has been shown to identify patients with high platelet aggregation despite P2Y12 inhibition, also known as high-on-treatment platelet reactivity (HPR), who have increased risk for ischemic cardiovascular events. However, this platelet function assay has not been sufficiently validated. Patients who instead have excessive platelet inhibition are at risk for bleeding but predictive value of the Plateletworks® assay regarding bleeding has not been shown. Clopidogrel is today largely replaced by newer P2Y12 inhibitors such as ticagrelor due to improved outcome. The drug uptake after a 180 mg loading dose (LD) of ticagrelor has, however, not been thoroughly investigated in patients with non ST-segment elevation myocardial infarction (NSTEMI). Patients with ST-segment elevation myocardial infarction (STEMI) have a slower uptake of ticagrelor, likely due to morphine-induced inhibition of gastrointestinal motility.

Aims I: To identify patients pretreated with clopidogrel at increased risk for adverse clinical events after coronary angiography; **II:** To investigate the relation between platelet reactivity and the 30-day incidence of bleeding complications, as defined by two relevant bleeding definitions in clopidogrel-treated patients; **III:** To evaluate the ticagrelor uptake and platelet P2Y12 inhibition in patients with NSTEMI not receiving opioids and compare these data with those of patients with stable coronary artery disease (SCAD); **IV:** To evaluate if the peripheral opioid antagonist methylnaltrexone could improve platelet inhibition after a LD of 180 mg ticagrelor in morphine treated patients with STEMI.

Methods: Study I and II were observational, prospective studies based on the same cohort of 491 patients on clopidogrel who underwent coronary angiography. Platelet aggregation was measured with adenosine diphosphate-induced platelet function testing (Plateletworks®) and a cut-off was established for prediction of the respective primary outcome variable. In **Study I,** Patients were followed for three months, and the primary endpoint was myocardial infarction. In **Study II,** the primary endpoint was incidence of bleeding within 30 days as defined by the BARC and ARMYDA-BLEEDS bleeding definitions. **Study III** was an observational, prospective study on 40 NSTEMI patients and 20 controls. Both groups received a 180 mg ticagrelor LD and blood samples were taken pre-dose and 1, 2, 3, 4, 5, and 6 hours post LD. Plasma concentrations of ticagrelor and its active metabolite AR-C124910XX were analyzed as well as platelet inhibition. The primary endpoint was the time to maximal ticagrelor concentration (T_{max}). **Study IV** was a multicenter, prospective, randomized, controlled trial in STEMI patients treated with morphine and ticagrelor. Patients were randomized to a blinded intravenous injection of either methylnaltrexone or 0.9% sodium chloride. The proportion of patients with HPR and the plasma concentrations of ticagrelor and AR-C124910XX were assessed at baseline, one, and two hours.

Results I: A cut-off of 82.3% platelet aggregation was found to predict myocardial infarction and thus defined HPR. In total 39.9% (n=196/491) had HPR. At three months follow-up the event rates of myocardial infarction (MI) and rehospitalization, respectively, were higher in patients with HPR (5.1% vs. 1.7%, p=0.03; and 23.0% vs. 14.2%, p=0.01, respectively). **II:** In total, 474 patients of the initial cohort of 491 were included. Patients in the lowest platelet aggregation quartile had a higher frequency of ≥type 2 BARC bleeding and ARMYDA-BLEEDS defined bleeding within 30 days compared with the highest quartile (16.9% vs. 6.7%, p=0.014, and 8.5% vs. 1.7%, p=0.016, respectively). **III:** The T_{max} of ticagrelor did not significantly differ between NSTEMI patients and the controls (2.0h [1.0-3.0]) vs. 2.0h [2.0-3.0], p=0.393). HPR was at one hour seen in 15% of the NSTEMI patients versus 10% of the controls (p=1.0) and at two hours in 3% of the NSTEMI patients compared with none of the controls (p=1.0). **IV:** A total of 82 patients received either methylnaltrexone (n=43) or placebo (n= 39). Methylnaltrexone administration did not significantly affect prevalence of HPR at two hours after inclusion, the primary end-point, compared with placebo (54% vs. 51%, p=0.84). Plasma concentrations of ticagrelor and its active metabolite did not differ significantly between the groups over time.

Conclusions I: Testing with Plateletworks® identified patients at increased risk of myocardial infarction and rehospitalization within three months after coronary angiography. **II:** Patients in the lowest platelet aggregation quartile had a significantly higher incidence of bleeding according to BARC and ARMYDA-BLEEDS definitions within 30 days **III:** The uptake of ticagrelor was not significantly slower in NSTEMI patients compared with the SCAD controls with adequate onset of platelet inhibition in both groups. **IV:** Methylnaltrexone did not significantly improve platelet reactivity or plasma concentrations of orally administered ticagrelor in STEMI patients receiving morphine.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bakgrund och syfte: I samband med undersökning av hjärtats kranskärl respektive ballongvidgning och stentbehandling av förträngningar i kranskärlen behandlas patienter rutinmässigt med läkemedel som hämmar blodplättarnas förmåga att klibba ihop sig. Tidigare studier har visat att dessa blodplättshämmande läkemedel även minskar risken för blodproppsbildning i kranskärlen och andra pulsådror och därmed minskar risken för komplikationer i samband med och efter kranskärlsundersökningen/behandlingen. Under lång tid har det blodplättshämmande läkemedlet acetylsalicylsyra (Trombyl®, Bamyl®, etc) utgjort grunden i behandlingen av patienter med kranskärlssjukdom. Senare studier har dock visat att ”dubbel blodplättshämning” med tillägg av läkemedlet klopidogrel (Plavix®) ytterligare minskar komplikationsrisken varför denna kombinationsbehandling sedan millennieskiftet rutinmässigt använts. Det har dock framkommit att en standarddos av klopidogrel hos en del av patienterna inte alltid ger den förväntade hämningen av blodplättarna och att dessa patienter därmed har en ökning av risken för blodproppar och insjuknande i hjärt-kärlsjukdom. Graden av blodplättshämning efter en standarddos av klopidogrel går dock att mäta med analysmetoder som är olika väl studerade. **Studie I** syftade till att med en enkel men relativt undermåligt utvärderad mätmetod **(**Plateletworks®) mäta graden av hämning hos blodplättarna efter en standarddos klopidogrel hos patienter som genomgick kranskärlsundersökningen och behandling samt att följa upp ifall analysresultaten kunde förutse återinsjuknande i hjärtinfarkt och annan hjärt-kärlsjukdom inom tre månader.

Patienter med pågående behandling med läkemedel som påverkar blodets förmåga att levra sig (koagulera), inklusive blodplättshämmande läkemedel, löper en ökad risk att drabbas av blödningar. Risken ökar med högre grad av hämning, vilket även funktionsanalyser kan påvisa. **Studie II** utgick från samma patientgrupp som **Studie I**, men syftade istället till att utvärdera om analys med Plateletworks® kunde identifiera de patienter som fick blödningskomplikationer inom 30 dagar efter kranskärlsröntgen. Eftersom frekvensen av blödningar är väldigt beroende av vilka definitioner som används för att definiera vad som ska räknas som blödning använde vi i **Studie II** två olika blödningsdefinitioner, inklusive en relativ nyligen etablerad standardiserad blödningsdefinition.

Hos patienter med akut och instabil kranskärlssjukdom inklusive hjärtinfarkt har klopidogrel på senare år i hög grad ersatts av nya blodplättshämmande läkemedel såsom tikagrelor (Brilique®) i tillägg till acetylsalicylsyra då dessa nyare hämmare har ett snabbare anslag och är mer potenta vilket har visat sig ytterligare minska risken för komplikationer jämfört med dubbel blodplättshämning med klopidogrel och acetylsalicylsyra. Tidigare studier har dock antytt att en standarddos tikagrelor hos vissa patienter inte alltid ger en rask hämning av blodplättarna, vilket skulle kunna medföra ett sämre skydd mot blodproppar. **Studie III** syftade till att i en undergrupp av hjärtinfarktpatienter med “icke ST-höjningsinfarkt”, så kallad NSTEMI, studera tiden det tog för den högsta läkemedelskoncentrationen tikagrelor att uppnås i blodet och att jämföra resultaten med läkemedelsupptagen hos patienter med stabil kranskärlssjukdom.

Patienter med den mest utbredda formen av hjärtinfarkt (ST-höjningsinfarkt; STEMI) erhåller ofta redan i ambulansen rutinmässigt en laddningsdos tikagrelor i tablettform för att få en snabbt insättande hämning av blodplättarna inför kranskärlsundersökning och behandling. Denna patientgrupp får i ambulansen ofta även smärtstillande behandling med morfin. Studier antyder dock att morfinets negativa påverkan på mag-tarmkanalens motorik och rörlighet orsakar ett försämrat läkemedelsupptag och minskar tidig effekt av tikagrelor, vilket skulle kunna medföra ökad risk för kliniska komplikationer. Läkemedlet metylnaltrexon (Relistor®) skulle möjligen kunna förbättra upptaget av tikagrelor då det motverkar morfineffekten i mag-tarmkanalen utan att ta bort den smärtlindrande centrala effekten. **Studie IV** syftade till att utvärdera detta hos patienter med STEMI som lottades (randomiserades) efter samtycke till metylnatrexon eller verkningslöst placebo och därefter mättes tiden det tog till att uppnå god effekt av läkemedlet tikagrelor.

Metod: Studie I och II baserades båda på samma grupp av 491 patienter som alla erhöll behandling med klopidogrel och acetylsalicylsyra inför kranskärlsundersökning och behandling. Blodplättarnas hämning av

klopidogrel mättes med Plateletworks®. I **studie I** följdes patienterna med avseende på eventuellt insjuknande i hjärtinfarkt inom tre månader från och med att patienterna kom med i studien. **Studie II** inkluderade totalt 474 av de ursprungliga 491 patienterna och dessa följdes med avseende på blödningshändelser inom 30 dagar från att de kom med i studien. **Studie III** inkluderade totalt 40 NSTEMI patienter och 20 kontrollpatienter med stabil kranskärlssjukdom. Båda grupperna erhöll en standarddos av 180 mg tikagrelor och blodprover togs för att mäta läkemedelsupptaget och blodplättshämningen just innan tikagrelordosen, samt vid varje timme under 6 timmar efteråt för att upptäcka en eventuell skillnad i tiden det tog till att uppnå maximal tikagrelorkoncentration. **Studie IV** inkluderade totalt 82 patienter med ST-höjningsinfarkt, som alla behandlats med morfin och tikagrelor. Lottning (randomisering) gjordes mellan metylnaltrexon eller placebo (koksalt), som gavs direkt i blodet och läkemedelsupptaget och blodplättshämningen mättes då respektive patient kom med i studien och efter en och två timmar.

Resultat och slutsats I: Testning av blodplättsfunktionen med Plateletworks® identifierade patienter med undermålig behandlingseffekt av klopidogrel och denna grupp, som utgjorde 39,9% av de 491 inkluderade patienterna, drabbades i signifikant högre grad av en hjärtinfarkt inom tre månader jämfört med de patienter som hade god effekt av klopidogrel. **II.** De 474 patienterna delades in i fyra grupper (kvartiler) baserat på graden av blodplättshämning av klopidogrel som analyserades med Plateletworks®. Patienter i kvartilen med högst hämningsgrad drabbades i signifikant högre utsträckning av blödningskomplikationer inom 30 dagar efter kranskärlsröntgen jämfört med den kvartil som hade minst blodplättshämmande effekt av klopidogrel, oavsett vilken blödningsdefinition som användes. **III.** Tiden att uppnå maximal läkemedelskoncentration av tikagrelor skiljde sig inte mellan patienter med NSTEMI jämfört med patienter med stabil kranskärlssjukdom och inom två timmar hade nästan alla patienter i båda grupperna en tillräckligt hög blodplättshämning. Således var upptaget och läkemedelseffekten bra i båda grupperna. **IV:** De 43 patienter som lottades till metylnaltrexon hade inte högre grad av trombocythämning av tikagrelor två timmar efter att metylnaltrexon gavs jämfört med de 39 patienter som lottades till placebo. Läkemedelskoncentrationen av tikagrelor skiljde sig inte heller signifikant mellan grupperna. Med andra ord hade injektionen av metylnaltrexon ingen positiv effekt på läkemedelsupptaget av tikagrelor hos patienter med ST-höjningsinfarkt som erhållit morfin och framtida studier bör söka efter andra alternativ till att förbättra läkemedelsupptaget hos denna patientkategori.

LIST OF SCIENTIFIC PAPERS

I. **Holm M,** Dalén M, Tornvall P, van der Linden J.
Point-of-care testing of clopidogrel-mediated platelet inhibition and risk for cardiovascular events after coronary angiography with or without percutaneous coronary intervention.
Blood Coagulation & Fibrinolysis 2014;25(6):577-84

II. **Holm M,** Tornvall P, Dalén M, van der Linden J.
Correlation Between Point-of-care Platelet Function Testing and Bleeding after Coronary Angiography according to Two Different Definitions for Bleeding.
American Journal of Cardiology 2014;114(9):1347-53.

III. **Holm M,** Tornvall P, Westerberg J, Rihan Hye S, van der linden J.
Ticagrelor pharmacokinetics and pharmacodynamics in patients with NSTEMI after a 180-mg loading dose.
Platelets 2017;28:706-711

IV. **Holm M**, Tornvall P, Henareh L, Jensen U, Golster N, Alström P, Santos-Pardo I, Witt N, Fedchenko N, Venetsanos D, Beck O, van der Linden J.
Methylnaltrexone to imprOVE platelet inhibition of ticagrelor in morphine-treated patients with ST-segMENT elevation myocardial infarction (MOVEMENT -trial)
Submitted

CONTENTS

LIST OF ABBREVIATIONS

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ARMYDA-BLEEDS	Antiplatelet therapy for reduction of myocardial damage during angioplasty-bleeding
BARC	The bleeding academic research consortium
DAPT	Dual antiplatelet therapy
ELISA	enzyme-linked immunosorbent assay
GP	Glycoprotein
HPR	High on-treatment platelet reactivity
LD	Loading dose
PCI	Percutaneous coronary intervention
PRI	Platelet reactivity index
MEA	Multiple electrode aggregometry
NCDR	American national cardiovascular data registry
NSTEMI	Non-ST-segment elevation myocardial infarction
STEMI	ST-segment elevation myocardial infarction
SCAD	Stable coronary artery disease
T_{max}	Time to maximum drug concentration
TxA_2	Tromboxane A_2
VAS	Visual analogue scale
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor

1 INTRODUCTION AND BACKGROUND

1.1 PLATELETS IN PRIMARY HEMOSTASIS

Platelet inhibition is a corner stone in the treatment of acute coronary syndrome (ACS) as platelet activation and aggregation are pivotal in the generation of arterial thrombosis. In case of atherosclerotic plaque rupture, a very prothrombotic surface is exposed to blood. The traditional model of thrombus formation begins with platelet interaction with subendothelial tissue and prothrombotic factors like von Willebrand factor (vWF), fibronectin, collagen, and tissue factor. This interaction causes platelet activation and adherence to the damaged vessel, i.e. initiation of primary haemostasis. Normally, the healthy endothelium prevents this by expressing antithrombotic compounds such as prostacyclin-I_2 and nitric oxide (1). After adhesion, the process of platelet activation is further amplified by production and release of several soluble platelet agonist, most importantly thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). TxA_2 promotes platelet activation and is produced by cyclo-oxygenase (COX) from arachidonic acid, a process targeted by aspirin and other non-steroid anti-inflammatory drugs (2). ADP is released from dense platelet granules and is very important in the process of thrombus formation as it interacts with P2Y-receptors on other circulating platelet, inducing a cascade reaction of platelet activation and aggregation. There are two key receptors in this group: P2Y1 and P2Y12 (3). The ADP-induced aggregation can be stimulated by both receptors, but is in fact dependent on their simultaneous activation (4). Activation of the G-protein coupled P2Y1-receptor leads to an initial phase of platelet activation by causing a change of the platelet shape and a weak platelet aggregation. This initial phase is followed by ADP interaction with the P2Y12-receptor (target for clopidogrel, ticagrelor, prasugrel, and cangrelor), which is more important for the growth and stabilization of the platelet aggregate. The ADP-P2Y12 binding activates the receptor coupled G-protein causing a complex intracellular signaling cascade (4), including inhibited phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (5). This results in an activation of fibrinogen receptor GP IIb/IIIa (also named integrin $\alpha_{IIb}\beta_3$), promoting enhanced platelet degranulation, release of more ADP, and stimulation of TxA_2 production (7). The GP IIb/IIIa receptor enables platelet aggregation via the cross-linking process where platelets adhere to each other using the soluble adhesive protein fibrinogen to build up the aggregate. This receptor is also used as a pharmacological target with GPIIb/IIIa inhibitors (6). Even though ADP and TxA_2 interact with different receptors, platelet activation through these agonists ultimately results in a platelet shape change, degranulation, and activation of fibrinogen receptors.

Another very important soluble platelet agonist is thrombin, generated at the site of endothelial injury. It induces platelet aggregation via activation of it specific protease-activated receptors (PAR), which is another pharmacological target (6). There is also a role of platelets in inflammation and atherosclerosis formation, partly through the expression of P-selectin from activated platelets (7). Measurements of P-selectin can be used to assess platelet function after agonist stimulation ex vivo (8).

The above described model reflects the core elements of the initiation of thrombus formation, where the stabilization of the initial platelet aggregate is dependent on soluble agonists, especially ADP (9). This model is, however, a simplification as platelet aggregation has proven to be a more dynamic process than initially thought. In stenosed arteries the lumen is reduced and passing blood is exposed to high shear rates, i.e. shear stress. The mechanism of platelet aggregation has been shown to differ according to the level of shear stress, where there appears to be a mechanism of biomechanical platelet activation. Under these conditions, the soluble platelet agonists including TxA_2, thrombin, and ADP probably play a "later" role in stabilization of the initial aggregated platelets (2). Moreover, in recent years new platelet receptors have been discovered, including several immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors, which regulate signal transduction in platelets. The exact impact on platelet activation and aggregation of these receptors is not fully understood (10). To provide a more thorough explanation of the recent discoveries of platelet activation is, however, not the scope of this thesis.

The secondary hemostasis, which secures and strengthens the platelet plug through the cleaving of fibrinogen to fibrin, is not described in this introduction.

1.2 DUAL ANTIPLATELET TREATMENT

In patients with acute coronary syndrome (ACS) and patients undergoing percutaneous coronary intervention (PCI), proper antiplatelet therapy is a cornerstone to reduce morbidity and mortality. Dual antiplatelet therapy (DAPT) with a combination of aspirin and an oral platelet P2Y12-inhibitor has been established in clinical use for almost 20 years (11). DAPT is with over 35 randomized large clinical trials including more than 225,000 patients, one of the most investigated treatment strategies in cardiovascular medicine (11).

Aspirin, or acetylsalicylic acid, irreversibly inhibits and inactivates the two COX-enzymes (COX-1, and COX-2) in platelets and endothelial cells. In platelets the inhibition of COX-1 disables the creation of TxA_2. A low dose aspirin treatment regime primarily inhibits the COX-1 dependent platelet activation via TxA_2, while it has little effect on the COX-2 function in e.g. endothelial cells. This inhibition of platelet COX-1 lowers the risk of atherothrombotic events and forms the cornerstone in the treatment of patients with established cardiovascular disease (12).

Clopidogrel is an oral intestinally absorbed thienopyridine prodrug with little to no effect on ADP-induced platelet activation in its inactive form. When orally administrated, 85% of the absorbed clopidogrel is hydrolyzed by esterases to an inactive metabolite (13). The remaining 15 % is in a two-step reaction, mediated by the hepatic cytochrome P-450 system, converted to the active but highly unstable metabolite R-130964. There are several hepatic CYP450 enzymes involved in this process, most notably CYP2C19, which affects both steps of the metabolic reaction that generates R-120964. When exposed to platelets, this active metabolite forms an irreversible binding to the platelet's P2Y12 receptors, inhibiting ADP-induced platelet aggregation (14).

In 2001, the landmark clopidogrel in unstable angina to prevent recurrent events (CURE) trial showed that DAPT consisting of clopidogrel in addition to aspirin reduced the composite endpoint of myocardial infarction, stroke or cardiovascular death (15). The subsequent PCI-CURE study, which examined the subgroup of patients in the CURE trial who underwent PCI, showed that this treatment regime reduced the risk of cardiovascular death, myocardial infarction, or urgent target-vessel revascularization within 30 days with about a third compared with aspirin alone (16). Treatment with clopidogrel was shown safer with fewer side effects than the previously introduced P2Y12-inbitor ticlopidine and thus replaced it in clinical practice (11).

The antiplatelet response to clopidogrel is, however, very heterogeneous between patients. Many patients get a suboptimal platelet inhibition, i.e. a high on-treatment platelet reactivity (HPR), which is a strong risk factor for cardiovascular death, myocardial infarction, and stent thrombosis after PCI (17). In one meta-analysis of 25 studies with 3688 patients, the mean prevalence of HPR was 21% (95% CI 17-25%), but with a significant heterogeneity between studies. Despite different prevalence, HPR was associated with an increased risk for adverse cardiovascular events in most of the included studies (18). The response variability to clopidogrel treatment depends mainly on the generated amount of active metabolite, which may be due to clinical factors like non-compliance, or drug-drug interference with for example proton pump inhibitors. Other factors include impaired intestinal absorption or changed platelet function caused by diabetes, increased BMI, or ongoing acute coronary syndrome. Moreover, genetically caused loss-of-function in the CYP2C19 enzyme is known to cause HPR (19).

1.3 NEWER MORE EFFICIENT P2Y12 INHIBITORS

Due to the problems with heterogeneous antiplatelet response on clopidogrel, newer and more efficient platelet inhibiting drugs are now in clinical use in the setting of acute coronary syndrome, including the more potent P2Y12-receptor antagonists ticagrelor, prasugrel, and cangrelor, whereby the latter is a short-acting intravenous drug (6). Ticagrelor reversibly binds to platelets thereby inhibiting ADP-mediated platelet activation and aggregation. It does not need metabolic changes for its inhibitory effect but has active metabolites, most importantly AR-C124910XX (20). An oral loading dose (LD) of 180 mg ticagrelor results in a high degree of platelet P2Y12 inhibition (80-90%), normally within around two hours (21-23). This is both a stronger and more rapid platelet inhibiting effect compared with clopidogrel, making ticagrelor a more attractive alternative in the situation of ACS, where a rapid onset of platelet inhibition is beneficial. The landmark study of Platelet Inhibition and Patient Outcomes (PLATO) showed benefits of DAPT using aspirin and ticagrelor over aspirin and clopidogrel in most subgroups of ACS patients. This is why today ticagrelor is routinely given to patients with ACS before coronary angiography, in the absence of contraindications (24).

The potent P2Y12 inhibitors prasugrel and cangrelor have also been shown to reduce ischemic events compared with clopidogrel in ACS patients (25, 26), but as these P2Y12

inhibitors are not the main focus of this thesis the interested reader can find background reading on this elsewhere (6, 11).

1.4 INCREASED BLEEDING RISK

In contrast to patients on P2Y12-inhibitors who have HPR, those with a high grade of platelet inhibition are at risk for bleeding, which is associated with increased morbidity, mortality, and high costs (27, 28). The introduction of newer more potent platelet inhibitors has a trade-off between decreased thrombotic complications and increased bleeding risk, which is an important consideration in for example the decision of treatment duration (11).

Contrary to ischemic events such as cardiac death, myocardial infarction, or stent thrombosis, which all have clear definitions, bleeding definitions are very heterogeneous (28). Many bleeding definitions have been suggested over the years and the perhaps most commonly used is the Thrombolysis in Myocardial Infarction (TIMI) bleeding criteria, which were established in the 1980s (29). The original version of the TIMI bleeding has been adapted to better fit certain specific scenarios, such as surgery with coronary artery bypass grafting where bleeding is very common (28). Also, many other bleeding definitions have been established and used in varying frequency, which has increased variance in the incidence of bleeding events between studies. In an attempt to standardize bleeding definitions used in cardiovascular studies, the Bleeding Academic Research Consortium (BARC) published a consensus report including a bleeding definition to be used in future clinical trials (28). BARC standardized endpoint definitions were considered "…an important way to improve the quality and efficiency of clinical trials…" according to the corresponding editorial in Circulation (30). These definitions are nevertheless in need of further data-driven validation as they were based on consensus (30, 31). Also, the European Society of Cardiology Working Group on Thrombosis suggested that bleeding events in acute coronary syndromes and percutaneous coronary interventions should "be reported using more than one bleeding scale, one of which should be the BARC bleeding definition" (31). For this reason, **Study II,** which focused on bleeding events, included the BARC bleeding definition. It also includes the bleeding definition from the previously published Antiplatelet Therapy for Reduction of Myocardial Damage During Angioplasty-Bleeding (ARMYDA-BLEEDS) study, where patients in the lowest quartile of platelet aggregation according to platelet function testing had a significantly higher incidence of bleeding complications compared with the highest quartile of platelet aggregation (32).

1.5 EVALUATION OF PLATELET P2Y12 INHIBITION

Although patients with HPR are at greater risk for adverse cardiovascular events, it is not recommended to routinely screen for HPR among the patients receiving P2Y12-inhibiting drugs prior to angiography and PCI. The reason for this is simple. Even though on-treatment platelet reactivity has been shown an independent and reliable risk predictor of cardiovascular events, a benefit of individualized antiplatelet therapy on the basis of platelet function testing

has not been successfully proven (33). Moreover, standardization of the definitions of HPR has been an issue, as it depends on the type of testing equipment used.

Many platelet function tests are available for evaluation of platelet inhibition and for the detection of HPR. The Plateletworks® assay, which relies on single platelet counting with a conventional cell counter before and after agonist stimulation in vitro, has shown predictive value for adverse thrombotic/ischemic events after PCI (34). This test has, compared with other available more common platelet function tests, such as Multiplate®, VerifyNow®, VASP-P assay®, and light transmittance aggregometry (LTA), not been validated in sufficiently large study samples (35). In **Study I and II,** this platelet function was used to evaluate P2Y12 platelet inhibition. The results of this assay are available within minutes, but a major disadvantage is that the platelet function testing needs to be conducted within 10 minutes after blood sampling (34).

Multiplate® rely on multiple electrode aggregometry (MEA), where an impedance aggregometer measure electrical impedance between two electrodes immersed in whole blood with hirudin anticoagulation. Stirring of the blood is performed in a semi-automatic fashion and after the addition of an agonist such as ADP, platelet aggregation inhibition of the agonist-specific receptor is measured. The results are available within a few minutes, and the results of the assay have been shown to have predictive value for both thrombotic and bleeding events in patients on P2Y12-inhibitors (36). Testing with MEA was conducted to evaluate platelet inhibition with ticagrelor in **Study III**. A disadvantage with MEA is that other platelet inhibiting drugs, such as GP IIb/IIIa inhibitors affect the results, i.e. it is not entirely P2Y12-specific. Moreover, the method requires some laboratory experience to conduct.

Another method to assess platelet P2Y12-receptor inhibition is measurement of VASP phosphorylation, which is very specific for the P2Y12 receptor inhibition. As mentioned above, VASP is a second messenger in the P2Y12-receptor signaling and the ratio of phosphorylated and dephosphorylated VASP directly and selectively measures platelet P2Y12 inhibition. This is a major advantage, as other platelet inhibiting drugs such as GP IIb/IIIa inhibitors does not affect the testing results. A disadvantage with the VASP assay is that specialized laboratory equipment and staff experienced with flow cytometric analyses are needed (36). In **Study IV**, a novel version of the VASP assay relying on enzyme-linked immunosorbent assay (ELISA) was used, as it does not require a flow cytometer for the analysis and significantly lowered the analysis time requirements. Moreover, it enabled freezing of blood samples after initial activation and lysis before later centralized analysis, as previously described (37).

1.6 IMPAIRED ONSET OF PLATELET P2Y12-INHIBITION IN ACS

A steady state of platelet inhibition is normally achieved at around 6 hours after a LD of clopidogrel (38, 39). However, in patients with ST-segment myocardial infarction (STEMI), the bioavailability of clopidogrel has been shown to be significantly decreased compared with

healthy controls (40). This may be due to a stress-related decrease in gastrointestinal motility including decreased gastric emptying and decreased small intestinal transit (41), which could explain the lower bioavailability of clopidogrel as drug absorption rate in the gastro-intestinal tract is largely determined by gastric emptying(42). However, opioids such as morphine, which is often administered to relief severe pain in STEMI patients, have also been reported to delay gastric emptying (43). This negatively impacts the uptake of clopidogrel, which was shown in a small randomized study of healthy volunteers, where morphine use significantly decreased the clopidogrel uptake and antiplatelet action (44). As the newer P2Y12 inhibitor ticagrelor has a peak concentration at around 2 hours after a LD of 180 mg ticagrelor, its use could perhaps counter the problem of belated uptake and effect in STEMI. The data on the uptake of ticagrelor originate mainly from healthy volunteers and patients with stable coronary artery disease (45-47). In ACS patients, however, the uptake has shown to be more unpredictable, especially in patients with ST-segment elevation myocardial infarction (STEMI) (48, 49).

Among ACS patients in Sweden, non-ST elevation myocardial infarction (NSTEMI) represented the most common group during 2014 (50). In Sweden, these patients are usually given a LD of 180 mg ticagrelor upon diagnosis, according to European Society of Cardiology guidelines (51). When the planning for **Study III** was conducted, the pharmacokinetic properties of ticagrelor in the acute phase had not been thoroughly studied in ACS patients with NSTEMI. A small previous study evaluated several different ticagrelor loading and maintenance doses and a LD of 180 mg ticagrelor to P2Y12-naïve patients (n=7) resulted in a mean time to maximal ticagrelor concentration (T_{max}) of 3 hours (52). Except for that study, the pharmacokinetics of a 180 mg LD of ticagrelor had not been evaluated in patients with NSTEMI. The idea behind **Study III** was that patients with NSTEMI, like those with STEMI might have a delayed onset of platelet inhibition, possibly due to an increased stress response and impaired gastrointestinal peristalsis.

A LD of ticagrelor is currently recommended on top of aspirin as anti-platelet therapy in patients with ST-segment elevation myocardial infarction (STEMI) (11, 24). However, a delayed onset of action, i.e. high prevalence of early HPR within the first hours have also been demonstrated in STEMI patients after orally administered ticagrelor (48, 49). In the "Administration of Ticagrelor in the Cath Lab or in the Ambulance for New ST Elevation Myocardial Infarction to Open the Coronary Artery" (ATLANTIC) study, pre-hospital ticagrelor administration did not improve pre-PCI coronary reperfusion, but stent-thrombosis was significantly lower at 30 days, when compared with in-hospital ticagrelor (53). This is an indication that improvement of ticagrelor action with an earlier LD resulting in earlier antiplatelet effect is of benefit in STEMI patients. Today, most STEMI patients in Stockholm receive a 180 mg ticagrelor LD in addition to aspirin in the prehospital setting. An early onset of ticagrelor effect in STEMI patients is considered important as an adequate platelet inhibition improves clinical outcome.

Morphine has also emerged as a predictor of belated antiplatelet effect after intake of the newer P2Y12 inhibitors prasugrel or ticagrelor (48). In a sub-study of the ATLANTIC study, use of morphine in patients with STEMI was shown to delay the onset of platelet inhibition after a 180 mg ticagrelor LD (37). These authors hypothesized that this interaction may have had an impact on the negative primary outcome of the ATLANTIC study, as there was an observed interaction between morphine treatment and ST-segment elevation resolution (53). In another recent trial, patients with acute myocardial infarction (64% STEMI) randomized to administration of morphine had a delayed uptake and antiplatelet response to ticagrelor, when compared with placebo (54).

The morphine-induced delay in gastric emptying can be reduced with the opioid antagonist naloxone, which has been verified in morphine-treated women during labor (55). However, a drawback with naloxone is that it passes the blood-brain barrier (BBB), and thus attenuates the pain-reducing morphine effect. Conversely, the opioid antagonist methylnaltrexone does not affect the morphine-mediated central analgesic effects due to a very limited passage over the BBB, and thus primarily acts as a peripheral morphine antagonist (56). The possible effect of this drug on the uptake of ticagrelor was evaluated in **Study IV.**

2 AIMS

The specific aims were:

- To investigate the ability of the Plateletworks® assay to identify patients pretreated with clopidogrel at increased risk for adverse clinical events after coronary angiography with or without PCI.

- To investigate the relation between on-treatment platelet reactivity assessed with the Plateletworks® assay and the 30-day incidence of bleeding complications, as defined by the BARC and ARMYDA- BLEEDS definitions, after coronary angiography with and without PCI.

- To evaluate the ticagrelor uptake and platelet P2Y12 inhibition in patients with NSTEMI not receiving opioids and compare these data with those of patients with SCAD.

- To evaluate if the peripheral opioid antagonist methylnaltrexone could improve platelet inhibition after a LD of 180 mg ticagrelor in morphine treated patients with STEMI.

3 METHODS

3.1 STUDY DESIGN AND PATIENT SELECTION

Study I and II were observational, prospective, single center studies based on the same cohort of 491 adult patients with ACS and patients with established coronary artery disease. All patients were on treatment with aspirin and clopidogrel before undergoing acute or elective coronary angiography with or without PCI between October 2006 and May 2011 at the Karolinska University Hospital in Solna, Sweden. **Study I** included the whole cohort, while in **Study II** 16 patients were excluded due to GP IIb/IIIa inhibitor use prior to the platelet function test or prasugrel use resulting in a cohort of 474 patients.

Study III (NCT02292277), was a prospective, open-label, observational, single-center, phase IV pharmacokinetic and pharmacodynamic study performed at Södersjukhuset between October 2014 and October 2015, and the. Patients eligible for the study were P2Y12-inhibitor naïve patients with NSTEMI presenting at the emergency room. Inclusion criteria were as follows: 1) Diagnosis of NSTEMI (i.e., relevant symptoms associated with ischemic ECG changes (not categorized as STEMI) and/or relevantly increased cardiac markers). 2) An indication for a 180-mg ticagrelor LD. Exclusion criteria for the NSTEMI group were: 1) Ticagrelor contraindication; 2) Age <18 years; 3) Administration of any P2Y12 inhibitor during the week before inclusion; 4) Treatment with glycoprotein IIb/IIIa antagonists within 48 hours before inclusion; 5) Ongoing morphine treatment. The control group of **Study III** consisted of P2Y12-inhibitor naïve patients with SCAD, who underwent elective diagnostic coronary angiography at the cardiac catheterization laboratory at Södersjukhuset. Inclusion criteria for the SCAD control group were as follows: 1) Documented stable coronary artery disease; 2) Administration of a 180-mg ticagrelor LD. Exclusion criteria for the control group were: 1) Ticagrelor contraindication; 2) ACS within the last 3 months; 3) Age <18 years; 4) Administration of any P2Y12 inhibitor during the week before inclusion; 5) Treatment with glycoprotein IIb/IIIa antagonists within 48 hours before inclusion; 6) Ongoing morphine treatment.

Study IV (EU-no. 2015-002910-65 and NCT02942550) was a prospective, single-blinded, randomized, placebo-controlled, multicenter trial performed at Södersjukhuset and Karolinska University Hospital Huddinge in Stockholm, Sweden. Patient inclusion was performed between November 2016 and December 2017. Patients considered for inclusion in the study were P2Y12-inhibitor naïve patients with STEMI presenting at the cardiac catheterization laboratory. Inclusion criteria were: 1) Diagnosis of STEMI including presence of typical symptoms, e.g. chest pain for more than 15 minutes together with new ST-segment elevation (>1mm (0.1mV) in at least two contiguous leads in the absence of left branch bundle block (LBBB) or signs or left ventricular hypertrophy on a 12-lead ECG; 2) Intake of a 180 mg oral LD of ticagrelor given before initiation of coronary angiography; 3) Analgesic treatment with intravenous morphine administered before initiation of coronary angiography. Exclusion criteria were: 1) Cardiac arrest; 2) Body weight >114 kg; 3) Vomiting after

ticagrelor intake; 4) Treatment with naloxone before inclusion or during the sampling period; 5) Inability to understand study outline and instructions; 6) Any methylnaltrexone bromide contraindication, including known hypersensitivity to the active substance or to any of the excipients and/or known or suspected mechanical gastro-intestinal obstruction or other acute surgical abdominal conditions; 7) Age <18 years; 8) Women in fertile age; 9) Administration of ticagrelor, clopidogrel, or prasugrel within 7 days before onset of STEMI symptoms; 10) Treatment with cangrelor; 11) Ongoing long-term opioid treatment.

3.2 CLINICAL INTERVENTIONS AND PROCEDURES

In Study I and II, all patients not previously on clopidogrel and/or aspirin treatment received a LD of clopidogrel (150 to 800 mg) in addition to aspirin (300 to 500-mg LD, followed by 75 mg/day) before coronary angiography. If PCI was performed, a daily maintenance dose of clopidogrel 75 mg was post-procedurally recommended in addition to aspirin for one year in patients receiving drug-eluting stents, whereas 3 months of dual antiplatelet treatment was recommended to patients receiving bare-metal stents. Patients already receiving clopidogrel treatment for >5 days before coronary angiography did not receive additional LD, but continued with their daily maintenance dose (75 mg once daily). Ten patients were receiving warfarin treatment, which was discontinued ≥7 days before coronary angiography. All interventions were performed according to international guidelines (57, 58). The femoral approach was used in all but 32 interventions, in which the radial approach was used, and unfractionated heparin was given in weight-adjusted doses (50 to 100 IE/kg). The sheath size was 6 Fr. A vascular closure device (Angio-Seal®; St. Jude Medical, St. Paul, Minnesota) was used in 247 patients. A compression assist device (Femostop®; St. Jude Medical) was used in the rest of the cohort and in patients with vascular closure devices when required for hemostasis. Use of periprocedural antiplatelet agents other than clopidogrel and aspirin, for example, glycoprotein IIb/IIIa inhibitors, was at the discretion of the interventionists.

In **Study III**, NSTEMI patients were included in the study upon arrival to the emergency room before receiving a 180-mg LD of integral ticagrelor pills immediately after NSTEMI diagnosis, as prescribed by the responsible physician. For the control group, the responsible physician decided to administer a LD of 180 mg ticagrelor after examining the coronary anatomy and before initiation of PCI.

Study IV included P2Y12-inhibitor naïve patients with STEMI presenting at the respective cardiac catheterization laboratory. All interventions and additional medication were performed at the discretion of the interventional cardiologists.

3.3 STUDY INTERVENTIONS

In **Study I and II**, a 4-ml blood sample was drawn from the arterial line at the start of each coronary angiography procedure for assessment of platelet function. A research nurse, who was well familiar with the testing equipment and had received training from the manufacturer, conducted all blood sampling and subsequent platelet function testing. The

physicians responsible for the patients during their hospital stays were not aware of the platelet function test results. Written informed consent was obtained from all patients.

In **Study III**, samples of venous blood were collected for pharmacokinetic assessment into lithium heparin tubes and placed on ice at the time-points shown in TABLE 1. The blood samples were then centrifuged at 1500 g at 4ºC for 10 min within 30 min of blood sampling. The resulting plasma samples were within 30 min of centrifugation stored at the Södersjukhuset's biobank below −20ºC until analyzed. Samples of venous blood were taken into hirudin tubes for pharmacodynamic evaluation, at the time-points shown in TABLE 1. The first 3–5mL of blood was discarded to avoid spontaneous platelet activation.

TABLE 1. Blood sampling for pharmacokinetic and pharmacodynamic assessment in Study III

	Time (h) relative to ticagrelor LD						
	Pre-dose	1	2	3	4	5	6
Ticagrelor pharmacokinetic sampling	x	x	x	x	x	x	x
ADP-induced platelet aggregation	x	x	x	x	x	x	x

In **Study IV**, oral consent was given upon arrival to the cardiac catheterization laboratory. Patients who fulfilled all the inclusion criteria and no exclusion criteria were randomized to either active treatment with methylnaltrexone or placebo, as specified below. All patients were asked to leave a written informed consent after completion of PCI. Randomization was performed thorough pre-sealed envelopes in a 1:1 fashion in blocks of four patients. To facilitate reproducibility, randomization was performed with the tool available at www.randomization.com, which enables simple block randomization using equal fixed block sizes. Stratification was performed for the two participating centers and also for inferior versus anterior or lateral STEMI.

In **Study IV**, blood samples were taken for pharmacodynamics and pharmacokinetic evaluation. This was done at the start of the coronary angiography from the arterial line to avoid possible sampling failure that could conceivably delay the PCI. The responsible personnel in the cardiac catheterization laboratory administered the study drug methylnaltrexone (Relistor®) or placebo after initial blood sampling. Methylnaltrexone was given as a single intravenous injection of 8 mg (0.4 ml solution) to patients weighing 38–61 kg or 12 mg (0.6 ml solution) to patients weighing 62–114 kg. The placebo treatment of 0.9% sodium chloride was given as a single intravenous injection of 0.4 or 0.6 ml according to the same weight schedule as the study drug. The study drug or placebo was administered using an unlabeled injection syringe, thus blinding patients to their respective treatment. Patients were asked to state self-estimated pain using a visual analogue scale (VAS) at baseline, at one, and two hours.

3.3.1 Assessment of platelet inhibition

In **Study I and II**, assessment of ADP-induced platelet aggregation was performed with the Plateletworks® assay (Helena laboratories, Beaumont, Texas). The test was performed <10 minutes after blood sampling. The baseline platelet count was obtained by the addition of 1 ml whole blood to the first tube, primed with the synthetic anticoagulant ethylenediaminetetraacetic acid (EDTA). One milliliter of whole blood was then added to the second tube, containing citrate and adenosine diphosphate (20 µmol), inducing platelet aggregation (FIGURE 1). For each tube, the platelet count was then measured with a cell counter (ABX Micros 60; Horiba ABX Diagnostics, Holliston, Massachusetts). Because platelet aggregates exceed normal platelet size, it is possible for the cell counter to discriminate between aggregated and non-aggregated platelets on the basis of size. The difference in platelet count between the two samples was used as a measurement of platelet aggregation.

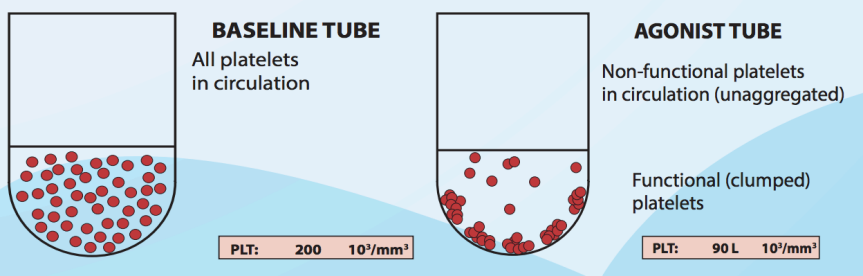

FIGURE 1. Agonist-induced platelet aggregation with examples of platelet count (reprinted with permission from Helena laboratories, Beaumont, Texas)

For **Study III**, analysis of ADP-induced platelet aggregation in whole blood was performed with multiple electrode aggregometry (MEA) using 6.5 µmol ADP as agonist within 2 hours of blood sampling. All materials used were obtained from the manufacturer (Roche Diagnostics International Ltd, Rotkreuz, Switzerland). High on-treatment platelet reactivity (HPR) was defined as > 46 ADP-induced aggregation units (AU) (59).

In **Study IV**, an initial baseline blood sample was taken from the arterial line and further blood sampling was performed at one and two hours after the patients received either methylnaltrexone or placebo. Samples of venous blood were taken into 0.109 M trisodium citrate tubes for pharmacodynamic evaluation. Blood samples were stored at room temperature and within 48 hours activated and lysed by the participating research nurses according to the manufacturer's instructions. The blood samples were then immediately frozen and stored below -20°C until analysis, as previously described (37). Analysis of P2Y12 inhibition blinded for study drug/placebo was performed centrally at Södersjukhuset by determination of platelet reactivity index (PRI) with an ELISA-based assay for the measurement of phosphorylated vasodilator-associated stimulated phosphoprotein (VASP) (CY-QUANT VASP/P2Y12, BioCytex, Marseilles, France).

3.3.2 Measurements of drug concentrations

In **Study III**, plasma concentrations of ticagrelor and its active metabolite AR-C124910XX were determined by validated methods (high-performance liquid chromatography and tandem mass spectrometry detection; LC-MS/MS) at a certified laboratory (Covance Central Laboratories, Indianapolis, Indiana, USA). The lower limits of detection were 2.50 ng/mL for both ticagrelor and AR-C124910XX.

For pharmacokinetic assessment in **Study IV**, blood samples were collected into lithium-heparin tubes and then cooled prior to centrifugation at 1500 g for 10 min at 4ºC. The resulting plasma samples were then stored at the Södersjukhuset's biobank below −20ºC until analyzed. Plasma concentrations of ticagrelor and its active metabolite, AR-C124910XX, were determined by a liquid chromatography – high resolution mass spectrometry method (LC-HRMS) and followed an earlier published procedure (60). Plasma concentrations of morphine were analyzed using the same methods. The used instrument was an UHPLC-Q Exactive high-resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a Dionex 3000 UltiMate LC system consisting of an ultra-high pressure dual pump, an auto-sampler, solvent degasser, and a thermostated column oven. The TraceFinder software v4.2 was used for instrument control and data evaluation.

After thawing, 0.1 mL of plasma was added into a 7 mL glass test tube. Thereafter, 0.2 mL of acetonitrile containing internal standards (10 ng ticagrelor-d7, 10 ng ticagrelor metabolite-d7, 8 ng morphine-d3) was added during vortexing. The prepared sample was centrifuged at 3400g for 5 minutes and 150 µL was transferred into a new glass-test tube. After evaporation to dryness in a vacuum centrifuge the residue was re-dissolved in 60 µL of 50% acetonitrile (10% for morphine) and transferred into an auto-sampler vial.

A volume of 1 µL was injected into the LC-HRMS system. Chromatographic separation was achieved using a Hypersil C18 column (particle size 1.9 µm, 2.1 mm x 100 mm, Thermo Scientific) operating at a column temperature of 40°C, and using gradient elution with a buffer A (0.1 % formic acid in water) and a buffer B (0.1% formic acid in acetonitrile) with a flow rate of 0.5 mL/min. The MS was operated in full scan positive electrospray ionization mode at 70,000 resolution setting. Extracted ion chromatograms with 10 ppm tolerance were used for peak area measurements. The exact masses of the protonated molecules were m/z 523.1934 for ticagrelor and 530.2373 for the d7 analogue, m/z 479.1671 for ticagrelor metabolite and 486.2111 for the d7 analogue, m/z 286.1438 for morphine and 289.1626 for the d3 analogue.

Calibration curves using fortified plasma standards were using the following concentrations: 5, 30, 100, 200, 600, 1200, 2000 ng/mL for ticagrelor and ticagrelor dealkylated metabolite (ALSAChim, Strasbourg, France), and 2, 12, 40, 80 ng/mL for morphine (Cerriliant Co, Round Rock, Texas, USA). Internal standards were from the same companies. The correlation coefficients (r) of the respective calibration curves generated during the validation was >0.999 for all three analytes. The results of the quality controls run together with the

study samples are described in TABLE 2. The lower limits of detection were 5.0 ng/mL for both ticagrelor and AR-C124910XX.

TABLE 2. **Drug concentration analysis quality control**				
Analyte	Assigned concentration, ng/mL	Measured concentration, ng/mL	Coefficient of variation (%)	n
Ticagrelor	15	17.0	5.5	10
	180	195	5.1	10
	1000	1131	4.6	10
AR-C124910XX	15	14.0	6.4	10
	180	188	1.9	10
	1000	1069	1.6	10
Morphine	6	5.6	2.5	10
	72	69	2.2	10

3.4 FOLLOW-UP AND END-POINTS

The primary end-point in **Study I** was myocardial infarction (ischemic symptoms and a spontaneous increase in biochemical cardiac markers, i.e. not periprocedural or postprocedural infarction). Moreover, secondary end-points included definite stent thrombosis (confirmed via angiography), death, and a composite endpoint (nonfatal myocardial infarction, all-cause death, or definite stent thrombosis) as in the Do Platelet Function Assays Predict Clinical Outcomes in Clopidogrel-Pretreated Patients Undergoing Elective PCI (POPULAR) study (34). Other secondary end-points were rehospitalization due to cardiovascular causes, rehospitalization due to chest pain specifically, reangiography, and confirmed stenosis in another coronary vessel. Patients were followed for three months after coronary angiography. Clinical outcome data were collected from the electronic medical record system in Stockholm, which also is continuously and accurately updated regarding all deaths in Sweden via the electronic online Swedish Population Register. Further follow-up was done via the Swedish Web-system for Enhancement and Development of Evidence-based care in Heart disease Evaluated According to Recommended Therapies (SWEDEHEART) registry, which continuously gathers information about patient's clinical presentation, complications from interventions, and outcomes for all coronary procedures, including surgical interventions. The compliance of clopidogrel during the follow-up was monitored in all patients via follow-up phone calls. One person was responsible for collecting of follow-up data and was not aware of platelet function data.

For **Study II**, data on in-hospital bleeding events were prospectively acquired, including location and extent, laboratory data, imaging data, medications, and treatment. The data on out of hospital bleeding events that did not require direct visits to health care professionals were registered at later routine follow-up visits. The study database and patients' medical records were re-examined for every bleeding event by two researchers blinded to platelet aggregation to classify them according to the bleeding definitions. The primary outcome was the 30-day incidence of bleeding complications after coronary angiography in relation to quartile distribution of on-treatment platelet reactivity measured by Plateletworks®. Bleeding was defined according the criteria of BARC. In essence, this bleeding definition classifies bleeding events as: **Type 0** (no bleeding); **Type 1** (minor bleeding which does not require

treatment by a healthcare professional), **type 2** (any overt actionable sign of hemorrhage that requires intervention, hospitalization, increased level of care, or prompting evaluation); **Type 3a** (Overt bleeding causing a hemoglobin drop of 30 to <50 g/L, or require transfusion); **Type 3b** (Overt bleeding causing: a hemoglobin drop of ≥50 g/L, cardiac tamponade, need for surgical intervention, need for vasoactive agents); **Type 3c** (intracranial hemorrhage, intraocular bleeding compromising vision); **Type 4** (CABG-related intracranial bleeding within 48 hours, reoperation after sternum closure to control bleeding, ≥5 red packed blood cells transfused within 48h, ≥2L chest tube output within 24h); **Type 5** (5a probable fatal and 5b definite fatal bleeding) (28). Moreover, **Study II** included the bleeding definition used in the ARMYDA-BLEEDS study (>10-cm hematoma, pseudoaneurysm, arteriovenous fistula, or major Thrombolysis In Myocardial Infarction (TIMI) bleeding criterion: intracranial or associated with a decrease in hemoglobin ≥50 g/L or >15% drop in hematocrit) (32). The National Cardiovascular Data Registry (NCDR) Cath- PCI Registry model was used for retrospective evaluation of preprocedural risk for bleeding. This predictive risk score is based on the variables ST-segment elevation myocardial infarction, age, body mass index, previous PCI, chronic kidney disease, shock, cardiac arrest <24 hours, gender, hemoglobin level, and PCI status (61). After returning to a referring hospital, 1 patient died from momentary iatrogenic lung bleeding during a chest drainage procedure, which aimed to evacuate a pleural effusion. This bleeding event was not regarded as an outcome for this study, because according to the autopsy protocol, it was a direct result of a surgical trauma.

In **Study III**, the primary endpoint was the time to maximum concentration (T_{max}) of ticagrelor after a 180 mg LD. Secondary endpoints were T_{max} of the active ticagrelor metabolite AR-C124910XX, evaluation of the relationship between plasma concentrations of ticagrelor and the pharmacodynamic response after a 180-mg ticagrelor LD, and frequency of HPR at 2 hours after a 180-mg ticagrelor LD. The safety endpoint was the occurrence of any serious adverse events during the sampling period.

In **Study IV**, the primary endpoint was the prevalence of HPR, defined as PRI ≥50% determined by the VASP assay (62) two hours after randomization and subsequent intravenous injection of either study drug or placebo. Secondary endpoints were: 1) Differences in ticagrelor and AR-C124910XX concentrations at one and two hours after randomization; 2) Difference in PRI at one and two hours after randomization; 3) Difference in patients´ subjective pain according to VAS.

3.5 ETHICS

All studies were conducted in accordance with the Declaration of Helsinki. **For Study I, II, and III,** written informed consent was obtained from all patients prior to any study-related procedures. The regional human research ethics committee in Stockholm, Sweden, approved the studies (reference numbers **Study I and II**: 2006/272-31/2 and 2013/604-32, **Study III**: 2014/1174-31/1 and 2014/2131-32).

In **Study IV**, an initial oral consent was given upon arrival to the cardiac catheterization laboratory. All patients were asked to leave a written informed consent after completion of PCI. **Study IV** was approved by the Swedish Medical Product Agency (EU-no. 2015-002910-65) and the regional ethical review board in Stockholm (reference numbers 2015/1911-31/4, 2016/838-32, 2016/1983-32, 2016/1824-32, and 2017/413-32).

3.6 STATISTICS

3.6.1 Sample size calculations

The sample size calculation for **Study I** was primarily based on a similar study conducted by Matetzky et al., which included 70 patients with STEMI. Their study was based on testing with ADP-induced aggregometry to evaluate platelet reactivity, and 88% of the events occurred in the quartile with clopidogrel resistance (63). For **Study I**, with a more unselected patient population, a lower frequency of patients with STEMI was expected. Internal data from the Karolinska University Hospital had previously shown an incidence around 5% of adverse cardiovascular events within 12 months after PCI. In **Study I**, an assumption based on the study by Matetzky et al. was made that 80% of the events would be in the two quartiles with the highest platelet aggregation while on clopidogrel treatment (64). The power calculation showed that with 90% power it would require 424 patients to demonstrate a significant difference between the groups ($P<0.05$).

For **Study II,** a sample size calculation was done on the basis of the ARMYDA-BLEEDS study, which showed 10.1% and 1.3% incidence rates of bleeding in the first and fourth platelet aggregation quartiles, respectively (32). For **Study II**, assuming that the bleeding event rate would be similar, it would require 85 patients in each platelet aggregation quartile (a total of 340 patients) to show a statistical difference between the first and the fourth quartiles ($p <0.05$, 80% power).

For **Study III**, the median T_{max} in the SCAD control group was before the study expected at approximately 2 hours after a 180 mg LD, with a range between 1 and 8 hours (47). A rough standard deviation estimate of 1.25 hours was obtained from this range using the formula $\frac{\ln(\max Tmax)-\ln(\min Tmax)}{k}$ from a statistical textbook by Dixon et al (65). A statistician estimated k to 9.5 from a natural distribution table. The estimated standard deviation of 0.22 on the logarithm scale was then calculated using the calculation above. For the study group with NSTEMI, a larger standard deviation can be expected and was estimated with the following formula provided by the statistician: $\sqrt{(0.22^2)\times 2} = 0.31$. To make a sample size calculation possible, the above calculated standard deviations were anti-logged using the exponential function and found to be 1.25 hours and 1.36 hours, respectively. For the power calculation, a 50% delay in median ticagrelor T_{max} (1 hour delay) was considered, as this can be regarded as a clinical significant difference. A power calculation with the above described time to T_{max} and estimated standard deviations, showed that a study with 80% power would require 40 patients in the study group and 20 patients in the control group.

For **Study IV,** The sample size calculation was based on results from a previous sub-study of the ATLANTIC trial (37), which showed that STEMI patients treated with morphine had a delayed onset of platelet inhibition after a 180 mg LD of ticagrelor. In patients treated with morphine, 14 out of 22 patients (64%) had a high on treatment platelet reactivity (HPR), defined as VASP-PRI ≥50%, 3 hours after study inclusion (around 2 hours after PCI). Among the STEMI patients who did not receive morphine 3 out of 15 patients (20%) had a HPR 3 hours after study inclusion. A sample size calculation showed that at least 25 patients would be needed in each group to obtain a significant difference between the groups ($p<0.05$, 90% power). To allow for stratification according to inferior or lateral/anterior STEMI, we aimed to include 40 patients in each group.

3.6.2 Statistical analyses

In **Study I-III**, continuous variables were expressed as mean and SD for normally distributed continuous variables, and compared using the Student's t-test, whereas non-normally distributed variables were expressed as median with interquartile range (IQR) and compared with the Mann-Whitney U-test/Wilcoxon rank-sum test. Categorical variables were in **Study I-IV** presented as no. (%) and compared using the $\chi 2$-test or the Fisher's exact test. A two-tailed P-value <0.05 was considered significant. In **Study I and II**, receiver operating characteristic (ROC) curve analysis was performed to establish a cutoff of platelet aggregation with the Plateletworks® assay for the prediction of the respective endpoints.

Statistical analyses were performed using SPSS version 20.0 in **Study I**, version 21.0 in **Study II**, version 22.0 in **Study III**, and version 24.0 in **Study IV** (IBM Corp, Armonk, NY). Moreover, Stata statistical software: Release 14 (StataCorp LP, College Station, TX) was used in **Study IV**.

In **Study I**, Spearman's rank correlation was used to compare variables that were not normally distributed. Unadjusted odds ratio (OR) calculations for the primary and secondary end-points were performed. Survival analysis was performed via Kaplan–Meier curve, and differences between groups were analyzed with the log-rank test. To identify potential confounders and variables that correlated with HPR, a logistic regression analysis was performed. The variables included were age, sex, BMI, diabetes, smoking, LD or no LD, and co-medication (including proton pump inhibitors and statins).

For **Study II**, Patients were divided into quartiles according to ADP-induced platelet aggregation. Normal distribution for continuous variables was tested with the Shapiro-Wilk test. To identify possible variables associated with bleeding, an initial univariate Cox regression analysis was performed. Variables with p values <0.10 in the univariate analysis were considered for the stepwise forward and backward manual multivariate Cox regression analysis. The bleeding events in the different quartiles over time were visualized with Kaplan-Meier curves.

In **Study III,** Continuous variables were tested for normality using the Shapiro-Wilk test. For inference, the Student's t-test was used for normally distributed variables and the Mann–

Whitney U-test for non-normally distributed variables. Categorical variables were described as numbers with percentages and compared with either the $\chi 2$-test or Fisher's exact test. Calculations of the area under the curves (AUC) were performed using the trapezoid rule for the ticagrelor and AR-C124910XX concentrations. A Spearman's correlation analysis between the ticagrelor concentrations and the platelet aggregation was performed.

In **Study IV**, a conservative approach was taken where all continuous variables were described using median and IQR and tested using non-parametric tests due to the relatively small sample size, and because many variables were non-normally distributed.

4 RESULTS

4.1 STUDY I

In total, 491 patients on aspirin and clopidogrel were included and clinical outcome was available for all patients except one (0.2%). ROC curve analysis assessed if platelet function testing could differentiate between patients with and without myocardial infarction (primary endpoint) within the 3 months follow-up (FIGURE 2). The area under the curve (AUC) was 0.60 (95% CI 0.49–0.70) with a sensitivity of 66.7% and a specificity of 60.9%. The optimal cutoff was found to be 82.3% platelet aggregation. Patients were grouped into HPR and normal on-treatment platelet reactivity using this cutoff.

Demographic characteristics are listed in TABLE 2. In total, 196 patients (39.9%) had HPR (≥82.3% on-treatment platelet aggregation). Patients with HPR group had significantly higher BMI (28.3±4.4 vs. 26.7±4.6, p<0.001). Moreover, PCI was more common in the HPR group (62.8 vs. 51.9%, p=0.02) and they received a clopidogrel LD more frequently (78.1 vs. 67.1%, p=0.01). Analysis with logistic regression showed that independent predictors of HPR were age calculated for a 10-year increase (OR, 1.26; CI, 1.03–1.54; p=0.02), clopidogrel LD received (OR, 1.73; CI, 1.04–2.87; p=0.04), and BMI using a 10-unit increase (OR, 2.34; CI, 1.43–3.82; p= 0.001).

As depicted in TABLE 3, the primary end-point myocardial infarction within the three months follow-up was significantly more frequent in patients with HPR (5.1 vs. 1.7%; OR, 3.12; CI, 1.05–9.27; p=0.03). Moreover, rehospitalization within three months for cardiovascular causes was significantly more common in patients with HPR (23.0 vs. 14.2%, OR 1.80, CI 1.13–2.86, p=0.01, TABLE 3).

The difference in the incidence of myocardial infarction is also illustrated in FIGURE 3. Myocardial infarction was more common in patients receiving a clopidogrel LD compared with those already on maintenance therapy at inclusion (14 of 351 vs. 1 of 140; p=0.06). Of the 276 patients who underwent PCI, 9 (3.3%) experienced a myocardial infarction within 3 months, compared with 6 (2.8%) of the 215 patients who underwent coronary angiography without PCI (p= 0.76).

TABLE 2. *Demographic characteristics including comparisons between patients with and without HPR*

Characteristic	Total cohort* (n=491)	On-treatment platelet reactivity: Normal, <82.3% aggregation* (n=295, 60.1%)	On-treatment platelet reactivity: High, ≥82.3% Aggregation* (n=196, 39.9%)	p value
Clinical parameters				
Age, years	65.0 ± 10.7	64.4 ± 10.1	66.0 ± 11.4	0.11
Male gender	382/491 (77.8)	226 (76.6)	156 (79.6)	0.44
BMI, kg/m^2	27.3 ± 4.6	26.7 ± 4.6	28.3 ± 4.4	**<0.001**
Hypertension†	268/490 (54.7)	156 (53.1)	112 (57.1)	0.37
Diabetes mellitus	122/491 (24.8)	71 (24.1)	51 (26.0)	0.62
Current smoking	95/491 (19.3)	57 (19.3)	38 (19.4)	0.99
Family history‡	208/481 (43.2)	123 (42.7)	85 (44.0)	0.77
Prior myocardial infarction	146/490 (29.7)	87 (29.5)	59 (30.1)	0.89
Prior PCI	105/491 (21.4)	60 (20.3)	45 (23.0)	0.49
Prior CABG	71/490 (14.5)	46 (15.6)	22 (12.8)	0.37
Medication				
Aspirin	470/491 (95.7)	282 (95.6)	188 (95.9)	0.86
GP IIb/IIIa-inh. prior to Plateteletworks® testing	16/491 (3.3)	12 (4.1)	4 (2.0)	0.22
Received clopidogrel LD	351/491 (71.5)	198 (67.1)	153 (78.1)	**0.01**
-800 mg	1/491 (0.2)	1 (0.3	0 (0.0)	1.0
-600 mg	98/491 (20.0)	56 (19.0)	42 (21.4)	0.51
-450 mg	5/491 (1.0)	3 (1.0)	2 (1.0)	1.0
-300 mg	244/491 (49.7)	137 (46.4)	107 (54.6)	0.08
-150 mg	3/491 (0.6)	1 (0.3)	2 (1.0)	0.57
Time from LD to coronary angiography				
<6 hours	20/432 (4.6)	6 (2.4)	14 (7.7)	**<0.01**
6-24 hours	92/432 (21.3)	40 (15.9)	52 (28.7)	**0.001**
Already on clopidogrel maintenance dose	140/491 (28.5)	97 (32.9)	43 (21.9)	**0.01**
Lipid-lowering drug	417/491 (84.9)	244 (85.9)	173 (83.6)	0.47
Proton pump inhibitors	79/491 (16.1)	48 (16.9)	31 (15.0)	0.57
Laboratory data				
Platelet count, x10^9/L	220.8 ± 59.9	220.8 ± 59.9	219.9 ± 56.2	0.87
Procedural characteristics				
Acute coronary angiography	352/491 (71.7)	213 (72.2)	139 (70.9)	0.76
PCI performed	276/491 (56.2)	153 (51.9)	123 (62.8)	**0.02**

*****Data are presented as mean ± SD for quantitative variables, and as no. (%) for qualitative variables.

† Defined as documented and treated hypertension.

‡ Defined as documented family history or stated by patients themselves. Regarded significant with first degree relatives <50 years with cardiovascular disease.

Abbreviations: BMI, body mass index; CABG, coronary artery bypass graft surgery; HPR, high on-treatment platelet reactivity; PCI, percutaneous coronary intervention.

TABLE 3. *Clinical outcome at 3 months post coronary angiography*

	On-treatment platelet reactivity			
Clinical event (≤ 3 months)	**Normal, <82.3% aggregation*** (n=295, 60.1%)	**High, ≥82.3% Aggregation*** (n=196, 39.9%)	**OR (95% CI)**	**p value**
Primary end-point				
Myocardial infarction	5 (1.7)	10 (5.1)	3.12 (1.05-9.27)	**0.03**
Secondary end-points				
Stent thrombosis	3 (1.0)	4 (2.0)	2.03 (0.45-9.16)	0.45
Death	5 (1.7)	7 (3.6)	2.15 (0.67-6.87)	0.24
Composite end-point†	11 (3.7)	14 (7.1)	1.99 (0.88-4.47)	0.09
Re-hospitalization‡	42 (14.2)	45 (23.0)	1.80 (1.13-2.86)	**0.01**
Re-hospitalization due to chest pain	30 (10.2)	33 (16.8)	1.79 (1.05-3.04)	**0.03**
Re-angiography	21 (7.1)	18 (9.2)	1.32 (0.68-2.55)	0.41
Stenosis in another vessel	6 (2.0)	8 (4.1)	2.05 (0.70-6.00)	0.18

*Data are presented as No. (%).
† Includes all-cause death, as well as non-fatal myocardial infarction and stent thrombosis.
‡ Re-hospitalization due to cardiovascular causes.
Abbreviations: OR, odds ratio; CI, confidence interval.

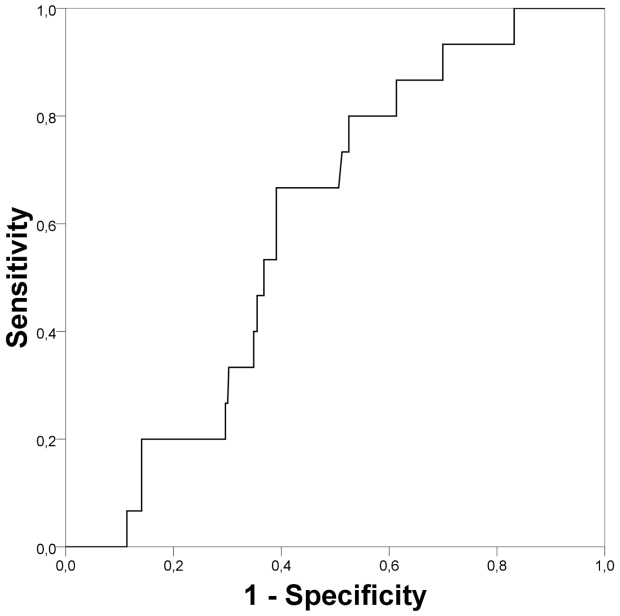

FIGURE 2. Receiver operating characteristics (ROC) curve. ROC curve analysis showing correlation between platelet aggregation and myocardial infarction (primary endpoint).

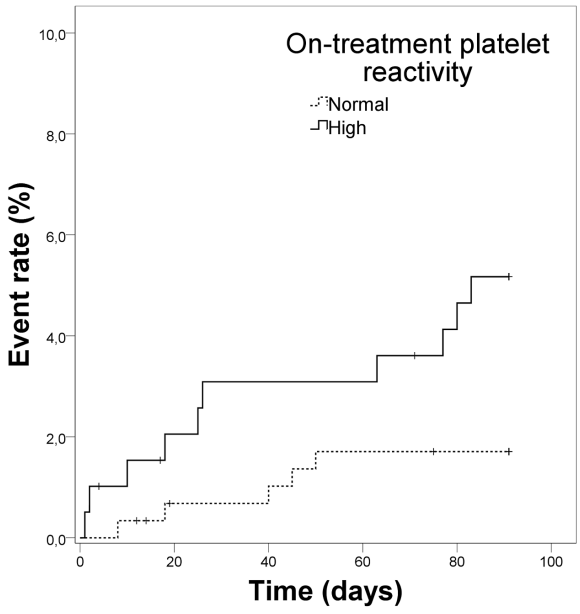

FIGURE 3. Kaplan-Meier curve
The graph shows the cumulative incidence of myocardial infarction within three months. Patients with on-treatment platelet reactivity had a significantly higher incidence, log-rank: p=0.03.

4.2 STUDY II

The patients (n=474) were divided into quartiles according to on-treatment platelet reactivity. Demographic data are shown in TABLE 4. The clopidogrel LDs were found evenly distributed between the different quartiles. The time from clopidogrel LD to coronary angiography was higher in quartile 1 compared with quartile 4.

Procedural characteristics and clinical data are shown in TABLE 5. STEMI was significantly more common in the highest quartile of platelet aggregation compared with the lowest quartile.

Data on bleeding events within 30 days were available in all patients but one (0.2%). Two patients had bladder or urethral bleeding complications, three had gastrointestinal bleedings, one had an intracranial bleeding, one had an eye bleeding, and the remaining bleeding complications were entry-site bleedings of different degree. The most extensive bleeding was included in the analysis if more than on bleeding event occurred within 30 days.

The incidence of type ≥1 BARC bleeding was 26.8% (127 of 474 patients; TABLE 6). Patients who had type ≥1 BARC bleeding had a higher median NCDR CathPCI risk score compared with those without bleeding (70 [IQR 55 - 80] vs. 60 [IQR 50 - 75], p=0.019). Median platelet aggregation was lower in patients with type ≥1 BARC bleeding compared with those without bleeding (51.0% [IQR 32.8% - 84.0%] vs. 79.1% [IQR 52.5% - 92.0%], p= 0.001). As shown in TABLE 6, Patients in the lowest quartile of platelet aggregation had a higher incidence of ≥1 BARC bleeding compared with the fourth quartile and the third quartile, respectively.

The incidence of type ≥2 BARC bleeding within 30 days was 10.8% (51 of 474 patients; TABLE 6). Patients who had type ≥2 BARC bleeding had a higher median NCDR CathPCI risk score compared with those with type 0 or type 1 bleeding (70 [IQR 60 - 85] vs. 65 [IQR 50 - 75], p= 0.035). The median platelet aggregation was lower in patients with type ≥2 BARC bleeding compared with patients with type 0 or type 1 bleeding (59.9% [IQR 1.5% - 83.8%] vs. 76.9% [IQR 48.0% - 91.1%], p= 0.005). As shown in TABLE 6, the first platelet aggregation quartile had a higher frequency of type ≥2 BARC bleeding within 30 days compared with the fourth quartile and the third quartile, respectively. Clopidogrel-naïve patients did not have a significantly different incidence of type ≥2 BARC bleeding compared with those already on a clopidogrel maintenance dose (10.7% vs. 10.9%, p= 0.932). The frequency of type ≥2 BARC bleeding complications over time is depicted in FIGURE 4.

The first quartile of platelet aggregation predicted type ≥2 BARC bleeding in the multivariate Cox regression, when adjusted for NCDR the other included variables (TABLE 7). The NCDR risk score did also significantly predict type ≥2 BARC bleeding complications in the model, adjusted for the other included variables. ROC curve analysis showed a significant correlation between type ≥2 BARC bleeding and on-treatment platelet aggregation, with an area under the curve of 0.62 (95% confidence interval [CI] 0.54 - 0.70, p= 0.005). The optimal cutoff was 76.7% platelet aggregation, with 71% sensitivity and 51% specificity. The incidence of

type ≥2 BARC bleeding was 14.7% (36 of 245 patients) in patients with ≤76.7% platelet aggregation and 6.6% (15 of 229 patients) in patients with >76.7% platelet aggregation (relative risk 2.2, 95% CI 1.3 - 4.0, p= 0.004).

As listed in TABLE 6, the incidence of ARMYDABLEEDS-defined bleeding within 30 days was 4.6% (22 of 474 patients). Patients who had an ARMYDA-BLEEDS-defined bleeding event within 30 days had an equal median NCDR CathPCI risk score compared with those without bleeding (65 [IQR 60 to 76.25] vs. 65 [IQR 50 to 80], p=1.00). Median platelet aggregation was lower in patients with ARMYDABLEEDS-defined bleeding compared with patients without bleeding (50.1% [IQR 31.8% - 75.3%] vs. 76.4% [IQR 47.0% - 91.0%], p= 0.016). As shown in TABLE 6, patients in the lowest platelet aggregation quartile had a higher occurrence of ARMYDABLEEDS-defined bleeding within 30 days compared with the third quartile and the fourth quartile, respectively. ARMYDA-BLEEDS-defined bleeding did not differ significantly between clopidogrel-naïve patients and those already on clopidogrel maintenance treatment (4.2% vs. 5.8%, p= 0.429). The cumulative incidence of ARMYDA-BLEEDS-defined bleeding events over time is depicted in FIGURE 5.

In the multivariate Cox regression the lowest quartile of platelet aggregation predicted increased risk for ARMYDA-BLEEDS-defined bleeding within 30 days, when adjusted for the other included variables (TABLE 7). The ROC curve analysis platelet function testing was able to distinguish between patients with and without ARMYDA-BLEEDS-defined bleeding, with an area under the curve of 0.65 (95% CI 0.54 - 0.76, p= 0.016). The optimal platelet aggregation cutoff was found at 74.8% platelet aggregation, with 77% sensitivity and 52% specificity. The incidence of ARMYDABLEEDS- defined bleeding within 30 days was 7.3% (17 of 233 patients) in patients with ≤74.8%platelet aggregation and 2.1% (5 of 241 patients) in patients with >74.8% platelet aggregation (relative risk 3.5, 95% CI 1.3 - 9.4, p= 0.007).

TABLE 4. *Demographic characteristics*

Characteristic	1st Quartile (n = 118)	2nd Quartile (n = 119	3rd Quartile (n = 117)	4th Quartile (n = 120)	*P*-value*
Median [range] platelet aggregation	29% [0-46%]	63% [47-75%]	84% [75-91%]	96% [91-100%]	
Age (years)	63.9 ± 8.9	65.0 ± 11.2	65.9 ± 11.8	65.5 ± 10.9	0.20
Women	28 (24%)	31 (26%)	23 (20%)	23 (19%)	0.39
BMI (kg/m^2)	26.3 ± 5.0	26.9 ± 3.9	27.3 ± 5.4	28.7 ± 4.4	**<0.001**
Hypertension†	65 (55%)	62 (52%)	58 (50%)	73 (61%)	0.37
Diabetes mellitus	23 (19%)	35 (29%)	29 (25%)	32 (27%)	0.19
Current smokers	19 (16%)	24 (20%)	27 (23%)	21 (18%)	0.77
Prior myocardial infarction	33 (28%)	36 (30%)	36 (31%)	36 (30%)	0.73
Prior percutaneous coronary intervention	25 (21%)	20 (17%)	29 (25%)	26 (22%)	0.93
Prior coronary bypass	20 (17%)	18 (15%)	16 (14%)	15 (13%)	0.44
NCDR® CathPCI Bleeding Risk (61)					
Total score (points)	63.3 ± 20.4	65.8 ± 20.1	64.3 ± 21.3	65.0 ± 21.7	0.63
- Low risk (<25)	-	1 (1%)	5 (4%)	3 (3%)	0.25
- Medium risk (26-65)	66 (56%)	64 (54%)	63 (54%)	64 (53%)	0.69
- High risk (>65)	52 (44%)	54 (45%)	49 (42%)	53 (44%)	0.99
Medication					
Clopidogrel LD (mg)					
150	-	1 (1%)	1 (1%)	1 (1%)	0.32
300	51 (43%)	59 (50%)	60 (51%)	64 (53%)	0.12
450	-	1 (1%)	2 (2%)	2 (17%)	0.50
600	22 (19%)	22 (18%)	22 (19%)	28 (23%)	0.38
800	-	1 (1%)	-	-	N/A
Time (hours) from LD to coronary angiography	47 [24 / 97]	69 [35 / 122]	45 [22 / 100]	27 [16 / 52]	**<0.001**
Time (hours) from last dose to coronary angiography	4.5 [2.5 / 6.0]	5.0 [3.0 / 6.0]	5.5 [3.5 / 6.5]	5.0 [3.0 / 7.0]	0.107
Clopidogrel maintenance treatment (months)	3 [1 / 3]	3 [0 / 3]	3 [0 / 3]	3 [1 / 3]	
Lipid-lowering drugs	102 (86%)	102 (86%)	96 (82%)	102 (85%)	0.75
Proton pump inhibitors	16 (14%)	23 (19%)	21 (18%)	16 (13%)	0.96
Fondaparinux	43 (36%)	47 (39%)	49 (42%)	52 (43%)	0.28
Bivalirudin	-	-	2 (2%)	1 (1%)	1.00

Data are expressed by number (percentage) for categorical variables, and as mean ±SD or median [25th / 75th percentile] for continuous variables. Quartiles were established for the percentage of platelet aggregation measured by adenosine diphosphate-induced single-platelet aggregation.

Abbreviations: LD, loading dose.

*Comparison between quartile 1 and quartile 4. †Defined as documented and treated hypertension.

TABLE 5. *Procedural characteristics*

Characteristic	1st Quartile (n = 118)	2nd Quartile (n = 119	3rd Quartile (n = 117)	4th Quartile (n = 120)	*P*-value*
Median [range] platelet aggregation	29% [0-46%]	63% [47-75%]	84% [75-91%]	96% [91-100%]	
Procedural characteristics					
Femoral approach	114 (97%)	112 (94%)	108 (92%)	110 (92%)	0.11
Radial approach	4 (3%)	7 (6%)	9 (8%)	10 (8%)	0.11
Vascular closing device	58 (49%)	57 (48%)	64 (55%)	68 (57%)	0.25
PCI performed	70 (59%)	56 (47%)	64 (55%)	75 (63%)	0.62
GP IIb/IIIa inhibitors	14 (12%)	9 (8%)	16 (14%)	14 (12%)	0.96
Laboratory data					
Platelet count, $x10^9$/L	220.9 ± 59.5	220.4 ± 51.4	219.1 ± 69.3	221.1 ± 53.8	0.98
Hemoglobin (g/L)	138.0 ± 16.4	136.8 ± 16.4	138.6 ± 16.1	140.8 ± 16.5	0.19
Hematocrit	0.39 ± 0.04	0.38 ± 0.05	0.38 ± 0.05	0.40 ± 0.04	0.16
Creatinine clearance (ml/min)†	86.6 ± 25.9	83.1 ± 29.2	87.5 ± 35.0	93.3 ± 31.7	0.11
Diagnosis at discharge					
STEMI	2 (2%)	2 (2%)	5 (4%)	11 (9%)	**0.01**
NSTEMI/unstable angina	68 (58%)	66 (55%)	67 (57%)	68 (57%)	0.88
Stable angina	36 (31%)	31 (26%)	31 (26%)	34 (38%)	0.71
Unspecific chest pain	5 (4%)	12 (10%)	6 (5%)	3 (3%)	0.50
Other‡	6 (5%)	6 (5%)	8 (7%)	4 (3%)	0.54

Data are expressed by number (percentage) for categorical variables, or as mean ±SD for continuous variables. Quartiles were established for the percentage of platelet aggregation measured by adenosine diphosphate-induced single-platelet aggregation. *Abbreviations:* GP, glycoprotein; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction; NSTEMI, non ST-elevation myocardial infarction. *Comparison between quartile 1 and quartile 4. †Estimated with Cockcroft-Gault equation. ‡Includes heart failure, aortic stenosis, arterio-ventricular block, and tachycardia.

TABLE 6. *Quartile distribution of bleeding events.*

	Total (n = 474)	1st Quartile (n = 118)	2nd Quartile (n = 119)	3rd Quartile (n = 117)	4th Quartile (n = 120)	*P*-value*
Median [range] of platelet aggregation	75% [0-100%]	29% [0-46%]	63% [47-75%]	84% [75-91%]	96% [91-100%]	
BARC bleeding						
Type 1	76 (16%)	28 (24%)	20 (17%)	16 (14%)	12 (10%)	**<0.01**
Type 2	39 (8%)	14 (12%)	12 (10%)	5 (4%)	8 (7%)	0.17
Type 3a	5 (1%)	3 (3%)	1 (1%)	1 (1%)	-	0.12
3b	5 (1%)	2 (2%)	1 (1%)	2 (2%)	-	0.25
3c	1 (1%)	-	-	1 (1%)	-	N/A
Type 4	1 (1%)	1 (1%)	-	-	-	0.50
Type 5a	-	-	-	-	-	N/A
5b	-	-	-	-	-	N/A
≥ Type 1	127 (27%)	48 (41%)	34 (29%)	25 (21%)	20 (17%)	**<0.001**
≥ Type 2	51 (11%)	20 (17%)	14 (12%)	9 (8%)†	8 (7%)	**0.014**
ARMYDA-BLEEDS defined bleeding						
Major TIMI bleeding	6 (1%)	4 (3%)	1 (1%)	1 (1%)	-	0.06
Entry site complications						
>10 cm hematoma	14 (3%)	5 (4%)	4 (3%)	3 (3%)	2 (2%)	0.28
Pseudoaneurysm	6 (1%)	1 (1%)	3 (3%)	-	2 (2%)	1.0
AV-fistula	-	-	-	-	-	N/A
Combined end point‡	22 (5%)	10 (8%)	7 (6%)	3 (3%)§	2 (2%)	**0.016**

Data are expressed by number (percentage) for categorical variables, or as mean ±SD for continuous variables. Quartiles were established for the percentage of platelet aggregation measured by adenosine diphosphate-induced single-platelet aggregation. *Abbreviations:* AV, arterio-venous; TIMI, Thrombolysis in Myocardial Infarction.

* Comparison between quartile 1 and quartile 4 †p = **0.031** (quartile 1 vs quartile 3). ‡ >10cm hematoma, pseudo-aneurysm, AV-fistula, or major TIMI bleeding (4 patients had 2 events each). § p = **0.048** (quartile 1 vs quartile 3).

TABLE 7. *Cox regression analysis*

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
BARC ≥ type 2 bleeding				
Quartile 1 (platelet aggregation)	1.99 (1.14 – 3.50)	**0.016**	2.08 (1.19 – 3.66)	**0.011**
NCDR risk score (10-point increase)*	1.17 (1.03 – 1.33)	**0.014**	1.18 (1.03 – 1.35)	**0.014**
Fondaparinux	1.23 (0.71 – 2.14)	0.457	1.04 (0.59 – 1.83)	0.902
Periprocedural GP IIb/IIIa inhibitors	1.26 (0.57- 2.79)	0.572	1.19 (0.54 – 2.65)	0.666
Diabetes	0.54 (0.26 – 1.15)	0.112	-	-
Bivalirudin	0.05 (0.0 – 2.01x10$^{6)}$	0.698	-	-
Angioseal	1.11 (0.64 – 1.93)	0.71	-	-
ARMYDA-BLEEDS defined bleeding				
Quartile 1 (platelet aggregation)	2.55 (1.10 – 5.89)	**0.034**	2.54 (1.10 – 5.90)	**0.029**
NCDR risk score (10-point increase) *	1.01 (0.83 – 1.24)	0.900	1.02 (0.82 – 1.26)	0.868
Fondaparinux	1.02 (0.44 – 2.40)	0.956	1.02 (0.42 – 2.50)	0.964
Periprocedural GP IIb/IIIa inhibitors	1.77 (0.60 – 5.22)	0.303	1.74 (0.59 – 5.14)	0.318
Diabetes	0.47 (0.14 – 1.58)	0.220	-	-
Bivalirudin	0.05 (0.0 – 7.1x10^{8})	0.801	-	-
Angioseal	0.63 (0.27 – 1.46)	0.28	-	-

***** The NCDR Cath PCI Bleeding Risk Score (61) is based on the following variables: STEMI, age, BMI, Previous PCI, chronic kidney disease, shock, cardiac arrest <24h, gender, hemoglobin levels, and PCI status.

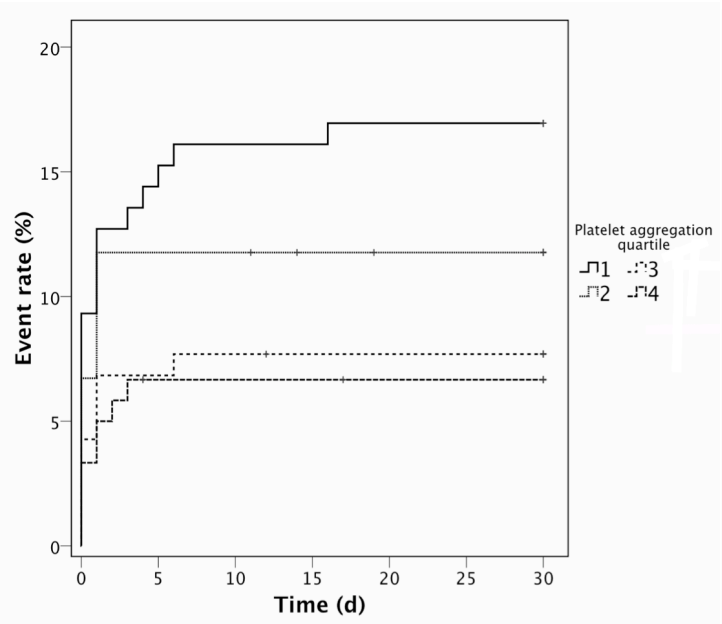

FIGURE 4. Frequency of ≥ type 2 BARC bleeding over time according to platelet aggregation quartiles.

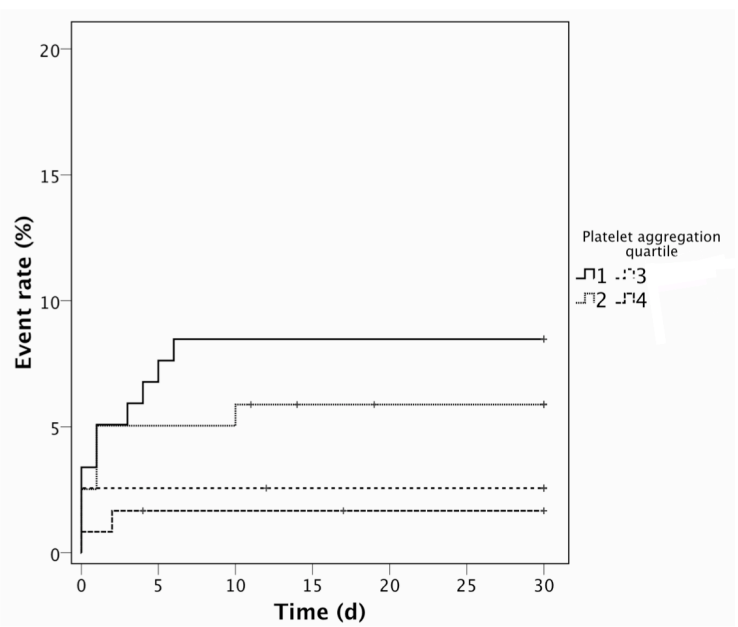

FIGURE 5. Frequency of ARMYDA-BLEEDS defined bleeding over time according to platelet aggregation quartiles.

4.3 STUDY III

The final study cohort included 40 NSTEMI patients and 20 SCAD controls (FIGURE 6). Baseline characteristics are shown in TABLE 8. Diabetes mellitus was significantly more common in the NSTEMI group. The median GRACE risk score (66) of the NSTEMI patients was 121.5 (IQR 105-142) with 25% (n = 10) having a high score (> 140), 45% (n = 18) having an intermediate score (109-140), and 30% (n = 12) having a low score. Of the included NSTEMI patients, 75% (n = 30) underwent coronary angiography during their primary admission. Of these, 73% (n = 22) underwent PCI and one patient was referred for coronary artery by-pass surgery. The median time from ticagrelor LD to coronary angiography was 27 hours (IQR 23.75 - 48 hours).

The pharmacokinetic parameters of ticagrelor and its active metabolite AR-C124910XX are shown in TABLE 9. None of the variables differed statistically significant between the NSTEMI patients and the SCAD controls, including the primary endpoint median T_{max} for ticagrelor (2 hours [1 – 3]) vs. 2 hours [2 – 3], p= 0.393). FIGURE 7 shows box plots of the ticagrelor concentrations. The AR-C124910XX plasma concentrations after the ticagrelor LD are depicted in FIGURE 8.

The pharmacodynamic response is shown in TABLE 10 and FIGURE 9. Both the NSTEMI patients and the SCAD controls had a fast onset of platelet inhibition by ticagrelor, with 15% (6 patients) and 10% prevalence (2 patients) of HPR at 1 hour (p= 0.707), and 2.5% (1 patient) and 0% at 2 hours (p = 1.0), respectively. The median platelet aggregation units were significantly higher in the NSTEMI group at 3 hours post the ticagrelor LD (p= 0.048). Furthermore, the median aggregation units was borderline statistically significantly higher in NSTEMI patients at 5 and 6 hours post the ticagrelor LD (p = 0.056, and p= 0.051, respectively), but without any prevalence of HPR.

There was a significant correlation between the ticagrelor concentrations and platelet aggregation during the first 6 hours after the 180 mg ticagrelor LD with data from all patients (Spearman's correlation coefficient −0.549, p< 0.001, TABLE 11). There were also significant correlations between the ticagrelor concentrations and platelet aggregation when assessed at one, two, three, and four hours post the 180-mg ticagrelor LD, with the strongest correlation at 1 hour. No serious adverse events occurred during the sampling period, but one NSTEMI patient had cardiogenic shock at study inclusion and underwent PCI within 2 hours.

Table 8. Demographic characteristics

Patient Characteristics	**NSTEMI patients** (n=40)	**SCAD controls** (n=20)	**p value**
Clinical Parameters			
Age, years	64.9 ± 10.7	65.9 ± 10.5	0.732
Male gender	28 (70%)	16 (80%)	0.541
BMI, kg/m^2	27.3 ± 3.9	26.1 ± 2.7	0.169
Current Smoker	9 (23%)	3 (15%)	0.734
Diabetes mellitus	16 (40%)	2 (10%)	**0.019**
Hypertension	23 (58%)	11 (55%)	1.0
Prior MI	5 (13%)	5 (25%)	0.278
Prior PCI	5 (13%)	9 (45%)	**0.009**
Prior CABG	3 (7.5%)	0 (0%)	0.544
Prior non-hemorrhagic stroke	2 (5%)	0 (0%)	0.548
COPD	2 (5%)	0 (0%)	0.548
Laboratory data			
Platelet count, $x10^9/L$	225.0 ± 51.4	225.0 ± 48.6	0.994
Hemoglobin, g/L	145.2 ± 13.0	143.9 ± 17.2	0.750
eGFR*, ml/min	94.3 ± 34.0	83.9 ± 25.5	0.233
Medication at inclusion			
Cholesterol lowering medication	14 (35%)	19 (95%)	**<0.001**
Aspirin	39 (97.5%)	20 (100%)	1.0

Data are presented as mean ± standard deviation for continuous variables, and as frequency (percentage) for categorical variables. Continuous variables were tested with the Student´s t-test and categorical variables with the Fisher's exact test.

*eGFR was calculated by using Cockcroft-Gault formula.

Abbreviations: BMI = body mass index; CABG = coronary artery bypass graft; COPD = Chronic obstructive pulmonary disease; EVF = erythrocyte volume fraction; eGRF = estimated glomerular filtration rate; MI = myocardial infarction; NSTEMI = non-ST elevated myocardial infarction; SCAD = stable coronary artery disease.

Table 9. Pharmacokinetic parameters

	NSTEMI patients (n=40)	**SCAD controls** (n=20)	**p value**
Ticagrelor			
C_{max} (ng/mL)	1285 (1006 – 2610)	1230 (958 – 1720)	0.819
$AUC_{0\text{-}6h}$ (ng x h/ml)	4561 (3258 – 5640)	3971 (3494 – 4960)	0.703
T_{max} (hours)	2.0 (1.0 – 3.0)	2.0 (2.0 – 3.0)	0.393
AR-C124910XX			
C_{max} (ng/mL)	308 (235 – 399)	267 (245 – 355)	0.526
$AUC_{0\text{-}6}$ (ng x h/ml)	1170 (868 – 1601)	987 (757 – 1225)	0.187
T_{max} (hours)	3 (2.0– 4.0)	3 (2.5 – 4.0)	0.289

Values are expressed as median with IQR, 25th – 75th percentile. Interference was tested with the Mann-Whitney U test. Abbreviations: AUC0-6h = Area under the curve from 0 hours to 6 hours; Cmax = maximum (peak) plasma concentration; NSTEMI = non-ST elevated myocardial infarction; SCAD = stable coronary artery disease; T_{max} = Time to peak plasma concentration.

Table 10. Pharmacodynamic response

Pharmacodynamic Parameters	NSTEMI (n=40)	SCAD controls (n=20)	P-value
Sample 0h			
Platelet aggregation (AU)	71.5 (52.5 – 84.0)	66.5 (57.5 – 72.0)	0.578
Sample 1h			
Platelet aggregation (AU)	17.0 (15 – 26.5)	25.5 (17.5 – 32.0)	0.075
HPR	6 (15%)	2 (10%)	0.707
Sample 2h			
Platelet aggregation (AU)	17.0 (13.5 – 23.0)	17.5 (12.5 – 22.0)	0.567
HPR	1 (3%)	0 (0%)	1.0
Sample 3h			
Platelet aggregation (AU)	17.0 (13.0 – 22.0)	13.0 (9.5 – 17.5)	**0.048**
HPR	0 (0%)	0 (0%)	N/A
Sample 4h			
Platelet aggregation (AU)	15.5 (12.5 – 23.0)	14.0 (11.5 – 15.5)	0.198
HPR	0 (0%)	0 (0%)	N/A
Sample 5h			
Platelet aggregation (AU)	17.0 (14.0 – 23.0)	13.0 (11.5 – 18.0)	0.056
HPR	0 (0%)	0 (0%)	N/A
Sample 6h			
Platelet aggregation (AU)	17.0 (12.0 – 23.0)	13.5 (10.0 – 16.5)	0.051
HPR	0 (0%)	0 (0%)	N/A
Area under the curve (AUC) 0-6h (AU x h)	132 (112 – 160)	128 (103 – 152)	0.411

Data are presented as median (interquartile range, 25^{th} - 75^{th} percentile) for quantitative variables, and as frequency (percentage) for qualitative variables. Continuous variables were tested with the Mann-Whitney U-test and categorical variables were tested with the Fisher's exact test. Abbreviations: AUC = area under the curve; HPR = high on-treatment platelet reactivity; NSTEMI = non-ST elevated myocardial infarction; SCAD = stable coronary artery disease.

Table 11. Correlation between ticagrelor concentrations and platelet aggregation

	Total cohort (n=60)	NSTEMI (n=40)	SCAD controls (n=20)
Sample 0h			
Correlation coefficient*	N/A	N/A	N/A
P value	N/A	N/A	N/A
Sample 1h			
Correlation coefficient	−0.666	−0.505	−0.827
P value	**<0.001**	**<0.001**	**<0.001**
Sample 2h			
Correlation coefficient	−0.391	−0.360	−0.384
P value	**0.002**	**0.023**	0.095
Sample 3h			
Correlation coefficient	−0.364	−0.232	−0.533
P value	**0.004**	0.150	**0.016**
Sample 4h			
Correlation coefficient	−0.296	−0.256	−0.248
P value	**0.021**	0.111	0.291
Sample 5h			
Correlation coefficient	−0.023	0.034	−0.128
P value	0.862	0.837	0.592
Sample 6h			
Correlation coefficient	−0.059	0.100	−0.147
P value	0.654	0.540	0.536
Samples 1,2,3,4,5, and 6 hours			
Correlation coefficient	−0.549	−0.505	−0.636
P value	**<0.001**	**<0.001**	**<0.001**

*Correlation analyses were performed with Spearman´s correlation.
Abbreviations: NSTEMI = non-ST elevated myocardial infarction; SCAD = stable coronary artery disease; N/A = Not applicable

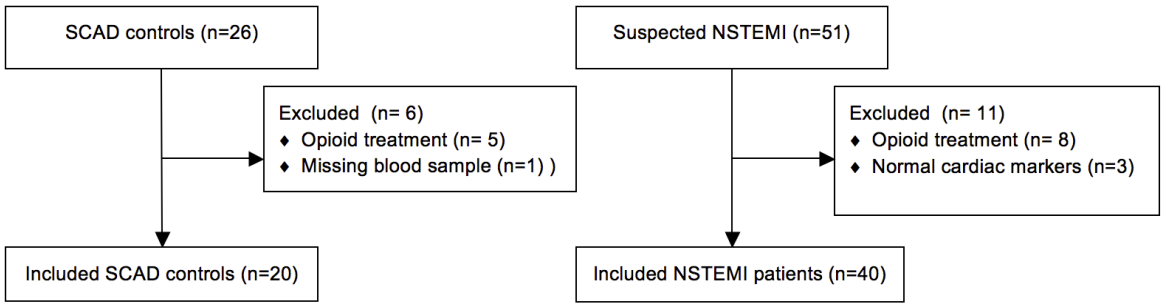

Figure 6. Study inclusion chart
NSTEMI indicates non ST-segment elevation myocardial infarction. SCAD indicates stable coronary artery disease.

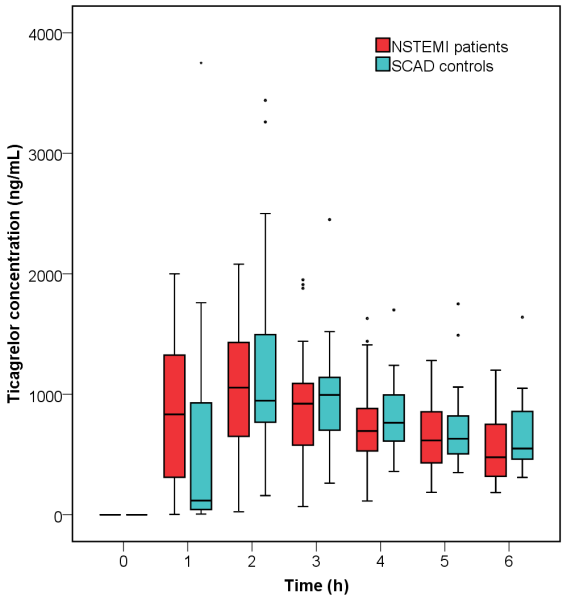

Figure 7. Ticagrelor concentrations
Ticagrelor concentrations at the different sampling time points shown in a boxplot. Center lines represent median and boxes 25th and 75th percentile

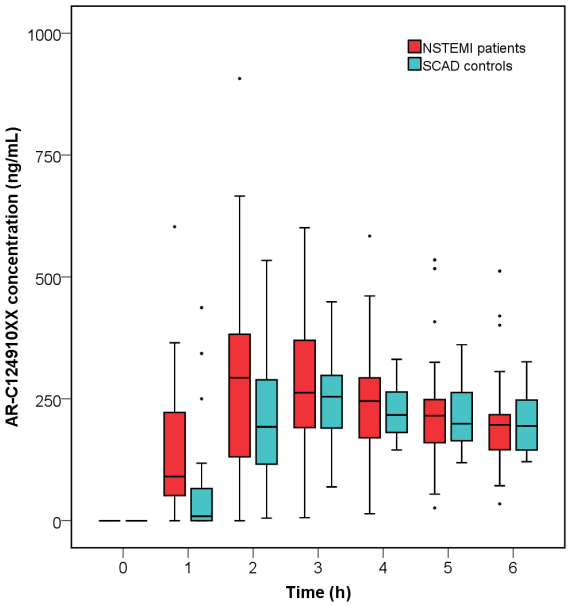

Figure 8. AR-C124910XX concentrations
Boxplot depicting concentrations of the active metabolite AR-C124910XX. Center lines represent median and boxes 25th and 75th percentile.

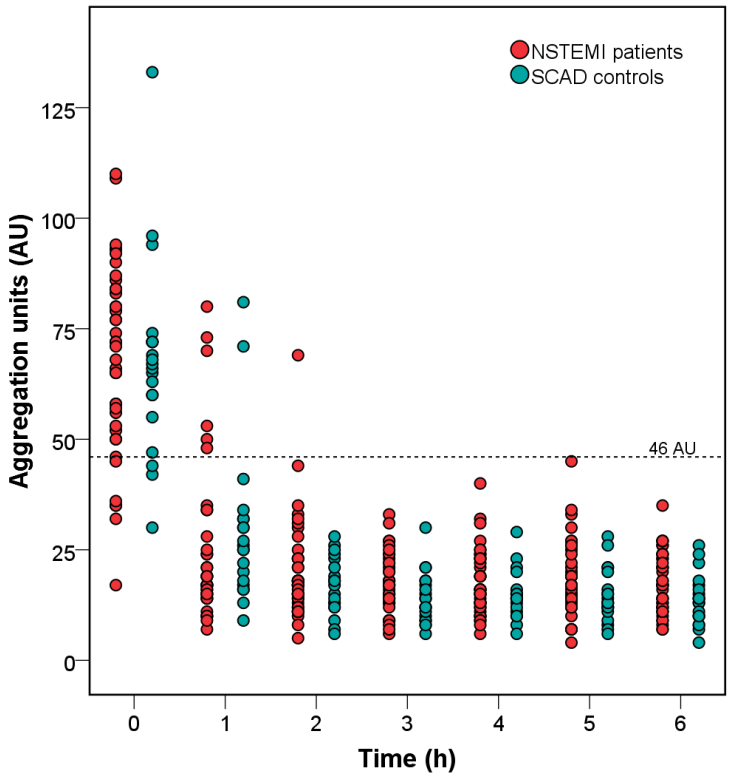

Figure 9. Pharmacodynamic response
Individual values of platelet reactivity at 0, 1, 2, 3, 4, 5, and 6, hours post the 180 mg ticagrelor LD, as assessed with ADP-induced multiple electrode aggregometry and shown in aggregation units (AU). The high on-treatment platelet reactivity (HPR) threshold is depicted as 46 AU. There were no significant differences in HPR at all time-points between the groups, whereas the AU was just significantly higher in NSTEMI patients at 3 hours ($p = 0.048$).

4.4 STUDY IV

A total of 95 STEMI patients were included in the study, which represents 31% of the 302 patients who presented with STEMI at the cardiac catheterization laboratories on the participating hospitals during the study period. The final study cohort consisted of 82 patients who were randomly assigned to receive either methylnaltrexone (n= 43) or placebo (n= 39), as shown in FIGURE 10.

The baseline characteristics were well balanced between the groups, as shown in TABLE 12. Procedural characteristics are listed in TABLE 13. The median time from the ticagrelor LD to study intervention and the median additional morphine administered did not differ significantly between the methylnaltrexone group and the placebo group.

As shown in TABLE 14 and FIGURE 11, the prevalence of HPR at two hours (primary outcome variable) did not differ significantly between patients randomized to methylnaltrexone compared to placebo (54% vs. 51%, p=0.84). Assessment with VASP showed no significant difference in platelet reactivity index (PRI%) between methylnaltrexone and placebo at baseline (p= 0.29), at one (p= 0.066), and at two hours (p= 0.38) after the study intervention, respectively.

The drug concentration analyses of morphine, ticagrelor, and the main active metabolite of ticagrelor, AR-C124910XX, are shown in TABLE 15 including comparisons between the groups. Plasma morphine concentrations (p= 0.94) did not differ significantly between the groups. Administration of methylnaltrexone when compared with placebo did not effect in a significant difference in ticagrelor or AR-C124910XX concentration at any of the time-points.

There was no significant difference in patient self-estimated pain between patients receiving methylnaltrexone and placebo at baseline (median pain level (IQR) 3 (2-5) vs. 2 (1-5, p=0.092) or at one (0 (0-2) vs. 2 (2-5), p = 0.36) or two hours after the study intervention (0 (0-1) vs. 0 (0-1), p = 0.28). Moreover, the difference (Δ) in pain between baseline and 1 or 2 hours did not differ significantly between the groups (2 (0.4) vs. 1 (0-3), p = 0.085 and 2 (0-5) vs. (2 (0-4), p=0.25, respectively).

Adverse events within 48 hours of study inclusion were registered. Life threatening arrhythmia, defined as need for cardiopulmonary resuscitation, or non-sustained ventricular tachycardia did not differ significantly between patients receiving methylnaltrexone or placebo (12% vs. 3%, p= 0.20; 60% vs. 49%, p=0.29). Death of any cause occurred in 5% of patients with methylnaltrexone compared with none of the placebo patients (p= 0.50), while the corresponding incidences of peri/postprocedural pulmonary edema were 0% and 6% (p=0.24), respectively. No patients experienced stent thrombosis, stroke, or bleedings other than minor access site.

TABLE 12. Demographic characteristics

Characteristic	Placebo (n=39)	Methylnaltrexone (n=43)	p value
Demographic/clinical			
Age, yr	69 (58, 77)	64 (60, 73)	0.45
Age >75 yr	12 (31)	7 (16)	0.12
Male gender	34 (87)	35 (81)	0.47
BMI	26.9 (25.0 - 29.1)	26.3 (24.1 - 28.1)	0.27
BMI > 25	29 (74)	27 (63)	0.26
Hypertension	17 (44)	22 (51)	0.49
Diabetes Mellitus	6 (15)	6 (14)	0.85
Dyslipidaemia	8 (21)	9 (21)	0.96
Current smoker	5 (13)	13 (30)	0.06
Prior AMI	4 (10)	4 (9)	1.00
Prior PCI	4 (10)	4 (9)	1.00
Prior CABG	0 (0)	1 (2)	1.00
Prior non-haemorrhagic stroke	0 (0)	2 (5)	0.50
Peripheral arterial disease	1 (3)	1 (2)	1.00
Chronic renal failure	2 (5)	2 (5)	1.00
COPD	2 (5)	4 (9)	0.68
Laboratory data			
Creatinine, µmol/L	83 (72 - 98)	82 (74 - 98)	0.92
eGFR*, ml/min	72 (59 - 84)	73 (58 - 82)	0.97
Hemoglobin g/L	147 (134 - 154)	147 (134 - 155)	0.88
Platelet count, x10^9	248 (201 - 288)	247 (208 - 285)	0.94
Prehospital medication			
Morphine dose, mg	6 (5 - 10)	6 (5 - 10)	0.63
Ondansetrone	8 (21)	3 (7)	0.07
Metoklopramide	3 (8)	4 (9)	0.79

Continuous variables are described as median (IQR) and were tested with the Wilcoxon rank-sum test. Categorical variables are described as no. (%) and were tested the $\chi 2$-test, or if needed with Fisher's exact test.
*eGFR was calculated using the Cockcroft-Gault formula.
Abbreviations: AMI = acute myocardial infarction;BMI = body mass index; CABG = coronary artery bypass graft; COPD = Chronic obstructive pulmonary disease; eGRF = estimated glomerular filtration rate; IQR = Interquartile range; PCI = Percutaneous coronary intervention.

TABLE 13. Procedural characteristics

Procedural characteristics	Placebo (n=39)	Methylnaltrexone (n=43)	p value
Clinical presentation			
Inferior STEMI	18 (46)	20 (47)	0.97
Systolic blood pressure, mmHg	140 (125 - 155)	135 (120 - 152)	0.58
Cardiogenic shock	0 (0)	1 (2)	1.00
Pulmonary edema	2 (6)	0 (0)	0.24
Procedural aspects			
Time from ticagrelor LD to study intervention, min	41 (31 - 50)	45.5 (37 - 60)	0.16
GP IIb/IIIa inhibitors	1 (3)	2 (5)	1.00
Heparin dose, IU	5000 (3000 - 8000)	5000 (3000 - 8000)	0.75
Enoxaparin	1 (3)	0 (0)	0.48
Bivalirudin	16 (41)	18 (42)	0.94
Thrombus aspiration	3 (8)	6 (14)	0.49
No. of stents used	1 (1, 2)	1 (1, 2)	0.92
Additional procedural medication			
Ondansetron	3 (8)	4 (9)	1.00
Metoklopramid	8 (21)	8 (19)	1.00
Morphine dose, mg	0 (0 - 3)	2 (0 - 5)	0.25

Continuous variables are described as median (IQR) and were tested with the Wilcoxon rank-sum test. Categorical variables are described as no. (%) and were tested the $\chi 2$-test, or if needed with Fisher's exact test.
Abbreviations: GP = glycoprotein; IU = international unit; IQR = Interquartile range; STEMI = ST-segment elevation myocardial infarction;;

TABLE 14. Pharmacodynamic evaluation

	Placebo (n=39)	Methylnaltrexone (n=43)	p value
Platelet function testing			
PRI% at baseline	86.4 (67.0 - 92.8)	90.3 (68.7 -93.8)	0.29
PRI% at 1h	59.0 (27.9 - 89.3)	84.9 (43.0 -92.6)	0.066
PRI% at 2h	57.8 (20.9 - 84.1)	63.2 (24.3 - 89.2)	0.38
ΔPRI% 0h-1h	14.9 (-1.40 - 39.0)	3.32 (-0.72 - 23.0)	0.18
ΔPRI% 0h-2h	16.4 (-0.04 - 45.1)	11.6 (0.82 - 37.9)	0.73
HPR* at baseline	30 (83)	38 (90)	0.35
HPR at 1h	18 (53)	29 (72)	0.082
HPR at 2h	18 (51)	21 (54)	0.84

Continuous variables are described as median (IQR) and were tested with the Wilcoxon rank-sum test. Categorical variables are described as no. (%) and were tested the $\chi 2$-test, or if needed with Fisher's exact test. The difference (Δ) in PRI% between the time points 0-1h and 0-2h is presented. Positive values suggest decreased PRI% levels.

*HPR is defined as PRI ≥ 50%.

Abbreviations: HPR= High on-treatment platelet reactivity; IQR= Interquartile range; PRI= Platelet Reactivity Index.

TABLE 15. Pharmacokinetic evaluation

	Placebo (n=39)	Methylnaltrexone (n=43)	p value
Drug concentration analyses (ng/mL)			
Morphine at baseline	12.8 (9.37 - 22.0)	13.2 (6.27 - 28.5)	0.94
Ticagrelor at baseline	0 (0 - 33.1)	0 (0 - 36.6)	0.42
Ticagrelor at 1h	41.1 (0 - 571)	39.2 (0 - 154)	0.41
Ticagrelor at 2h	88.3 (15.2 - 820)	105 (0 - 518)	0.88
Δticagrelor 1h-0h	22.7 (0 - 211)	27.5 (0 - 101)	0.81
Δticagrelor 2h-0h	40.2 (0 - 432)	94.6 (0 - 289)	0.57
AR-C124910XX at 0h	0 (0 - 0)	0 (0 - 0)	0.94
AR-C124910XX at 1h	0 (0 - 52.9)	0 (0 - 6.81)	0.17
AR-C124910XX at 2h	5.49 (0 - 104)	6.14 (0 - 55.9)	0.95
ΔAR-C124910XX 1h-0h	0 (0 - 46.7)	0 (0 - 6.81)	0.15
ΔAR-C124910XX 2h-0h	2.63 (0 - 94.8)	6.14 (0 - 54.1)	0.71

Continuous variables are described as median (IQR) and were tested with the Wilcoxon rank-sum test. Categorical variables are described as no. (%) and were tested the $\chi 2$-test, or if needed with Fisher's exact test. The lower level of detection for ticagrelor and AR-C124910XX concentrations were 5 ng/mL.

The difference (Δ) in ticagrelor and AR-C124910XX concentrations, respectively, between the time points 0-1h and 0-2h is presented. Positive values suggest increased concentrations.

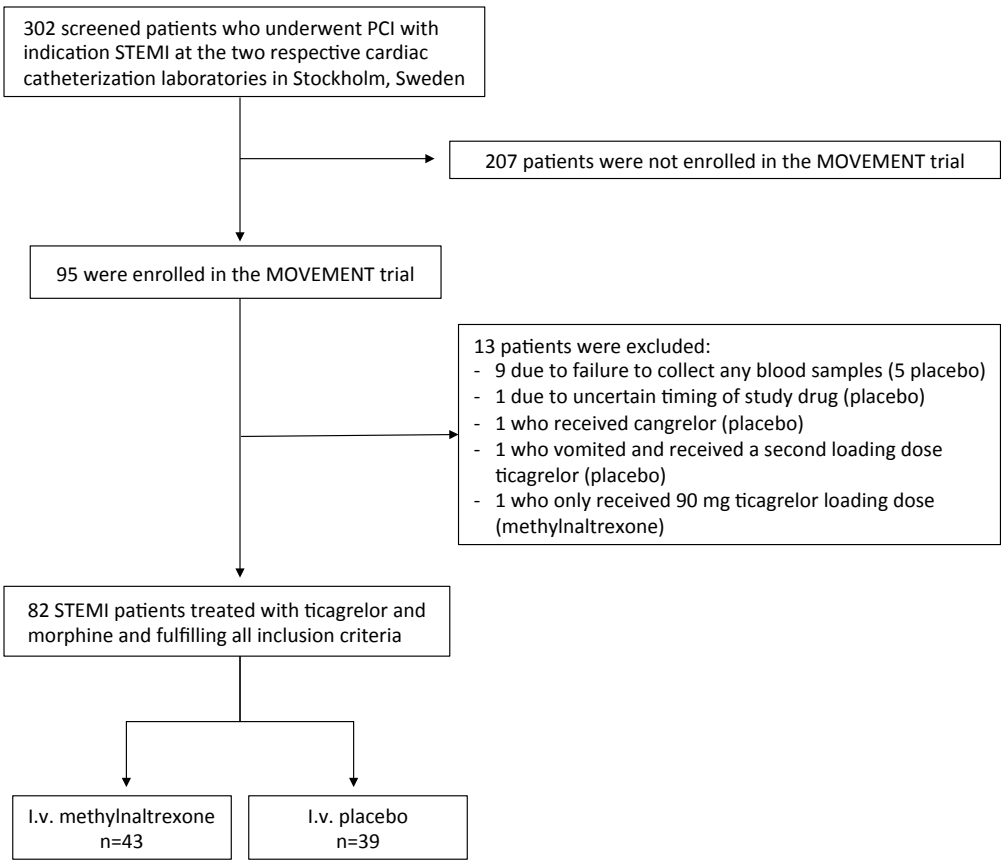

FIGURE 10. Enrollment and randomization.
Patients who presented with ST-segment elevation myocardial infarction at the respective cardiac catheterization laboratories were considered for study inclusion. The figure show the number of patients enrolled in the study and randomized.

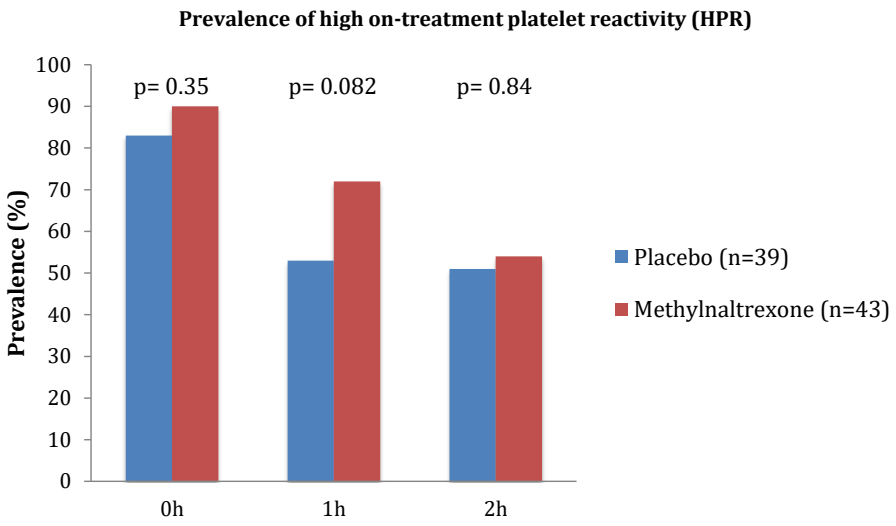

FIGURE 11. Prevalence of high on-treatment platelet reactivity.
Defined as ≥ 50% PRI with the VASP assay before inclusion, 1 hour, and 2 hours after study intervention. Differences in the prevalence in HPR were tested with the $\chi 2$-test.

5 DISCUSSION

The major findings in this thesis were as follows. Patients with impaired response to clopidogrel, i.e. HPR, according to platelet function testing with Plateletworks® shortly before coronary angiography/PCI had a significantly higher incidence of myocardial infarction within 3 months. Those in the lowest quartile of platelet aggregation with the Plateletworks® assay had a significantly higher incidence of bleeding within 30 days, according to two relevant bleeding definitions. An oral LD of 180 mg ticagrelor resulted in an early maximal drug concentration, in median at two hours, and a rapid onset of platelet inhibition both in NSTEMI patients and patients with SCAD. Randomized intravenous administration of the peripheral opioid antagonist methylnaltrexone did not significantly improve the delayed antiplatelet effect or drug uptake after a 180 mg ticagrelor in morphine treated patients with STEMI.

5.1 PREDICTIVE VALUE OF THE PLATELETWORKS® ASSAY

In **Study I**, patients with HPR, according to platelet function testing with the Plateletworks® assay, had a significantly higher incidence of myocardial infarction within three months after coronary angiography compared with those with an adequate response to clopidogrel. Moreover, the patients with HPR had a higher BMI and also a higher incidence of rehospitalization due to cardiovascular events within three months. The optimal cut-off for the prediction of myocardial infarction within three months was found to be 82.3% platelet aggregation with a rather modest AUC of 0.60 in the ROC curve analysis. The underlying sensitivity of 66.7% and specificity of 60.9% were also not very high. To our knowledge, the only previous study establishing a cut-off for Plateletworks® for prediction of the risk for ischemic cardiovascular events found a similar optimal cut-off at 80.5%. The authors found a similar sensitivity and specificity of 63% and 58.5%, respectively, and an AUC of 0.61 in their ROC curve analysis (34). Moreover, the incidence of HPR was in that study very similar to **Study I** (43.2% vs. 39.9%) (34). These findings might indicate reproducibility of the Plateletworks® assay, but with a rather modest predictability in both studies. In contrast to that study, there was only a non-significant tendency (p= 0.09) in **Study I** of patients in the HPR group with the combined end-point of all-cause death, nonfatal myocardial infarction, or stent thrombosis (34). This might be due to a type I error, as the other study included more patients (n=606), or possibly unknown confounding. It might also be due to the shorter follow-up of our study (3 months vs. 1 year). However, we cannot exclude that it is due to a failure of the Plateletworks® assay to properly identify all HPR patient at risk for recurrent cardiovascular events, especially since combined endpoints often are beneficial to use in smaller studies.

Study I had a significant higher ratio of patients who underwent PCI in the HPR group. The PCI procedure may in this unselected cohort indicate a more severe coronary disease among the HPR patients, which have had an impact on the risk for myocardial infarction. As the incidence of myocardial infarction within three months did not differ between patients who underwent PCI and those who underwent coronary angiography without PCI, the correlation

between HPR and myocardial infarction is **Study I** is likely not caused by the different rate of PCI. Moreover, the incidence of HPR was not significantly different in patients undergoing elective PCI patients, compared to acute PCI patients (45.1% vs. 44.8%; p= 0.96). We did not use stent thrombosis as a primary end-point as only 56% of the patients in **Study I** underwent PCI.

In **Study II**, we assessed the predictive ability of the Plateletworks® assay with regards to bleeding. This was proven successful, and we were able to show a significant correlation with bleeding events using two relevant definitions (BARC ≥ type 2, ARMYDA-BLEEDS). Conversely, the abovementioned study did not find any predictive value of the Plateletworks® assay with regard to bleeding events (34), possibly due to a less sensitive bleeding definition.

As the event rate in **Study II** was significantly higher than in **Study I**, we were able to adjust for potential confounders with a time-dependent multivariate Cox regression. In the regression model, we found that platelet aggregation in the lowest quartile significantly predicted bleeding defined with both the included bleeding definitions, when adjusted for the other included variables. We also established cut-offs for each bleeding definition at 76.7% platelet aggregation (BARC ≥ type 2) and 74.8% platelet aggregation (ARMYDA-BLEEDS). However, as with the cutoff established in **Study I**, this dichotomous approach resulted in only modest sensitivity and specificity.

In **Study II**, we chose the BARC ≥ type 2 bleeding definition for two reasons. First, it is the currently recommended standardized bleeding definition. Second, this bleeding definition has been correlated with increased mortality in patients undergoing PCI and it is thus very relevant (67). It is also, however, of interest to present the incidence of BARC type 1 bleeding. Even though it represents less serious and maybe even clinically unimportant bleeding, e.g. trivial nose bleeds, superficial skin bleeding etc., such events have been associated with premature drug cessation, which may in turn negatively impact outcome (68). The event rate of BARC ≥ type 2 was 11% in the overall cohort, which could be considered rather high. When considering this event rate, one has to take into account that BARC type 2 bleedings is the dominant event in the combined BARC ≥ type 2. BARC type 2 includes any overt actionable sign of hemorrhage, which may not be so infrequent in patients who underwent coronary angiography using the femoral approach. However, the rate of more serious bleedings (BARC ≥ type 3) was 2.5%.

In **Study II**, using the ARMYDA-BLEEDS bleeding definition we found a bleeding incidence of 4.8%, which is rather similar to the 4.6% incidence in the ARMYDA-BLEEDS study (32). Furthermore, the correlation between the lowest quartile of platelet function and bleeding in that study was also found in **Study II.** Comparing the ARMYDA-BLEEDS and BARC ≥ type 2 bleeding definitions, the incidence was much higher with the latter. This may indicate that the BARC bleeding definition has a higher sensitivity than the ARMYDA-BLEEDS bleeding definition.

Another interesting finding of **Study II** was that most of the bleeding events occurred within the first 7 days after coronary angiography, regardless of definition used (FIGURE 4 and FIGURE 5). This may probably be due to a relatively high proportion of early procedurally related bleeding events but may be of interest to further evaluate in future studies.

5.2 BLEEDING EVENTS VS. THROMBOSIS

Like Odysseus navigated between Scylla and Charybdis, currently known pharmacological drugs used to avoid thrombotic events all have the drawback of increased bleeding risk. The therapeutic window is rather narrow, and it is not possible to use platelet inhibiting drugs to reduce incidence of ischemic events without increasing bleeding risk. The risk of thrombosis does, however, not decrease linearly with lowered on-treatment platelet reactivity. Conversely, the benefit with decreasing platelet reactivity with antiplatelet drugs is greatest when the cut-off for HPR is passed. In the case of "excessive" platelet inhibition, i.e. when the cut-off for low on-treatment platelet reactivity is passed, a meta-analysis has shown a 1.7-fold higher risk for major bleeding without any further significant decrease in the incidence of stent thrombosis, compared with patients with platelet reactivity above LPR, but below HPR (69). A schematic figure of a possible therapeutic window for the Plateletworks® assay is shown in FIGURE 12 including the cut-offs established in **Study I and II.**

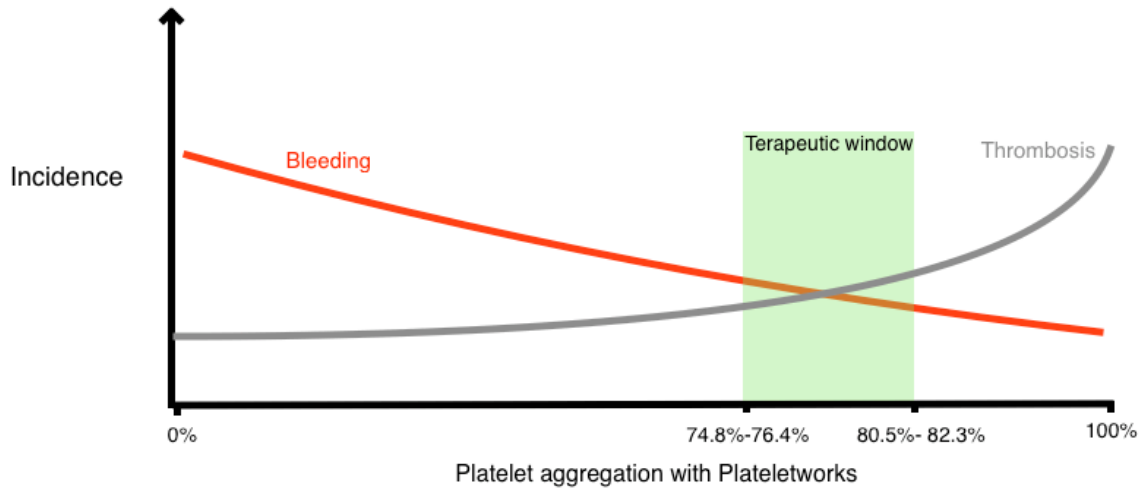

FIGURE 12. Thrombosis risk vs. bleeding risk on P2Y12 inhibition with clopidogrel. Schematic figure (not in scale) showing the cut-offs for the Plateletworks® assay established in **Study I** (82.3%), from a previously published paper (80.5%) (34), and the cut-offs for bleeding established in **Study II** (74.8% for the ARMYDA-BLEEDS bleeding definition and 76.4% for the BARC ≥ type 2 bleeding).

5.3 PLATELET FUNCTION TESTING IN CLINICAL PRACTICE

The use of a platelet function test in clinical practice could in theory be of value to identify patients with HPR and thus at risk for recurrent cardiovascular events. This may theoretically enable an intervention that reverses this increased risk (other more potent drug, higher dose etc.). Adjusting treatment according to on-treatment platelet reactivity has indeed been tried

in patients with SCAD, however, without any significant improvement of clinical outcomes (70-72). Already back in 2014, the ESC guidelines stated that measurement of on-treatment platelet reactivity to monitor treatment response should not be routinely used but limited to clinical research. The guidelines did, though, state that platelet function testing "may be considered in specific high-risk situations (e.g. history of stent thrombosis; compliance issue; suspicion of resistance; high bleeding risk) (33).

In 2016, the "Assessment of a Normal versus Tailored dose of prasugrel after stenting in patients Aged >75 years to Reduce the Composite of bleeding, stent Thrombosis and Ischemic Complications" (ANTARCTIC) study was published, where elderly patients who underwent PCI due to ACS were included. Despite use of monitoring of platelet function and treatment adjustment in this high-risk patient cohort, there was no benefit on clinical outcome. This further strengthens the position that routine platelet function testing to find patients with HPR is without value in current clinical practice. It should, however, be noted that platelet function testing may be considered following P2Y12 inhibitor discontinuation to shorten the time window to important/acute major surgery such as CABG (51). This approach is not discussed in detail, as it is not the subject of this thesis. Moreover, the use of a platelet function test to de-escalate the platelet inhibition from more potent P2Y12 inhibitors back to clopidogrel is possibly a future use of these assays and discussed in more detail below under future perspectives.

5.4 IMPAIRED ONSET OF TICAGRELOR IN ACS

In **Study I**, the time from LD of clopidogrel to coronary angiography is presented in intervals of <6 hours and 6-24 hours (TABLE 2), as previous studies have shown that a steady state of platelet inhibition is achieved at around 6 hours after a LD of clopidogrel (38, 39). With this in mind, it was expected that a LD of clopidogrel less than 6 hours before coronary angiography was significantly more common in patients with HPR. However, the frequency of a LD of clopidogrel 6-24 hours prior to coronary angiography was also significantly higher in patients with HPR, even though these patients should have steady state of platelet inhibition. This could possibly be explained by impaired gastrointestinal absorption, maybe due to morphine use in close relation with the clopidogrel administration. Data on this were, however, not available. In **Study II,** patients in the lowest quartile of platelet aggregation had a higher median time from the LD clopidogrel to coronary angiography, compared with the highest quartile (TABLE 4). This could possibly be due to a significantly higher frequency of STEMI in the highest quartile of platelet aggregation (TABLE 5), but as the overall rate of STEMI was low it might also be due to the unknown frequency of morphine administration.

In **Study III**, we were able to show that NSTEMI patients not receiving opioids had an early uptake of ticagrelor and adequate onset of platelet inhibition, which did not significantly differ from the control group of patients with SCAD. We did not include NSTEMI patients who had been administered morphine due to its negatively impact on ticagrelor uptake in patients with ACS (54), as our aim was to evaluate any possible negative impact on ticagrelor uptake by the condition of NSTEMI in itself. During 2014, 71% of the NSTEMI patients in

Sweden were on ticagrelor at discharge from the hospital, according to the data available when we initiated **Study III**. Thus, it was of clinical value to demonstrate that a ticagrelor LD results in an early and adequate drug uptake and platelet inhibition, especially for the subgroup of NSTEMI patients at very high risk (hemodynamic instability/cardiogenic shock, refractory chest pain, life-threatening arrhythmias, mechanical complications of MI, acute heart failure, or recurrent dynamic ST-T-wave changes, particularly intermittent ST-elevation), who according to guidelines should undergo invasive evaluation with coronary angiography within 2 hours (51). The impact of morphine on ticagrelor uptake was investigated in a recent randomized trial (54), where ACS patients were randomized to morphine or placebo before the ticagrelor LD. Median T_{max} of ticagrelor was at 2 hours in the placebo arm, compared with 4 hours for patients receiving morphine. Thus, the placebo arm of that study had a similar ticagrelor uptake as the NSTEMI and the SCAD patients of **Study III.**

5.5 OPTIMIZING THE EARLY TICAGRELOR EFFECT IN STEMI

Pain relief is very important in patients with ACS as reduced pain results in decreased sympathetic activation, and consequently lowers heart rate and blood pressure. This potentially positive effect of morphine has, however, not been shown to improve outcome in ACS patients (73). Conversely, morphine administration has in ACS patients been shown to correlate with negative outcome, including increased mortality (53, 74, 75). This could possibly be attributed to a morphine-induced impairment of uptake and effect of oral P2Y12 inhibitors, which was also stated in the 2017 European Society of Cardiology STEMI guidelines (76). In **Study IV**, we failed to show any beneficial effect of intravenous administration of the peripheral opioid antagonist methylnaltrexone on the ticagrelor uptake and onset of effect in STEMI patients. To our knowledge, the use of methylnaltexone or any other opioid antagonist has not been previously studied in morphine-treated patients with STEMI. Thus, there is a lack of directly comparable results. However, there is a recently published abstract of a cross-over study on patients with coronary artery disease confirmed angiograpically who were randomized to either administration of intravenous methylnaltrexone or placebo. The patients then received intravenous morphine and shortly afterwards a standard 180 mg ticagrelor LD. In line with the results of **Study IV**, the authors of that study did not find any benefit of methylnaltrexone on ticagrelor uptake and antiplatelet effect (77). Thus, methylnaltrexone did not result in any beneficial effects neither in the more controlled non-acute setting of that trial, nor in the acute setting of STEMI according to **Study IV**. The strategy of administering methylnaltrexone to improve the uptake of ticagrelor in morphine-treated STEMI patients should not be used in clinical practice.

In **Study IV**, a majority of patients had HPR two hours after the study intervention according to testing with VASP. This is in line with previous studies of platelet aggregation in ACS patients, separately presenting morphine use and platelet function testing with the VASP assay (37, 54).

5.6 LIMITATIONS

In **Study I,** the variables age, the use of a clopidogrel LD, and BMI significantly predicted the presence of HPR. It was not deemed valuable to perform a multivariate analysis with myocardial infarction due the low event rate, which might be considered a limitation. Another limitation is that aspirin compliance or resistance was not evaluated. During inclusion of the study cohort for **Study I and II** we had an "all-comer" approach, i.e. we did not only include for example PCI patients. While this makes the external validity better, it might also have increased the risk of introducing unknown bias not controlled for. Moreover, in these studies only one platelet function test was used to evaluate the treatment effect of clopidogrel. However, in a much larger study, the Plateletworks® assay had the highest predictive value for a composite clinical endpoint (all-cause death, nonfatal acute myocardial infarction, stent thrombosis, and ischemic stroke) in a backward regression model (34). The assay has also been shown to correlate well with the more established laboratory based light transmittance aggregometry platelet function test (78). Still, it might have been of additional value to use parallel testing with another more established platelet function assay.

In both **Study III**, and **Study IV**, only one platelet function test was used (MEA and VASP, respectively). Both these tests are more validated than Plateletworks® (36), but it may still have been of value to have added parallel testing with a second platelet function test. However, this would have, especially for **Study IV**, greatly reduced the feasibility of the studies. Moreover, the results of the platelet function tests are in **study III and IV** in line with the ticagrelor and ARC124910XX concentrations, which were analyzed with very exact methods.

Study III excluded opioid-treated patients and the results are probably not applicable in NSTEMI patients on opioid treatment. Moreover, only P2Y12-naïve patients were and included and the results should possibly not be generalized to patients already on P2Y12-inhibition.

One limitation with **Study IV** was the in-hospital study inclusion and methylnaltrexone/placebo injection, which was chosen as the Swedish Medical Product Agency requires a physician asking for study participation. With this in mind, prehospital patient inclusion was considered too difficult to achieve. It cannot be ruled out that a prehospital administration of methylnaltrexone concurrent with morphine might have resulted in improved uptake of ticagrelor in patients with STEMI. Moreover, blood sampling was conducted up to two hours after study inclusion, both to facilitate feasibility of the study and as the primary aim was to assess the early ticagrelor effect. Nevertheless, a later significant difference in onset of ticagrelor effect might have occurred. This is, however, perhaps not very likely considering previously presented data without a significant difference between methylnaltrexone and placebo up to 6 hours after the ticagrelor LD (77). Moreover, in another recent trial a significant difference in the prevalence of HPR between morphine and placebo was seen already at 30 minutes with the VASP assay (54).

5.7 FUTURE PERSPECTIVES

The use of a platelet function assay to guide patients toward stronger platelet inhibition has, as discussed above, not been consistently proven successful in reducing cardiovascular events. Conversely, platelet function testing has instead been suggested useful in "de-escalating" the platelet function treatment in ACS patients from a newer P2Y12 inhibitor back to clopidogrel during the phase of maintenance treatment. After de-escalation back to clopidogrel, platelet function testing may be used to find presence of HPR or not. The hypothesis is that the need for stronger P2Y12 inhibition is greater early after stenting and there is evidence that around 80% of the stent thromboses occur within the first month after coronary stenting (79). This strategy of de-escalating P2Y12 treatment and then evaluating the prevalence with HPR using platelet function testing is currently being investigated in a large ongoing trial (80).

Although not the scope of this thesis, the approach of deciding the type of P2Y12 treatment on the basis of genetic testing with focus on the metabolism of clopidogrel should also be mentioned in this context. P2Y12 treatment according to genetic testing was in a recently published study found to reduce the incidence of a composite endpoint within one year including both ischemic events (cardiovascular death, non-fatal myocardial infarction, and non-fatal stroke) and bleeding events (BARC type 3 or higher) compared with patients on "standard care" (81). This approach is interesting, although the "standard care" arm of ACS patients in that study had a rather high frequency of clopidogrel treatment (50.7%), even though the benefit of ticagrelor over clopidogrel is well established in patients with ACS (24) and indeed recommended in European guidelines in the absence of contraindications (11). As ticagrelor was more frequently used in the genotype-guided group, simple swapping from clopidogrel to ticagrelor instead of genetically testing may possibly have the same effect, but this is a subject for future studies. There is an interesting ongoing study (NCT01742117) evaluating the role of genetic testing in SCAD patients, where clopidogrel is still used today. The investigators use genetic testing to identify patients with CYP2C19 loss-of-function and swap these patients to ticagrelor. That study will provide very interesting data on the use of genetic testing to guide P2Y12 inhibitor treatment.

The optimal way to counteract the morphine-induced delay in ticagrelor among STEMI patients needs to be evaluated using other strategies than intravenous methylnaltrexone in further studies. Possible strategies may include the use of the novel i.v. P2Y12 receptor inhibitor cangrelor to overcome early HPR (25) although to our knowledge, there are not clinical studies directly comparing this strategy with standard ticagrelor use. Possible alternative interventions include the addition of other antiplatelet drugs such as GP IIb/IIIa receptor inhibitors, crushing or chewing of the ticagrelor tablets (82, 83), or perhaps use of pain-relieving drugs other than opioids, which do not reduce the uptake of oral P2Y12-receptor inhibitors.

6 CONCLUSIONS

The specific conclusions were:

- Testing with the Plateletworks® assay at the start of coronary angiography identified the patients with HPR at significantly increased risk of myocardial infarction and rehospitalization due to cardiovascular causes within 3 months of the procedure, as compared with patients with a normal clopidogrel response.

- Clopidogrel-treated patients with low on-treatment platelet reactivity, according to platelet function testing with Plateletworks® at the time of intervention, had a significantly higher incidence of bleeding defined according to BARC and ARMYDABLEEDS <30 days after coronary angiography with and without PCI.

- NSTEMI patients not receiving opioids have a fast and adequate uptake of ticagrelor and onset of platelet inhibition, which was not significantly slower compared with the SCAD control group.

- The peripheral opioid antagonist methylnaltrexone did not improve platelet reactivity or plasma concentrations of orally administered ticagrelor in STEMI patients receiving morphine. The strategy of administering methylnaltrexone to improve the uptake of ticagrelor in morphine-treated STEMI patients should not be used in clinical practice.

7 ACKNOWLEDGEMENTS

I am sincerely grateful to all involved in completion of this work, and in particular to:

- Professor **Jan van der Linden,** my main supervisor, for all your patience, optimism, friendship, and never-ending support. Without your knowledge and, most of all, always cheerful attitude, I would not have been able to finish this project. Thank you for believing in me and for giving me the opportunity to become a researcher under your supervision. I am forever grateful for all your help and for introducing me to the field of cardiovascular research!

- Professor **Per Tornvall,** my co-supervisor, for all your support and help in conducting this project. I have always valued your excellent advice and guidance, which many times have given me different perspectives on things. I have really appreciated to have you as my co-supervisor!

- Research nurse **Kristina Kilsand**, for your invaluable help with patient inclusion and data collection during 6 years for the first two studies in this thesis. Without you, this project would truly not have been possible.

- **Nanna Golster, Julia Westerberg, Shaym Rihan-Hye, Elias Karlsson, and Elias Hasselström** for your excellent help with the study related procedures for Study III and IV during your respective master thesis.

- The staff and colleagues at the unit for interventional cardiology at Södersjukhuset for all your help during the later phase of the project. You have always made me feel welcome and you have participated greatly in the last two studies and I thank you for your ability to take time for research-related procedures, which was especially noticable during Study IV.

- The research nurses **Runa Sundelin, Lis Kohlström, Gunilla Förstedt, Eva-Lotta Nylund, and Gun Wedeen.** You have all been very important for this project as the last study was finished with your excellent help!

- The staff and collegues at the unit for interventional cardiology at Karolinska University Hospital, Huddinge for help with patient inclusion for Study IV.

- My co-authors **Loghman Henareh, Ulf Jensen, Patrik Alström, Irene Santos-Pardo, Nils Witt, Nikolai Fedchenko, Dimitrios Venetsanos, Olof Beck, and Magnus Dalén,** and for our cooperation and for all your advice! Many of you have also given excellent advice on study design and included patients in Study IV, which I sincerely thank you for!

- All the staff at the cardiac intensive care unit at Södersjukhuset, for all your help with taking and handling blood samples for Study IV. You took time to do these study related procedures despite an often full ward and at times very high stress. Thank you so much for all your help!

- **Sabina** and **Christoffer** at the department of Clinical Pharmacology at Karolinska University Hospital Huddinge, for your help with drug concentration analyses for study IV.

- All the colleagues and staff at the emergency department at Södersjukhuset for excellent help with identification of patients eligible for inclusion and for always making me feel welcome!

- **Fuad Bahram** and the rest of the staff at Forskningscentrum Södersjukhuset for all your help during all my stressful times in the laboratory!

- Associate Professor **Ulrik Sartipy,** for excellent statistical advice.

- **Marie van der Linden** for with great patience lending me your husband during weekends and sometimes late nights.

- All the patients participating in Study I-IV.

- My parents, **Barbro** and **Lennart**, and my brother **Jakob** for endless support and encouragement.

- **Sofi** for all your love and endless support. I love you!

This work was made available by financial support from

Regional agreement on medical training and clinical research (ALF)

Astra Zeneca

The Swedish Heart and Lung Foundation

Karolinska Institutet

8 REFERENCES

1. Ruggeri ZM. Platelets in atherothrombosis. Nat Med. 2002;8(11):1227-34.
2. McFadyen JD, Jackson SP. Differentiating haemostasis from thrombosis for therapeutic benefit. Thromb Haemost. 2013;110(5):859-67.
3. Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. J Clin Invest. 2001;107(12):1591-8.
4. Kim S, Kunapuli SP. P2Y12 receptor in platelet activation. Platelets. 2011;22(1):54-8.
5. Schwarz UR, Geiger J, Walter U, Eigenthaler M. Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets--definition and detection of ticlopidine/clopidogrel effects. Thromb Haemost. 1999;82(3):1145-52.
6. Patrono C, Morais J, Baigent C, Collet JP, Fitzgerald D, Halvorsen S, et al. Antiplatelet Agents for the Treatment and Prevention of Coronary Atherothrombosis. J Am Coll Cardiol. 2017;70(14):1760-76.
7. Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007;357(24):2482-94.
8. Thomas MR, Wijeyeratne YD, May JA, Johnson A, Heptinstall S, Fox SC. A platelet P-selectin test predicts adverse cardiovascular events in patients with acute coronary syndromes treated with aspirin and clopidogrel. Platelets. 2014;25(8):612-8.
9. Jackson SP. The growing complexity of platelet aggregation. Blood. 2007;109(12):5087-95.
10. Coxon CH, Geer MJ, Senis YA. ITIM receptors: more than just inhibitors of platelet activation. Blood. 2017;129(26):3407-18.
11. Valgimigli M, Bueno H, Byrne RA, Collet JP, Costa F, Jeppsson A, et al. 2017 ESC focused update on dual antiplatelet therapy in coronary artery disease developed in collaboration with EACTS: The Task Force for dual antiplatelet therapy in coronary artery disease of the European Society of Cardiology (ESC) and of the European Association for Cardio-Thoracic Surgery (EACTS). Eur Heart J. 2018;39(3):213-60.
12. Bhatt DL. Intensifying platelet inhibition--navigating between Scylla and Charybdis. N Engl J Med. United States2007. p. 2078-81.
13. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, et al. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. J Am Coll Cardiol. 2007;49(14):1505-16.
14. Bernlochner I, Byrne RA, Kastrati A, Sibbing D. The future of platelet function testing to guide therapy in clopidogrel low and enhanced responders. Expert Rev Cardiovasc Ther. 2011;9(8):999-1014.
15. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med. 2001;345(7):494-502.

16. Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, et al. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. Lancet. 2001;358(9281):527-33.

17. Aradi D, Komocsi A, Vorobcsuk A, Rideg O, Tokes-Fuzesi M, Magyarlaki T, et al. Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: systematic review and meta-analysis. Am Heart J. 2010;160(3):543-51.

18. Snoep JD, Hovens MM, Eikenboom JC, van der Bom JG, Jukema JW, Huisman MV. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. Am Heart J. 2007;154(2):221-31.

19. Ahmad T, Voora D, Becker RC. The pharmacogenetics of antiplatelet agents: towards personalized therapy? Nat Rev Cardiol. 2011;8(10):560-71.

20. Teng R. Pharmacokinetic, pharmacodynamic and pharmacogenetic profile of the oral antiplatelet agent ticagrelor. Clin Pharmacokinet. 2012;51(5):305-18.

21. Gurbel PA, Bliden KP, Butler K, Tantry US, Gesheff T, Wei C, et al. Randomized double-blind assessment of the ONSET and OFFSET of the antiplatelet effects of ticagrelor versus clopidogrel in patients with stable coronary artery disease: the ONSET/OFFSET study. Circulation. 2009;120(25):2577-85.

22. Teng R, Kujacic M, Hsia J. Evaluation of the Pharmacokinetic Interaction Between Ticagrelor and Venlafaxine, a Cytochrome P-450 2D6 Substrate, in Healthy Subjects. Clin Ther. 2014.

23. Teng R, Kujacic M, Hsia J. Pharmacokinetic interaction study of ticagrelor and cyclosporine in healthy volunteers. Clin Drug Investig. 2014;34(8):529-36.

24. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2009;361(11):1045-57.

25. Bhatt DL, Stone GW, Mahaffey KW, Gibson CM, Steg PG, Hamm CW, et al. Effect of platelet inhibition with cangrelor during PCI on ischemic events. N Engl J Med. 2013;368(14):1303-13.

26. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2007;357(20):2001-15.

27. Ndrepepa G, Berger PB, Mehilli J, Seyfarth M, Neumann FJ, Schomig A, et al. Periprocedural bleeding and 1-year outcome after percutaneous coronary interventions: appropriateness of including bleeding as a component of a quadruple end point. J Am Coll Cardiol. 2008;51(7):690-7.

28. Mehran R, Rao SV, Bhatt DL, Gibson CM, Caixeta A, Eikelboom J, et al. Standardized bleeding definitions for cardiovascular clinical trials: a consensus report from the Bleeding Academic Research Consortium. Circulation. 2011;123(23):2736-47.

29. Chesebro JH, Knatterud G, Roberts R, Borer J, Cohen LS, Dalen J, et al. Thrombolysis in Myocardial Infarction (TIMI) Trial, Phase I: A comparison between

intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. Circulation. 1987;76(1):142-54.

30. Hicks KA, Stockbridge NL, Targum SL, Temple RJ. Bleeding Academic Research Consortium consensus report: the Food and Drug Administration perspective. Circulation. United States2011. p. 2664-5.

31. Steg PG, Huber K, Andreotti F, Arnesen H, Atar D, Badimon L, et al. Bleeding in acute coronary syndromes and percutaneous coronary interventions: position paper by the Working Group on Thrombosis of the European Society of Cardiology. Eur Heart J. 2011;32(15):1854-64.

32. Patti G, Pasceri V, Vizzi V, Ricottini E, Di Sciascio G. Usefulness of platelet response to clopidogrel by point-of-care testing to predict bleeding outcomes in patients undergoing percutaneous coronary intervention (from the Antiplatelet Therapy for Reduction of Myocardial Damage During Angioplasty-Bleeding Study). Am J Cardiol. 2011;107(7):995-1000.

33. Authors/Task Force m, Windecker S, Kolh P, Alfonso F, Collet JP, Cremer J, et al. 2014 ESC/EACTS Guidelines on myocardial revascularization: The Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS)Developed with the special contribution of the European Association of Percutaneous Cardiovascular Interventions (EAPCI). Eur Heart J. 2014.

34. Breet NJ, van Werkum JW, Bouman HJ, Kelder JC, Ruven HJ, Bal ET, et al. Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. JAMA. 2010;303(8):754-62.

35. Aradi D, Collet JP, Mair J, Plebani M, Merkely B, Jaffe AS, et al. Platelet function testing in acute cardiac care - is there a role for prediction or prevention of stent thrombosis and bleeding? Thromb Haemost. 2014;113(1).

36. Aradi D, Storey RF, Komocsi A, Trenk D, Gulba D, Kiss RG, et al. Expert position paper on the role of platelet function testing in patients undergoing percutaneous coronary intervention. Eur Heart J. 2014;35(4):209-15.

37. Silvain J, Storey RF, Cayla G, Esteve JB, Dillinger JG, Rousseau H, et al. P2Y12 receptor inhibition and effect of morphine in patients undergoing primary PCI for ST-segment elevation myocardial infarction. The PRIVATE-ATLANTIC study. Thromb Haemost. 2016;116(2):369-78.

38. Montalescot G, Sideris G, Meuleman C, Bal-dit-Sollier C, Lellouche N, Steg PG, et al. A randomized comparison of high clopidogrel loading doses in patients with non-ST-segment elevation acute coronary syndromes: the ALBION (Assessment of the Best Loading Dose of Clopidogrel to Blunt Platelet Activation, Inflammation and Ongoing Necrosis) trial. J Am Coll Cardiol. 2006;48(5):931-8.

39. Steinhubl SR, Berger PB, Mann JT, 3rd, Fry ET, DeLago A, Wilmer C, et al. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA. 2002;288(19):2411-20.

40. Heestermans AA, van Werkum JW, Taubert D, Seesing TH, von Beckerath N, Hackeng CM, et al. Impaired bioavailability of clopidogrel in patients with a ST-segment elevation myocardial infarction. Thromb Res. 2008;122(6):776-81.

41. Stengel A, Tache Y. Neuroendocrine control of the gut during stress: corticotropin-releasing factor signaling pathways in the spotlight. Annu Rev Physiol. 2009;71:219-39.

42. Roberts DJ, Hall RI. Drug absorption, distribution, metabolism and excretion considerations in critically ill adults. Expert Opin Drug Metab Toxicol. 2013;9(9):1067-84.

43. Parkman HP, Hasler WL, Fisher RS, American Gastroenterological A. American Gastroenterological Association technical review on the diagnosis and treatment of gastroparesis. Gastroenterology. 2004;127(5):1592-622.

44. Hobl EL, Stimpfl T, Ebner J, Schoergenhofer C, Derhaschnig U, Sunder-Plassmann R, et al. Morphine decreases clopidogrel concentrations and effects: a randomized, double-blind, placebo-controlled trial. J Am Coll Cardiol. 2014;63(7):630-5.

45. Husted S, Emanuelsson H, Heptinstall S, Sandset PM, Wickens M, Peters G. Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin. Eur Heart J. 2006;27(9):1038-47.

46. Cannon CP, Husted S, Harrington RA, Scirica BM, Emanuelsson H, Peters G, et al. Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non-ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial. J Am Coll Cardiol. 2007;50(19):1844-51.

47. Husted SE, Storey RF, Bliden K, Tantry US, Hoimark L, Butler K, et al. Pharmacokinetics and pharmacodynamics of ticagrelor in patients with stable coronary artery disease: results from the ONSET-OFFSET and RESPOND studies. Clin Pharmacokinet. 2012;51(6):397-409.

48. Parodi G, Valenti R, Bellandi B, Migliorini A, Marcucci R, Comito V, et al. Comparison of prasugrel and ticagrelor loading doses in ST-segment elevation myocardial infarction patients: RAPID (Rapid Activity of Platelet Inhibitor Drugs) primary PCI study. J Am Coll Cardiol. 2013;61(15):1601-6.

49. Alexopoulos D, Xanthopoulou I, Gkizas V, Kassimis G, Theodoropoulos KC, Makris G, et al. Randomized assessment of ticagrelor versus prasugrel antiplatelet effects in patients with ST-segment-elevation myocardial infarction. Circ Cardiovasc Interv. 2012;5(6):797-804.

50. SWEDEHEART Annual report 2014 http://www.ucr.uu.se/swedeheart/index.php/dokument-sh/arsrapporter(Issued in 2015) [cited Mars 13, 2016].

51. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC). Eur Heart J. 2016;37(3):267-315.

52. Storey RF, Husted S, Harrington RA, Heptinstall S, Wilcox RG, Peters G, et al. Inhibition of platelet aggregation by AZD6140, a reversible oral P2Y12 receptor antagonist, compared with clopidogrel in patients with acute coronary syndromes. J Am Coll Cardiol. 2007;50(19):1852-6.

53. Montalescot G, van 't Hof AW, Lapostolle F, Silvain J, Lassen JF, Bolognese L, et al. Prehospital ticagrelor in ST-segment elevation myocardial infarction. N Engl J Med. 2014;371(11):1016-27.

54. Kubica J, Adamski P, Ostrowska M, Sikora J, Kubica JM, Sroka WD, et al. Morphine delays and attenuates ticagrelor exposure and action in patients with myocardial infarction: the randomized, double-blind, placebo-controlled IMPRESSION trial. Eur Heart J. 2016;37(3):245-52.

55. Frame WT, Allison RH, Moir DD, Nimmo WS. Effect of naloxone on gastric emptying during labour. Br J Anaesth. 1984;56(3):263-6.

56. Rotshteyn Y, Boyd TA, Yuan CS. Methylnaltrexone bromide: research update of pharmacokinetics following parenteral administration. Expert Opin Drug Metab Toxicol. 2011;7(2):227-35.

57. Smith SC, Jr., Feldman TE, Hirshfeld JW, Jr., Jacobs AK, Kern MJ, King SB, 3rd, et al. ACC/AHA/SCAI 2005 guideline update for percutaneous coronary intervention--summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (ACC/AHA/SCAI Writing Committee to update the 2001 Guidelines for Percutaneous Coronary Intervention). Catheter Cardiovasc Interv. 2006;67(1):87-112.

58. Kushner FG, Hand M, Smith SC, Jr., King SB, 3rd, Anderson JL, Antman EM, et al. 2009 focused updates: ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction (updating the 2004 guideline and 2007 focused update) and ACC/AHA/SCAI guidelines on percutaneous coronary intervention (updating the 2005 guideline and 2007 focused update): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Catheter Cardiovasc Interv. 2009;74(7):E25-68.

59. Tantry US, Bonello L, Aradi D, Price MJ, Jeong YH, Angiolillo DJ, et al. Consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding. J Am Coll Cardiol. 2013;62(24):2261-73.

60. Sillen H, Cook M, Davis P. Determination of ticagrelor and two metabolites in plasma samples by liquid chromatography and mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2010;878(25):2299-306.

61. Rao SV, McCoy LA, Spertus JA, Krone RJ, Singh M, Fitzgerald S, et al. An updated bleeding model to predict the risk of post-procedure bleeding among patients undergoing percutaneous coronary intervention: a report using an expanded bleeding definition from the National Cardiovascular Data Registry CathPCI Registry. JACC Cardiovasc Interv. 2013;6(9):897-904.

62. Barragan P, Bouvier JL, Roquebert PO, Macaluso G, Commeau P, Comet B, et al. Resistance to thienopyridines: clinical detection of coronary stent thrombosis by monitoring of vasodilator-stimulated phosphoprotein phosphorylation. Catheter Cardiovasc Interv. 2003;59(3):295-302.

63. Matetzky S, Shenkman B, Guetta V, Shechter M, Beinart R, Bienart R, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation. 2004;109(25):3171-5.

64. Matetzky S, Shenkman B, Guetta V, Shechter M, Bienart R, Goldenberg I, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation. 2004;109(25):3171-5.

65. Dixon WJ, Massey FJ. Introduction to Statistical Analysis. . 4th Edition ed: McGraw-Hill Companies; 1983: 142.

66. Hamm CW, Bassand JP, Agewall S, Bax J, Boersma E, Bueno H, et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J. 2011;32(23):2999-3054.

67. Ndrepepa G, Schuster T, Hadamitzky M, Byrne RA, Mehilli J, Neumann FJ, et al. Validation of the Bleeding Academic Research Consortium definition of bleeding in patients with coronary artery disease undergoing percutaneous coronary intervention. Circulation. 2012;125(11):1424-31.

68. Roy P, Bonello L, Torguson R, de Labriolle A, Lemesle G, Slottow TL, et al. Impact of "nuisance" bleeding on clopidogrel compliance in patients undergoing intracoronary drug-eluting stent implantation. Am J Cardiol. 2008;102(12):1614-7.

69. Aradi D, Kirtane A, Bonello L, Gurbel PA, Tantry US, Huber K, et al. Bleeding and stent thrombosis on P2Y12-inhibitors: collaborative analysis on the role of platelet reactivity for risk stratification after percutaneous coronary intervention. Eur Heart J. 2015;36(27):1762-71.

70. Price MJ, Berger PB, Teirstein PS, Tanguay JF, Angiolillo DJ, Spriggs D, et al. Standard- vs high-dose clopidogrel based on platelet function testing after percutaneous coronary intervention: the GRAVITAS randomized trial. JAMA. 2011;305(11):1097-105.

71. Trenk D, Stone GW, Gawaz M, Kastrati A, Angiolillo DJ, Muller U, et al. A randomized trial of prasugrel versus clopidogrel in patients with high platelet reactivity on clopidogrel after elective percutaneous coronary intervention with implantation of drug-eluting stents: results of the TRIGGER-PCI (Testing Platelet Reactivity In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel) study. J Am Coll Cardiol. 2012;59(24):2159-64.

72. Collet JP, Cuisset T, Range G, Cayla G, Elhadad S, Pouillot C, et al. Bedside monitoring to adjust antiplatelet therapy for coronary stenting. N Engl J Med. 2012;367(22):2100-9.

73. McCarthy CP, Mullins KV, Sidhu SS, Schulman SP, McEvoy JW. The on- and off-target effects of morphine in acute coronary syndrome: A narrative review. Am Heart J. 2016;176:114-21.

74. Meine TJ, Roe MT, Chen AY, Patel MR, Washam JB, Ohman EM, et al. Association of intravenous morphine use and outcomes in acute coronary syndromes: results from the CRUSADE Quality Improvement Initiative. Am Heart J. 2005;149(6):1043-9.

75. de Waha S, Eitel I, Desch S, Fuernau G, Lurz P, Urban D, et al. Intravenous morphine administration and reperfusion success in ST-elevation myocardial infarction: insights from cardiac magnetic resonance imaging. Clin Res Cardiol. 2015;104(9):727-34.

76. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J. 2018;39(2):119-77.

77. Franchi F, Rollini F, Park Y, Hu J, Kureti M, Rivas J, et al. Abstract 19115: Effect of the Peripheral Opioid Receptor Antagonist Methylnaltrexone on the Pharmacokinetic and Pharmacodynamic Profiles of Ticagrelor in Patients Receiving Morphine: A Prospective Randomized Placebo-Controlled Trial. Circulation. 2016;134(Suppl 1):A19115-A.

78. van Werkum JW, Kleibeuker M, Postma S, Bouman HJ, Elsenberg E, ten Berg JM, et al. A comparison between the Plateletworks (TM)-assay and light transmittance aggregometry for monitoring the inhibitory effects of clopidogrel. Int J Cardiol. 2010;140(1):123-6.

79. Palmerini T, Benedetto U, Biondi-Zoccai G, Della Riva D, Bacchi-Reggiani L, Smits PC, et al. Long-Term Safety of Drug-Eluting and Bare-Metal Stents: Evidence From a Comprehensive Network Meta-Analysis. J Am Coll Cardiol. 2015;65(23):2496-507.

80. Sibbing D, Aradi D, Jacobshagen C, Gross L, Trenk D, Geisler T, et al. A randomised trial on platelet function-guided de-escalation of antiplatelet treatment in ACS patients undergoing PCI. Rationale and design of the Testing Responsiveness to Platelet Inhibition on Chronic Antiplatelet Treatment for Acute Coronary Syndromes (TROPICAL-ACS) Trial. Thromb Haemost. 2017;117(1):188-95.

81. Notarangelo FM, Maglietta G, Bevilacqua P, Cereda M, Merlini PA, Villani GQ, et al. Pharmacogenomic Approach to Selecting Antiplatelet Therapy in Acute Coronary Syndromes: PHARMCLO trial. J Am Coll Cardiol. 2018.

82. Venetsanos D, Sederholm Lawesson S, Swahn E, Alfredsson J. Chewed ticagrelor tablets provide faster platelet inhibition compared to integral tablets: The inhibition of platelet aggregation after administration of three different ticagrelor formulations (IPAAD-Tica) study, a randomised controlled trial. Thromb Res. 2017;149:88-94.

83. Parodi G, Xanthopoulou I, Bellandi B, Gkizas V, Valenti R, Karanikas S, et al. Ticagrelor crushed tablets administration in STEMI patients: the MOJITO study. J Am Coll Cardiol. 2015;65(5):511-2.