Monitoring and optimizing antiplatelet therapy in patients undergoing percutaneous coronary intervention

PhD thesis

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ABBREVIATIONS

5HT – serotonin

ACS – acute coronary syndrome

ADP – adenosine-diphosphate

ApoE – apolipoprotein E

ARC – Academic Research Consortium

ASA – acetylsalicylic-acid

ATP – adenosine-triphosphate

AUC – area under the curve

CAD – coronary artery disease

CD40L – cluster of differentiation 40 ligand

CI – confidence interval

COX – cyclooxygenase

CRP – C reactive protein

CYP – cytochrome P450

DAPT – dual antiplatelet therapy

ELISA – enzyme linked immunosorbent assay

$EP_{1\text{-}4}$ – prostaglandin E2 receptor family

ESC – European Society of Cardiology

FXIII – factor XIII

GP – glycoprotein

HR – hazard ratio

IP – prostacyclin (prostaglandin I2) receptor

IQR – interquartile range

LDL – low density lipoprotein

LOWESS – locally weighted scatterplot smoothing

LTA – light transmission aggregometry

MEA – multiple electrode aggregometry

NO – nitric-oxide

OR – odds ratio

PAD – peripheral artery disease

PAI_1 – plasminogen activator inhibitor 1

PAR – protease-activated receptor

PCI – percutaneous coronary intervention

PDE - phosphodiesterase

PDGF – platelet derived growth factor

PF_4 – platelet factor 4

PF – platelet function

PGE_1 – prostaglandin E1

PI_3K - phosphatidylinositol-3 kinase

PKA – protein kinase A

PKC – protein kinase C

PLA_2 – phospholipase A2

PLT - platelet

PPP – platelet poor plasma

PRP – platelet rich plasma

PsNR – pseudo non-responder

RCT – randomized controlled trial

RNR – real non-responder

SCAD – stable coronary artery disease

TIA – transient ischemic attack

TIMI – thrombolysis in myocardial infarction

TP – thromboxane protein

TRAP – thrombin receptor activating peptide

TXA_2 – thromboxane A2

TXB_2 – thromboxane B2

U - unit

VASP – vasodilator-stimulated phosphoprotein

vWF – von Willebrand factor

1. INTRODUCTION

Cardiovascular disease is the principal cause of death in the western societies. Clinical manifestations of coronary artery disease include stable coronary artery disease and acute coronary syndromes. Revascularization by percutaneous coronary intervention became one of the most important therapeutic possibilities in both forms of ischemic heart disease. To maximize its clinical benefit, many procedural and pharmaceutical improvements have been carried out in the past two decades, of which introduction of dual antiplatelet therapy with aspirin and clopidogrel to prevent thrombotic events after percutaneous coronary intervention proved to be essential. Recently, novel, more potent antiplatelet agents turned up and the clinical utility of platelet function testing and tailored antiplatelet therapy became an extensively researched field in cardiology.

1.1. Role of platelets in ischemic heart disease

1.1.1. Central pathophysiological role of platelets in atherosclerosis

Atherosclerosis, the disease of the vascular intima characterized by intimal lipid accumulation and plaque formation, affects principally the large and medium-sized elastic and muscular arteries. Atherogenesis starts with the loss of intact endothelial function due to several different noxae [1]. In its physiological state, endothelium is a substantive organ ensuring normal blood flow conditions, trans-endothelial transports and equilibrium of vasoactive substances and hemostasis. Different forms of endothelial injury increase its adhesiveness, permeability and procoagulant properties and also, result in intensified platelet adherence and aggregation [1]. Platelets play an important role in the initiation of atherosclerosis via several surface glycoproteins (e.g. GPIbα, GPIIb/IIIa, and β3 integrin) which enables platelet "rolling" even on the structurally intact endothelium, then a firm platelet-vessel wall interaction followed by leukocyte accretion and release of several mediators [2-4]. The earliest type of atherosclerotic lesion is the fatty streak, which is a pure inflammatory lesion [5]. Several data supports how platelets contribute to vascular inflammation via interactions with inflammatory cells. Platelet activation results in expression of several inflammatory receptors, enrolment of leukocytes and monocytes, formation of platelet-leukocyte aggregates [6-11] and release of active biomolecules and chemokines from the platelet granules [12].

Permanent activation of inflammatory cells and platelets results in smooth-muscle cell migration into the intima and fatty streaks progress into intermediate and advanced lesions, leading to wall thickening and lumen narrowing of the arteries. With further progression, atherosclerotic lesions tend to form a fibrous cap (mediated by distinct growths factors and decreased connective-tissue degradation) [1] covering a necrotic core containing leukocytes, lipid, and debris.

Stable advanced lesions usually have uniformly dense fibrous caps. In contrast, plaques may become unstable with thinning of the fibrous cap at the shoulder region of the atheroma due to permanent activation of macrophages releasing proteolytic enzymes [13]. The most dangerous consequences of unstable plaques are plaque rupture occurring at thinning of the fibrous cap and plaque erosion [14,15] followed by intra plaque hemorrhage, activation of platelets and the coagulation cascade, resulting in thrombus formation and occlusion of the artery. Active disruption is related to extrinsic factors (e.g. rheological circumstances) and passive disruption is due to several intrinsic factors (large lipid core, high cholesteryl ester content, high density of macrophages and low density of smooth muscle cells, narrowed fibrous cap and high tissue factor concentration [16]) defining the stable or unstable characteristics of the plaque. The potentially dangerous lesions are often non occlusive and thus are difficult to diagnose by angiography. The other mechanism of plaque injury is erosion, which occurs mainly in women [17] and is caused by endothelial denudation. Causative role of erosion can be as high as 40% of acute coronary syndromes and 25% of myocardial infarctions [18].

1.1.2. Platelets in atherothrombosis: adhesion, activation and aggregation

Platelets play central roles in physiologic and pathologic processes of primary and secondary hemostasis. Primary hemostasis is the rapid formation of a platelet plug at the injured/alternated vessel wall. Secondary hemostasis is the parallel activation of the coagulation cascade resulting in formation of a fibrin strand further strengthening the primary thrombus. The initial step in primary hemostasis is the adhesion of platelets to the exposed subendothelial matrix with surface glycoproteins. The adhesive process can be initiated via the collagen receptor GPIa/IIa complex, but under high shear conditions platelet adhesion is mediated by the soluble plasma protein von Willebrand factor through the GPIb/V/IX complex [19]. Primary adhesion activates intracellular signaling

pathways (outside-in signaling) leading to intracellular Ca^{2+} elevation and activation of intracellular kinases (e.g. PKA, PKC, PI_3K, see Figure 1) [20]. This results in cytoskeletal and membrane rearrangement, morphological changes (shape change), secretion of several mediators (e.g. TXA_2) and also, exhaustion of the α-granules (fibrinogen, plasminogen, fibronectin, vitronectin, thrombospondin, PF_4, PAI_1, and PDGF) and dense granules (e.g. ADP, ATP, serotonin, epinephrine, Ca^{2+}). The consequent conformational changes of certain membrane glycoproteins (inside-out signaling) lead to exposure of binding sites and enables interaction of soluble adhesive plasma proteins fibrinogen and vWF with the membrane GPIIb/IIIa complex [21]. This results in further conformational change of the receptor with subsequent activation of several intracellular kinases leading to bridge formation between the adjacent platelets leading to formation of aggregates [22]. After initial activation, amplification loops represented by accelerated TXA_2 and ADP production ensure recruitment and rapid formation of platelet rich thrombi. The most important platelet activating receptors are thrombin receptors (PAR_1 and PAR_4), purinergic receptors ($P2X_1$, $P2Y_1$ and $P2Y_{12}$), collagen receptors (GPIb/V/IX, GPIa/IIa, and GPVI), TXA_2 receptors (TP), $5HT_{2A}$ receptors, α_2-adrenergic receptors, and the prostaglandin E_2 receptors ($EP_{1\text{-}4}$) while the most important inhibitory receptors are the prostacyclin (PGI_2) and NO receptors. The main platelet activating receptors and intracellular signaling pathways are summarized in Figure 1.

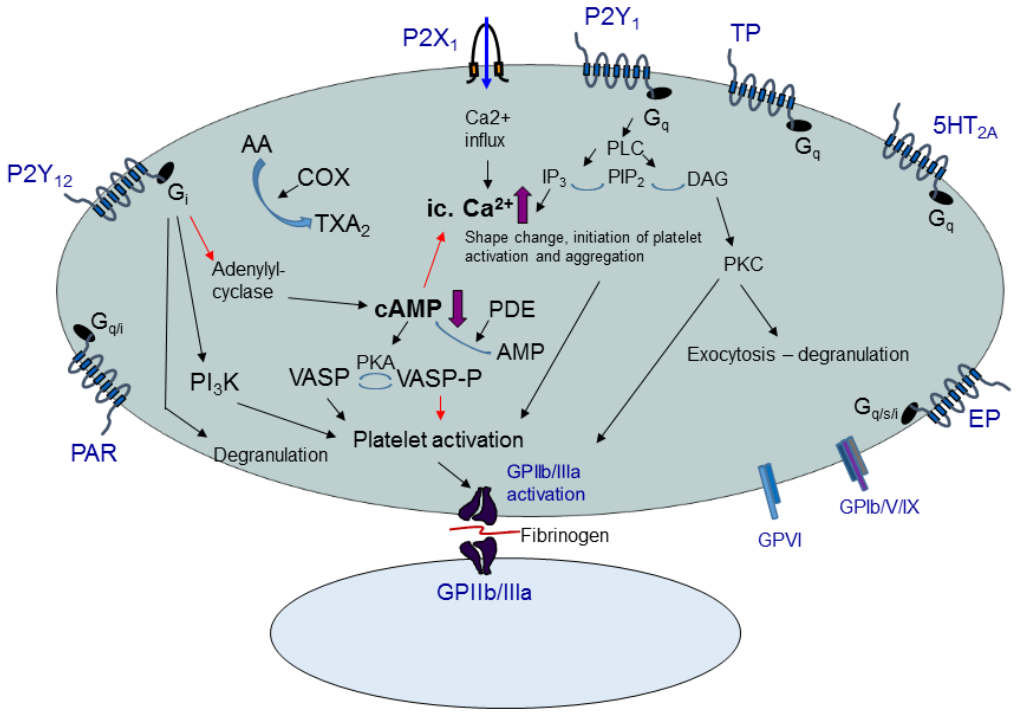

Figure 1. Main platelet activating receptors and pathways. Black arrows indicate activation, red arrows denote inhibition. Bent arrows indicate transformation/metabolization. After activation of a stimulatory surface receptor, one of the most important consequences is the elevation of intracellular Ca^{2+} level, activation of intracellular kinases (eg. PKA, PKC, PI_3K) and decrease of intracellular cAMP (which enhances the Ca_{2+} uptake into the sarcoplasmic reticulum). These processes result in shape change, degranulation, membrane rearrangement and exposure of hidden binding sites of certain membrane glycoproteins enabling the platelets to anchor to the subendothelial collagen and adhere to adjacent platelets. The purinergic $P2X_1$ and $P2Y_1$ receptors mediate mainly the initiation of platelet activation by increasing the intracellular Ca^{2+} level resulting in shape change of the platelet. The $P2Y_{12}$ receptor, activated by ADP, represents the major amplification loop of platelet activation leading to the formation of a stable platelet aggregate. $5HT_{2A}$: serotonin receptor, AA: arachidonic-acid, AMP: adenosine-monophosphate, cAMP: cyclic-adenosine-monophosphate, COX: cyclooxygenase, EP: prostaglandin receptor, G_i (inhibitory)-, G_q- and G_s (stimulatory): different subtypes of the G protein according to the α subunit, Gp: glycoprotein, IP_3: inositol trisphosphate, PAR: protease activated receptor, PDE: phosphodiesterase, PI_3K: phosphatidylinositol-3-kinase, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipase C, $P2X_1$, $P2Y_1$ and $P2Y_{12}$: subtypes of the purinergic receptors, TXA_2: thromboxane A_2, VASP: vasodilator-stimulated phosphoprotein, VASP-P: phosphorylation of vasodilator-stimulated phosphoprotein

1.2. Antiplatelet therapy in coronary artery disease

1.2.1. Antiplatelet agents

1.2.1.1. COX1 inhibitors

Synthetized in the late nineteenth century, aspirin (acetylsalicylic-acid) is the oldest antiplatelet drug [23]. Its platelet inhibitory effect was discovered in the mid-1960s, and since then it has become the cornerstone of antiplatelet therapy in primary and secondary prevention of ischemic heart disease [24,25]. ASA covalently binds to and irreversibly inhibits the cyclooxygenase enzymes, exerting 50-100 fold higher affinity for the COX_1 than to the COX_2 enzyme [26]. COX inhibition leads to impaired TXA_2 synthesis from arachidonic-acid, which is released from the membrane constituent phospholipids by PLA_2 following cell activation [23]. As TXA_2 is a potent vasoconstrictor and platelet activating agent via the platelet TP receptor, inhibiting this pathway profoundly decreases platelet activity and aggregability. Impaired platelet function caused by ASA is obtained during the life span of the platelet (7-10 days). Though aspirin has a short plasma half-life (15-20 minutes), constant administration of low dose aspirin results in continuous platelet inhibition due to low daily platelet turn-over [27]. Therefore, the platelet inhibitory effect of aspirin is not linearly dose-dependent in contrast to its gastrointestinal toxicity. ASA has several COX independent antithrombotic effects as well, such as inhibition of thrombin generation [28] and enhancement of fibrinolysis [29], contributing to the favorable therapeutic effect of aspirin. The mechanism of action of the currently available and developmental antiplatelet agents are summarized in Figure 3.

1.2.1.2. $P2Y_{12}$ receptor inhibitors

According to our current understanding, the $P2Y_{12}$ receptor has a central role in almost all platelet functions, being responsible for the major amplification loop of platelet activation and thus is a very promising target for antithrombotic drugs. Platelet aggregatory response provoked by ADP is initiated by the $P2Y_1$ receptor resulting in shape change and initial activation and aggregation of adjacent platelets. $P2Y_{12}$ receptor is liable for the ADP-dependent amplification of secretion, procoagulant activity, aggregation and finally for stabilization of the platelet thrombus [30]. Since the results of early studies with ticlopidine [31] and those of the large clinical studies with

clopidogrel [32,33], additional use of a $P2Y_{12}$ blocking agent to ASA has been used to prevent ischemic events after percutaneous coronary intervention .

Thienopyridines. Thienopyridines covalently bind to the $P2Y_{12}$ receptor leading to irreversible inhibition of platelet function for the life span of the platelet (7-10 days). The first generation ticlopidine was soon replaced by the second generation clopidogrel due to ticlopidine's adverse side effects (i.e neutropenia and thrombotic thrombocytopenic purpura). Clopidogrel's favorable therapeutic effect is attributed to its antiplatelet and anti-inflammatory profile. Beside irreversible $P2Y_{12}$ blockade, clopidogrel has also been reported to attenuate platelet–leukocyte aggregate formation [34], periprocedural increase of CRP levels [35], and to reduce P-selectin and CD40L expression [36] and the rate of thrombin formation [37]. However, clopidogrel has wide inter-individual efficacy in terms of platelet inhibition. The potential causative roles in its background included pharmacokinetic, pharmacodynamic, clinical, genetic and cellular mechanisms as well as the patient's compliance [38] (see also below). Recently, the third generation, more potent prasugrel has been introduced in the clinical practice [39,40]. Prasugrel has a more rapid onset of action and a more extent platelet inhibitory effect and proved to be superior to clopidogrel in large clinical studies, though at a price of increased bleeding risk [41]. All thienopyridines are prodrugs and require metabolization via the hepatic CYP enzymes to pass into its biologically active metabolite. The activating steps and the involved hepatic enzymes somewhat differ at each drug, resulting in different pharmacokinetic and -dynamic features, and explains the distinct drug-drug interference profile of the compounds (Figure 2, Table 1).

Nucleotide/nucleoside analogues. These drugs are active agents not requiring metabolic transformation, thus having immediate antiplatelet effect upon intravenous administration or intestinal absorption. Ticagrelor is an orally administered reversible allosteric inhibitor of the $P2Y_{12}$ receptor. Ticagrelor proved to be favorable compared to clopidogrel in large clinical studies exerting a more expressed and faster antiplatelet effect [42]. Cangrelor recently got approval based on the results of the CHAMPION-PHOENIX study, where it significantly reduced the rate of ischemic events during PCI, with no significant increase in severe bleeding [43]. This intravenously administered ATP derivative is used to achieve immediate and complete platelet inhibition before

PCI and may be used in patients who are unable to swallow and/or in bridging therapy before surgery in patients requiring $P2Y_{12}$ inhibitor therapy [44]. After cessation of its administration normal platelet function is restored within 1 hour [43,44].

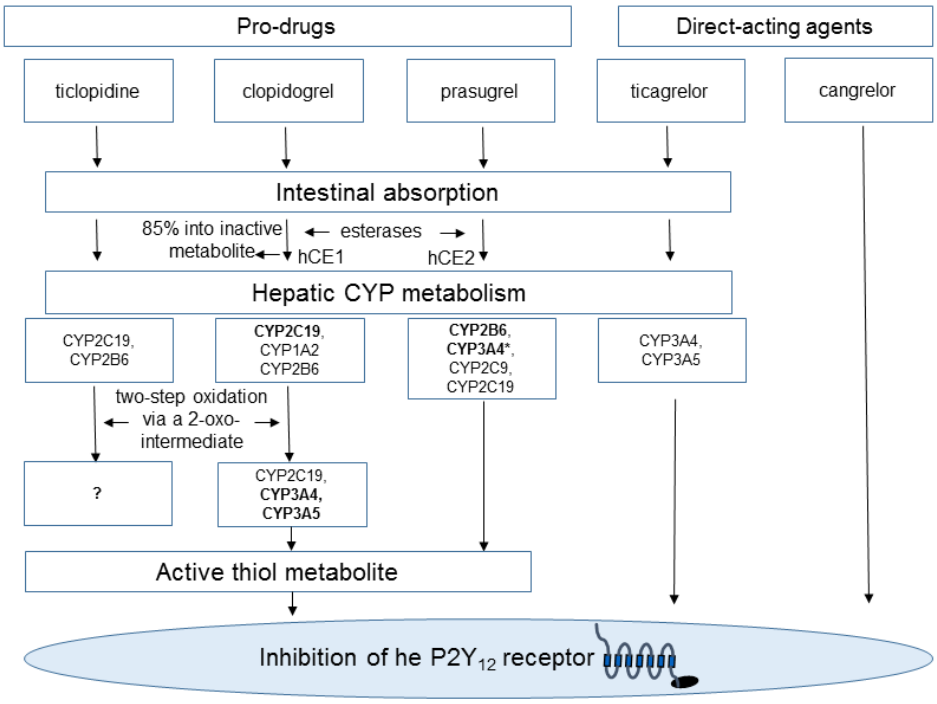

Figure 2. Metabolization of the $P2Y_{12}$ inhibitors. After absorption, ticlopidine undergoes extensive metabolization by the hepatic CYP enzymes. The active metabolite is formed in a two-step oxidation, through the intermediate 2-oxo-ticlopidine [45]. The CYP enzymes involved in the second oxidation step are not identified. Clopidogrel, after intestinal absorption, is extensively hydrolyzed by plasma esterases into an inactive compound. The remaining clopidogrel fraction undergoes a two-step oxidation process in the liver to turn into its bioactive form via the intermediate 2-oxo-clopidogrel [45]. Prasugrel is also a pro-drug, but its activation process is started by plasma esterases and includes only one CYP-dependent step, which importantly, is mediated mainly by the intestinal CYP3A enzymes (*) [45]. This explains the lack of drug-drug interactions reported with the use of prasugrel. Ticagrelor is an *ab ovo* active agent, though 30-40% of its action is linked to its active, bioequivalent metabolite transformed by hepatic CYP enzymes. Cangrelor is also an active compound administered intravenously, and its metabolization is independent from the hepatic CYP enzymes. Bold letters indicate major enzymes of the metabolic steps. hCE1: human carboxylesterase 1 (primarily synthetized by the liver), hCE2: human carboxylesterase 2 (primarily synthetized by the intestine). CYP: cytochrome p450 enzymes.

Table 1. Pharmacological profiles of currently approved $P2Y_{12}$ inhibitors.

	ticlopidine	clopidogrel	prasugrel	ticagrelor	cangrelor
Group based on chemical structure	1st generation thienopyridine	2nd generation thienopyridine	3rd generation thienopyridine	cyclo-pentyltriazolo-pyrimidine	ATP-analogue
Way of administration	Oral (bid)	Oral	Oral	Oral (bid)	Intravenous
Receptor inhibition	Irreversible	Irreversible	Irreversible	Reversible	Reversible
Onset of action	6 h	2-8 h	30 min-4 h	30 min-2 h	Seconds
Offset of action	7-10 d	7-10 d	7-10 d	3-5 d	cca. 60 min
Main enzymes of CYP metabolization	CYP2C19, CYP2B6, CYP3A4 (?)	CYP1A2, CYP2B6, CYP2C19, CYP3A4, CYP3A5	CYP2B6, CYP3A4	CYP3A4, CYP3A5	-
Reported significant drug-drug interactions	yes	yes	no	yes	no

1.2.1.3. GPIIb/IIIa receptor inhibitors

The platelet specific GPIIb/IIIa receptor is the final common pathway of platelet aggregation, being the receptor for fibrinogen with the highest affinity and binding also fibronectin, vitronectin and vWF [46]. Blocking this final step by intravenously administered GPIIb/IIIa inhibitors results in very efficient platelet inhibition, but also in increased risk of bleeding. These antiplatelets have different pharmacological features. Abciximab is a monoclonal antibody fragment and causes impaired platelet function for several days [47]. In contrast, eptifibatide (a low-molecular weight heptapeptide) and tirofiban (a non-peptide tyrosine derivative) shows more rapid dissociation from the GPIIb/IIIa receptor and restoration of normal hemostatic function after their cessation is expected after 3-4 hours [47]. In current clinical practice, the use of GPIIb/IIIa inhibitors is limited to bail-out situations -in patients with low bleeding and high thrombotic risk- for a short period of time during and immediately before and after PCI in patients with ACS [48].

1.2.1.4. PDE inhibitors

Phosphodiesterase enzyme breaks down the intracellular cAMP resulting in enhanced platelet activity by increasing the intracellular Ca^{2+} level. By inhibiting PDE, cilostazol and dipyridamole effectively lowers platelet reactivity. Dipyridamole is primarily approved for the secondary prevention of transient ischemic attack. Cilostazol is also a

direct arterial vasodilator mainly used in patients with PAD [49]. It was also tested in former studies, whether adding these agents to DAPT could overcome high on-treatment platelet reactivity in PCI treated patients, though due to controversial results, they did not bring any break-through in antithrombotic treatment [50-52].

1.2.1.5. Antiplatelets under development

There are further promising targets for platelet inhibition. Thrombin, the most potent agonist activates platelets through the PAR receptor family. Human platelets express PAR_1 and PAR_4 receptors. By inhibiting the PAR_1 receptor, only the effects of thrombin on platelets are prevented without impairing effects on coagulation or other functions. The recently approved PAR_1 inhibitor in clinical use is vorapaxar [53]. Other antiplatelet agents being under clinical investigation/development include compounds targeting the P2Y1 receptor, the 5-HT receptors, the prostaglandin receptors (IP, EP_{1-4}), the GPVI main collagen receptor, the GPIb-VWF axis, the thromboxane receptors and the phosphatidylinositol-3-kinase intracellular pathway (see Figure 3, indicated with orange color) [54,55].

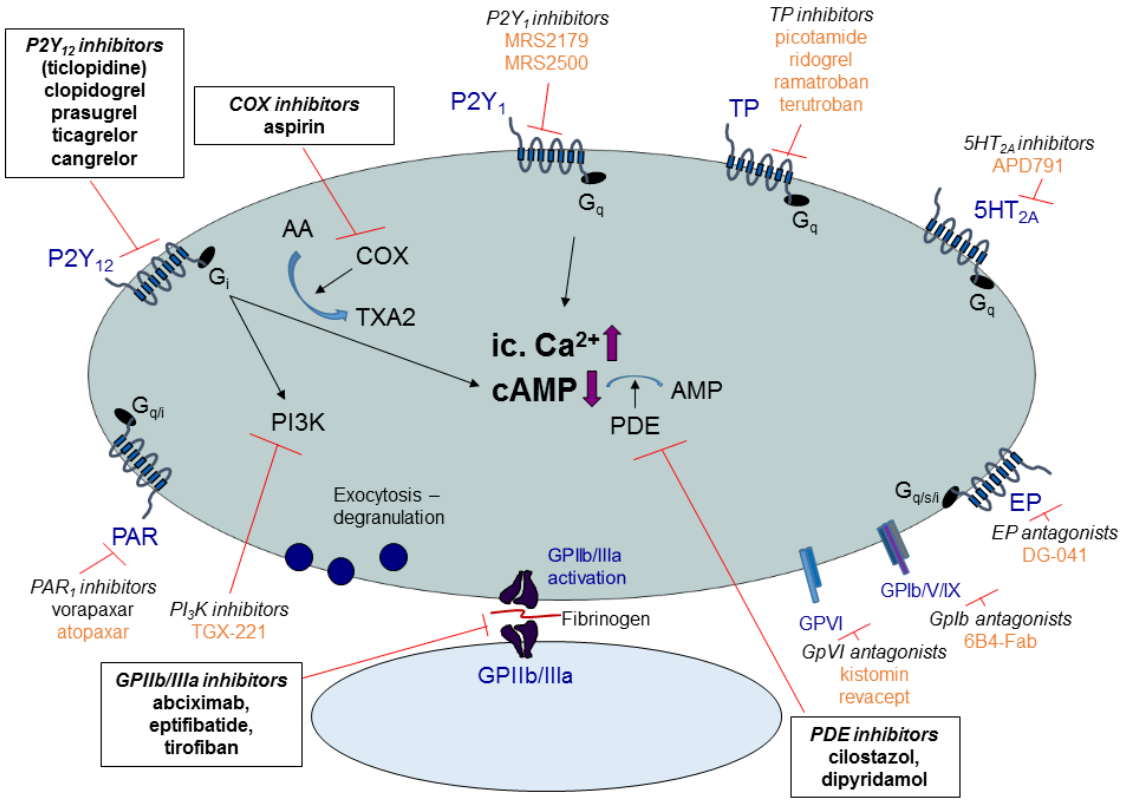

Figure 3. **The antiplatelet agents' sites of action.** Currently approved drugs are indicated with black bold text in frame, agents under development are signed with orange color. Black arrows denote stimulation, red lines depict inhibition. Thromboxane protein receptor inhibitors have not been proven to be superior to aspirin up to date [56-58]. The $P2Y_1$ inhibitors MRS2179 and MRS2500 and the PI3K inhibitor TGX-221 are only for research use currently [59,60]. The safety trial of additional use of the serotonin antagonist APD-791 beside clopidogrel/aspirin therapy has not yet started patient recruitment (NCT02034292). The EP3 prostaglandin receptor antagonist DG-041 has only been investigated in Phase I clinical trials [61]. The GPIb antibody 6B4-Fab and the GPVI antagonist kistomin and revacept were evaluated only in preclinical studies up to date [62-64]. The evaluation of PAR-1 receptor antagonist atopaxar was suspended after phase II clinical trials due to safety concerns [65,66] . For abbreviations see legend of Figure 1.

1.2.2. Antiplatelet strategies and current clinical guidelines in patients with SCAD

The antiplatelet regimens in SCAD are summarized in Table 2. ASA treatment is indicated before elective stenting and a loading dose is recommended if the patient is not pre-treated. Clopidogrel loading is not recommended routinely, until coronary anatomy is not known. After elective PCI, dual antiplatelet treatment is recommended with aspirin and clopidogrel for at least 1 month after BMS deployment and 6-12 months after DES insertion. General use of GPIIb/IIIa inhibitors and prasugrel or

ticagrelor is not recommended in these patients, as they may increase bleeding risk [67]. In SCAD lifelong administration of low dose aspirin (75-150 mg daily) is recommended in all patients. In case of aspirin intolerance or allergy, clopidogrel may be considered. Use of platelet function testing is recommended only in specific, high risk situations when it may have potential therapeutic consequences [48,67].

Table 2. Recommendations for antiplatelet treatment in patients with SCAD undergoing PCI based on the current ESC guidelines [48,67].

Recommendations for antiplatelet therapy in SCAD patients	**Class**[1]	**Level**[2]
Pretreatment		
Clopidogrel 600 mg loading dose is recommended in elective PCI patients once coronary anatomy is known and decision to proceed with PCI is made (≥2 hours before PCI).	I	A
Pretreatment with clopidogrel may be considered in patients with high probability for significant CAD.	IIb	C
In patients already on a 75 mg clopidogrel, a new loading dose of 600 mg or more may be considered once the indication for PCI is confirmed.	IIb	C
During PCI		
ASA is indicated for elective stenting.	I	B
ASA loading (oral 150-300 mg, i.v. 80-150 mg) is recommended if not pretreated.	I	C
Clopidogrel is recommended for elective stenting (≥600 mg loading, 75 mg maintenance dose).	I	A
GPIIb/IIIa inhibitors may be used only for bail-out.	IIa	C
After stenting		
DAPT is indicated for at least 1 month after BMS implantation.	I	A
DAPT is indicated for 6 months after DES implantation.	I	B
Shorter DAPT duration (<6 months) may be considered after DES implantation in patients at high bleeding risk.	IIb	A
Longer DAPT duration (>6 months) may be used in patients at high ischemic and low bleeding risk.	IIb	C
Life-long single antiplatelet therapy, usually ASA, is recommended.	I	A
Use of PF testing		
PF testing may be used in specific or high risk situations (e.g. history stent thrombosis; compliance issue; suspicion of resistance; high bleeding risk) if results may change the treatment strategy.	IIb	C
Routine platelet function testing to adjust antiplatelet therapy before or after elective stenting is not recommended.	III	A

1.2.3. Antiplatelet strategies and current clinical guidelines in ACS

In patients with STEMI, $P2Y_{12}$ inhibitor therapy should be initiated orally as early as possible before angiography, usually at first medical contact (Table 3). Upstream use of

GPIIb/IIIa antagonists may also be considered in high risk patients undergoing transfer that delays PCI. ASA loading should be performed in all cases. According to the current guidelines [39,48], the first choice of $P2Y_{12}$ inhibitors are prasugrel or ticagrelor because of their more rapid onset of action and greater platelet inhibitory potency compared to clopidogrel [41,42]. Clopidogrel should be used only in lack of availability of the newer agents or in case of contraindications.

Table 3. Recommendations for antiplatelet treatment in patients with STEMI undergoing primary PCI based on the actual ESC guidelines [39,48].

Recommendations for antiplatelet therapy in STEMI patients	**Class[1]**	**Level[2]**
Pretreatment		
It is recommended to give P2Y12 inhibitors at the time of first medical contact.	I	B
Upstream use of a GPIIb/IIIa inhibitor (vs. in-lab use) may be considered in high-risk patients undergoing transfer for primary PCI.	IIb	B
During and after PCI		
ASA is recommended for all patients without contraindications at an initial oral loading dose of 150–300 mg (or 80–150 mg i.v.) and at a maintenance dose of 75–100 mg daily long-term regardless of treatment strategy.	I	A
A P2Y12 inhibitor is recommended in addition to ASA and maintained over 12 months unless there are contraindications such as excessive risk of bleeding. Options are:	I	A
• Prasugrel (60 mg loading dose, 10 mg daily dose) if no contraindication	I	B
• Ticagrelor (180 mg loading dose, 90 mg twice daily) if no contraindication	I	B
• Clopidogrel (600 mg loading dose, 75 mg daily dose), only when prasugrel or ticagrelor are not available or are contraindicated	I	B
GPIIb/IIIa inhibitors should be considered for bail-out or evidence of no-reflow or a thrombotic complication.	IIa	C

In NSTE-ACS, recommendations for antiplatelet treatment are similar, with the exception of upstream use of GPIIb/IIIa inhibitors and pre-treatment of prasugrel, which regimens are not recommended in patients in whom coronary artery anatomy is not known (Table 4).

After any type of acute coronary syndrome treated with PCI, fibrinolysis or medically, long term dual antiplatelet treatment is recommended. Regarding aspirin, low dose maintenance therapy should be used indefinitely due to its well established benefits in secondary prevention. In case of aspirin intolerance or allergy, clopidogrel

might be used instead. Duration of parallel $P2Y_{12}$ therapy is usually recommended to last up to 12 month.

Table 4. Recommendations for antiplatelet treatment in patients with NSTE-ACS undergoing PCI according to current ESC guidelines [40,48].

Recommendations for antiplatelet therapy in NSTE-ACS patients	Class[1]	Level[2]
Pretreatment		
Pre-treatment with prasugrel in patients in whom coronary anatomy is not known, is not recommended.	III	B
Pre-treatment with GPIIb/IIIa antagonists in patients in whom coronary anatomy is not known, is not recommended.	III	A
During and after PCI		
ASA is recommended for all patients without contraindications at an initial oral loading dose of 150–300 mg (or 80–150 mg i.v.), and at a maintenance dose of 75–100 mg daily long-term regardless of treatment strategy.	I	A
A P2Y12 inhibitor is recommended in addition to ASA, and maintained over 12 months unless there are contraindications such as excessive risk of bleeding. Options are:	I	A
• Prasugrel (60 mg loading dose, 10 mg daily dose) in patients in whom coronary anatomy is known and who are proceeding to PCI if no contraindication	I	B
• Ticagrelor (180 mg loading dose, 90 mg twice daily) for patients at moderate-to-high risk of ischemic events, regardless of initial treatment strategy including those pre-treated with clopidogrel if no contraindication	I	B
• Clopidogrel (600 mg loading dose, 75 mg daily dose), only when prasugrel or ticagrelor are not available or are contraindicated	I	B
GPIIb/IIIa antagonists should be considered for bail-out situation or thrombotic complications.	IIa	C

1.3. Laboratory monitoring of platelet function in current cardiology practice

There are several laboratory methods to measure platelet function, most of which were primarily developed to screen and detect hereditary and acquired platelet/primary hemostasis disorders. Since the use of dual antiplatelet therapy results in acquired platelet dysfunction, most of these tests were challenged in monitoring the efficacy of antiplatelet therapy, though with various success. The more functional aggregometry tests give more information about the overall platelet reactivity and are less specific for the inhibitory effect of a given drug. In contrast, more specific tests assessing the inhibitory effect of a drug at the subcellular level might be less informative about the overall function of the activation-aggregation cascade. In the next session I will provide a non-exhaustive overview of the most often used platelet function tests in cardiology practice. The most widely applied tests are summarized in Table 7 (see below).

1.3.1. Aggregometry methods

Platelet activation finally results in platelet aggregation, which can be detected by several functional aggregometry methods. These tests measure the ability of different platelet agonists to induce platelet activation and aggregation *in vitro*.

1.3.1.1. Light transmission aggregometry (Born aggregometry)

The gold standard of aggregometry methods is light transmission aggregometry, also referred as the Born-method, introduced by Gustavo Born more than 50 years ago [68,69]. The method detects the decrease in optical density of the platelet rich plasma which occurs when the platelets in it aggregate due to activation with an agonist. Change in optical density is depicted as a function of time. PRP is prepared by centrifugation and is basically free of other blood cellular elements. The standard used in the measurement is the platelet poor plasma of the same subject (PPP, prepared by further centrifugation), representing the minimum of optical density (or the maximum of light transmittance).

In spite of the wide-spread use of LTA for detecting platelet function, it is often criticized because of comparability difficulties of the obtained results. The method is fairly non-standardized, concerning pre-analytical processes (blood sampling, preparation of PRP, circumstances and time of sample storage until the measurement), agonists of different sources and concentrations, as well as the settings of the recorder [70] or the measurement parameter to be evaluated (e.g. maximal aggregation, final aggregation and disaggregation) [71,72]. The most widely used agonists are collagen, epinephrine, ADP, TRAP, and arachidonic acid.

Most initial data about the association of high on-treatment platelet reactivity and recurrent ischemic events in CAD patients after PCI raised from studies using the LTA method [73-75]. Moreover, in a comparative platelet function study, LTA was able to predict recurrent ischemic events during 1 year follow-up [76]. However, because of the non-standardized, labor-intensive procedure of the LTA method it is not advised for monitoring the efficacy of dual antiplatelet therapy in every-day clinical practice according to the latest consensus document [77].

1.3.1.2. VerifyNow method

The VerifyNow is a semi-automated, standardized point-of-care method to measure platelet aggregation in whole blood. Activated platelets aggregate to fibrinogen-coated microbeads, decreasing the optical density of the sample. The $P2Y_{12}$ assay includes ADP agonist along with prostaglandin E_1, which increases the sensitivity of the test and can be successfully used to assess $P2Y_{12}$ therapy [78-80]. The VerifyNow Aspirin test uses arachidonic-acid agonist and assesses the thromboxane A_2 mediated activation pathway. VerifyNow has been widely and successfully used to measure the efficacy of DAPT and its association with clinical outcome and the benefit of tailored antiplatelet therapy in patients undergoing PCI [81-84].

1.3.1.3. Multiple electrode aggregometry (Multiplate)

Multiple electrode aggregometry or impedance aggregometry is based on detection of impedance change caused by the adherence of platelets after activation with an agonist to multiple electrode-pairs immersed in the whole blood sample. The change of impedance is depicted as a function of time, and the area under the curve value given in units (U) indicates the measure of platelet reactivity [85]. Multiplate is a semi-automated, standardized point-of-care device, with the following available assays: ADP test and high sensitive ADP test (with use of PGE_1 to eliminate false-positive results) to monitor $P2Y_{12}$ inhibitor therapy, ASPI test (arachidonic-acid agonist) to monitor ASA therapy, and other non-specific assays such as TRAP test (TRAP-6 agonist), and COL test (collagen agonist), RISTO test (ristocetin agonist) to examine the vWF and GpIb dependent aggregation (e.g. to detect von Willebrand's disease). The Multiplate ADP test was widely used in clinical studies assessing the efficacy of dual antiplatelet therapy [86] and it was found to be capable to predict ischemic [87] or bleeding events [88,89] in PCI treated patients.

1.3.1.4. Plateletworks

In this standardized, point-of-care method, aggregation testing in whole blood is based on the comparison of a platelet count performed before and after provoking platelet activation, using different agonists. Though, the method is not widely used and/or investigated, it was successfully tested in a comparative platelet function study, where its results were associated with occurrence of ischemic events during 12 months follow-

up [76]. Also, it was found to be capable to identify patients at higher risk of myocardial infarction and rehospitalization within 3 months after coronary angiography [90].

1.3.1.5. Platelet function analyzer 100 (PFA-100)

This automated point-of-care method was developed to screen primary hemostatic disorders (e.g. von Willebrand's disease). In this device, whole blood sample flows at high shear rates through an aperture in a membrane which is coated with distinct agonists and leads to rapid occlusion of the aperture, referred as the closure time. The COX inhibitors usually prolong the closure time of the collagen/epinephrine cartridge, therefore the method has often been used to measure functional aspirin resistance [91]. However, clopidogrel's efficacy [92] and its association with clinical outcomes cannot be assessed with this method [76].

1.3.1.6. Impact-R (cone and plate(let) analyzer - CAP)

By this method the anticoagulated whole blood sample is added to a polystyrene well. Platelet activation is induced by application of shear stress and aggregates are visualized and quantified by staining. The results are expressed as percentage of well surface covered by aggregates (SC) as an index of adhesion and average aggregate size (AS) as an index of aggregation [93]. Agonist-induced aggregation was suggested to be capable to monitor antiplatelet therapy [94]. However, in a comparative platelet function testing study, Impact-R failed to discriminate between patients with and without major cardiovascular events at 1 year follow-up after PCI [76].

1.3.2. Other methods to detect platelet activation

There are several other tests to measure platelet activation by detecting appearance of activation markers on the platelets' surface or soluble activation markers in the plasma (representing cleaved and shed surface glycoprotein fragments), and structural changes of membrane or intracellular regulatory proteins.

1.3.2.1. Flow cytometry

Flow cytometry can measure several aspects of platelet activation [95]. After stimulation of platelets by an agonist, different markers of activation can be measured with the help of fluorescent antibodies (e.g. P-selectin exposure, activated GPIIb/IIIa expression, binding of fibrinogen). In a comparative study, ADP induced aggregometry

(VerifyNow, LTA and MEA) and flow cytometry results correlated significantly in PCI treated patients on DAPT [96]. In contrast, after AA induction, correlations were only partially observed. Also, association of platelet activation detected by flow cytometry with clinical outcome is fairly unknown yet. Furthermore, due to expense of the unit and need for labor-intensive sample preparation, flow cytometry is not practical for monitoring DAPT at point-of care.

1.3.2.2. VASP test

Over a decade ago, an important new technique has been introduced by using fixed and permeabilized platelets and specific labeling of the intra-cellular regulatory protein vasodilator-stimulated phosphoprotein [97]. It is phosphorylated through the cAMP biochemical cascade, indicating $P2Y_{12}$ receptor inhibition and its dephosphorylation indicates $P2Y_{12}$ receptor activity [98], thus the VASP assay can be used to monitor $P2Y_{12}$ inhibitor therapy. Results of this assay correlate well with those of distinct platelet aggregometry methods [99-101]. Though VASP assay was described to be capable to predict adverse ischemic events after PCI [102], its discriminative capacity was lower than that of the aggregometry methods [103].

1.3.2.3. Measurement of soluble activation markers

An alternative way to assess platelet function is to find a reliable plasma marker that reflects platelet activation and is a specific marker of the platelet, is not affected by artefacts of sample collection, and is measureable by a reproducible and simple laboratory technique. Possible candidate molecules can be substances that are released from the platelet's granules, molecules that are expressed on and shed from the platelet's surface, and secreted metabolic molecules [104]. In spite of high former interest, none of these markers could fulfill the above mentioned criteria, thus their clinical utility is controversial at this point [105].

1.3.2.4. Thromboelastography (TEG, ROTEM)

This is a viscoelastic hemostatic assay, which analyzes clot formation (time to initial fibrin generation), clot elasticity development (clot strength) and the process of fibrinolysis. Elasticity of the clot is influenced by several factors, such as the contractile force of platelets during clot retraction (which is the major determinant), platelet and fibrinogen concentration, hematocrit, FXIII and the thrombin generation during

coagulation [106]. TEG is most widely used in surgery and anesthesiology and was less extensively investigated in monitoring DAPT therapy in cardiology patients, however some data suggested that clot strength and rapid fibrin formation beside DAPT were risk factors of recurrent ischemic events in patients after PCI [73].

1.4. Definition and clinical significance of high on-treatment platelet reactivity

1.4.1. Clinical and laboratory approach to the efficacy of antiplatelet therapy

Since the introduction of percutaneous coronary intervention in the therapeutic toolbar of coronary artery disease, many procedural and pharmaceutical improvements have been carried out. The spread of drug eluting stents successfully decreased the occurrence of restenosis, though they brought along a new complication in the form of late and very late stent thrombosis. Regarding antiplatelet therapy, which is a *sine qua non* in the success of percutaneous coronary intervention and intracoronary stenting, it has been proven, that adding clopidogrel to aspirin was more effective in preventing recurrent ischemic events in the long term compared to aspirin monotherapy [32,33]. Furthermore, DAPT was shown to decrease the long term rate of both ischemic and bleeding events compared to aspirin combined with an anticoagulant (heparin or coumarin) [107,108] or an oral GPIIb/IIIa inhibitor [109]. However, in spite of the application of dual antiplatelet therapy after PCI, ischemic events such as stent thrombosis and myocardial infarction still continued to occur, therefore the terms "treatment failure" and "aspirin and clopidogrel resistance" emerged. Treatment failure refers to the state, when an adverse clinical event/condition recurs despite the administration of a drug to avoid it. In the literature, "aspirin and clopidogrel resistance" referred to the condition, when the lack of aspirin/clopidogrel's effect was justified by a laboratory platelet function test. However, in pharmacology, drug resistance means inability of a drug to hit its therapeutic target (e.g. receptor, enzyme or regulatory protein) either from a pharmacokinetic (e.g. impaired intestinal absorption leading to suboptimal concentration of the drug at the effect site) or from a pharmacodynamic (meaning that the target protein itself is impaired/altered) cause. Usually, platelet function tests are not capable to assess these details of antiplatelet mechanism of action. Thus, laboratory proven aspirin and clopidogrel "resistance" was replaced by the term "high on-treatment platelet reactivity" (HPR) or "residual platelet reactivity/activity"

(RPR/RPA). With respect to the applied antiplatelet therapy and platelet function test we may differentiate “high on-clopidogrel and high on-aspirin platelet reactivity” (HCPR and HAPR) or “dual high on-treatment platelet reactivity”.

1.4.2. Clinical significance of measuring aspirin-effect and high on-aspirin platelet reactivity (HAPR)

Measuring the efficacy of aspirin treatment proved to be more complicated and less reliable from the methodological aspect, than that of the $P2Y_{12}$ inhibitors [110,111]. Methods to monitor aspirin therapy include measuring of serum TXB_2 (the stable metabolite of TXA_2) concentrations or detecting the urine TXB_2 or 11-dehydro-thromboxane-B_2 level [111]. However, these measurements are complicated and these metabolites might be generated through COX-1 independent pathways as well, thus they rather reflect an overall inflammatory state, than the measure of aspirin-effect. Therefore, the inhibitory effect of aspirin most widely is measured indirectly in platelet function tests using arachidonic-acid as an agonist (via the induction of TXA_2 generation and consequent platelet activation) [112]. Many non-specific antagonists (ADP, collagen, and epinephrine) are also rely on TXA_2 generation as an amplification loop, therefore were used in the laboratory determination of aspirin response. However these tests overestimate the prevalence of true aspirin non-response [110,113].

To date, the predictive value of measuring aspirin-effect in association with clinical outcome is controversial. In early studies, the “aspirin-resistant” phenotype was associated with higher risk of ischemic events, however in these studies patients were on aspirin monotherapy usually and non-aspirin specific platelet function tests were used [114-117]. Therefore, these tests rather identified patients with a “hyper-reactive platelet phenotype”, than measured aspirin’s platelet inhibitory effect via the COX_1 inhibition.

Recently, the largest clinical trial investigating high on-treatment platelet reactivity on aspirin and clopidogrel so far found no association between on-aspirin treatment platelet reactivity assessed by VerifyNow Aspirin test and all cause death, stent thrombosis or myocardial infarction, though HAPR was inversely related to bleeding [81]. Similarly, another large scale study failed to link high residual platelet reactivity on aspirin measured by PFA-100 to clinical outcome [118]. On the other

hand, recent data from a large scale, one center registry suggested, that patients with HAPR identified by the Multiplate ASPI test (using AA induction) showed a significantly higher risk of death or ST at 1 year and HAPR was an independent predictor of the combined primary outcome [119]. Similarly, dual high on-treatment platelet reactivity to aspirin and clopidogrel was associated with a higher risk for atherothrombotic events and identifies patients at highest thrombotic risk [120,121].

Whether or not HAPR is associated with clinical outcome, the benefit of aspirin dose adjustment based on laboratory efficacy testing is questionable anyway. Several clinical data justified that overcoming HAPR by increasing aspirin dose only increased the risk of bleeding, without further improving ischemic outcomes [122,123]. Since such a therapeutic modification is not recommended, reasonableness of assessing HAPR currently remains debated.

In summary, in contrast to high on-clopidogrel platelet reactivity, the term "high on-aspirin treatment platelet reactivity" and "aspirin resistance" are less clearly defined and have a widely variable prevalence in the literature [124], and though some data suggest, that HAPR along with HCPR might be helpful in thrombosis risk stratification, its clinical significance remains controversial. Consequently, the following part of this thesis will focus on high platelet reactivity on $P2Y_{12}$ inhibitor therapy.

1.4.3. High on-clopidogrel platelet reactivity (HCPR) and high platelet reactivity on $P2Y_{12}$ inhibitor therapy (HPR)

In the past decade, extensive research has been focused on the association of laboratory certified HCPR and recurrent ischemic events resulting in growing evidence, that HCPR and HPR are independent risk factors of thrombotic events after PCI [77,81,125]. The largest studies (N>300) investigating the existence and strength of an association between on-treatment platelet reactivity and clinical outcome are summarized in Table 5.

Table 5. Major studies linking on-treatment platelet reactivity with clinical outcome

First author (year), Study name	No. of patients and presenting clinical syndrome	Platelet function test(s)/applied $P2Y_{12}$ inhibitor	Cut-off for HPR	Cut-off for LPR	OR [95% CI] for ischemic event(s)*	OR [95% CI] for bleeding event(s)*	Follow-up time
Sibbing (2010) [88]	308 NSTEMI /STEMI 2245 UAP/SCAD	Multiplate ADP/ clopidogrel	>46 U	<19 U	6.44 [2.38–17.38] for def ST**	2.6 [1.3–5.2] for in hospital TIMI major bleeding	30 days
Geisler (2010) [126]	514 SCAD 505 ACS	LTA 20 µM ADP/ clopidogrel	Upper tertile border for FA >42.5%	NR	HR 1.05 [1.01–1.08] for early (<30 days) stent thrombosis 2.21 [1.31–3.73] for combined CV end point 2.31 [1.1–4.84] for all 3 month ST	NR	3 months
Breet (2010), POPular [76]	1069 elective PCI	LTA 5 µM ADP LTA 20 µM ADP VerifyNow $P2Y_{12}$ Plateletworks/ clopidogrel	MA ≥42.9% MA≥ 64.5% ≥ 236 PRU ≥ 80.5%	NR	2.09 [1.34-3.25] 2.05 [1.32-3.19] 2.53 [1.63-3.91] 2.22 [1.25-3.93] for combined CV end point	No association was found between TIMI major or minor bleeding and any of the PF tests.	1 year
Park (2011) [127]	1586 SCAD 1264 ACS	VerifyNow $P2Y_{12}$/ Clopidogrel	>235 PRU and/or % inhibition <15	<235 PRU	HR 1.33 [0.88–2.01] for combined CV end point 1.45 [0.27–7.92] for def/prob ST	HR 0.78 [0.35–1.69] for TIMI major bleeding	2 years
Bonello (2012) [128]	128 STEMI 100 NSTEMI 73 UAP	VASP/ prasugrel	≥53.5 % PRI	<16 % PRI	1.44 [1.2–1.72] per 10% increase for def/prob ST	0.75 [0.59–0.96] per 10 % increase for TIMI major and minor bleeding	1 year
Siller-Matula (2012), PEGASUS-PCI [129]	274 elective PCI 67 NSTE-ACS 73 STEMI	Multiplate ADP+PGE_1/ clopidogrel	≥48 U	NR	36.9 [4.3–319] for def/prob ST	No predictive ability was found for TIMI major bleeding.	1 year
Cuisset (2013), POBA [130]	1542 ACS	VASP/ 25% prasugrel 75% clopidogrel	NR	≤10 % PRI	NR	4.7 [2.7-8.3] for BARC bleeding	6 months
Stone (2013), ADAPT-DES [81]	4147 SCAD 2373 UAP 1250 NSTEMI 813 STEMI	VerifyNow $P2Y_{12}$/ Clopidogrel	>208 PRU	<95 PRU	HR 2.54 [1.55–4.16] for def/prob ST, HR 1.42 [1.09–1.86] for MI	HR 1.52 [1.17-1.97] for clinically relevant bleeding	1 year

ACS: acute coronary syndrome; ADP: adenosine-diphosphate; BARC: Bleeding Academic Research Consortium; CI: confidence interval; def: definite; FA: final aggregation; HPR: high on-treatment platelet reactivity; HR: hazard ratio; LPR: low platelet reactivity; LTA: light transmission aggregometry; NR: not reported; NSTEMI: non-ST elevation myocardial infarction; OR: odds ratio; PRI: platelet reactivity index; prob: probable; SCAD: stable coronary artery disease; STEMI: ST elevation myocardial infarction; TIMI: thrombolysis in myocardial infarction; UAP: unstable angina pectoris; VASP: vasodilator-stimulated phosphoprotein.*Odds ratios are given unless otherwise indicated. **All ST are defined according to Academic Research Consortium criteria.

Recognition of an association between HPR (mostly on clopidogrel) and clinical outcome as well as wide inter-individual variability of platelet inhibition and relatively high prevalence of HCPR raised doubt in one-size-fits all dosing method and led to the development of more potent $P2Y_{12}$ receptor inhibitors. However, the price to pay for

more expressed platelet inhibition is an increased bleeding risk in unselected patient populations [41,42]. Consequently, current recommendations exclude patients with stable coronary artery disease and ACS patients at high bleeding risk from the benefit of these novel $P2Y_{12}$ inhibitors [48,131,132]. These patients remain subjects of clopidogrel treatment. Notably, the prevalence of high on-treatment platelet reactivity (HPR) only diminished but not vanished with the use of novel antiplatelets [133,134].

On the other hand, recently published data from the ADAPT-DES study highlighted further interesting aspects of platelet function testing, verifying that overcoming high on-treatment platelet reactivity at any price might profoundly increase bleeding risk and through that may counter-balance the favorable effects of intense platelet inhibition. The authors discussed it as a potential explanation of the lack of association between HPR and mortality [81]. However, association of low platelet reactivity on dual antiplatelet therapy with higher bleeding risk [88] and the concept of a "therapeutic window" of $P2Y_{12}$ inhibitor treatment was already introduced in earlier studies [77,89]. The fact that prognostic significance of bleeding consequences is equally important with that of ischemic events—particularly with the spreading use of more potent $P2Y_{12}$ inhibitors [77,81]— further expands the space for platelet function testing.

1.4.4. Poor response to antiplatelet agents - determining factors of HPR

Factors associated with high on-clopidogrel platelet reactivity have already been investigated by a large number of studies [38] (Table 6). Although certain genetic polymorphisms (CYP2C19 loss of function, CYP3A4 and CYP3A5, and ABCB1 gene polymorphisms), drug-drug interactions (e.g. CCBs and PPIs) and accompanying clinical risk factors such as higher body mass index (BMI), reduced LVF, diabetes mellitus, chronic kidney disease, female gender and smoking have already been linked to HPR measured by various platelet function tests, there is a lot of discrepancy among study results. This discrepancy might derive from the use of distinct platelet function tests assessing different aspects of platelet activation/aggregation and variable cut-off values as well as from inclusion of platelet reactivity as categorical or continuous parameter. Moreover, there is a gap of knowledge regarding the response variability to newer antiplatelet agents.

Table 6. Response variability to $P2Y_{12}$ therapy

Determinants of HPR	**References**
Clinical factors	
Age	[135-137]
Gender	[137-139]
BMI	[103,136,140,141]
ACS	[142,143]
Renal failure	[137,142,144,145]
Reduced LVF	[142,146]
Smoking	[139,147,148]
Inflammation	[149-151]
Underdosing	[152,153]
Compliance	[154]
Genetic factors	
CYP2C19*2 loss of function	[155-157]
CYP3A4, A5 gene variants	[158-160]
ABCB1 gene variants	[161]
Drug-drug interactions	
PPI (mostly omeprazole)	[162-164]
CCB	[135,165,166]

1.4.5. Tailored antiplatelet therapy

Due to extensive research, several methods for platelet function testing underwent clinical validation beside the gold standard optical aggregometry and clinically determined cut-off values have been established [76,77,87]. Nevertheless, platelet function testing has a fairly variable, but generally low predictive value depending on the applied platelet function test, investigated clinical end point, follow-up time and clinical presentation of coronary artery disease (CAD) [77,125]. The most recent consensus document advices only a few platelet testing method for assessing platelet reactivity in cardiology practice [77]. The most widely used and investigated platelet function tests are summarized in Table 7.

Table 7. The most widely used platelet function tests to measure the efficacy of DAPT.

	Multiplate 6.4 µM ADP	**VerifyNow P2Y12 20 µM ADP + PGE1**	**VASP 20 µM ADP + PGE1**	**LTA* variable ADP cc-s**
Point-of-care/ standardization	++/yes	+++/yes	-/yes	-/no
$P2Y_{12}$ receptor specificity	+	++	+++	+
Measurement time	< 10 min	< 10 min	2-3 h	< 10 min
Cut-off for HPR	> 46 U	> 208 PRU	> 53% PRI	a, 5 µM ADP induced MA > 42.9% b, 20 µM ADP induced MA > 64.5%
Name of study or first author, derivative patient cohort, N (%ACS)	Sibbing [88], general PCI, 2533 (12.16% MI)	ADAPT-DES [81], general PCI, 8582 (51.7)	Frere [167], 195 (100% NSTE-ACS)	POPular [76], elective PCI (0)
Predictive ability for stent thrombosis, OR [95% CI]**	6.44 [2.38-17.38]	HR 2.54 [1.55–4.16]	PPV 12% NPV 99%	a,2.09 [1.34-3.25]*** b, 3.85 [1.18-12.58]
Cut-off for LPR	< 19 U	< 95 PRU (upper border of the 1st quintile)	< 10% PRI	10 µM ADP induced MA< 40% (upper border of the 1st quartile)
Name of study or first author, derivative patient cohort, N (%ACS)	Sibbing [88], general PCI, 2533 (12.16%MI)	Kirtane [168], general PCI 8582 (51.7)	POBA [130], 1542 (100% NSTE-ACS)	Cuisset [169], 597 (100)
Predictive ability for bleeding, OR [95% CI]	2.6 [1.3–5.2]	1.48 [1.21–1.81]	4.7 [2.7-8.3]	6.6% vs. 1.4 % 1st quartile vs. the others

ADP: adenosine-diphosphate, HPR: high on-treatment platelet reactivity, HR: hazard ratio, MA: maximal aggregation, NPV: negative predictive value, NSTE-ACS: non-ST elevation acute coronary syndrome, OR: odds ratio, PCI: percutaneous coronary intervention, PPV: positive predictive value, PRI: platelet reactivity index, PRU: platelet reactivity unit. *The latest consensus paper does not recommend the use of LTA due to its non-standardized features. .**Odds ratios are given unless otherwise indicated. *** OR is given to a combined CV end point.

Parallel to the assessment of distinct platelet function tests, several randomized controlled trials have been conducted to investigate the benefit of tailored antiplatelet therapy based on platelet function testing. These RCTs and the main prospective cohort studies with the same object are summarized in Table 8. The first RCT investigating this question was the GRAVITAS trial closing with neutral results, suggesting that doubling the clopidogrel dose in patients with HPR on standard 75 mg clopidogrel does not improve clinical outcome [84]. The TRIGGER-PCI trial, which investigated the effect of prasugrel therapy introduction in HPR patients on 75 mg clopidogrel in comparison to continuation of conventional therapy was ceased on half-way of the planned study period due to unexpectedly low event rates [83]. In the TRILOGY-ACS platelet function substudy, where medically treated NSTE-ACS patients were recruited,

randomization to 10 mg prasugrel or conventional clopidogrel therapy did not lead to any clinical benefit during 30 month follow-up. However, platelet function in the prasugrel arm was significantly decreased compared to the clopidogrel arm during the whole study period. This result suggests that maximizing platelet inhibition is not so favorable and has less significance in patients without revascularization [170].

Table 8. Major studies investigating the clinical benefit of tailored antiplatelet therapy.

Study name and design (year)	Patient number (ACS/STEMI %)	Platelet function test (cut-off, HPR rate)	Therapeutic modification if HPR	Comparison	Primary end point	OR/HR [95%CI] for primary end point and for ST
GRAVITAS Randomized controlled trial (2010) [84]	2214 (40/0.4)	VerifyNow $P2Y_{12}$ (>230 PRU, 41%)	High dose clopidogrel (50%)	75 vs 150 mg clopidogrel	CV death, MI or ST at 6 month	HR 1.01 [0.58-1.76] HR 0.63 [0.21-1.93]**
TRIGGER-PCI Randomized controlled trial (2012) [83]	423 (0/0)	VerifyNow $P2Y_{12}$ (>208 PRU, 100%)	75 mg clopidogrel (50%) 10 mg prasugrel (50%)	75 mg clopidogrel vs 10 mg prasugrel	CV death or MI at 6 month	NE NE**
TRILOGY-ACS Randomized controlled trial (2012) [170]	2564 (100/0)	VerifyNow $P2Y_{12}$ * (>208 PRU, 46%) (>230 PRU, 39%)	75 mg clopidogrel (50%) 10 mg prasugrel (50%)	75 mg clopidogrel vs 10 mg prasugrel	CV death, MI or stroke at 30 month	HR 1.43 [1.07-1.89] NR
ARCTIC Randomized controlled trial (2013) [82]	2440 (27/0)	VerifyNow $P2Y_{12}$ (34.5%) VerifyNow Aspirin (7.6%)	High dose clopidogrel (80%) Prasugrel (12%) High dose ASA (85%)	Guided vs conventional arm wo PF testing	All-cause death, MI, urgent revasc., stroke, ST at 1 year	HR 1.13 [0.98-1.29] HR 1.54 [0.56-3.18]***
RECLOSE-2-ACS Prospective registry (2011) [171]	1789 (100/46)	LTA 10 µM ADP (14%)	High dose clopidogrel (NR) Ticlopidine (NR)	Guided HPR vs no HPR	CV death, MI, urgent revasc., stroke at 2 years	OR 1.80 [1.21-2.68] OR 2.18 [1.19-3.97]***
MADONNA Prospective registry (2012) [172]	798 (37/12)	Multiplate ADP (26%)	High dose clopidogrel (12%) Prasugrel (14%)	Non-guided arm wo therapy modification vs guided	Def/prob ST at 30 days	OR 7.9 [1.08-69.2]
ISAR-HPR Historical control cohort study (2014) [173]	999 (50/15)	Multiplate ADP (100%)	High dose clopidogrel (15%) Prasugrel (20%)	Guided vs conventional cohort wo therapy modification	All-cause death or ST at 30 days	HR 0.71 [0.39-1.30] HR 0.31 [0.11-0.87]**
PÉCS REGISTRY Prospective registry (2014) [174]	741 (100/48)	Multiplate ADP (29.5%)	High dose clopidogrel (58%) Prasugrel (42%)	High dose clopidogrel vs prasugrel group	All-cause death, MI, ST or stroke at 1 year	OR 2.67 [1.20-5.96] OR 1.96 [0.50-7.58]**

* Randomization before PF testing. ** Definite/probable stent thrombosis *** All stent thromboses. NE: not estimated due to insufficient data. NR: not reported. See abbreviations above at Table 5 and 7.

The largest trial studying tailored antiplatelet therapy up to date was the ARCTIC trial [82]. This trial investigated different therapy modification algorithms (including repeated clopidogrel loading dose and elevated -150 mg- clopidogrel maintenance dose

administration, additional GPIIb/IIIa inhibitor application or in a smaller fraction of patients, switch to prasugrel maintenance treatment with the use of an initial prasugrel loading dose) based on platelet function testing and compared the clinical outcome of these patients with that of a conventionally treated arm on 75 mg clopidogrel, where no platelet function testing was applied. In this trial therapy modification did not improve the clinical outcome of the patients compared to the conventionally treated arm. However, heterogeneous therapy modification algorithms were used according to the treating physician and therapy adjustment in the opposite direction (dose reduction or switch to a less potent antiplatelet drug) was also performed when too intense platelet inhibition was noticed.

In summary, the results of the above clinical studies do not support the concept of tailored antiplatelet therapy based on routine platelet function testing. However, these studies were conducted completely (TRIGGER-PCI) or mainly in elective PCI cohorts (GRAVITAS 60.2%, ARCTIC 73%) representing rather low-intermediate risk patients or in medically treated NSTE-ACS patients. Consequently, there are relatively few data on the role of tailored antiplatelet therapy in invasively treated ACS patients with the highest thrombotic risk.

Though routine platelet function testing is not recommended by the current guidelines, more recent data suggest that tailored antiplatelet therapy may reduce thrombotic and bleeding risk in the ACS patient cohort [174]. Currently, therapeutic decisions at the individual level are further aggravated by widened supply of antiplatelet agents, different biological drug profiles, considerable efficacy/safety issues and the pronounced heterogeneity of cardiovascular patients. Thus, monitoring platelet reactivity beside obvious clinical parameters might provide important contributory data to identify patients at higher ischemic or bleeding risk in the acute setting of CAD.

2. OBJECTIVES

As we reviewed in the introduction section, there is a lack of knowledge regarding the clinical benefit of platelet function testing and tailored antiplatelet therapy in acute coronary syndrome patients at high thrombotic risk. Therefore, first we aimed to investigate the incidence of high on-treatment platelet reactivity, the effect of intensified antiplatelet therapy and long term follow-up of platelet function in patients with myocardial infarction and stable coronary artery disease. Secondly, we analyzed predictors of high on-clopidogrel platelet reactivity measured by a clinically recently available point-of-care method in a high thrombotic risk cohort represented by acute coronary syndrome patients. Detailed objectives of our studies were the followings:

1. To assess platelet function values and the rate of high on-clopidogrel platelet reactivity (HCPR) measured with light transmission aggregometry among patients with myocardial infarction (MI) and patients with stable coronary artery disease (SCAD).

2. To assess the influencing clinical and demographical factors of platelet reactivity measured by LTA.

3. To optimize antiplatelet therapy in patients with HCPR until laboratory certified platelet inhibition is achieved in patients with MI and SCAD.

4. To conduct a long-term follow up of individually tailored antiplatelet therapy with repeated platelet function testing and registration of clinical outcome data in patients with MI and SCAD.

5. To identify predictors of HCPR measured with the point-of-care multiple electrode aggregometry in the acute coronary syndrome patient cohort with high thrombotic risk.

6. To develop a HCPR risk prediction model and perform HCPR risk stratification of patients with ACS.

3. METHODS

To answer the above questions we conducted two prospective studies on separate patient cohorts.

3.1. Monitoring and optimizing antiplatelet therapy in patients with myocardial infarction and stable coronary artery disease

3.1.1. Patient population and study design

We enrolled 200 patients into our study: 133 patients with myocardial infarction (MI) (105 patients with ST elevation myocardial infarction and 28 patients with non-ST elevation myocardial infarction) and 67 patients with stable coronary artery disease (SCAD). All patients underwent PCI and intracoronary stenting (bare metal stent or drug eluting stent implantation).

A loading dose of 600 mg clopidogrel was given to all patients in the MI group and to clopidogrel naive patients in the SCAD group. MI patients were given 75 or 150 mg clopidogrel maintenance dose based on the physician's decision. Determining factors were age, gender, body weight, and comorbidities of the patients. According to our current institutional practice, higher doses of clopidogrel were applied in the first 30 days after PCI, then standard 75 mg clopidogrel for the whole study period. In the SCAD group, clopidogrel maintenance dose was 75 mg daily. All patients were given ≥100 mg aspirin daily (in 5 cases 300 mg daily dose was applied according to the physician's decision.)

The exclusion criteria were as follows: history of severe renal or hepatic disease, hematological or hemostatic disorders, acute or chronic inflammatory disease, active malignancy and active bleeding. The study protocol was approved by the institutional ethics review committee, and a written informed consent was obtained from all patients before enrolment.

3.1.2. Aggregometry measurements

Platelet function was measured with LTA (Carat TX4, Budapest, Hungary) 72 hours after PCI in the MI group and 24 hours after PCI in the SCAD group. Measurement

details are described previously [175] . Briefly, blood was drawn from the cubital vein into citrate-anticoagulated tubes (final concentration was 3.2%). Platelet rich plasma was separated by centrifugation at 980 rpm for 10 minutes. Platelet poor plasma was prepared by further centrifugation at 3000 rpm for 10 minutes. Platelet agonists were 10 μM epinephrine (EPI), 1 μg/ml collagen (COLL) and 0,5 μg/ml AA to assess overall platelet reactivity and 1,25 μM; 5 μM; 10 μM ADP to assess clopidogrel's effectiveness specifically. Measuring time was 7 minutes and maximal aggregation was determined in percentage. Control LTA was performed 5 days after therapeutic adjustments. The intra- and inter-assay CVs of 5 μM induced aggregation were 7.98% and 3.34%, respectively.

3.1.3. Patient follow-up

Patients were re-interviewed and repeated LTA was performed at 6 and 12 month. The investigators were in close contact with the patients during the study period; patients were fully informed about the importance of the applied antiplatelet therapy and were asked to report modifications of their medication. The number of participants gradually decreased at the consecutive control points: at 6 month 102 MI and 48 SCAD patients were available for control LTA testing and interviewing. At 12 month 91 MI and 44 SCAD patients completed the study.

3.2. Determining factors of high on-treatment platelet reactivity in patients with acute coronary syndrome

3.2.1. Patient population and study design

In this study, we enrolled 463 consecutive ACS patients referred for urgent coronary angiography. Indications were as follows: 334 cases with ST segment elevation myocardial infarction (STEMI), 110 patients with non-ST segment elevation myocardial infarction (NSTEMI) and 19 cases with unstable angina (UAP). 95.9% of the population underwent percutaneous coronary intervention and 93.9% had coronary stent implantation. Exclusion criteria were history of cerebrovascular disease (transient ischemic attack or stroke), history of major gastrointestinal bleeding and contraindication to $P2Y_{12}$ inhibitor treatment. Acetylsalicylic acid loading and maintenance treatment was applied according to current guidelines [48]. A single

loading dose of 600 mg clopidogrel was given to all patients prior to or at the time of angiography/PCI.

Use of glycoprotein IIb/IIIa receptor (GPIIb/IIIa) inhibitors was left to operator's discretion. GPIIb/IIIa inhibitor applied was eptifibatide exclusively. Unfractionated heparin was administered for both diagnostics and percutaneous coronary intervention (70 IU/kg bolus for diagnostics and PCI with planned use of a GPIIb/IIIa inhibitor or 100 IU/kg bolus for PCI without planned use of a GPIIb/IIIa inhibitor). Coronary angiography and percutaneous coronary intervention were then performed using standard techniques. The study protocol was approved by the institutional ethics committee on human research and was conducted according to the principles of the Declaration of Helsinki.

3.2.2. Platelet function testing

Platelet function was measured in whole blood with multiple electrode aggregometry (Multiplate analyzer, Roche, Basel, Switzerland) 12 to 36 hours after 600 mg clopidogrel loading. When the GPIIb/IIIa inhibitor eptifibatide was applied during the PCI, based on the pharmacokinetic features of the drug, platelet function test was postponed to ensure a 24-hour time lag from cessation of eptifibatide administration. Measurement details were described previously [85]. Briefly, blood was drawn from the antecubital vein into hirudin-anticoagulated tubes (specified concentration >15 μg/mL). All samples were stored at room temperature and rested at least 30 minutes before the measurement as advised by the manufacturer. After twofold dilution with 0.9% w/v saline, samples were stirred for 3 minutes in the test cuvettes at 37°C. Then platelet agonist adenosine-diphosphate (ADP) was added at 6.4 μM final concentration. Increasing impedance is transformed to arbitrary aggregation units and is plotted against time continuously for 6 minutes. Platelet aggregation defined with MEA is quantified as area under the curve (AUC) of arbitrary aggregation units (U). The definition of high on-clopidogrel-treatment platelet reactivity (HPR) was based on the consensus paper of the Working Group on On-Treatment Platelet Reactivity, using >46 U as the cut-off value [77]. Below this threshold, platelet inhibition was considered to be efficient (no HPR). Platelet function testing was measured by an assistant who was blinded to patient

clinical characteristics and laboratory measurements. Investigators making statistical analyses were not involved in the data acquisition.

3.2.3. Other laboratory measurements

Hematology testing was performed on a Sysmex XE 2100 analyzer (Sysmex Europe GmbH, Hamburg, Germany). Blood glucose and high sensitive C-reactive protein (hs-CRP) levels were measured by a Modular Analytics EVO Cobas 6000 analyzer (Roche, Basel, Switzerland), whereas troponin I was evaluated using Architect Stat high sensitive troponin I immunoassay (Abbott, Abbott Park, IL). All measurements were carried out according to manufacturers' instructions.

3.3. Statistical methods used in the studies

Formal sample size calculations were not applied because all cohort studies are ongoing studies. Furthermore, for derivation studies of risk prediction models, there are no generally accepted approaches to estimate the sample size requirements. Analyses were performed on all data acquired during the study period. Categorical variables in 2×2 contingency tables were assessed using Fisher's exact test. Categorical data in 2×k contingency tables were analyzed using the unordered chi-squared test or, to detect linear trend, the Cochran-Armitage test (chi-squared test for trend). Continuous parameters were examined for normality with the Shapiro-Wilks W and the D'Agostino Pearson test. As none of the investigated continuous variables showed normal distribution, the Mann–Whitney test was applied for inter-group comparisons. The Wilcoxon signed-rank test or repeated measures ANOVA test was used for repeated measures. To adjust for differences in demographic data between patients with stable coronary artery disease and myocardial infarction, ANCOVA analysis was used. Adjustment was performed for the following covariates: age, gender, BMI, smoking, hypertension, diabetes mellitus, hypercholesterolemia, elapsed time between clopidogrel loading and platelet function testing, history of myocardial infarction, previous PCI or CABG operation, aspirin and clopidogrel medication on admission.

For the risk prediction model construction, logistic regression analysis was used. Presence of non-linear relationships of the continuous variables to log odds of HPR were explored using restricted cubic splines which were then evaluated graphically and

by formal Wald testing for linearity. Since none of the continuous predictors showed non-linear association with the log odds of the outcome, they were used as linear variables. Univariate logistic regression analysis was performed to identify parameters with a p value less than 0.2. These variables were entered into a backward multivariate logistic regression model. Clinical parameters that were previously found to be associated with HPR, such as BMI, diabetes mellitus and renal function were forced into the multivariate model irrespective of the univariate p value. In multivariate logistic regression, parameters with a p value above 0.1 on likelihood ratio testing were then sequentially removed. The likelihood ratio and the Hosmer-Lemeshow tests were used to assess model fit, whereas predictive power was evaluated by receiver operating characteristic (ROC) curve analysis. To adjust for different scales of the predictors in the multivariate model, significance of individual parameters was assessed using the adequacy index, a parameter which indicates the proportion of the total explained variation in the outcome (expressed in terms of -2 log likelihood chi square) that could be explained by a single predictor [176]. Since the performance of a prediction model in the derivation dataset may overestimate the true performance, we conducted internal validation using 10,000 bootstrap samples to assess optimism. Risk of HPR was stratified as low, intermediate or high based on sensitivity, specificity and cumulative frequency distribution analyses of the predicted probability. For each of the risk classes, interval likelihood ratios were calculated.

A two-tailed p value less than 0.05 was considered statistically significant.

All analyses were carried out with MedCalc 15.2 (MedCalc Software, Ostend, Belgium, available at http://www.medcalc.org) except for linearity testing of continuous variables and internal validation, which were performed with R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria, available at http://www.R-project.org) using the ‘rms’ package 4.2-1 (authored by Frank E Harrell Jr, available at http://biostat.mc.vanderbilt.edu/rms).

4. RESULTS

4.1. Monitoring and optimizing antiplatelet therapy in patients with myocardial infarction and stable coronary artery disease

Our first prospective study was conducted in patients with myocardial infarction (N=133) and stable coronary artery disease (N=67) undergoing percutaneous coronary intervention at our institute.

4.1.1. Patient characteristics

Baseline characteristics of the different patient groups are listed in Table 9. Age, prevalence of hypertension, hypercholesterinaemia, previous myocardial infarction, percutaneous coronary intervention and coronary artery bypass graft operation was significantly higher in the SCAD group, while ratio of smokers was higher in the MI group. Previous ACEI, beta-blocker, statin, aspirin and clopidogrel medication was significantly higher in the SCAD group.

Regarding initial $P2Y_{12}$ inhibitor therapy, in the MI group, 41 patients (30.8%) were on standard 75 mg clopidogrel maintenance dose and 92 individuals (69.2%) were on 150 mg, high dose clopidogrel (according to our current institutional practice, see above). In the SCAD group all patients were on standard clopidogrel therapy at start (Table 9).

Table 9. Demographic, clinical, and procedural characteristics of the MI and SCAD patients.

Variable	MI (n = 133)	SCAD (n = 67)	p Value*
Age, years, median (IQR)	59 (51-66)	66 (59-73)	<0.0001
Female, %	32.6	34.3	0.87
BMI, kg/m^2, median (IQR)	27.7 (24.9-31.1)	27.4 (24.9-30.0)	0.32
Medical history			
Hypertension, %	65.2	85	<0.01
Diabetes mellitus, %	26.7	35.8	0.25
Dyslipidaemia, %	22.9	47.8	<0.001
History of tobbacco use, %	57.9	28.4	<0.001
Previous myocardial infarction, %	11.1	32.8	<0.001
Previous PCI, %	16.3	44.8	<0.0001
Previous CABG, %	0.0	13.4	<0.0001

Medication on admission			
Acetylsalycilic acid, %	22.9	82.1	<0.0001
Clopidogrel, %	7.4	47.8	<0.0001
ACEI or ARB, %	44.4	91	<0.0001
β-blocker, %	36.3	76.1	<0.0001
Nitroglycerin, %	16.3	50.7	<0.0001
Lipid lowering agents, %	26.7	89.6	<0.0001
PPI medication at baseline			
On PPI, %	91	53.7	<0.0001
Pantoprazole, %	91	50.7	<0.0001
Lansoprazole, %	0.0	1.5	0.34
Rabeprazole, %	0.0	1.5	0.34
Procedural parameters			
Number of diseased vessels (%)			
1	48.9	50.7	1.00
2	25.9	29.9	0.62
3 or left main artery	25.2	19.4	0.38
Number of implanted stents, median (IQR)	1 (1-2)	1 (1-2)	0.46
Total stent length, mm, median (IQR)	32 (24-50)	28 (20-48)	0.23
Clopidogrel maintenance dose at baseline, %			
75 mg	30.8	100	<0.0001
150 mg	69.2	0	<0.0001

ACEI: angiotensin-converting enzyme inhibitor, ARB: angiotensin II receptor blocker, BMI: body mass index, CABG: coronary artery bypass graft surgery, IQR: interquartile range, MI: myocardial infarction, PCI: percutaneous coronary intervention, PPI: proton pump inhibitor, SCAD: stable coronary artery disease. Highlighted rows indicate statistical significance. * p values were calculated with the Mann-Whitney (continuous parameters) and the Fisher's exact (categorical parameters) tests.

4.1.2. Baseline platelet aggregations in the MI and SCAD patients

Baseline platelet reactivity in respect to most agonists was significantly higher in patients receiving 75 mg clopidogrel in the MI group than in the SCAD group (detailed demographic description and aggregation values of these subgroups are shown in Table 10).

Table 10. Demographic data and baseline platelet reactivity of SCAD and MI patients on 75 mg clopidogrel.

Variable	MI on 75 mg clopidogrel (n = 41)	SCAD (n = 67)	p value*
Age, years, median (IQR)	66 (57-72)	66 (59-73)	0.36
Female, %	48.8	34.3	0.16
BMI, kg/m^2, median (IQR)	25.7 (23.1-30.1)	27.4 (24.9-30)	0.16
Medical history			
Hypertension, %	68.3	85.0	0.05
Diabetes mellitus, %	21.9	35.8	0.14
Dyslipidaemia, %	17.1	47.8	<0.01
History of tobbacco use, %	48.8	28.4	0.04
Previous myocardial infarction, %	12.2	32.8	0.02
Previous PCI, %	14.6	44.8	<0.01
Previous CABG, %	0.0	13.43	0.01
Medication on admission			
Acetylsalycilic acid, %	26.8	82.1	<0.0001
Clopidogrel, %	9.8	47.8	<0.0001
ACEI or ARB, %	51.2	91.0	<0.0001
β-blocker, %	41.5	76.1	<0.001
Nitroglycerin, %	9.8	50.7	<0.0001
Lipid lowering agents, %	29.3	89.6	<0.0001
Procedural parameters			
Number of diseased vessels (%)			
1	41.5	50.7	0.43
2	34.1	29.9	0.67
3 or left main artery	24.4	19.4	0.63
Number of implanted stents, median (IQR)	2 (1-2)	1 (1-2)	0.33
Total stent length, mm, median (IQR)	35 (28-46)	28 (20-48)	0.24
Baseline platelet reactivity, MA %, median (IQR)			
1 µg/ml collagen	20 (12-31.5)	18.5 (10-35.5)	0.70
1,25 µM ADP	16 (9.8-25)	8 (2.3-15)	<0.001
5 µM ADP	43 (36-53)	39 (28-48)	0.02
10 µM ADP	54 (45.8-62.3)	46 (35.3-56.8)	<0.01
10 µM epinephrine	43 (30-59)	24 (17-46.8)	<0.01
0,5 µg/ml arachidonic acid	4 (1.8-6.3)	2 (1-4.8)	0.01

ACEI: angiotensin-converting enzyme inhibitor, ADP: adenosine-diphosphate, ARB: angiotensin II receptor blocker, BMI: body mass index, CABG: coronary artery bypass graft surgery, IQR: interquartile

range, MA: maximal aggregation, MI: myocardial infarction, PCI: percutaneous coronary intervention, PPI: proton pump inhibitor, SCAD: stable coronary artery disease. * p values were calculated with the Mann-Whitney (continuous parameters) and the Fisher's exact (categorical parameters) tests.

After adjustment to demographic parameters, the difference diminished in case of epinephrine and arachidonic-acid induced aggregations (p=0.359 and p=0.861, respectively), however, the difference remained significant with all concentrations of the ADP agonist (1.25ADP: p=0.005; 5ADP: p=0.046; 10ADP: p=0.023; Figure 4).

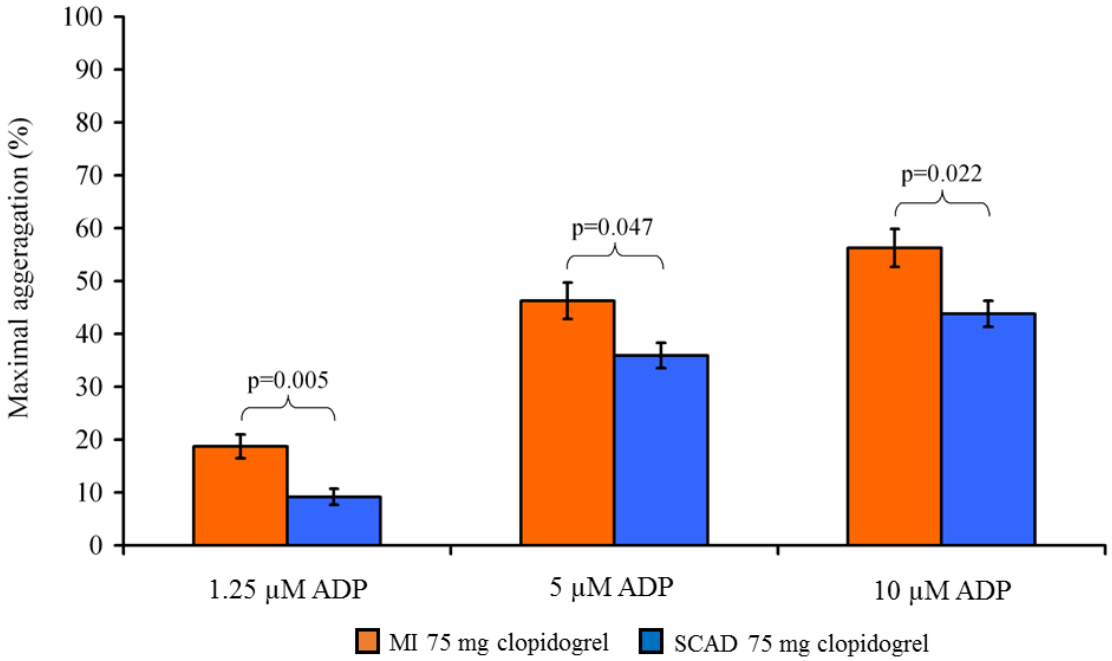

Figure 4. Baseline platelet reactivity of MI and SCAD patients being on identical 75 mg clopidogrel therapy. In the MI group platelet reactivity was significantly higher with all concentrations of ADP after adjustment for demographic differences. Results are expressed as mean (represented by columns) ± SEM (represented by bars). $p<0.05$ was considered significant.

Interestingly, patients receiving 150 mg clopidogrel compared to those taking the standard dose within the MI group, showed only a tendency of lower platelet reactivity, without reaching statistical significance. Moreover, after adjusting to demographic parameters, this tendency completely diminished regarding all agonists (Figure 5).

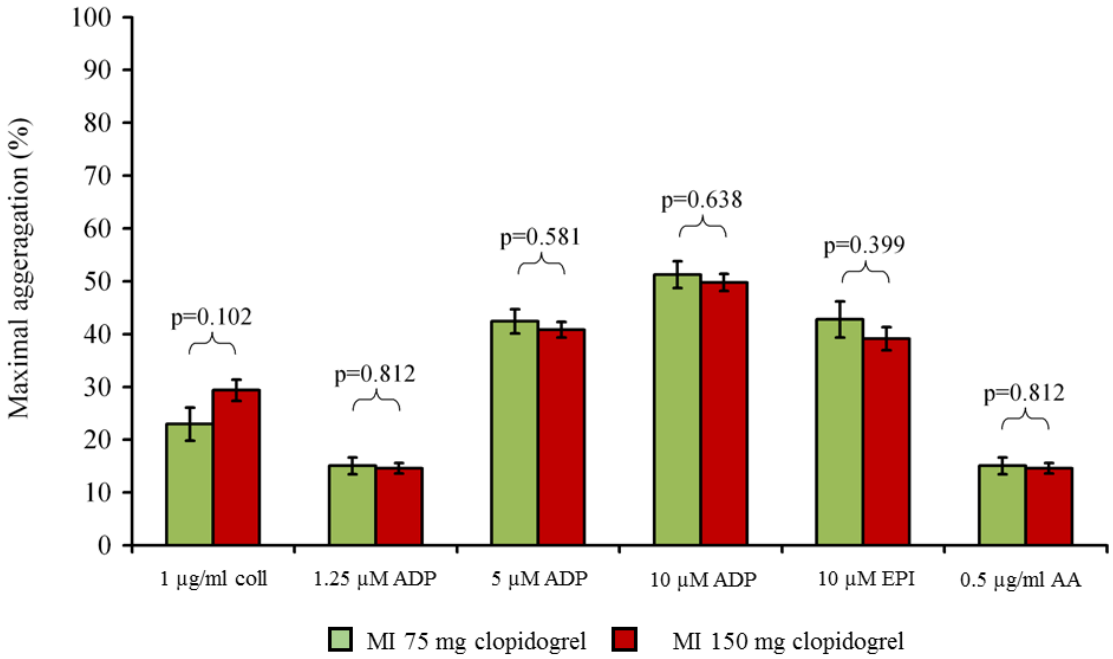

Figure 5. Platelet reactivity at baseline in the 75 mg and 150 mg clopidogrel subgroups of MI patients. After adjustment for covariates no difference was observed between the two subgroups. Results are expressed as mean (represented by columns) ± SEM (represented by bars). $p<0.05$ was considered significant.

Detailed characteristics of patient subgroups and aggregation values are shown in Table 11.

Table 11. Demographic data and baseline platelet reactivity of MI patients on 75 and 150 mg clopidogrel.

Variable	MI on 75 mg clo n = 41	MI on 150 mg clo n = 92	p value*
Age, years, median (IQR)	66 (57-72)	56 (50-64)	<0.001
Females, %	48.8	25	0.01
BMI, kg/m2, median (IQR)	25.7 (23.1-30.1)	28.5 (25.9-31.9)	<0.01
Medical history			
Hypertension, %	68.3	64.1	0.70
Diabetes mellitus, %	21.9	29.3	0.41
Dyslipidaemia, %	17.1	26.1	0.37
History of tobacco use %	48.8	61.9	0.18
Previous myocardial infarction, %	12.2	10.9	0.76
Previous PCI, %	14.6	16.3	1.00

Previous CABG, %	0.0	0.0	NE
Medication on admission			
Aspirin, %	26.8	20.7	0.50
Clopidogrel, %	9.8	6.5	0.50
ACEI or ARB, %	51.2	56.5	0.58
β-blocker, %	41.5	33.7	0.44
Nitroglycerine, %	9.8	10.9	1.00
Lipid-lowering agents, %	29.3	25	0.67
Procedural parameters			
Number of diseased vessels %			
1	41.5	52.2	0.27
2	34.1	22.8	0.20
3 or LM	24.4	25	1.00
Number of implanted stents, median (IQR)	2 (1-2)	1 (1-2)	0.50
Total stented length (mm), median (IQR)	35 (28-46)	31.5 (24-53.5)	0.47
Baseline platelet reactivity, MA %, median (IQR)			
1 µg/ml collagen	20 (13-30)	25 (12.5-44.5)	0.40
1,25 µM ADP	16 (10-25)	12 (6-21)	0.14
5 µM ADP	43 (36-53)	39 (29.5-50)	0.07
10 µM ADP	54 (46-62)	48 (37-59)	0.09
10 µM epinephrine	43 (30-58)	34.5 (20-55)	0.08
0,5 µg/ml arachidonic acid	4 (2-6)	3 (1-7)	0.67

ACEI: angiotensin-converting enzyme inhibitor, ADP: adenosine-diphosphate, ARB: angiotensin II receptor blocker, BMI: body mass index, CABG: coronary artery bypass graft surgery, IQR: interquartile range, MA: maximal aggregation, MI: myocardial infarction, NE: not evaluated because of insufficient data, PCI: percutaneous coronary intervention, PPI: proton pump inhibitor, SCAD: stable coronary artery disease. * p values were calculated with the Mann-Whitney (continuous parameters) and the Fisher's exact (categorical parameters) test.

4.1.3. Definition and ratio of high on-clopidogrel platelet reactivity based on LTA results in MI and SCAD patients

5 µM ADP induced maximal aggregation ($AGGR_{max\ 5ADP}$) was considered as the indicator of the efficacy of clopidogrel therapy with the cut-off value of $AGGR_{max}$ 5ADP>50 %. Above this value high on-clopidogrel platelet reactivity (HCPR), below this value no high on-clopidogrel platelet reactivity (no HCPR) was identified, independently of the applied clopidogrel maintenance dose.

The number of patients with high platelet reactivity tended to be higher in the MI than in the SCAD group (MI: 19.5% vs SCAD: 11.9%; p=0.232). Similarly to the baseline platelet aggregation data, proportion of HCPR patients within the MI group tended to be higher among patients on 75 mg maintenance dose compared to those being on 150 mg (26.8% vs. 16.3%, p=0.164).

4.1.4. Definition, ratio and management of clopidogrel pseudo and real non-responders

In HPR patients, antiplatelet therapy was adjusted as follows: in case of 75 mg clopidogrel, the drug dose was doubled. In patients with HPR already on 150 mg clopidogrel, 2x250 mg ticlopidine was induced, as the study was conducted before the prasugrel/ticagrelor era (patient management and therapeutic modifications are indicated in Figure 6).

Patients with high initial platelet reactivity on 75 mg but reaching normal platelet reactivity on 150 mg clopidogrel were defined as clopidogrel pseudo non-responders (PsNR). In contrast, patients with persisting high platelet reactivity even on 150 mg clopidogrel were defined as clopidogrel real non-responders (RNR, Figure 6).

The ratio of real non-responders was significantly higher in the MI group compared to SCAD group (MI: 18/133=13.5% *vs* SCAD: 2/67=2.9%; p=0.023). The ratio of pseudo non-responders also tended to be higher in the MI group but did not reach statistical significance (MI: 8/41=19.5% *vs* SCAD: 6/67=8.9%, p=0.143, Figure 6).

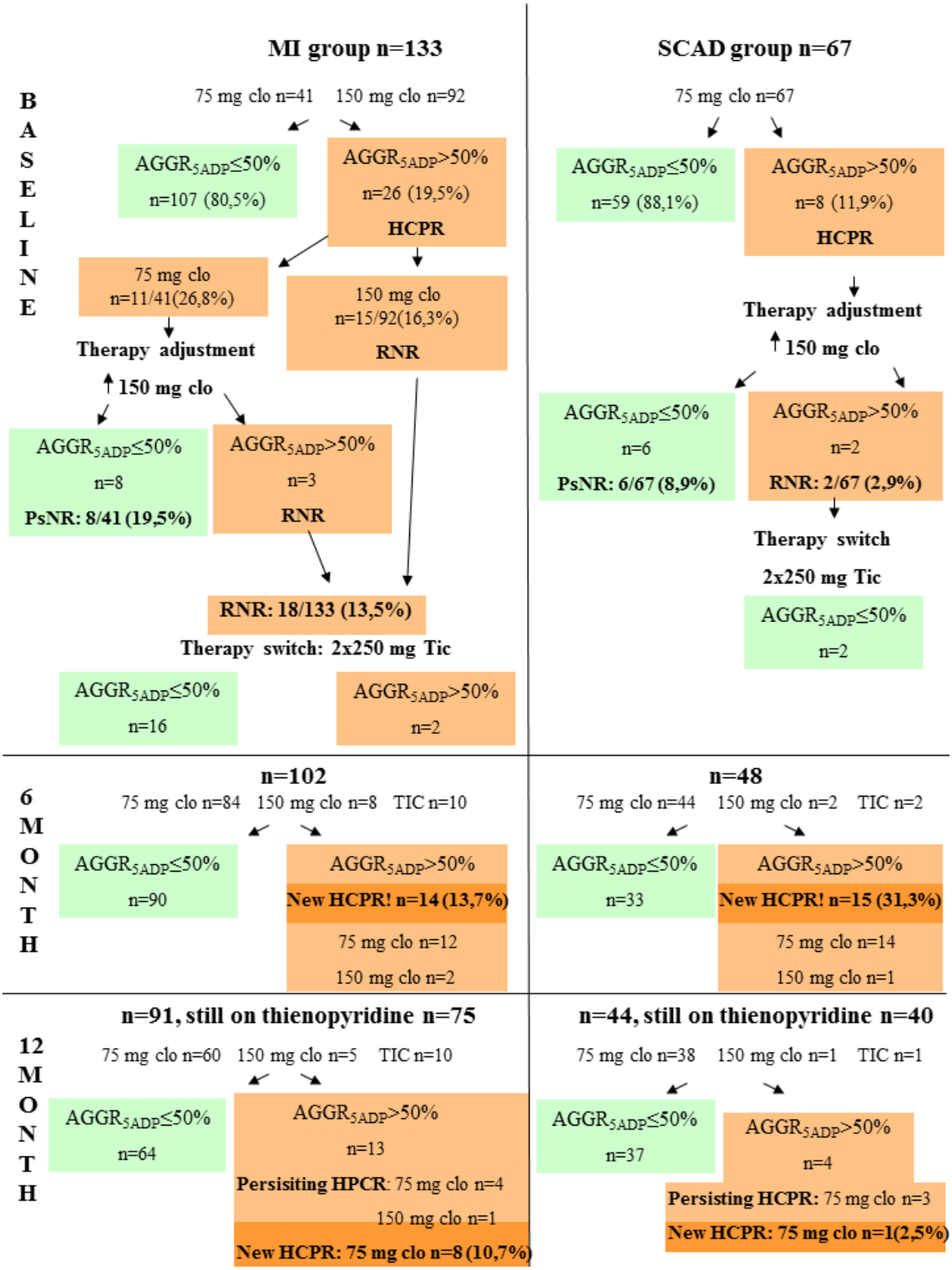

Figure 6. Classification, platelet reactivity and antiplatelet management of the MI and SCAD patient groups. Initial clopidogrel therapy, number of patients with HCPR, therapy modification and ratio of pseudo and real clopidogrel non-responders are shown in the MI and SCAD group at baseline, 6 month and 12 month. $AGGR_{5ADP}$: maximal 5μM ADP induced aggregation by LTA, clo: clopidogrel, HCPR: high on-clopidogrel platelet reactivity, MI: myocardial infarction, PsNR: pseudo non-responder, RNR: real non-responder, SCAD: stable coronary artery disease, TIC: ticlopidine.

4.1.5. Functional results of antiplatelet therapy modification

In pseudo non-responders clopidogrel dose doubling resulted in effective platelet inhibition. On the other hand, switch of therapy to ticlopidine also resulted in normal platelet reactivity in all patients in the SCAD group and in the majority of real non-responders in the MI group (16 out of 18 individuals, Figure 6). Platelet reactivity in MI and SCAD patients before and after therapy adjustment or switch is shown in Figure 7. Patients undergoing therapy intensification remained on modified therapy for the whole study period.

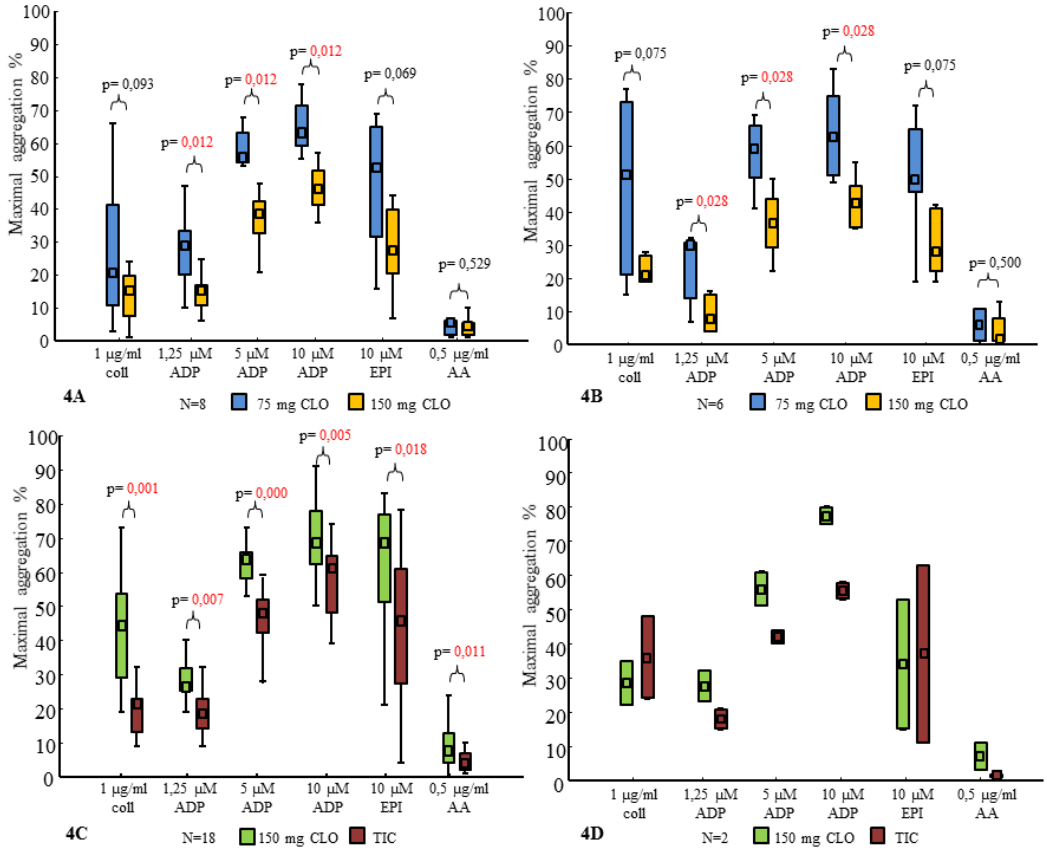

Figure 7. Platelet reactivity after modification of therapy in clopidogrel pseudo non-responders (PsNR) in the MI (A) and in the SCAD (B) group and in clopidogrel real non-responders (RNR) in the MI (C) and SCAD group (D). Clopidogrel dose doubling in PsNR patients resulted in significantly lower platelet aggregation induced by ADP in both patient groups (A and B). Platelet reactivity induced by collagen, epinephrine and arachidonic-acid also tended to be lower in PsNR patients. Platelet reactivity was significantly lower after ticlopidine induction induced by all agonists in RNR patients in the MI group (C) and was also substantially lower induced by ADP in the SCAD group (D, statistical analysis was not performed because of low case number). Results are expressed as median (represented by

squares), IQR (represented by boxes) and non-outlier range (represented by bars). $p<0.05$ was considered significant.

4.1.6. Long term follow-up of platelet function in MI and SCAD patients

In the MI group most of the patients (n=84) were already on standard 75 mg clopidogrel therapy by 6 month, however 8 patients received 150 mg clopidogrel and 10 patients received ticlopidine as a result of therapy intensification (Figure 6). At 12 month, 60 patients were on standard, 5 patients were on high dose clopidogrel and 10 patients were on ticlopidine. Platelet reactivity remained on the same level throughout 12 months follow-up in the MI group (Figure 8A).

In the SCAD group 44 patients were on standard, 2 patients were on high dose clopidogrel and 2 patients were on ticlopidine at 6 month. At 12 month, 38 patients on 75 mg, 1 patient on 150 mg clopidogrel and 1 patient on ticlopidine were available for retesting (Figure 6). In SCAD patients, platelet reactivity showed slight oscillation: at 6 months platelet aggregation was significantly higher compared to the 12 month values ($5ADP_{6month}$ vs. $5ADP_{12month}$ $p=0.005$; Figure 8B).

Platelet reactivity did not differ between the MI and SCAD group at 6 and 12 month and was also irrespective of the 75 mg or 150 mg clopidogrel maintenance therapy at 6 and at 12 months (data not shown).

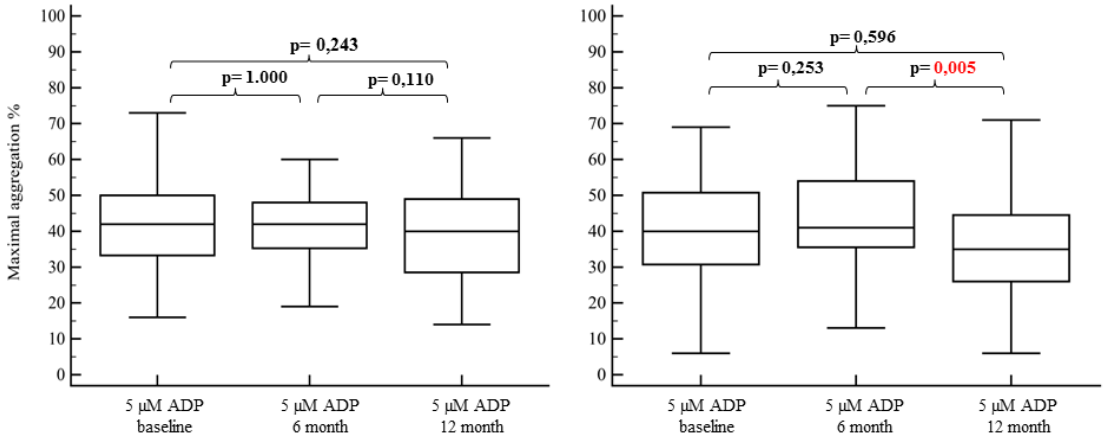

Figure 8. Long term follow-up results of platelet reactivity in MI (A) and SCAD (B) patients. In the MI group (N=71), platelet function remained approximately the same throughout 12 month follow-up period. In SCAD patients (N=39), platelet function showed modest oscillation during 12 month follow-up. At 6 month platelet reactivity tended to be higher compared to the baseline values and proved to be significantly higher than platelet reactivity at 12 month. Results are expressed as median (represented by squares), IQR (represented by boxes) and non-outlier range (represented by bars). $p<0.05$ was considered significant.

4.1.7. Incidence of new HCPR during 12 month follow-up in MI and SCAD patients

Despite the fact, that out of our 200 patients, 198 individuals were on effective antiplatelet therapy after modifications at baseline, there was a remarkable incidence of new HCPR at 6 month in both patient groups (Figure 9).

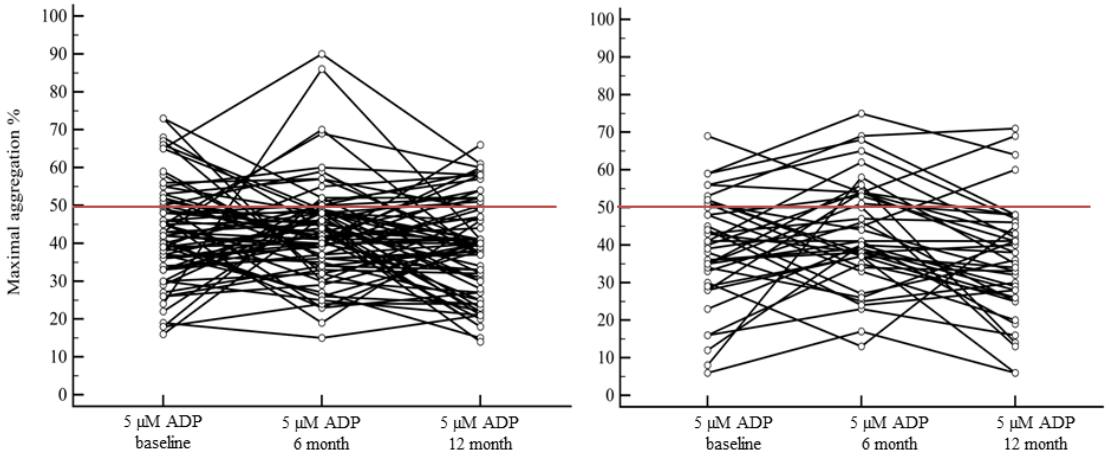

Figure 9. Individual platelet aggregation values induced by 5 µM ADP at baseline, 6 and 12 month in the MI (A) and in the SCAD (B) group. Patients were represented by separate dots and lines. To declare high on-treatment platelet reactivity, we used the $AGGR_{max\ 5ADP}$=50% cut-off value. There were a remarkable number of patients returning with HCPR at both measuring times in both patient groups. The incidence of new HCPR at 6 month is higher in the SCAD group, than in the MI group (in correlation with platelet function kinetics, Figure 8). In some patients, HPR persisted from 6 to 12 month in both groups. However, there were HCPR patients at 6 month who returned with normal platelet reactivity at 12 month without any therapeutic adjustment.

In the MI group new HCPR occurred in 12 patients receiving standard and in 2 patients receiving high dose clopidogrel therapy (13.7%, Figure 6). Interestingly, in the SCAD group incidence of new HCPR was unexpectedly high; 14 patients were on 75 mg and 1 patient was on 150 mg clopidogrel (Figure 6).

In these patients, further therapeutic interventions were not performed. Interestingly, the majority of patients with HCPR at 6 month, returned with normal platelet reactivity at 12 month (MI group: 8 patients on 75 mg and 1 patient on 150 mg clopidogrel; SCAD group: 11 patients on 75 mg and 1 patient on 150 mg clopidogrel). Nevertheless, HCPR persisted from 6 to 12 month in 4 patients being on 75 mg and 1 patient being on 150 mg clopidogrel in the MI group. Persisting HCPR from 6 to 12 month was also observed in 3 SCAD patients receiving 75 mg clopidogrel.

By 12 month, new HCPR was observed in 8 MI patients being on standard clopidogrel therapy and no further incidence of HCPR was observed on high dose clopidogrel therapy. The ratio of new HCPR at 12 month was substantially lower in the

SCAD group: only 1 patient on 75 mg clopidogrel returned with new HCPR and no further incidence of HCPR was observed on high dose clopidogrel therapy (Figure 6).

Interestingly, all clopidogrel real non-responders switched over to ticlopidine remained effectively inhibited during the whole follow-up period.

4.1.8. Clinical end points during 12 month follow up

During the study we documented clinical efficacy and safety endpoints (Table 12). The 12 month cumulative event rate was 8.3% in the MI and 1.5% in the SCAD group. During the 12 month follow-up no TIMI major or minor bleeding events were documented.

Table 12. Clinical outcome data during 12 month follow-up.

End point	MI (n = 133)	SCAD (n = 67)
Death from any cause *n*	4	1
Stroke *n*	1	0
Reinfarction *n*	0	0
Urgent revascularization *n*	2	0
Non-urgent target vessel revascularization *n*	4	0
Stent thrombosis *n*	0	0
TIMI minor bleeding *n*	0	0
TIMI major bleeding *n*	0	0

4.2. Determining factors of high on-treatment platelet reactivity in patients with acute coronary syndrome

In a second prospective cohort study, we investigated the predictors of HCPR measured by the point-of-care multiple electrode aggregometry.

4.2.1. Patient characteristics

Demographic, clinical, and procedural characteristics of the overall population of 463 patients and the subgroups with or without HPR are summarized in Table 13. Most of the parameters were similar in the HPR and no HPR groups. However, platelet count (PLT) and high sensitive C-reactive protein level measured upon admission were

statistically higher in the HPR group (Fisher's exact test, p=0.01 and p=0.02, respectively).

Table 13. Demographic, Clinical, and Procedural Characteristics.

Variable	Overall (n = 463)	No HCPR (n = 389)	HCPR (n = 74)	p Value*
Platelet reactivity, U, median (IQR)	29.0 (21.0-39.0)	27.0 (20.0-34.0)	56.5 (50.0-62.0)	<0.0001
Age, years, median (IQR)	61.0 (52.0-69.0)	61.0 (52.0-68.0)	60.5 (52.0-70.0)	0.55
Female, %	28.7	27.0	37.8	0.07
BMI, kg/m^2, median (IQR)	28.0 (25.0-31.0)	28.0 (25.0-31.0)	28.0 (26.0-31.0)	0.38
LVEF, %, median (IQR)	51.0 (43.0-59.0)	51.0 (44.0-59.0)	50.0 (40.0-57.0)	0.32
Medical history				
Hypertension, %	63.5	64.5	58.1	0.30
Diabetes mellitus, %	25.1	24.7	27.0	0.66
Dyslipidaemia, %	19.4	19.0	21.6	0.63
Current smoker, %	39.1	40.9	29.7	0.09
Previous myocardial infarction, %	13.6	13.6	13.5	1.00
Previous PCI, %	15.6	15.2	17.6	0.60
Previous CABG, %	2.6	2.6	2.7	1.00
Congestive heart failure, %	12.3	11.8	14.9	0.44
Peripheral artery disease, %	3.2	3.9	0.0	0.14
Thyroid dysfunction, %	5.2	5.4	4.1	0.78
Chronic kidney disease, %	18.4	19.3	13.5	0.32
Concomitant medication				
Acetylsalycilic acid, %	98.7	98.7	98.6	1.00
ACEI or ARB, %	88.6	89.2	85.1	0.32
Aldosterone receptor antagonist, %	27.2	26.0	33.8	0.20
β-blocker, %	89.2	89.7	86.5	0.42
Calcium channel blocker, %	6.5	6.9	4.1	0.45
Nitroglycerin, %	23.8	23.4	25.7	0.66
Statin, %	91.6	92.0	89.2	0.49
Proton pump inhibitor, %	93.7	93.3	95.9	0.60
Oral anti-diabetic, %	18.4	17.5	23.0	0.26
Insulin, %	6.7	6.2	9.5	0.31
Laboratory values upon admission				
RBC count, T/L, median, (IQR)	4.7 (4.3-5.0)	4.7 (4.3-5.0)	4.7 (4.5-5.0)	0.96

Hemoglobin, g/L, median, (IQR)	141.0 (130.0-152.0)	142.0 (130.0-152.0)	141.0 (128.0-151.0)	0.25
Hematocrit (IQR)	0.41 (0.38-0.44)	0.41 (0.38-0.44)	0.41 (0.38-0.44)	0.89
WBC count, G/L, median, (IQR)	10.2 (8.3-12.9)	10.0 (8.3-12.5)	10.8 (8.4-13.6)	0.13
Platelet count, G/L, median, (IQR)	236.0 (198.3-282.8)	232.0 (195.0-281.3)	250.5 (221.0-310.0)	0.01
MPV, fL, median, (IQR)	10.8 (10.2-11.4)	10.8 (10.2-11.4)	10.7 (10.3-11.2)	0.46
CRP, mg/L, median, (IQR)	4.4 (2.2-11.2)	4.1 (2.1-10.6)	7.4 (2.6-24.4)	0.02
Glucose, mmol/L, median, (IQR)	7.0 (5.9-8.8)	6.9 (5.9-8.6)	7.2 (6.0-9.2)	0.38
Troponin I, ng/mL, median, (IQR)†	1.9 (0.4-9.8)	1.9 (0.4-9.8)	1.8 (0.6-10.1)	0.69
Troponin I, categorical, %				0.26
TnI < 0.3 mg/mL	21.6	21.9	20.3	
0.3 ng/mL ≤ TnI ≤ 50.0 ng/mL	72.4	73.0	68.9	
TnI > 50.0 ng/mL	6.0	5.1	10.8	
Procedural parameters				
Indication for angiography (%)				0.65
STEMI	72.1	71.7	74.3	
NSTEMI	23.8	24.4	20.3	
UAP	4.1	3.9	5.4	
Number of diseased vessels (%)				0.55
1	39.1	38.6	41.9	
2	30.0	30.1	29.7	
3 or left main artery	30.9	31.4	28.4	
Therapeutic modality, %				0.86
POBA / thrombus aspiration only	1.9	2.1	1.4	
BMS implantation	76.0	76.1	75.7	
DES implantation‡	17.9	17.5	20.3	
CABG	1.1	1.0	1.4	
Medical therapy only	3.0	3.3	1.4	
Number of implanted stents, %				0.26
1	58.7	58.1	62.0	
2	30.5	30.4	31.0	
3	7.6	7.9	5.6	
4	2.1	2.2	1.4	
5	1.1	1.4	0.0	
Total stent length, mm, median (IQR)	33.0 (23.0-50.0)	33.0 (22.3-49.3)	36.0 (23.3-50.0)	0.96
Mean stent diam., mm, median (IQR)	3.0 (2.8-3.3)	3.0 (2.8-3.3)	3.0 (2.8-3.2)	0.30
Use of GPIIb/IIIa receptor inhibitor (%)	52.1	52.2	51.4	0.89

ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; BMS, bare metal stent; CABG, coronary artery bypass graft surgery; CRP, C-reactive protein; DES, drug eluting stent; diam., diameter; GPIIb/IIIa, glycoprotein IIb/IIIa; IQR, interquartile range; LVEF, left ventricular ejection fraction; MPV, mean platelet volume; NSTEMI, non-ST-segment elevation myocardial infarction; PCI, percutaneous coronary intervention; POBA, plain old balloon

angioplasty; HCPR, high on-clopidogrel platelet reactivity; RBC, red blood cell; STEMI, ST-segment elevation myocardial infarction; TnI, troponin I; UAP, unstable angina pectoris; WBC, white blood cell.

* p values refer to differences between the HPR and no HPR groups.

† values within detection limits: 0.01 ng/mL < TnI ≤ 50.0 ng/mL.

‡ implantation of at least one DES with or without parallel BMS placement.

4.2.2. Distribution of platelet aggregation values measured by MEA

The ADP-induced-platelet aggregation units (U) of the overall population showed a right-skewed, unimodal distribution with a median (M) of 29.0 U (IQR 21.0 to 39.0 U, Figure 10). Based on the >46 U cut-off value, the proportion of HCPR was 74/463=16.0%. The median platelet aggregation values in the HCPR and no HCPR groups were 56.5 U (IQR 50.0 to 62.0 U) and 27.0 U (IQR 20.0 to 34.0 U), respectively, Table 1. The ADP induced aggregation values did not differ in the GPIIb/IIIa inhibitor treated and untreated groups (M: 30 U, IQR: 22.0 to 40.0 U vs. M: 29 U, IQR: 20.0 to 39.0 U, p=0.31).

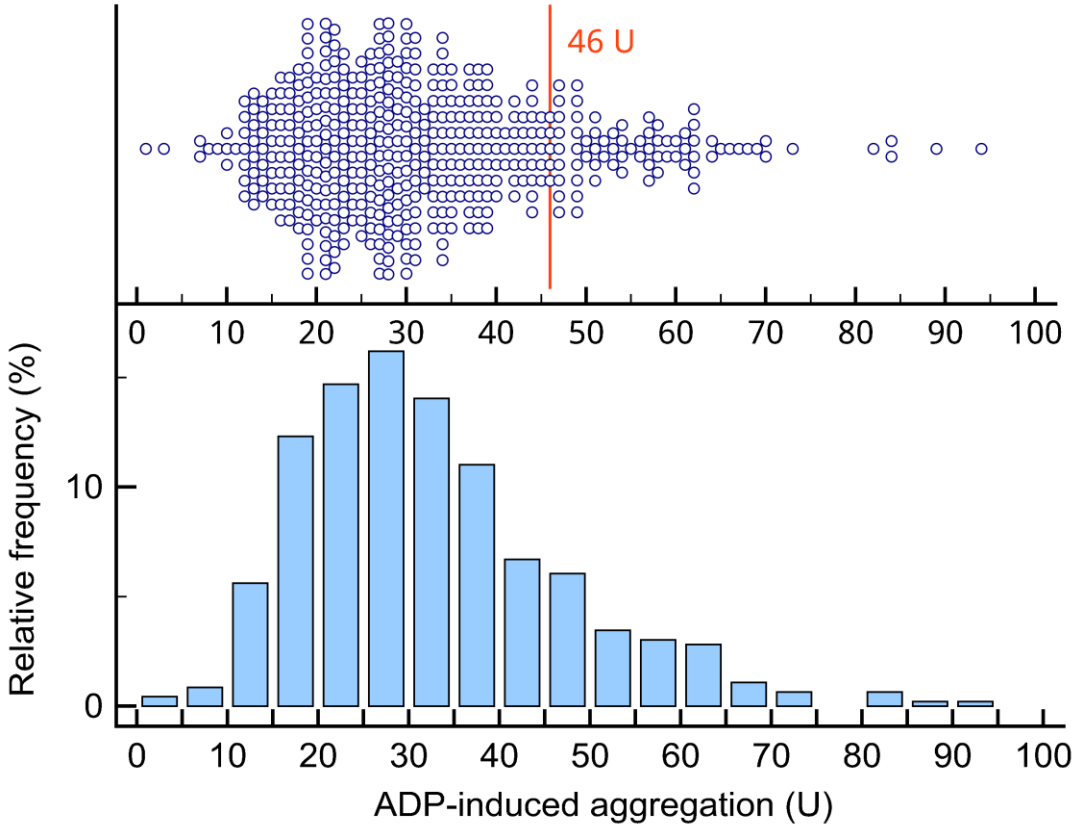

Figure 10. Distribution of ADP-induced platelet aggregation values measured by MEA. The upper part of the figure demonstrates the distribution of aggregation values (U) in the entire population with single dots representing patients. The vertical orange line indicates the cut-off value of 46 U between patients with and without high on-treatment platelet reactivity. The lower part of the figure shows the histogram of ADP-induced aggregation values in the overall population.

4.2.3. Factors related to HCPR, model construction

Univariate logistic regression analysis was performed to all parameters listed in Table 13. Variables univariately associated with HCPR with a p value less than 0.2 were as follows: PLT count (per G/L, p=0.0006), white blood cell count (WBC, per G/L, p=0.06), CRP level (per mg/L, p=0.03), troponin I level >50 ng/mL (p=0.07) upon admission, female gender (p=0.06) and non-smoking status (p=0.07). These factors and clinical parameters that were previously found to be associated with HCPR, such as diabetes mellitus, BMI and renal function were entered into the backward multivariate logistic regression model. Based on the analysis, PLT count (per G/l, odds ratio [OR]: 1.0073, 95% confidence interval [95% CI]: 1.0035 to 1.0112, p=0.0002), CRP level (per mg/L, OR, [95% CI]: 1.0077 [1.0016 to 1.0137], p=0.01) upon admission,

and current smoking (OR [95% CI]: 0.51 [0.29 to 0.89], p=0.02) proved to be predictors of HCPR (Table 14). According to the adequacy index, PLT count was the most powerful variable, while CRP level and active smoking had less pronounced effects (Table 14).

Table 14. Multivariate Predictors of High Platelet Reactivity.

Variable	Adequacy	Coefficient	Standard error	OR	95% CI	p Value
Platelet count, per G/L	0.52	0.0073	0.0019	1.0073	1.0035-1.0112	0.0002
CRP, per mg/L	0.19	0.0076	0.0031	1.0077	1.0016-1.0137	0.0127
Current smoking	0.15	-0.6747	0.2855	0.5093	0.2911-0.8912	0.0181
Constant		-3.4020	0.5249			<0.0001

CI indicates confidence interval; CRP, C-reactive protein; OR, odds ratio.

The likelihood ratio and Hosmer-Lemeshow tests showed good model fit (p<0.0001 and p=0.43, respectively, Table 15).

Table 15. Performance of the Risk Prediction Model.

Parameter	Value
Overall model fit	
Null model -2 log likelihood	406.869
Full model -2 log likelihood	384.257
Chi-square	22.612
Degree of freedom	3
Significance level (p)	<0.0001
ROC curve analysis	
Area under the ROC curve	0.665
Standard error	0.036
95% Confidence interval	0.620-0.708
Significance level (p)	<0.0001
Youden index	
Youden index J	0.307
Associated criterion	>0.167
Sensitivity, %	59.5

Specificity, %	71.2
Hosmer-Lemeshow test	
Chi-square	8.06
Degree of freedom	8
Significance level (p)	0.43

ROC indicates receiver operating characteristic.

The area under the ROC curve analysis revealed moderate predictive power (AUC=0.665, Figure 11).

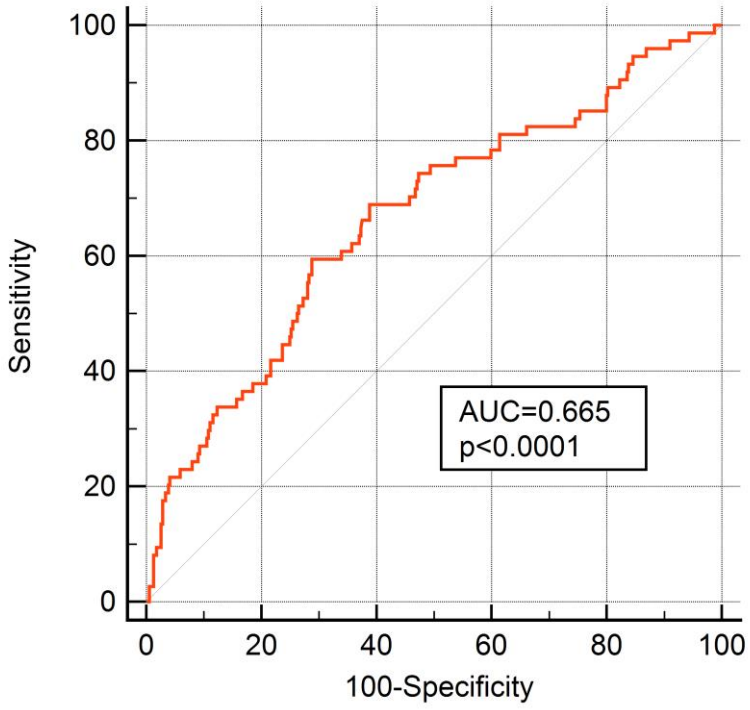

Figure 11. Discriminative capacity of the risk prediction model. The receiver operating characteristic curve analysis revealed moderate predictive power (AUC [95% CI]=0.67 [0.62 to 0.71], p<0.0001).

4.2.4. Internal validation

Since the performance of a model in the development dataset may overestimate the true performance, we conducted internal validation using 10,000 bootstrap samples. Predictive accuracy was characterized by the AUC value while calibration was assessed by means of the intercept and slope of the calibration line, as proposed by Cox[177]. Moreover, we evaluated calibration graphically by applying a LOWESS smoother on

scatterplots of predicted versus observed probabilities. Considering accuracy, optimism proved to be 0.013 resulting in an optimism-corrected AUC value of 0.653. Both the optimism-adjusted intercept and slope were on average correct (-0.102 and 0.930, respectively), not necessitating recalibration of the apparent model (Table 16, Figure 12).

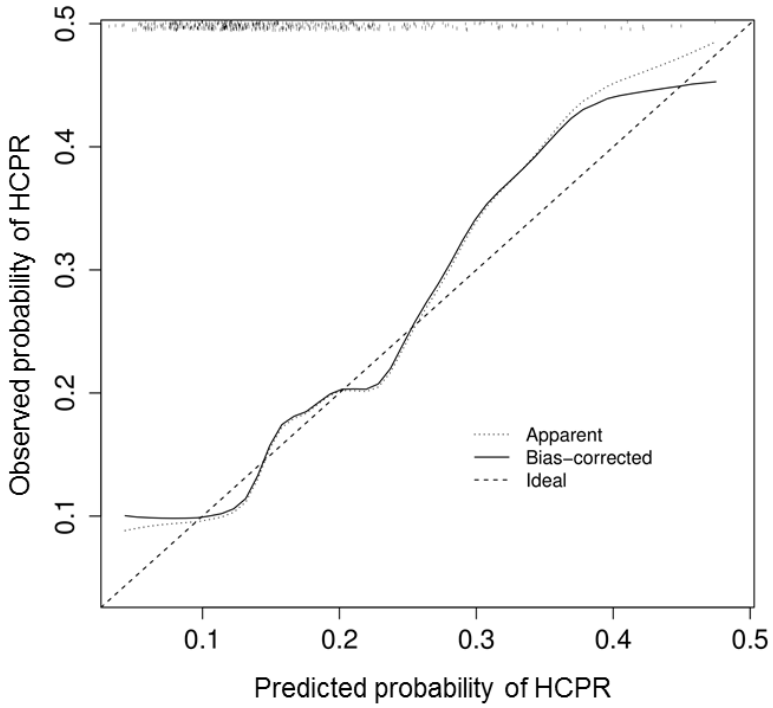

Figure 12. Calibration plot of the apparent and optimism-corrected models. Calibration curves were created using a LOWESS smoother on the scatterplot of expected versus observed risks. Differences between the predicted and actual event rates were small across the whole risk spectrum for the apparent model (Hosmer-Lemeshow test, p=0.43,). Internal validation using 10,000 bootstrap samples revealed only negligible optimism in calibration with a bias-corrected (validated) intercept of -0.102 and slope of 0.930. Thus, recalibration of the apparent model was not necessary. The rug plots across the top of the figure show the distribution of the predicted risk.

Table 16. Internal Validation of Predictive Accuracy and Calibration.

	AUC	Cox validation intercept	Cox validation slope
Apparent	0.665	0.000	1.000
Optimism*	0.013	0.102	0.070
Optimism-corrected*	0.653	-0.102	0.930

AUC indicates area under the receiver operating characteristic curve.

* Results are based on 10,000 bootstrap samples.

4.2.5. Risk stratification

Based on sensitivity, specificity and cumulative frequency distribution analyses of the predicted probability, three risk classes (low, intermediate, and high risk) were defined. Table 17 gives details of the classification parameters, the proportion of patients in the classes, event rates, and interval likelihood ratios for each of these risk strata. Using this classification, low- and high risk patients, who represent some 60% of the population, may be precisely identified. There is more than a fourfold increase in post-test probability of HCPR between patients of the low- and high risk strata (8.7% versus 35.7%). On the contrary, pre- and post-test probabilities are almost identical (16.0% versus 18.1%) in the intermediate risk group corresponding to some 40% of the cases.

Table 17. Interval Likelihood Ratios According to Risk Strata.

Risk class (predicted probability of HCPR)	HCPR	No HCPR	Number / percent at risk	Rate of HPR	Interval likelihood ratio	95% CI
Low risk (≤0.13)	18	190	208 / 44.9%	8.7%	0.498	0.329-0.754
Intermediate risk (>0.13 to ≤0.25)	36	163	199 / 43.0%	18.1%	1.161	0.894-1.508
High risk (>0.25)	20	36	56 / 12.1%	35.7%	2.920	1.795-4.752
Overall	74	389	463 / 100%	16.0%	NA	NA

HPR: high on-clopidogrel platelet reactivity; CI: confidence interval; NA: not applicable.

Using risk stratification, low, intermediate and high risk patients were successfully separated. As it is indicated in Figure 13, 95% confidence intervals of the interval likelihood ratios do not overlap each other suggesting clearly different levels of risk in the three strata.

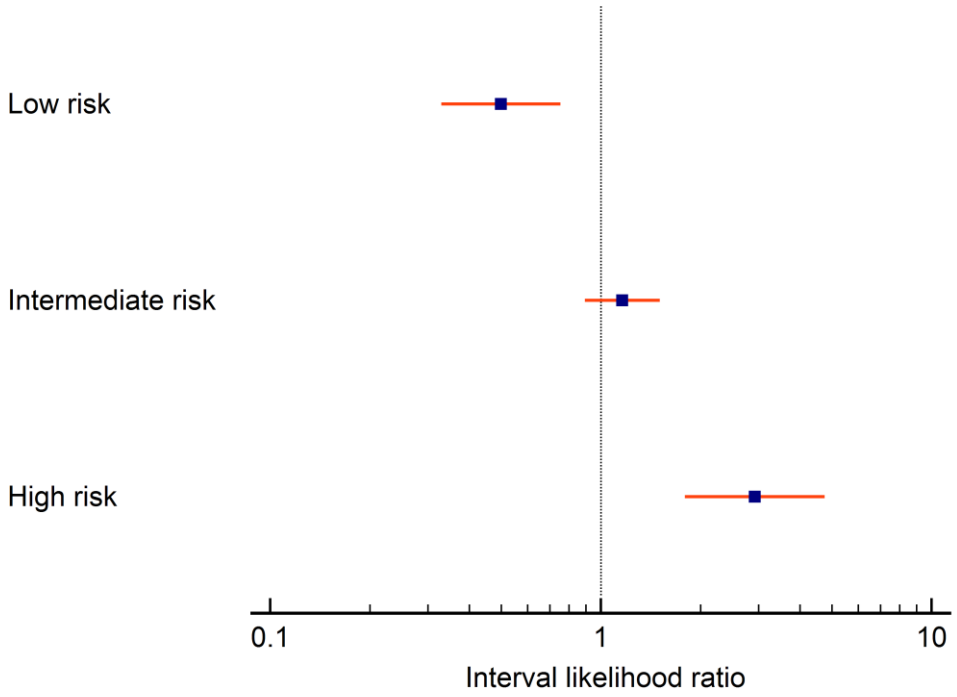

Figure 13. Interval likelihood ratios according to risk strata. Point estimates ranging from 0.50 to 2.92 with non-overlapping 95% confidence intervals indicate clearly separated risk levels.

Also, with increasing risk class, there is a monotonic rise in the observed rates of high on-clopidogrel platelet reactivity (Cochran-Armitage test, $p<0.0001$, Figure 14). Moreover, differences between the predicted and actual event rates were small across the risk strata.

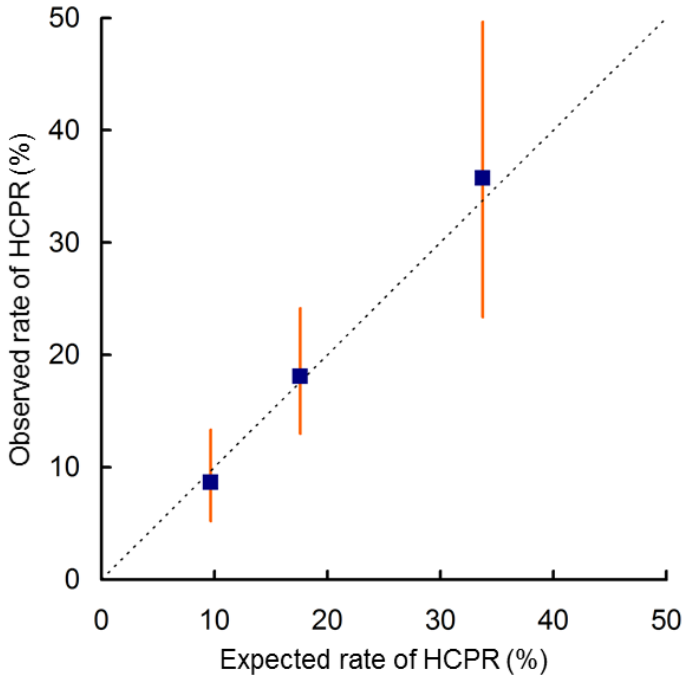

Figure 14. Observed rate of high on-clopidogrel platelet reactivity across the risk strata. The rate of HPR showed monotonic increase throughout subsequent risk classes (Cochran-Armitage test, $p<0.0001$). There is a fourfold rise in probability of HPR between patients of the low- and high risk groups (8.7% versus 35.7%). Error bars represent exact binomial 95% confidence intervals.

5. DISCUSSION

In the present work, we aimed to investigate the contribution of platelet function testing to treatment optimization and risk stratification of patients undergoing percutaneous coronary intervention.

In our first study, we examined the efficacy of clopidogrel therapy with the gold standard "in vitro" platelet function test in order to identify patients with high on-clopidogrel platelet reactivity with either acute or stable form of ischemic heart disease undergoing PCI. Thereafter gradual antiplatelet therapy adjustment was performed until reaching laboratory proven platelet inhibition.

Platelet aggregation measured by light transmission aggregometry

LTA is a cheap and reliable method in well-trained hands and under well-established circumstances and is successfully and widely used to monitor platelet reactivity in numerous laboratories [178]. Its predictive ability regarding ischemic events has already been well supported by several studies [73-75]. Importantly, in a large, comparative study investigating the association of different platelet function tests with clinical outcomes, it proved to be predictive to ischemic events during 12 months following PCI [76]. Moreover its predictive power regarding cardiac end points was at least comparable with that of the easily applicable point-of-care Verify Now method [179,180].

With the use of LTA testing in our first 200 patients, we found that baseline platelet reactivity was significantly higher in patients with myocardial infarction than in patients with stable coronary artery disease. After adjusting for demographic variances between the patient groups (such as age, gender rate, BMI, smoking habits, occurrence of hypertension, diabetes mellitus, dyslipidaemia, history of myocardial infarction, previous PCI or CABG operation, elapsed time between clopidogrel loading and platelet function testing, and antiplatelet medication on admission), the difference remained significant with all concentrations of the ADP agonist. Our finding of higher platelet reactivity in myocardial infarction goes along well with the literature [81,181-184]. Importantly, our definition of HPR was also in good agreement with the current

recommendation of the platelet expert panel (5 μM ADP induced MA >46%) [180,185]. However, the initial ratio of HCPR in our overall patient population (17%) was lower than generally known from previous studies (even ≥ 50%) [125,186,187]. The cause in its background might have been the high proportion of our MI patients being on 150 mg clopidogrel initially. However, unexpectedly, the platelet reactivity in MI patients receiving 150 mg clopidogrel was approximately on the same level as that of the patients on standard clopidogrel dose. Its possible explanation might be the selection of patients in whom standard clopidogrel dose is presumably less effective by considering the clinical influencing factors of response variability (acute form of cardiac disease, younger age, higher BMI, higher frequency of comorbidities [38,186,188]) and successful treatment of this population with the double dose. In the CURRENT-OASIS 7 study, administration of 150 mg clopidogrel in the first week in ACS patients treated with PCI was associated with a reduction in stent thrombosis compared with the standard dose [189]. This also supports our finding, that in lack of possibility to perform platelet function tests or to give prasugrel/ticagrelor (e.g. contraindication) the administration of 150 mg daily clopidogrel might be beneficial initially in high risk, acute cardiac patients selected by certain clinical parameters, because a portion of these patients have HPR beside 75 mg clopidogrel and need dose elevation to be effectively inhibited. 150 mg clopidogrel might also be a reasonable step in the gradual therapy intensification of MI patients if more potent drugs are contraindicated, as it may overcome HPR without increasing bleeding risk according to our observations and also supported by data from large clinical trials [84].

Increased incidence of high on-clopidogrel platelet reactivity in the acute setting

The ratio of HCPR even on 150 mg clopidogrel maintenance dose (clopidogrel real non-responders) was significantly higher among the MI patients, suggesting that these invasively treated acute cardiac patients may benefit from prospective platelet function testing because of their higher atherothrombotic burden and widened antiplatelet treatment possibilities. Notably, in good agreement with our findings, the prevalence of HPR was shown to be higher in patients with acute coronary syndrome than in those with stable CAD in a large patient cohort [81]. In the recent years robust evidence supported the association of high on-treatment ADP induced platelet reactivity and

ischemic outcomes in different cardiology patient groups [73-75,87,167,190]. Accepting HPR as a clinically relevant factor, we need to investigate its modifiability and whether its optimization improves the outcome in different clinical settings. However, up to date, large clinical trials investigating the effect of tailored antiplatelet therapy on clinical outcome closed with neutral results [82-84,170]. Importantly, these studies used only one platelet function test (Verify Now) and agonist (ADP) to assess platelet function and were conducted in heterogeneous, but mostly elective and low-intermediate risk patient populations. Also, there were great variances in terms of study designs, definitions of HPR, timing of platelet testing, and antiplatelet therapy modification algorithms as well as clinical endpoints and follow-up times. Consequently, there is a gap of knowledge regarding the role of platelet function testing and guided antiplatelet therapy in invasively treated ACS patients, especially in patients with STEMI, who are at the highest thrombotic risk. Furthermore, it cannot be excluded, that the use of other platelet function tests or use of more parallel agonists to compose a so-called „platelet reactivity index“ could be more sensitive in discriminating patients with HPR. Currently ongoing clinical trials (e.g. the TROPICAL-ACS, NCT01959451) will hopefully further expand our knowledge and will provide at least some of the lacking answers to these question. Also, more, carefully designed RCTs (e.g. parallel application of the most widely used PF tests, more homogeneous patient groups and therapy modification algorithms) are needed to gain additional information.

Therapy modification results in effective platelet inhibition

According to our repeated platelet function measurements, with switch of therapy (i.e. conversion to ticlopidine), we may overcome HCPR persisting even on 150 mg clopidogrel. This might be the result of different metabolic steps and enzymes being involved in the activation process of the distinct thienopyridines [45] (see also in the Introduction section, 1.2.1.2.). Interestingly, in patients with SCAD, therapy modification resulted in effective platelet inhibition in 100% of the patients; however optimal platelet inhibition could not be achieved even by therapy modification in a small portion of the MI patients with HCPR on 150 mg clopidogrel (2/19). It is likely,

however, that with switch to a novel, more potent agent even these patients would be efficiently treated.

Appearance of new HCPR during long term follow-up

There are relatively few and controversial data about long term platelet function kinetics [82-84]. In the TRILOGY-ACS platelet function substudy almost 50% of the patients on clopidogrel had HPR at the subsequent control points during 30 months follow up and the ratio of HPR patients proved to be stable over the time [191]. According to our results, platelet reactivity remained on the same level in the MI group throughout 12 month and proved to be modestly oscillating in the SCAD group. However, a new appearance of HPR at 6 and 12 months was observed in both patient groups independently from the clopidogrel maintenance dose. This finding may have clinical consequences suggesting, that on clopidogrel treatment platelet reactivity - beside wide-scale inter-individual variability - is not a stable parameter. It is determined by several clinical, genetic and cellular factors showing considerable alternation during follow-up [38,186,188]. Interestingly, platelet function in patients on clopidogrel altered in both directions spontaneously during long term follow up, contrarily induction of ticlopidine resulted in stable platelet inhibitory effect during 12 months follow-up. It also supports that clopidogrel's effectiveness is very vulnerable and thienopyridines using other metabolization pathways may exert not only more extensive but more stable long-term platelet inhibitory effect [45]. Importantly, appearance of new HPR and inter-patient variability of platelet function –however to a smaller extent- was also described recently in prasugrel treated patients, particularly among patients on lower prasugrel maintenance dose [133,134]. Appearance of HPR may have clinical significance in patients with DES as their endothelial regeneration may be prolonged resulting in higher risk for late and very late stent thrombosis [192]. Therefore re-evaluation of platelet reactivity may be useful in patients with DES or before the performance of a subsequent percutaneous coronary intervention or to assess the efficacy of the maintenance dose of an antiplatelet drug. Therefore, the right approach to platelet function testing may include early assessment to assess drug effectivity and re-evaluation to judge dose effectivity or to adjust proper maintenance dose of an antiplatelet agent.

Safety considerations during intensified therapy

In our patient population no TIMI major or minor bleeding consequences occurred in association with tailored antiplatelet therapy, which may suggest, that therapy intensification might be applied safely in a selected, well determined patient population. However re-evaluation of platelet function after modification of therapy might become desirable as there are data suggesting, that antiplatelet therapy intensification may increase bleeding risk in unselected patient population [41,42,189]. The ADAPT-DES study identified strong inverse relationship between high on-treatment platelet reactivity and clinically relevant bleeding as well as strong association of bleeding consequences with mortality; similar to that of stent thrombosis or myocardial infarction [193]. Consequently, increasing bleeding risk may counter-balance the favorable therapeutic effects of intensified platelet inhibition and beyond a given level may not improve survival - especially in low risk cardiac patients. Therefore, possible prediction of low on-treatment platelet reactivity (LPR) and associated bleeding risk with platelet testing may also gain importance besides the expanding use of more potent antithrombotic agents. Recent data suggest, that finding an optimal range in platelet inhibition (so called therapeutic window) would probably provide the best balance between ischemic and bleeding consequences and would maximize the neat clinical benefit of cardiac patients.

Not only were ACS patients less extensively investigated in tailored antiplatelet therapy studies, but also the predictors of HPR in this cohort have not been adequately clarified to date.

Almost a decade has passed, since a new point-of-care method, the multiple electrode aggregometry was introduced in clinical practice. Since then it has been used more and more extensively for successfully monitoring dual antiplatelet therapy, leading to establishment of a clinically predictive cut-off value [87]. Therefore, in our second study we aimed to identify simple clinical and easily accessible laboratory parameters that may be associated with HCPR defined by the established MEA

threshold in a large cohort of ACS patients (N=463). Thereafter, we developed a HCPR risk prediction model.

Predictors of HCPR measured by Multiplate in patients with ACS – laboratory parameters

Among several examined parameters, platelet count, CRP level upon admission, and current smoking proved to be predictors of HPR. According to our results, baseline platelet count, a routinely measured laboratory parameter, is a strong, continuous predictor of HPR, which is a novel finding to the best of our knowledge. Multiple electrode aggregometry measures platelet function in whole blood, where cell-cell interactions, micro-circumstances and the presence of a metal electrode surface could substantially influence platelet activation and aggregation. Platelet function measured by multiple electrode aggregometry has been reported to be dependent on interactions between platelets, red and white cells as compared to platelet function tests, where platelet aggregation occurs in cell purified, platelet rich plasma (e.g. light transmission aggregometry, LTA) [150,194]. Platelet aggregation values obtained by MEA have been reported to be significantly lower below the normal platelet count range [195], but were also described to be independent from thrombocyte count within the physiological limits in healthy, untreated patients [196]. Contrarily, we found an association even within the normal range after administration of 600 mg clopidogrel. Our patient population receiving dual antiplatelet therapy included patients with myocardial infarction mainly (95.9%) with high atherothrombotic burden, which was reported to be accompanied by elevated inflammatory markers [197]. Inflammatory conditions lead to increased platelet generation and turnover via IL-6 mediated thrombopoietin production and enhanced megakaryocyte proliferation [198], which may result in lower inhibitory effect of antiplatelet medication and higher *in vitro* platelet function even if platelet count is in the normal range.

Not only was platelet count associated with HPR in ACS patients in the present study, but it was also an independent predictor of 30-day stent thrombosis even in a large, general PCI cohort [87]. Moreover, in that study, 30-day stent thrombosis was associated only with platelet reactivity measured by MEA and platelet count and no other clinical parameter was found to be predictive for ST. As some of the platelet

indices, like mean platelet volume and platelet distribution width were also reported to be associated with HPR measured by MEA in a general PCI cohort [199], we also examined the relation of mean platelet volume and HPR, but no correlation was found in the present study (data not shown).

Our finding of baseline CRP level as predictor of HPR was in good agreement with that of other studies, where CRP was independently associated with HPR defined by different platelet function tests after elective PCI [137,150,200]. CRP level along with platelet count was also found to be higher in ACS patients with HPR than in those without measured by MEA in a recent study, however an independent association was not evaluated [174]. A direct relation between CRP levels and platelet count in humans has not been clarified yet; however, several possible mechanisms have been suggested. High CRP levels may lead to the activation of the clotting system [201] and increased thrombin generation may result in higher platelet activation. Inflammation indicated by elevated CRP level leads to down-regulation of several hepatic cytochrome P450 (CYP) isoenzymes involved in clopidogrel metabolization via inflammatory cytokines [202] which may result in impaired active metabolite production. Moreover, CRP is not only an inflammatory marker, but also known as a risk factor of cardiovascular disease and a predictor of recurrent ischemic events including stent thrombosis after PCI [203-206].

Clinical predictors of HCPR

According to our results, cigarette smoking was inversely associated with HCPR, further expanding the observation called the "smokers' paradox". This finding goes along with previous studies where smoking was also inversely related to HCPR defined by VerifyNow $P2Y_{12}$ assay [137,139] and light transmittance aggregometry [147]. Smoking is known to be an inducer of the CYP1A2 hepatic enzyme [207], which is also involved in the conversion of clopidogrel. Increased CYP1A2 enzyme activity may lead to enhanced active clopidogrel metabolite generation and more expressed clopidogrel pharmacokinetic and pharmacodynamic effects [148]. Moreover, according to a meta-analysis of major randomized clinical trials, the clinical benefit of clopidogrel therapy in reducing cardiovascular events was primarily seen in active smokers whereas the treatment effect was less favorable in non-smokers [208]. On the other hand, bleeding

risk was increased in clopidogrel treated current smokers compared to non-smokers [209].

Notably, none of the other numerous examined clinical and laboratory factors were associated with HPR, although platelet reactivity, mostly as a continuous variable, has been affiliated with several clinical parameters in a number of previous studies. The most important such factors are age, female gender, BMI, diabetes mellitus, renal failure, left ventricular function, acute coronary syndrome, some drug-drug interactions inhibiting the CYP2C19- and CYP3A4 enzymes [38,142,210], and in studies with whole blood platelet function tests, also leukocyte count [150,211]. The initial observations that raised concern of a potential interaction between PPIs and clopidogrel derived from in vitro studies showing that omeprazole-clopidogrel co-administration resulted in reduced active clopidogrel metabolite level and decreased in vitro platelet aggregation values, while pantoprazole had less of an interaction with clopidogrel [212]. However, the debated impact of this interaction on clinical outcome is not supported with data from randomized controlled trials [213,214]. In our *in vitro* study, we did not observe any correlation between HPR and PPI therapy (p=0.4). This might be explained by the almost exclusive use of pantoprazole and the originally high rate of PPI administration in our patient population.

With respect to other clinical parameters, associations with platelet aggregation as a continuous variable were expansively observed previously, though their roles as independent predictors of a clinically derived HPR were conflicting or were limited to a certain method or stable CAD population [141,150,215,216]. The relatively low number of identified predictors despite the large number of investigated variables in the present study could reflect inappropriate sample size and might also be responsible for the moderate distinctive capacity of the risk prediction model (see below).

Today, several point-of-care methods are available to measure platelet function in cardiac patients being on $P2Y_{12}$ inhibitor therapy (Verify Now, Multiplate, and Plateletworks). Yet, none of them has been shown to be more accurate than the others. Importantly, distinct platelet function tests (including both the point-of-care and the more labor-intensive methods, such as the LTA or the vasodilator-stimulated phosphoprotein phosphorylation test) analyze and reflect different aspects of physiological platelet activation and aggregation; hence factors associated with HPR

defined by distinct methods might be heterogeneous as well. Therefore, it is unlikely that predictors of HPR might be uniformly applicable and they should always be evaluated in the context of a certain method.

Rate of high on-clopidogrel platelet reactivity measured by MEA

We found a relatively low rate of high on-clopidogrel platelet reactivity (16.0%) in an ACS patient cohort compared to previous literature, where HPR rate was reported within a very broad range depending on the applied platelet function test, threshold of HPR, and investigated patient population [125]. Considering the time lag between clopidogrel loading and MEA testing in our study (which is a frequently used time frame), aggregation units most likely reflected the effect of a single 600 mg clopidogrel loading dose, potentially explaining our low HPR rate. The consensus cut-off used in our study (>46 U) was also based on aggregation values of patients after clopidogrel 600 mg loading dose [87] which may substantially differ in variability and in distribution from that of patients being on 75 mg maintenance therapy. It must be noted that when a more expressed or maximal drug effect is hypothesized, platelet inhibition may be less variable and the effect of different influencing factors might be less pronounced. Therefore, presence of HPR in the maintenance phase could be associated with more diverse variables [150], which might have an important effect on the long term efficacy of antiplatelet therapy.

Risk prediction model for HCPR

In our study, a risk prediction model of HCPR was built with the help of the identified predictors such as PLT count, CRP level and current smoking. Based on internal validation, the model proved to be only moderately optimistic, however its discriminative capacity was modest (AUC=0.665 and optimism corrected AUC=0.653). To achieve higher predictive power, identification of further potential predictors might be needed in the future. Though, with the help of the presented model, we could sufficiently classified patients into clearly separated low-, intermediate- and high risk categories. High-risk patients had more, than a fourfold increase in post-test probability of HCPR, than patients with low risk (8.7% vs 35.7%). Notably, the discriminative

capacity of our score was similar to that of found in other studies, where HPR risk prediction models were developed. Importantly, in these studies platelet function measurements were also timed 6-24 hours after 600 mg clopidogrel loading, though LTA was used to assess residual platelet aggregation. In the PREDICT-score, the authors included five clinical parameters used in a dichotomous fashion (namely age>65 years, left ventricular dysfunction, renal failure, diabetes mellitus, and acute coronary syndrome) and composed a cumulative score (ranging from 0-9). Patients in the highest stratum (score 7-9) had 3.3 times higher probability to have HPR, than patients with a score of 0. Moreover, higher residual platelet aggregation values were associated with higher adverse event rates. Similarly in the PREDICT-STABLE score, where only elective PCI patients were included, the score was composed of five dichotomously treated clinical parameter (age>65 years, left ventricular dysfunction, renal failure, diabetes mellitus, and high BMI) to compose a score ranging from 0-9. The ratio of HPR increased gradually with score levels (14% vs 43.3% in the lowest and highest stratum) and 12 month MACE was also associated with PREDICT-STABLE score (3.4% vs 10.3% between low and high score levels). Unfortunately, due to feasibility reasons, we could not link follow-up and clinical outcome data to HPR values in our study, therefore association of identified predictors and HPR with recurrent ischemic events could not have been analyzed. However, parallel evaluation of the risk prediction model for HPR and for clinical outcomes would provide further valuable data.

One of the major concerns regarding platelet function testing is their variable, but generally low positive predictive value. However, PPV is a diagnostic test statistic feature along with sensitivity, specificity, accuracy, and ROC curve c statistic. Platelet function testing can be assessed as either a diagnostic or a prognostic tool. It may be evaluated for its diagnostic performance in measuring the antiplatelet effect of a $P2Y_{12}$ inhibitor. Regarding this aspect, PF tests can be used to provide precise diagnostic evidence of P2Y12 inhibition [217]. However, the often challenged positive predictive value of platelet function testing depends on the prevalence of HPR in the population to be tested, thus limiting the measurement to those at high risk for HPR might increase the test's positive predictive value. On the other hand, PPV should not be used in the assessment of the predictive value of platelet function testing, as the prevalence of the clinical event that PF testing is expected to predict – namely stent thrombosis – is

generally low (1-2%) in the studied cohorts. Instead, in evaluating prognostic performance of PF testing, risk assessment tests should be used, which describe the multiplicative risk of the condition or hazard (using odds ratios and hazard ratios). Importantly, these tests are used to assess risk, not to diagnose whether or not a future event will occur. Thus, it is possible, that some patients with high risk will not experience the certain event, while patients in low risk stratum will suffer from it. Moreover, a statistically significant association does not necessarily mean a biologically significant association. Therefore, further statistical techniques are usually needed to verify that the test has an additive value to other established risk scores and it may improve the overall risk assessment (e.g. change in the ROC curve c statistic, or using net reclassification improvement (NRI) and integrated discrimination improvement (IDI) tests) [218]. Such additive prognostic value of PF testing has been nicely demonstrated in the large clinical trials. In the POPULAR study, addition of HPR to established clinical risk scores for predicting clinical outcome resulted in significant increase of the ROC c-statistic value. In the ADAPT-DES study, analysis of the NRI and IDI showed that platelet reactivity measured by VerifyNow was able to reclassify the risk of developing both ischemic and bleeding events beyond the baseline clinical characteristics of the patients.

Based on the foregoing, PF testing may have an important role in risk assessment and identification of patients who may profit from antiplatelet therapy intensification. Importantly, the prevalence of HPR has been shown to be higher in ACS patients compared to a stable CAD cohort [81], representing higher atherothrombotic risk of these patients. Since HPR rate proved to be moderate (16%) even in the ACS cohort in our study, identification of patients with high risk for HPR by the presented clinical model might enable more purposeful use of platelet function testing. By limiting the target population to be tested with the help of risk prediction models, HPR risk assessment might potentially contribute to a more conscious design of clinical trials investigating the effect of platelet function test guided antiplatelet therapy and a more purposeful use of platelet function testing. Our results also suggest that platelet function is a well determinable and modifiable clinical factor even in the acute presentation of CAD and its *in vitro* monitoring may contribute to select the adequate individual

therapy of a cardiac patient in a complex clinical setting. Applying proper therapy at the individual level may be the fair way to improve the clinical outcome of a large population.

In conclusion, to make a final judgement about the role of platelet function testing after percutaneous coronary intervention, further carefully designed large scale, randomized clinical trials are needed. Study design should be particularly deliberate considering antiplatelet loading and maintenance doses (for sake of comparability), timing of platelet testing (loading vs maintenance dose effect), the applied platelet function test, and should use predetermined cut-off values, therapy intensification algorithms and follow-up period as well as standard, well defined clinical end points.

6. CONCLUSIONS

6.1. We found an increased level of ADP induced platelet reactivity measured by light transmission aggregometry in patients with myocardial infarction compared to those with stable coronary artery disease, representing heightened atherothrombotic risk of the acute patient cohort.

6.2. In patients with myocardial infarction, initial, clinical risk profile but not platelet function test guided clopidogrel dose doubling resulted in the same inhibitory potency as the standard dose clopidogrel in patients with expectedly lower risk for high on-treatment platelet reactivity.

6.3. The ratio of patients, who had high on-clopidogrel platelet reactivity even on high dose clopidogrel (real non-responders) was significantly higher in the myocardial infarction group, suggesting that this patient cohort with higher thrombotic risk might benefit from prospective platelet function testing, especially in view of reported prevalence of high on-treatment platelet reactivity even beside the novel antiplatelet agents.

6.4. Therapy modification resulted in effective platelet inhibition in majority of the patients, according to *in vitro* testing. The potent inhibitory effect of therapy conversion proved to be stable during 12 month follow-up.

6.5. Among patients on clopidogrel therapy, appearance of new high on-clopidogrel platelet reactivity was observed during 12 months, irrespective of applied clopidogrel dose or representing clinical syndrome. This may suggest the vulnerability of clopidogrel effect in the long term, and highlights the potential significance of repeated evaluation of antiplatelet therapy.

6.6. We analyzed the association of a wide range of clinical and laboratory parameters with high on-clopidogrel platelet reactivity measured by multiple electrode

aggregometry in an acute coronary syndrome cohort and identified elevated platelet count and CRP level upon admission and a non-smoking status as predictors.

6.7. We developed and internally validated a risk score for high on-clopidogrel platelet reactivity, which enabled successful classification of the patients into low, intermediate and high risk strata. Patients with the highest risk had more than a fourfold increase in post-test probability of high on-clopidogrel platelet reactivity compared to those with low risk. Such a risk assessment of high on-treatment platelet reactivity might enable more targeted use of platelet function testing to identify patients who may benefit from therapy intensification.

6.8. With recent reclassification of on-treatment platelet reactivity into low, optimal and high categories, platelet function testing may be used to find a therapeutic window to optimize the balance of ischemic and bleeding consequences in patients on dual antiplatelet therapy.

7. SUMMARY

The great success of percutaneous coronary intervention in the treatment of CAD was enabled by induction of dual antiplatelet therapy to prevent thrombotic events. Due to variable platelet inhibition on clopidogrel therapy, it has been replaced by more potent P2Y12 inhibitors in invasively treated patients with acute coronary syndrome (ACS). Since with the use of these inhibitors bleeding events became more frequent, patients with SCAD and ACS patients at high risk for bleeding remained subject to clopidogrel treatment. High on-clopidogrel platelet reactivity (HCPR) is known as a predictor of recurrent ischemic events. Therefore, screening patients with HCPR and optimizing individual antiplatelet therapy might improve clinical outcome.

The aim of our study was to identify patients with HCPR and to induct an intensified antiplatelet therapy with a 12 months follow-up in patients with MI and SCAD. We also intended to identify predictors of HCPR and to develop a risk prediction model in an ACS cohort. We found that platelet reactivity and the rate of HCPR even on elevated clopidogrel dose is higher in patients with MI than in those with SCAD after PCI. HCPR can be functionally overcome by switch of therapy. Platelet inhibition in patients on dual antiplatelet therapy alternates over time and an incidence of new HPR irrespectively of antiplatelet therapy modification is observed in both patient groups. We found that platelet count, C-reactive protein level on admission, and non-smoking status were associated with HCPR. With the help of these determinants, a risk prediction model has been developed and internally validated. In summary, patients with MI may benefit from prospective platelet function testing because of higher thrombotic risk represented by elevated platelet aggregation values and higher rate of HCPR even on elevated clopidogrel dose. Moreover, therapy alteration leads to efficient platelet inhibition in majority of the MI patients. The risk prediction model with simply available parameters might help to identify individuals at high risk for HCPR and risk assessment might contribute to the more targeted use of platelet function testing. HCPR is a well detectable, predictable and modifiable parameter even in the acute presentations of CAD with high thrombotic risk and thus platelet function testing might contribute to improving the clinical outcome of this patient cohort.

8. ÖSSZEFOGLALÁS

A perkután koronária intervenció (PKI) terápiás sikerét az iszkémiás szívbetegség kezelésében a kettős trombocitagátló kezelés bevezetése tette teljessé a trombotikus szövődmények gyakoriságának csökkentésével. A clopidogrel helyét - a trombocitagátló potenciál nagyfokú varianciája miatt - hatékonyabb trombocitagátló szerek vették át az invazívan kezelt akut koronária szindrómás betegek terápiájában. Azonban az új szerek mellett észlelt magasabb vérzési rizikó miatt a betegek egy része nem részesülhet az általuk nyújtott terápiás előnyökben. A terápia melletti magas trombocita reaktivitás (MTR) és a kedvezőtlen klinikai kimenetel összefüggését felismerve felmerült, hogy a trombocita funkció mérése és MTR esetén a trombocitagátló terápia intenzifikálása javíthatná a betegek klinikai kimenetelét. Vizsgálataink célja a MTR gyakoriságának trombocita funkciós teszttel történő felmérése, a trombocitagátló terápia intenzifikálása és utánkövetése volt elektív és miokardiális infarktus (MI) miatt PKI-ra kerülő betegekben. Vizsgáltuk továbbá a MTR-t meghatározó klinikai és laboratóriumi tényezőket egy rizikóbecslő modell kifejlesztése céljából. Eredményeink alapján infarktusos betegekben magasabb trombocita reaktivitás észlelhető az elektív csoporthoz képest. Az infarktus csoportban az emelt dózisú clopidogrel kezelés mellett észlelt valódi non-reszponzió aránya szignifikánsan magasabb volt. A terápiamódosítás a betegek többségében hatékony trombocita gátláshoz vezet az *in vitro* mérések alapján. 12 hónapos utánkövetés során a betegek egy részében terápiamódosításhoz nem köthető, újonnan megjelenő MTR-t észleltünk. A MTR meghatározó tényezőit vizsgálva a felvételi trombocita számot és CRP szintet, valamint a nemdohányzó státuszt azonosítottuk prediktorként, melyek felhasználásával egy MTR rizikóbecslő modellt építettünk és sikeres rizikó stratifikációt végeztünk. Eredményeink alapján MI-ban a magasabb trombotikus rizikót jelképező trombocita hyperreaktivitás, a clopidogrel valódi non-reszponzió gyakoribb előfordulása és a trombocita reaktivitás terápiamódosítás általi befolyásolhatósága miatt a trombocita funkció prospektív mérése ezen betegekben kifejezett előnyökkel járhat. Akut koronária szindrómában a MTR rizikója néhány egyszerű paraméter segítségével megbecsülhető, a stratifikáció segítséget nyújthat a trombocita funkciós tesztek célzottabb felhasználásában és a terápia intenzifikálásából leginkább profitáló betegek azonosításában.

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10. OWN PUBLICATIONS

Publications related to the thesis:

Leé S, Vargová K, Hizoh I, Horváth Z, Gulácsi-Bárdos P, Sztupinszki Z, Apró A, Kovács A, Préda I, Tóth-Zsámboki E and others. High on clopidogrel treatment platelet reactivity is frequent in acute and rare in elective stenting and can be functionally overcome by switch of therapy. Thromb Res 2014;133(2):257-64. **IF: 2.447**

Lee S, Hizoh I, Kovacs A, Horvath Z, Kiss N, Toth-Zsamboki E, Kiss RG. Predictors of high on-clopidogrel platelet reactivity in patients with acute coronary syndrome. Platelets 2016;27(2):159-167. **IF: 2.982**

Leé S, Kiss RG, Noori E, Kiss N. Vérlemezkegátlás a kardiológiában – Új horizontok. Antiplatelet therapy in cardiology – new horizons. Cardiologia Hungarica 2011;41:46-52. Article in Hungarian.

Publications irrespective of the thesis:

Horvath Z, Csuka D, Vargova K, Kovacs A, Molnar AA, Gulacsi-Bardos P, **Lee S**, Varga L, Kiss RG, Preda I and others. Elevated C1rC1sC1inh levels independently predict atherosclerotic coronary heart disease. Mol Immunol 2012;54(1):8-13. **IF: 3.003**

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Vargová K, Pállinger É, Horváth Zs, Kovács A, **Leé S**, Gulácsi-Bárdos P, Falus A, Kiss RG, Préda I. A mikrovezikulumok szerepe cardiovascularis kórképekben. The role of

microvesicles in cardiovascular disease. Orvosképzés 2012;2:55-60. Article in Hungarian.

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