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Personalized antithrombotic treatment in high-risk patients with coronary artery disease

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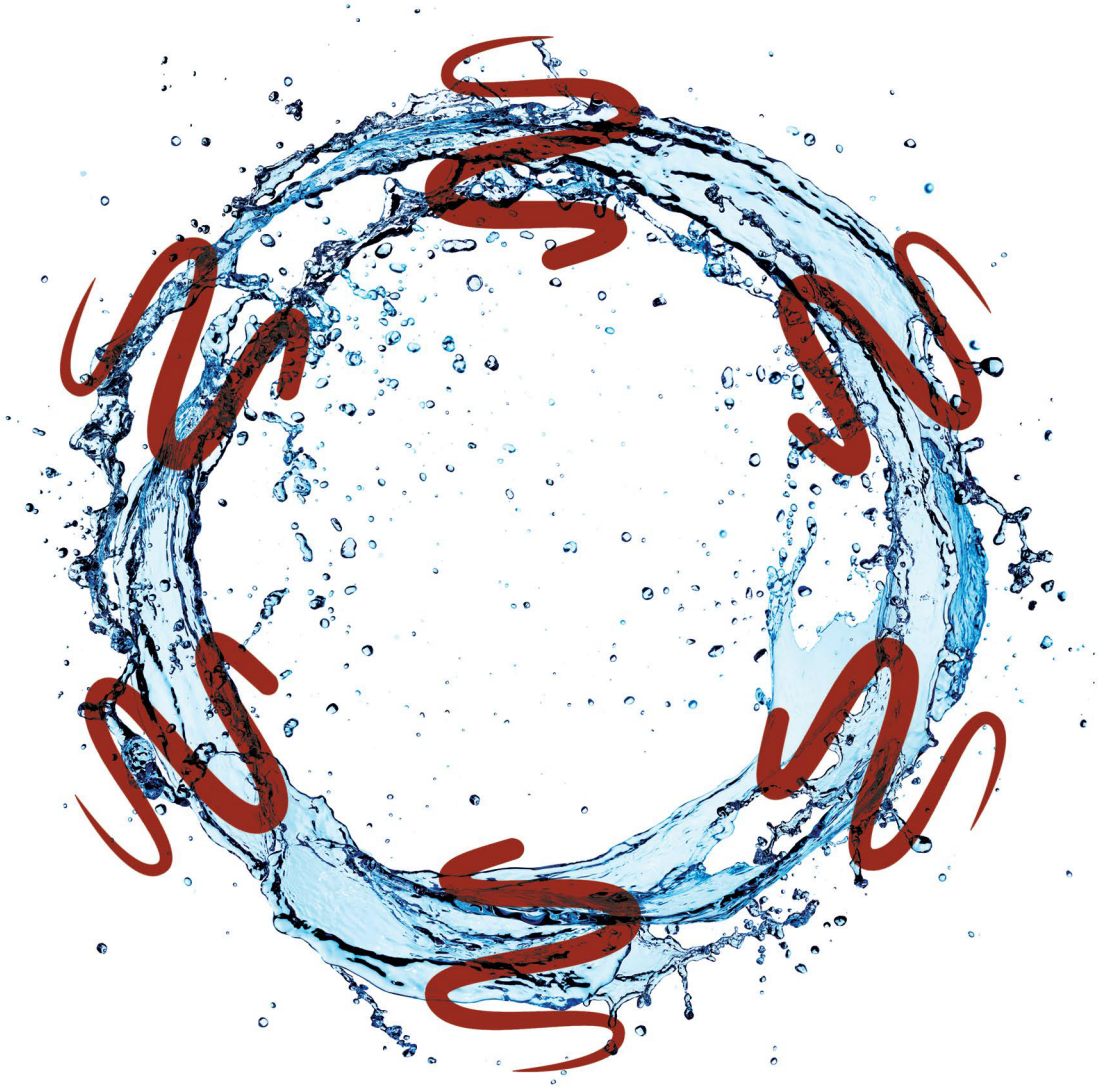
Personalized antithrombotic treatment
IN HIGH-RISK PATIENTS WITH
CORONARY ARTERY DISEASE



Renske H. Olie

Stellingen behorende bij het proefschrift:

Personalized antithrombotic treatment

in high-risk patients with coronary artery disease

1. Het goed kunnen identificeren van de patiënten met een verhoogd bloedingsrisico is de eerste stap in het terugdringen van het aantal bloedingscomplicaties. *(dit proefschrift)*

2. Globale stollingstesten zoals de trombinegeneratietest en ROTEM zijn veelbelovende testen om het bloedingsrisico bij duale trombocytenaggregatieremming in te schatten. *(dit proefschrift)*

3. De antitrombotische behandeling bij hoog-risico patiënten met coronairlijden moet worden gecontroleerd en aangepast om veilig tussen de Scylla van trombose en de Charybdis van bloeding te navigeren. *(dit proefschrift, naar analogie van F.R. Rosendaal, N Engl J Med. 1996;335(8):587-589)*

4. Triple antitrombotische therapie dient alleen nog voorgeschreven te worden aan patiënten met een zeer hoog ischemisch risico en een beperkt bloedingsrisico. *(dit proefschrift)*

5. Goede zorg voor kwetsbare patiënten, zoals ouderen waarbij sprake is van polyfarmacie, vraagt om samenhang, afstemming en samenwerking tussen patiënt (en/of mantelzorger), medisch specialist, huisarts, apotheker en verpleegkundige. *(Basisset medisch specialistische zorg 2022 – IGJ)*

6. De zorg voor hoog-risico patiënten na PCI behoort verder te gaan dan het voorschrijven van de standaard antitrombotische medicatie alleen. *(valorisatie)*

7. Zorg en wetenschap moeten geïntegreerd worden om werkelijk klinische vooruitgang te kunnen bereiken.

8. Om de Nederlandse gezondheidszorg in de toekomst toegankelijk en betaalbaar te houden zal veel meer moeten worden ingezet op het bevorderen van een gezonde leefstijl en het versterken van de zelfredzaamheid van mensen.

9. Either you run the day or the day runs you. *(Jim Rohn)*

10. Nulla tenaci invia est via – Voor de volhouder is geen weg onbegaanbaar

Renske Olie, 20 maart 2023

Personalized antithrombotic treatment in high-risk patients with coronary artery disease

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Personalized antithrombotic treatment in high-risk patients with coronary artery disease

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Chapter 1

General introduction and outline of this thesis

Parts published as:
Renske H. Olie, Paola E.J. van der Meijden, Henri M.H. Spronk, Hugo ten Cate
Antithrombotic Therapy: Prevention and Treatment of Atherosclerosis and Atherothrombosis

Handb Exp Pharmacol. 2022;270:103-30

Coagulation cascade - Atherothrombosis

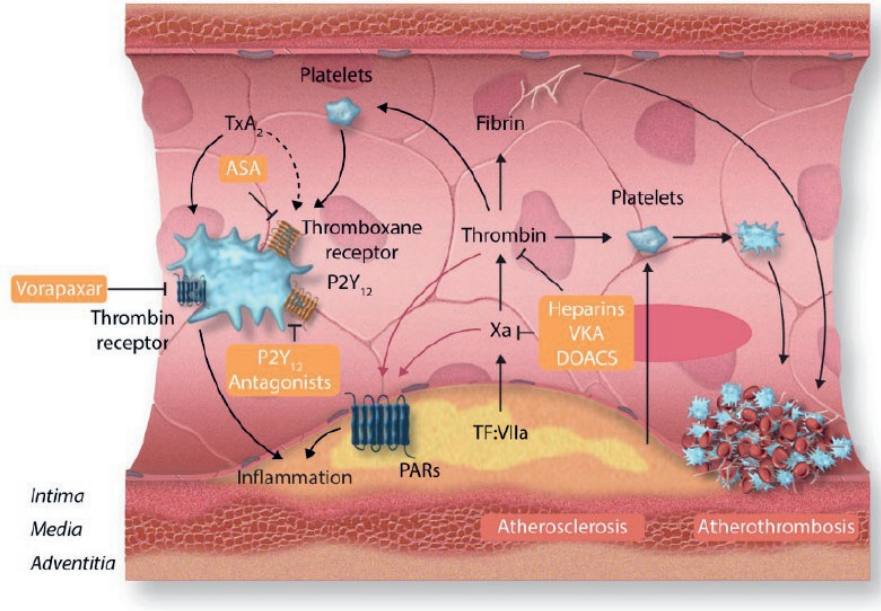
Atherosclerosis is a multifactorial vascular disease that develops in the course of a lifetime. Numerous risk factors for atherosclerosis have been identified, mostly inflicting pro-inflammatory effects, hence the term 'chronic inflammatory disease'[1]. Atherothrombosis is the consequence of atherosclerosis, in which haemostatic mechanisms are triggered by rupture or erosion of plaques to form a clot. The blood coagulation system consists of coordinated platelet and thrombin generation pathways that together lead to thrombus formation. After rupture or erosion of atherosclerotic plaques, platelets adhere to exposed subendothelial collagen and von Willebrand factor (vWF). Activated platelets release (amongst other substances) ADP and thromboxane A2 (TXA2) to recruit and activate other platelets to form a haemostatic plug[2]. Simultaneously with platelet activation and aggregation, tissue factor in the plaque binds to factor VII(a) in blood, triggering blood coagulation resulting in generation of thrombin. Thrombin is a key enzyme in haemostasis; it not only converts fibrinogen into fibrin to form a stable platelet-fibrin thrombus, but is also a potent platelet agonist via activation of protease-activated receptors (PARs)-1 and 4. Furthermore, thrombin also drives several feedback loops that include activation of factors V, VIII and XI to further amplify thrombin generation. This amplification phase of coagulation requires a negatively charged phospholipid surface providing phosphatidylserine (PS), on which the positively charged intrinsic tenase and prothrombinase complexes assemble[3]. Activated platelets at the site of plaque rupture primarily provide this phospholipid surface, and thereby, the platelet pathway and thrombin pathway are connected[4].

Antithrombotic therapy in coronary artery disease

The focus of this thesis is on high-risk patients requiring antithrombotic therapy after percutaneous coronary intervention (PCI) for either acute coronary syndrome (ACS) or chronic coronary syndrome (CCS). Dual antiplatelet therapy (DAPT) with aspirin and a P2Y12 inhibitor is the cornerstone of antithrombotic therapy for ischemic secondary prevention (Figure 1.1). Aspirin is the common name for acetylsalicylic acid, a compound that acts by inhibition of cycloocygenase-1 and the production of TXA2 in platelets[4]. Its intake results in irreversible inhibition of platelet activation and aggregation. Three oral P2Y12 inhibitors are currently available: the thienopyridines clopidogrel and prasugrel, and the direct acting drug ticagrelor. Clopidogrel is a prodrug that needs metabolization in the liver[5]. The formed active metabolite interferes with the binding of ADP to the P2Y12 receptor on the platelets. The formation of active

metabolite shows a large interindividual variation, partly explained by genetic polymorphisms encoding cytochrome P450 (CYP) 2C19, the hepatic enzyme involved in biotransformation of the prodrug clopidogrel into its active metabolite[6]. This pharmacodynamic variation translates into variation in clopidogrel effectiveness after PCI. Although prasugrel is a prodrug like clopidogrel, it only requires a single oxidation step to form its active metabolite, and it seems not to be affected by genetic variations in CYP enzymes. Compared to clopidogrel, its use is associated with an increase in the risk of major bleeding, especially among those with age >75 years or body weight <60 kg, and it is contraindicated in patients with prior stroke[7]. Ticagrelor, a direct acting, reversible P2Y12 receptor antagonist provides faster and stronger platelet inhibition compared to clopidogrel with less patient-to-patient variation[8].

Figure 1.1 Antithrombotic agents described in this thesis.



Antithrombotic and vascular-protective effects of anticoagulants and platelet inhibitors. Atherosclerosis-mediated vascular injury causes atherothrombosis through activation of coagulation and platelets. Fibrin formation can be diminished by anticoagulants including heparins, vitamin K antagonists, and direct oral anticoagulants. Platelets can be inhibited by aspirin (acetylsalicylic acid (ASA), effecting the thromboxane A2 receptor), P2Y12 receptor antagonists clopidogrel, prasugrel, or ticagrelor, or via inhibition of the thrombin receptor PAR1 by Vorapaxar. Inhibition of thrombin, factor Xa, and platelets will diminish cellular effects through attenuated activation of the protease-activated receptors PAR1 and PAR2 on endothelial cells, vascular smooth muscle cells, and macrophages[9]. (Figure from: Ten Cate H, Guzik TJ, Eikelboom J, Spronk HMH. Pleiotropic actions of factor Xa inhibition in cardiovascular prevention: Mechanistic insights and implications for anti-thrombotic treatment. Cardiovascular Research. 2021;117(9):2030-44, DOI: 10.1093/cvr/cvaa263. Reprinted by permission of Oxford University Press.)

The principle of combining aspirin with P2Y12 inhibition was established in the CURE trial, comparing the efficacy and safety of aspirin plus clopidogrel with aspirin alone in the secondary prevention of MACE in patients with CAD[10]. Since then, DAPT has become a cornerstone treatment in secondary prevention following ACS and/or PCI. After PCI with stent implantation, there is a risk of (acute) stent thrombosis leading to myocardial infarction, urgent revascularization or mortality. Depending on the setting of PCI (urgent or elective) and the bleeding risk, current ESC guidelines recommend DAPT for at least 1 to 12 months, or longer in selected cases at high ischemic risk[11,12].

Antithrombotic agents described in this thesis

Besides aspirin and the P2Y12 inhibitors (clopidogrel, prasugrel and ticagrelor), vorapaxar is another antiplatelet agent discussed in this thesis (Figure 1.1). Vorapaxar is an orally administered, competitive PAR-1 antagonist that blocks thrombin-mediated platelet activation via PAR-1, without inhibiting other modes of thrombin activity, such as fibrin formation, protein C activation and PAR-4 activation. Besides (dual) antiplatelet therapy indicated for coronary artery disease, some of these CAD patients have an indication (e.g. atrial fibrillation, venous thromboembolism) for oral anticoagulants. For many years, the vitamin K antagonists (VKAs) warfarin, acenocoumarol and phenprocoumon have been the cornerstone in oral anticoagulation treatment. VKAs inhibit vitamin K epoxide reductase, the enzyme regulating the recycling of inactive vitamin K into active vitamin K, thus inducing vitamin K depletion. Vitamin K is needed for the production of functionally active FII, FVII, FIX and FX, as well as protein C and protein S. Since 2012, four direct oral anticoagulants (DOACs) have been registered for clinical use; the direct thrombin inhibitor dabigatran, and the direct factor Xa inhibitors apixaban, rivaroxaban and edoxaban. Nowadays, guidelines recommend DOAC over VKA because of a lower rate of intracranial bleeding and easiness in use.

Bleeding complications and high risk patients

With the improving results of PCI due to better stents and antithrombotic treatment, increasingly complex patient populations with more comorbidities are treated. Although evidence on optimal treatment exists for most patients, high-risk patients with multiple clinical risk factors remain a challenging group. Both the bleeding risk and the risk for recurrent ischemic events are considerably increased in these patients, leading to the exclusion from or underrepresentation in large clinical trials. Therefore,

selecting the optimal antithrombotic combination therapy with appropriate duration of DAPT remains a challenge for the treating physician[13]. With advances in stent technology and broader use of potent P2Y12 inhibitors, thrombotic events have dramatically decreased, and consequently, prevention of bleeding complications has become a major goal; the price of gained efficacy with combination of several antithrombotic agents is always increased risk of (major) bleeding.

Identifying patients at risk for bleeding is a first important step towards prevention of bleeding complications. Recently, a consensus document from the Academic Research Consortium for High Bleeding Risk (ARC-HBR) was published, presenting a consensus definition of patients at high bleeding risk[14].

In this thesis, we used the Bleeding Academic Research Consortium (BARC) definition (Table 1.1) to report on these bleeding complications[15].

Table 1.1 Bleeding Academic Research Consortium (BARC) definition of bleeding.

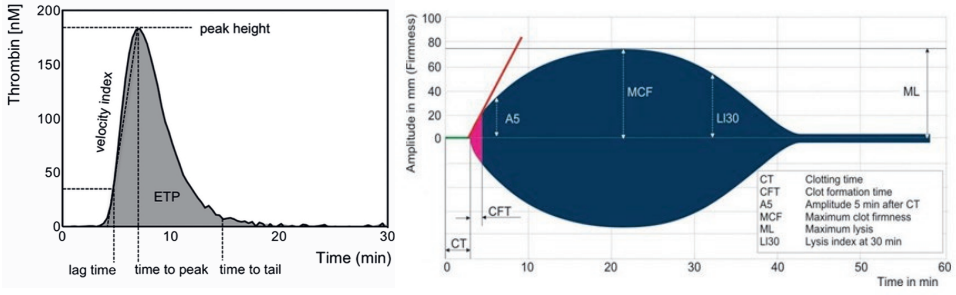
BARC type	Definition
0	No bleeding
1	Bleeding that is not actionable and does not cause the patient to seek unscheduled performance of studies, hospitalization, or treatment by a healthcare professional; may include episodes leading to self-discontinuation of medical therapy by the patient without consulting a healthcare professional
2	Any overt, actionable sign of hemorrhage (e.g. more bleeding than would be expected for a clinical circumstance, including bleeding found by imaging alone) that does not fit the criteria for type 3, 4, or 5, but does meet at least one of the following criteria: Requiring nonsurgical, medical intervention by a healthcare professional Leading to hospitalization or increased level of care, or Prompting evaluation
3	Overt bleeding plus hemoglobin drop of >3 g/dL (provided hemoglobin drop is related to bleed). Any transfusion with overt bleeding. Cardiac tamponade Bleeding requiring surgical intervention for control Bleeding requiring intravenous vasoactive agents Intracranial or intraocular bleeding
4	CABG-related bleeding
5	Fatal bleeding

Between 5% and 10% of patients with atrial fibrillation are referred for PCI, while vice versa, it is estimated that atrial fibrillation develops in up to 20% of patients with acute coronary syndromes. Thus, a considerable group of patients has an indication for both (dual) antiplatelet therapy and anticoagulant therapy. In observational studies, patients treated with triple therapy after an ACS were at high risk for bleeding. Several trials in this patient group have now documented a reduction in bleeding when a DOAC and a P2Y12 inhibitor are used together, as compared to VKA-based triple therapy[16].

Laboratory tests & genetics

A wide range of laboratory tests is available to evaluate the effect of antiplatelet and anticoagulant agents on haemostasis. On-treatment platelet reactivity to ADP can be measured by several platelet function tests (PFTs). Amongst the tests used in this thesis are the VerifyNow P2Y12, Multiplate ADP and light transmittance aggregometry (LTA) with ADP. Although the measurement principles differ per test, all PFTs aim to measure the platelet activation and aggregation potential upon stimulation with ADP. Because clopidogrel is a prodrug that needs to be metabolized in the liver, genetic polymorphisms encoding CYP2C19 translate into variation in clopidogrel effectiveness. In this thesis, we performed genotyping of the CYP2C19 polymorphism in whole blood, although nowadays also several point of care assays are available. The anticoagulant effect of VKA can be measured with the INR, while DOAC levels can be measured with specific anti-Xa tests for rivaroxaban, apixaban and edoxaban, or diluted thrombin time for dabigatran. Besides these specific assays that evaluate only one specific haemostasis pathway, two global assays of haemostasis are used in this thesis. Thrombin generation describes the formation and inactivation of thrombin and was performed in platelet poor plasma. The main parameters are the lag time, the endogenous thrombin potential (ETP; area under the thrombin generation curve) and the peak height (Figure 1.2). Rotational thromboelastometry (ROTEM) is a whole blood point-of-care viscoelastic assay that provides a quick overview of global haemostatic parameters. Besides the standard assays EXTEM, INTEM and FIBTEM, an additional tissue plasminogen activator (tPA) based ROTEM assay can be used to assess the dynamic properties of fibrinolysis after clot formation. Main parameters are clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF), and, specific to the tPA assay, lysis onset time (LOT) and lysis time (LT) (Figure 1.2).

Figure 1.2 Thrombin generation and rotational thromboelastometry (ROTEM).



Cohort studies and randomized trials

In this thesis, data are used from a prospective observational cohort study, initiated by the Thrombosis Expertise Centre of the Maastricht University Medical Centre and conducted from May 2014 to June 2019. Five-hundred twenty-four high-risk patients, treated with dual or triple antithrombotic therapy following PCI, were included and data was collected regarding comorbidities, medication, bleeding questionnaires and an extensive laboratory evaluation was performed. A subset of patients with an indication for both antiplatelet and anticoagulant therapy was included in the international, multi-centre, non-interventional WOEST2 registry (Clinicaltrials.gov NCT02635230), designed to obtain contemporary real-world data on outcomes in patients on dual or triple antithrombotic therapy following PCI. To study whether the beneficial effects of the PAR-1 antagonist vorapaxar can only be ascribed to more potent platelet inhibition or to an additional effect on thrombin generation we used plasma samples obtained from patients from three Norwegian centres participating in the Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events – Thrombolysis in Mycoardial Infarction 50-trial (TRA2°P-TIMI-50) (ClinicalTrials.gov number NCT00526474).

Outline of this thesis

Chapter 2 describes the role of the coagulation system in atherothrombosis, and introduces the different antithrombotic drugs currently available for treatment and prevention of atherothrombotic complications like coronary artery disease. **Chapter 3** introduces the cohort of high-risk patients treated with dual or triple antithrombotic therapy after PCI, that served as the basis for the research described in this thesis. In **chapter 4**, the impact of genetic variations on the results of platelet function tests in the group of patients treated with clopidogrel is explored. **Chapter 5 and 6** evaluate the value of two global assays of haemostasis, thrombin generation and rotational thromboelastometry respectively, as a method to identify bleeding risk in patients on dual antithrombotic therapy. **Chapter 7** focuses on the group of patients with an indication for both antiplatelet and anticoagulant therapy following PCI, where patient data of our cohort are included in the international WOEST2 registry. Dual versus triple antithrombotic therapy was compared with regard to bleeding and ischemic outcomes. In **Chapter 8** the effect of a PAR-1 receptor antagonist on platelet activation and coagulation biomarkers is evaluated.

Finally, **Chapter 9, Summary, general discussion and prospects,** provides an overview of the most important findings of this thesis, relates it to previous studies, and discusses its implications for both clinical practice and future research.

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Chapter 2

The coagulation system in atherothrombosis: Implications for new therapeutic strategies

Renske H. Olie, Paola E.J. van der Meijden, Hugo ten Cate

Res Pract Thromb Haemost 2018;2(2):188-198

Abstract

Clinical manifestations of atherosclerotic disease include coronary artery disease (CAD), peripheral artery disease (PAD) and stroke. Although the role of platelets is well established, evidence is now accumulating on the contribution of coagulation proteins to the processes of atherosclerosis and atherothrombosis. Coagulation proteins not only play a role in fibrin formation and platelet activation, but also mediate various biological and pathophysiologic processes through activation of protease-activated-receptors (PARs). Thus far, secondary prevention in patients with CAD/PAD has been the domain of antiplatelet therapy, however, residual atherothrombotic risks remain substantial. Therefore, combining antiplatelet and anticoagulant therapy has gained more attention. Recently, net clinical benefit of combining aspirin with low-dose rivaroxaban in patients with stable atherosclerotic disease has been demonstrated. In this review, based on the State of the Art lecture 'Clotting factors and atherothrombosis' presented at the ISTH Congress 2017, we highlight the role of coagulation proteins in the pathophysiology of atherothrombosis, and specifically focus on therapeutic strategies to decrease atherothrombotic events by optimization of vascular protection.

Introduction

In the broad category of arterial thrombosis, one can distinguish thrombosis related to atherosclerosis, called atherothrombosis, and arterial thromboembolism, e.g. as a consequence of atrial fibrillation. Clinical manifestations of atherothrombosis occur in the heart (coronary artery disease (CAD); myocardial infarction), the peripheral arterial vasculature (peripheral artery disease, PAD) and in the brain (ischemic stroke). Traditionally, the treatment of atherothrombotic disease consists of antiplatelet therapy, whereas treatment of arterial (and venous) *thromboembolism* is based on oral anticoagulants (either vitamin K antagonists (VKA) or direct oral anticoagulants (DOACs), also known as non-vitamin-K oral anticoagulants; NOACs). This differentiation is based on the important role of platelets in the etiology of the progression from atherosclerotic plaques to atherothrombotic occlusion[1]. Atherosclerosis is characterized by endothelial dysfunction and chronic inflammation[2,3]. Binding of monocytes to the activated endothelial cells of the arterial vessel wall and their subsequent translocation and differentiation into macrophages that internalize oxidized lipoproteins results in the formation of macrophage foam cells. These foam cells induce several processes, such as vascular smooth muscle cell (VSMC) proliferation, migration and fibrous cap formation, eventually leading to the formation of a fatty streak[4]. Atheroma, composed of a mixture of lipid in foam cells, smooth muscle cells and a fibrin cap, forms slowly growing atherosclerotic plaques in the lumen of the artery. Once this atherosclerotic plaque ruptures, collagen and tissue factor (TF) are exposed and trigger the activation of platelets and the coagulation cascade, respectively, thus leading to atherothrombotic occlusion of the artery. Although the role of platelets in this whole process is well established[1], evidence is now accumulating on the contribution of coagulation proteins to the processes of atherosclerosis and atherothrombosis. In this review, which is based on the State of the Art lecture 'Clotting factors and atherothrombosis' at the ISTH Congress 2017 in Berlin, we focus on the contribution of the coagulation system on atherogenesis and consecutive atherothrombotic events, explore the implications and potentials for treatment, and discuss recent and future developments in treatment of different clinical manifestations of atherothrombotic disease.

Pathophysiology

Mechanisms of thrombosis in atherosclerosis

The pathogenesis of atherothrombotic events starts with disruption of an atherosclerotic plaque, thereby exposing thrombogenic material to the blood[5].

Thrombus formation is driven by co-ordinated platelet and thrombin generation pathways. Platelets adhere to exposed subendothelial collagen and von Willebrand factor (vWF), become activated, and then release ADP and thromboxane A2 (TXA2), which activate other platelets. Platelet activation induces conformational changes in glycoprotein (GP) IIb/IIIa, which increases the affinity for its ligands fibrinogen and vWF, and thereby mediates platelet aggregation. Simultaneously with platelet activation and aggregation, the transmembrane receptor TF in the plaque binds to factor VII(a) in blood and coagulation is triggered, resulting in thrombin generation. Thrombin is a key enzyme in that it not only converts fibrinogen into fibrin, but also is a potent platelet agonist via activation of protease-activated receptor (PARs)-1 and 4. Furthermore, thrombin also drives several feedback loops that include activation of factors V, VIII and XI to further amplify thrombin generation; eventually, factor XIII is activated to cross-link fibrin to stabilize the clot. The amplification phase of coagulation requires a phospholipid surface providing phosphatidylserine (PS) on which the intrinsic tenase (a complex of factor IXa and factor VIIIa) and prothrombinase (factor Xa and factor Va) complexes assemble[6]. This phospholipid surface is primarily provided by activated platelets at the site of plaque rupture, and thereby, the platelet pathway and thrombin pathway are connected.

Pathological examination of coronary artery thrombi removed by thrombectomy or collected after autopsies of fatal myocardial infarction, has shown that thrombi in coronary arteries often have a white, platelet-rich head, which forms at the site of plaque rupture, while its distal extension is red in color, reflecting its fibrin- and erythrocyte-rich tail, which forms when blood flow is reduced[5,9,10]. Platelets dominate this process in the first 3-4 hours, whereas fibrin becomes the dominant component at a later time point. These studies confirm that both platelet and thrombin pathways are involved in thrombus formation on an atherosclerotic background.

Associations of coagulation factors with atherosclerotic plaque progression

Numerous coagulation proteins have been implicated in pro-inflammatory conditions, such as atherosclerosis. The presence of TF, considered the primary physiologic trigger of the coagulation cascade, in atherosclerotic lesions was shown by studies of Wilcox et al.[11,12]. TF was found on the membrane of macrophages and vascular smooth muscle cells, where it co-localizes with factor VII. Several clinical studies[13-15] indicate that levels of TF are significantly higher in lesions obtained from patients with unstable angina or myocardial infarction than in those from patients with stable coronary artery disease. The role of these coagulation proteins in atherosclerotic lesions was more extensively

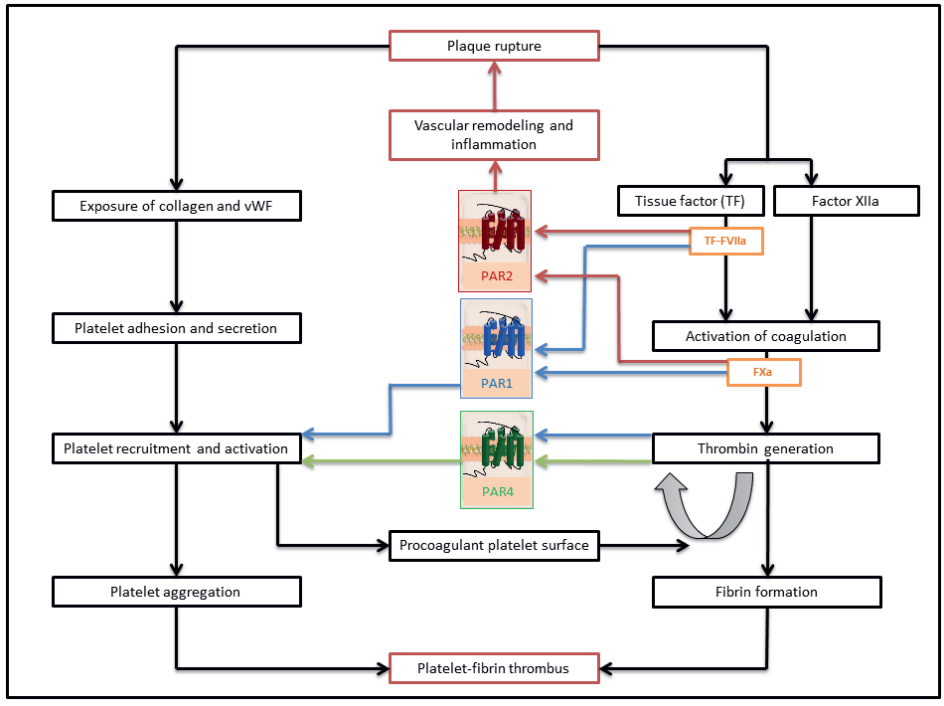
studied by Borissoff et al, who examined the presence and activity of relevant coagulation proteins in early and stable advanced atherosclerotic lesions[16]. TF, thrombin, factor X and FXII activities were significantly higher in early atherosclerotic lesions than in stable advanced atherosclerotic lesions. Furthermore, endogenous thrombin potential and thrombin-antithrombin (TAT) complex values indicated a procoagulant profile of early atherosclerotic lesions as compared to stable advanced atherosclerotic lesions. Even in subclinical atherosclerotic disease, such as individuals with increased carotid intima media thickness (IMT), a relation between TF and IMT as marker of early atherosclerosis has been documented[17].

The presence of coagulation components in atherosclerotic lesions suggests at least a role of these coagulation proteins in plaque thrombogenicity. Taking it one step further, the question rises whether some of these coagulation proteases like thrombin and factor Xa really drive atherogenesis up to the point of atherothrombosis. The fact that coagulation proteins are more present in early atherosclerotic lesions compared to advanced atherosclerotic lesions[16], supports an important role for these coagulation proteins in the initial development of atherosclerosis, rather than involvement limited to thrombus formation in unstable plaques solely. If so, one would expect some sort of protective effect of factor deficiencies like hemophilia against atherosclerotic disease. Although mouse models suggest that hypocoagulability in hemophilia protects against atherosclerosis, studies in patients with a factor VIII or IX deficiency clearly show that these patients have the same degree of atherosclerosis burden as the general population[18,19]. When explaining these apparently conflicting findings between mice and hemophilia patients, the use of factor concentrates in humans should be taken into account, although supplementation will never be complete. The signal of lower cardiovascular mortality in patients with hemophilia[20], albeit not significant (standardized mortality ratio 0.51, 95%CI 0.24-1.09), might not be explained by a reduction in atherosclerotic burden, but by reduced thrombus formation due to hypocoagulability, possibly in combination with increased plaque stability due to reduced thrombin generation[19].More recently, factor XI deficiency was shown to slow down atherogenesis in mice, with a prominent reduction of macrophage infiltration in the atherosclerotic lesions, as marker of reduction of inflammatory activity in the vessel wall of these mice[21]. Moreover, another coagulation protein of the contact activation system, factor XII, has recently been shown to play an important role in atherosclerotic lesion formation by functioning as a strong inducer of pro-inflammatory cytokine responses in macrophages[22]. Factor XIIa may under certain conditions not only contribute to arterial thrombus formation, but also contribute to inflammation through the activation of the inflammatory kallikrein-kinin system, as reviewed by Nickel et al.[23].

PAR activation

Besides their activity in coagulation, the TF-VIIa complex, factor Xa and thrombin each can signal through activation of PARs, and thereby, coagulation and inflammatory pathways are connected, as illustrated in Figure 2.1.

Figure 2.1 Pathways involved in arterial thrombus formation.



An atherothrombotic event starts with disruption of an atherosclerotic plaque, thereby exposing collagen, vWF, TF and factor XII to the blood. A dual pathway of platelet activation/aggregation and coagulation activation ultimately leads to formation of an arterial thrombus, composed of platelets and fibrin. The interplay between thrombin and platelets is illustrated via the activation of PARs, and via the procoagulant platelet surface as a site for assembly of the tenase and prothrombinase complexes, required for the propagation phase of coagulation. The activation of distinct PARs by plasma proteases initiates a series of cellular responses, including platelet activation and several proatherogenic processes such as vascular remodeling and inflammation. Red arrows represent PAR2-mediated processes, blue arrows represent PAR1-mediated processes, green arrows represent PAR4-mediated processes. Adapted from Weitz, JI. Thromb Haemost 2014; 112(5):924-931[7] and Ten Cate, H. Thromb Haemost 2017; 117(7):1265-1271[8]. Abbreviations; TF = tissue factor, vWF = von Willebrand factor, PAR = protease-activated receptor.

Via PAR-activation, the serine proteases from the clotting system are engaged in several processes, including leucocyte transmigration, vascular remodeling, angiogenesis and inflammation, which all contribute to the initial development of atherosclerosis[24]. With the exception of PAR2, all PARs are cleaved by thrombin,

whereas factor Xa initiates cellular responses through PAR2 and, to a lesser extent, PAR1 activation[25,26]. PAR1 is not only a potent platelet agonist, it also induces several proatherogenic cellular responses. Depending on the conditions of PAR activation, such as thrombin concentration, duration of activation and the location and conformation of the PAR receptor, thrombin can have opposing effects on a cell, as was reviewed in greater detail by Posma et al.[27]. PAR2, a receptor for factor Xa, but not for thrombin, seems to be a key player in vascular remodeling and inflammation[25]. Thus, the direct cellular effects of thrombin and factor Xa are responsible for several proatherogenic processes and these additional actions of thrombin and the other coagulation proteins open opportunities for therapeutic modulation. In particular, the potential to attenuate thrombo-inflammation by direct anticoagulants in the setting of secondary prevention of atherothrombotic events (see below), seems of interest[28].

Biomarkers in cardiovascular disease

Both isolated coagulation proteins and coagulation activation markers like D-dimer, TAT and prothrombin fragment 1.2 have been associated with arterial cardiovascular disease in numerous prospective studies and meta-analyses, and this has been extensively reviewed by Lowe et al.[29]. For example, plasma fibrinogen shows a strong and consistent association (OR 1.78, 95% CI 1.69-1.86) with coronary heart disease, although this may partially reflect its inflammatory marker status, as its concentration is increased under the influence of pro-inflammatory mediators such as interleukin-6[30]. Also more integral tests, like in vitro thrombin generation tests, have been studied in relation to cardiovascular disease[31]. Risks for (recurrent) cardiovascular events are highly variable among individual patients, and from that perspective, the use of biomarkers could potentially help to identify patients at greatest risk to stratify therapy accordingly. Although there is no evidence linking thrombin production to cardiovascular mortality, the downstream fibrin split product D-dimer is associated with cardiovascular events, including mortality in patients with PAD[32]. Contrary to venous thromboembolism (VTE), where the association of D-dimer levels with VTE-risk is strong enough to use it in clinical risk prediction, thus far, the associations of coagulation biomarkers with arterial thrombosis have not added significantly to risk prediction of cardiovascular events. Recently, a biomarker-based prediction model in patients with coronary heart disease (CHD) was published; the 'ABC-CHD' model (Age, Biomarkers, Clinical variables)[33]. However, only cardiac biomarkers, like N-terminal pro-B-type natriuretic peptide (NT-proBNP), high-sensitivity troponin T (hs-TnT) and LDL-cholesterol have been incorporated in this prediction model. Besides cardiac

biomarkers, markers of inflammatory activity, such as high-sensitivity C-reactive protein (hsCRP) or interleukin (IL)-6, or markers of hypercoagulability, like D-dimer levels, could be prognostic in the setting of patients with established arterial cardiovascular disease. Future studies using a combination of these different groups of biomarkers may increase the accuracy of risk prediction, and should explore their role in personalizing antithrombotic strategies in high-risk cardiovascular disease patients.

Therapeutic strategies in atherothrombotic disease

Based on the associations between the coagulation system and atherothrombosis, and above mentioned experimental studies, evidence is accumulating that the coagulation system plays an important role in atherogenesis, plaque (in)stability and consecutive atherothrombotic complications. Hence, if thrombin generation and other coagulation proteins like factor Xa play such an important role, does intervention in thrombin generation then modify atherosclerosis and thrombotic events? What clinical evidence do we have to support the idea that we should not only focus on platelet inhibition to prevent atherothrombotic disease, but also add some form of anticoagulant therapy?

Classically, secondary prevention of coronary events is the domain of antiplatelet therapy, either monotherapy with aspirin or dual antiplatelet therapy (DAPT) with ADP receptor blockers. Several alternative options for the 'standard antiplatelet regimen' with aspirin plus clopidogrel have emerged in recent years. More potent platelet P2Y12 ADP receptor blockers, prasugrel and ticagrelor, have been introduced in patients with coronary artery disease (CAD)[34,35]. Both were associated with significantly reduced rates of ischemic events, but with an approximately 30% increase in the risk of major bleeding. These newer antiplatelet agents have been implicated as standard of care in international guidelines, especially for acute coronary syndrome (ACS) patients with low estimated bleeding risks[36-39]. Extended duration of dual therapy with ticagrelor and aspirin beyond the first year in patients with prior myocardial infarction reduced the composite endpoint of cardiovascular death, stroke and myocardial infarction, however, this benefit comes with a significant increase in major bleeding and no overall mortality benefit[40]. Despite intensification of antiplatelet therapy in recent years, there remains an approximately 10% risk for recurrent ischemic events at one year after coronary events[34,35]. Even higher seems the risk for cardiovascular events in patients with symptomatic peripheral artery disease (PAD)[41,42]; current guidelines[43,44] recommend monotherapy with aspirin or clopidogrel to reduce the risk of myocardial infarction (MI), stroke, or vascular death, but despite the use of antiplatelet therapy an

approximately 20% risk for recurrent ischemic events, rehospitalization or death at one year remains[41]. In PAD patients, ticagrelor was not superior to clopidogrel in reducing major adverse cardiovascular events (MACE) and acute limb ischemia[45]. According to analysis of PAD patients in the CHARISMA trial, DAPT versus aspirin alone was not able to reduce this high risk for recurrent events in PAD patients, whereas the risk of minor bleeding was increased[46]. In a subgroup analysis of patients with a history of myocardial infarction and concomitant PAD in the PEGASUS trial, the combination of ticagrelor and aspirin reduced both MACE and major adverse limb events (MALE) compared to aspirin alone[47]. However, in this subgroup of patients with concomitant PAD and CAD treated with ticagrelor and aspirin, the incidence of MACE during 3 years follow-up was still as high as 15.2%. Thus, despite antiplatelet therapy, high morbidity and mortality remains in all patients with atherosclerosis, with the highest rates in those with PAD. Moreover, patients with PAD or CAD often have polyvascular disease, and those patients have an even higher risk of morbidity and mortality than patients with only one affected vascular bed[41,42].

With blockade of the TXA2 pathway and the P2Y12 receptor, platelets can still be activated by thrombin, via the PAR-1 receptor. In an attempt to further optimize antiplatelet therapy, the selective PAR-1 antagonist vorapaxar was developed. Vorapaxar blocks thrombin-mediated platelet activation, but does not inhibit other modes of thrombin activity, such as fibrin formation, protein C activation and PAR-4 activation[48]. The combination of three antiplatelet agents in stable ACS patients in the TRA-2°P-TIMI 50 study, involving aspirin, clopidogrel and the PAR-1 inhibitor vorapaxar, led to significant reduction in rates of ischemic cardiovascular events, but at the price of increased major bleeding[49]. In a subgroup analysis of PAD patients, the addition of vorapaxar to standard therapy with aspirin and/or clopidogrel did not reduce the risk of CV death, myocardial infarction or stroke when compared with placebo[49,50]. However, it did result in significant risk reduction in limb outcomes, such as acute limb ischemia, (non-) urgent peripheral revascularization and hospitalization. However, again, intensification of antiplatelet therapy was accompanied by an increased risk of bleeding, especially intracranial bleeding[50]. Hence, according to current guidelines, the overall clinical benefit of vorapaxar added to standard antiplatelet therapy in PAD patients is uncertain[43]. Nevertheless, the beneficial effect of vorapaxar on prevention of both urgent revascularizations (i.e. acute limb ischemia due to atherothrombosis) and non-urgent revascularizations (i.e. progression of atherosclerotic disease) raises the question whether there could be an additional non-platelet-mediated effect of vorapaxar on the vascular endothelium. Since PAR-1 on endothelial cells and VSMCs

mediates mitogenic effects[27,51], vorapaxar might be effective in reducing vascular remodeling and consecutive progression of atherosclerosis[50].

Standard treatment of patients at risk of (recurrent) atherothrombotic disease with antiplatelet agents exclusively, allows the thrombin pathway to have a persistent proatherogenic and prothrombotic effect. While platelet activation can persist despite antiplatelet treatment via thrombin-induced activation of PAR1 and PAR4, activation of PAR2 by factor Xa and the TF-VIIa complex may promote a pro-inflammatory condition in the vessel wall. Therefore, ongoing research focuses on identifying the optimal 'dual-pathway' approach; a combination of antiplatelet and anticoagulant agents, maximizing the efficacy in reduction of thrombotic events with smallest risk of bleeding.

Until recently, vitamin K antagonists were the only available oral anticoagulants evaluated for long-term treatment of patients with coronary artery disease. Their efficacy in reducing myocardial infarction and stroke was quite convincingly demonstrated in the Warfarin and Aspirin ReInfarction Study (WARIS)[52] and the Anticoagulants in the Secondary Prevention of Events in Coronary Thrombosis (ASPECT) study[53]. However, despite efficacy in the setting of secondary prevention of cardiovascular disease, the combination of aspirin and warfarin increased bleeding and did not reduce mortality[54-56]. Moreover, after introduction of DAPT, therapy with VKA in the setting of ACS or percutaneous coronary intervention (PCI) was practically abandoned due to more effective protection of DAPT, in particular against in-stent thrombosis[57,58]. In patients with PAD, the combination of aspirin and warfarin did not reduce major cardiovascular complications and markedly increased bleeding[59]. However, a post hoc analysis that excluded patients with fatal bleeding and hemorrhagic stroke from the co-primary outcome demonstrated risk reductions that were more favorable with combination therapy than with antiplatelet therapy alone, suggesting that the excess bleeding neutralized the potential benefit of combination therapy in PAD patients[59].

Nonetheless, the results with warfarin had provided proof-of-principle that inhibition of coagulation may be of additional benefit in atherothrombotic disease, a process previously thought to be predominantly platelet-driven. Therefore, after the introduction of direct oral anticoagulants (DOACs), several phase II and later two phase III trials evaluated their role in prevention of recurrent ischemia in stabilized ACS patients. The APPRAISE-2 trial (APixaban for Prevention of Acute Ischaemic Events) was terminated early after recruitment of almost 7400 patients, because of an increase in major bleeding with apixaban at a dose of 5 mg twice daily, without a reduction in

recurrent ischemic events[60]. However, the ATLAS-2 trial (Anti-Xa Therapy to Lower Cardiovascular Events in Addition to Standard Therapy in Subjects with Acute Coronary Syndrome) was carried to completion and met its primary objective[61]. In this study, 15.526 patients with a recent acute coronary syndrome were randomized to receive twice-daily doses of either 2.5 mg or 5 mg of rivaroxaban or placebo, for the majority of patients (93%) on top of DAPT with aspirin and a thienopyridine. The two doses of rivaroxaban significantly reduced the primary efficacy endpoint, a composite of MI, death from cardiovascular causes or stroke, with the 2.5 mg B.I.D. dose also showing a survival benefit. Nevertheless, both doses of rivaroxaban increased the rates of major bleeding and intracranial hemorrhage. Even the lower dose of rivaroxaban (2.5 mg B.I.D.) tested in ATLAS-2 was still associated with a three-fold increase in major bleeding and a doubling of the rate of intracranial hemorrhage. However, there was no significant increase in fatal bleeding, and the lower dose of rivaroxaban reduced overall and cardiovascular mortality, thereby resulting in survival benefit. Because of these findings, rivaroxaban 2.5 mg B.I.D. is licensed for this indication in Europe. Although promising, the exact role for DOACs in ACS management seems to be complicated, due the increased bleeding risk, uncertainty about the competing antithrombotic options in post-ACS patients and uncertainty about the combination with other platelet inhibitors than clopidogrel, such as prasugrel, ticagrelor and vorapaxar[58].

Nevertheless, the findings with rivaroxaban in ATLAS-2 have opened new avenues for research, because they provide a proof of concept that anticoagulants may complement current antiplatelet drugs to reduce ischemic events in patients with atherosclerotic disease. Thus, several phase II and phase III studies are underway, studying the role of DOACs in patients with atherothrombotic disease. To overcome the increased bleeding risk of triple therapy in ATLAS-2, the GEMINI-ACS-1 trial was set up to explore the safety of a dual pathway antithrombotic therapy approach with low-dose rivaroxaban (2.5 mg B.I.D), in place of aspirin, together with a P2Y12 inhibitor (clopidogrel or ticagrelor) in 3037 post-acute ACS patients[62]. Compared to patients in the standard treatment arm with aspirin and a P2Y12 inhibitor, patients in the rivaroxaban plus P2Y12 inhibitor arm had similar rates of clinically significant bleeding. Although clearly undersized for ischemic events, the composite ischemic endpoint of cardiovascular death, myocardial infarction, stroke or stent thrombosis was similar in both groups.

Very recently, results of the COMPASS-trial (Cardiovascular OutcoMes for People using Anticoagulation StrategieS) were published[63].This study tested the hypothesis of anticoagulation as a superior strategy as compared to antiplatelet therapy alone in

secondary cardiovascular prevention[64]. Following a planned interim analysis the Data Monitoring Committee recommended to stop the trial early as the primary endpoint, a composite of cardiovascular death, stroke or MI, had reached its prespecified criteria for superiority. Among patients with stable cardiovascular disease, either CAD or PAD, those assigned to rivaroxaban 2.5 mg B.I.D. plus aspirin had a 24% risk reduction for the composite of cardiovascular death, stroke or myocardial infarction compared to those assigned to aspirin alone (HR 0.76, 95% CI 0.66-0.86). Although there was an increase in major bleeding (HR 1.70, 95% CI 1.40-2.05), there was no significant difference in intracranial or fatal bleeding between these two groups. Treatment with a combination of very-low dose rivaroxaban and aspirin provided a clear net clinical benefit, both in patients with CAD and PAD. For the latter, a group of patients with either peripheral artery disease or carotid artery disease, not only major adverse cardiovascular events (MACE) were significantly reduced, but there was also a significant reduction in major adverse limb events (MALE) and amputations for patients treated with the combination of rivaroxaban and aspirin[65]. As described above, no other pharmaceutical therapy in PAD patients had shown such a clear reduction in both cardiovascular and limb events thus far (Table 2.1). The relative risk reduction with combination therapy compared to aspirin alone was demonstrated in all patient groups, including patients with carotid artery disease, PAD or CAD[65,66].

Pleiotropic effects of anticoagulants

What is at the basis of this beneficial effect of adding low-dose rivaroxaban to aspirin? It is likely that the benefit of factor Xa and thrombin inhibition in this setting reflects not only attenuation of coagulation, but also inhibition of thrombin-mediated platelet activation, and suppression of several processes leading to atherogenesis, vascular inflammation and plaque instability. Thus, DOACs might offer 'vascular protection' that goes beyond thrombosis prevention. The term vascular protection refers to the spectrum of effects that are potentially mitigated by inhibiting the sequelae of inflammation, cell proliferation, tissue remodeling, destabilization of atherosclerotic plaques, ultimately leading to plaque rupture and atherothrombosis. The observation in COMPASS that the benefit of the combination was evident right from the start might reflect atherosclerotic plaque stabilization. However, the curves continue to divert throughout the whole study, suggesting that not only existing atherosclerotic lesions are stabilized, but also pro-inflammatory activities in the development of atherosclerosis are inhibited. Such additional effects of DOACs have been strongly suggested by several preclinical models. In animal studies, dabigatran reduced the size of atherosclerotic lesions and enhanced plaque stability[67,68].

Table 2.1 **Schematic presentation of different therapeutic strategies in patients with stable coronary artery disease (CAD) (unless indicated as "post-ACS") and stable peripheral artery disease (PAD), with their outcomes on major adverse cardiovascular and limb events (MACE, MALE) and bleeding.**

	Stable coronary artery disease (CAD); outcome		Stable peripheral artery disease (PAD); outcome				Bleeding complications in PAD and CAD patients	
Therapeuric strategy	**MACE**	**Ref.**	**MACE**	**Ref.**	**MALE**	**Ref.**	**Bleeding**	**Ref.**
Aspirin	↓	[96]	↓/–	[96,97]	↓	[98]	↑	[96–98]
Monotherapy compared to aspirin								
Clopidogrel	↓	CAPRIE [99]	↓	CAPRIE[99]	n/a	n/a	↑	CAPRIE[99]
Ticagrelor	n/a –	n/a	↓[b]	EUCLID[45]	–[b]	EUCLID[45]	↑	EUCLID[45]
Rivaroxaban 5 mg B.I.D.		COMPASS[63,66]	–	COMPASS[63,65]	↓	COMPASS[63,65]	↑↑	COMPASS[63,65,66]
Combination therapy compared to aspirin								
Aspirin +clopidogrel	↓	CHARISMA[100]	–	CHARISMA[46]	–	CHARISMA[46]	↑↑	CHARISMA[46,100]
Aspirin +ticagrelor	↓↓	PEGASUS- TIMI 54[40]	↓↓[a]	PEGASUS- TIMI 54[47]	↓↓[a]	PEGASUS-TIMI 54[47]	↑↑	PEGASUS-TIMI 54[40,47]
Aspirin/DAPT + vorapaxar	↓↓↓	TRA2°P-TIMI 50[49]	–	TRA2°P-TIMI 50[49,50]	↓↓	TRA2°P-TIMI 50[49,50]	↑↑↑	TRA2°P-TIMI 50[49,50]
Aspirin + VKA	↓↓↓	WARIS, ASPECT[52,53]	–	WAVE[59]	↓	WAVE[59]	↑↑↑	WARIS, ASPECT[52,53] WAVE[59]
DAPT + rivaroxaban 2.5 mg B.I.D. *(post-ACS)*	↓↓↓↓	ATLAS ACS2-TIMI 51[61]	n/a	n/a	n/a	n/a	↑↑↑	ATLAS ACS 2-TIMI 51[61]
Aspirin + rivaroxaban 2.5 mg B.I.D.	↓↓↓↓	COMPASS[63,66]	↓↓↓↓	COMPASS[63,65]	↓↓↓↓	COMPASS[63,65]	↑↑	COMPASS[63,65,66]

Study population, control groups, and definition of primary efficacy and safety outcomes are highly variable between different studies. This table is based on the authors' interpretation of clinical trials and meta-analyses, and is clearly not based on head-to-head comparisons of the different therapeutic strategies. (↓) to (↓↓↓↓) indicates modest to strong decrease in MACE/MALE. (↑) to (↑↑↑↑) indicates modest to strong increase in bleeding complications. (–) indicates no beneficial effect compared to aspirin monotherapy. (↓/–) indicates contradictory results. [a]In patients with concomitant PAD and CAD. [b]No direct comparison with aspirin monotherapy, but compared to clopidogrel monotherapy. ACS, acute coronary syndrome; B.I.D., bis in die, twice a day; DAPT, dual antiplatelet therapy; MACE, major adverse cardiovascular events; MALE, major adverse limb events; n/a., not available and/or not applicable; ref., reference; VKA, vitamin K antagonist.

Comparable animal studies with direct Xa inhibitors showed similar beneficial effects on atherosclerosis[69,70]. These beneficial effects of DOACs on atherosclerosis and atherothrombosis are thought to be due to inhibition of thrombin's PAR-mediated signaling[24,27]. These pleiotropic effects have also been suggested in other models, such as in myocardial ischemia-reperfusion models, where rivaroxaban improved cardiac function through the reduction of inflammation in the left ventricle of mice[71]. Factor Xa is thought to elicit a pro-inflammatory response by directly activating PAR-2 receptors, whereas inhibition of factor Xa, and thereby attenuating thrombo-inflammation, might be part of the beneficial effect of rivaroxaban in this setting. Whether such pleiotropic effects would also appear in the human vasculature is not yet elucidated. However, the marked reduction in major adverse limb events and amputations in the PAD subset from the COMPASS trial may hint towards an effect on atherosclerosis rather than "simply" on thrombosis[65]. However, this hypothesis of vascular protection does not explain why it was just the combination arm in COMPASS that was superior to aspirin, and the rivaroxaban only arm was not. Two possible explanations come to mind when trying to explain these findings. COMPASS-PAD showed a significant reduction in MALE in the rivaroxaban monotherapy arm compared to the aspirin monotherapy arm, however, for MACE only a non-significant trend (HR 0.86, 95%CI 0.69-1.08) was reported, which might have been a result of premature termination of the COMPASS trial. The second explanation may be potential synergism of the combination of two drugs that act on different pathways; anti-thrombotic (aspirin), anticoagulant (rivaroxaban) and anti-inflammatory (both). Future studies should further explore the opportunities and challenges of combined anticoagulant and antiplatelet agents in prevention and treatment of patients with atherosclerotic disease. These studies should focus on the identification of highest risk patients, for example with polyvascular bed involvement, who are likely to benefit the most of optimization of antithrombotic strategies.

Precautions

Anticoagulation, and thus inhibition of coagulation proteases, not only attenuates fibrin formation, but may also influence other biological and pathophysiologic processes. For example, besides impairing the function of several coagulation factors, the VKAs inhibit vascular vitamin K-dependent proteins, such as Matrix Gla Protein and osteocalcin, resulting in calcification of arteries[72]. However, the clinical importance of VKA-induced vascular calcifications remains unclear, because the evident beneficial effect, due to the high efficacy of VKA in stroke prevention, may outweigh the potential harmful effects

of VKA-associated vascular calcification. Another point of concern, is that pathology studies have suggested that the use of VKAs is an independent risk factor for plaque instability in patients with coronary artery disease[73] and stroke[74] due to intraplaque hemorrhage. Whether DOACs have the same negative effect on plaque stability is currently unknown. Conflicting results have been published on the risk of acute myocardial infarction (MI) with the direct thrombin inhibitor dabigatran. While some meta-analyses of randomized trials[75,76] and a recently published large retrospective cohort study[77] concluded that the use of dabigatran was associated with an increased risk of MI, a post-hoc analysis of revised data from the RELY trial[78] and other meta-analyses did not confirm this finding[79]. The potential underlying mechanisms of the suggested increase in MI associated with dabigatran is not clear. Some speculated that the combination of TF- and contact-activation-generated thrombin at the site of coronary plaque rupture might overwhelm the local concentrations of dabigatran[80]. Ex vivo, plasma samples from warfarin-administered patients generated lower peak thrombin levels than those from dabigatran-administered patients, and the authors speculate that the reduced ability of dabigatran to counter the high concentrations of thrombin that are generated by TF after a ruptured atherosclerotic plaque might explain the difference in MI in patients on dabigatran compared to warfarin[81].

Future perspectives

The results of the COMPASS trial might change the field of antithrombotic treatment in patients with atherosclerotic disease. Future studies will have to focus on further implementation and challenges of combined anticoagulant and antiplatelet agents, for example a head-to-head comparison between the addition of a second antiplatelet drug versus a very low dose of a factor-Xa inhibitor to aspirin. Moreover, these future studies should focus on the identification of patient groups that are likely to benefit the most of combining anticoagulant and antiplatelet treatment.

Besides, the search for potential new anticoagulants will continue. The contribution of the proteins of the contact system (factors VIII, IX, XI, XII, prekallikrein and high-molecular-weight kininogen) to the process of atherothrombosis have recently gained more attention[82]. Not only fibrin formation and the strength of the fibrin clot is stimulated by several steps of the contact system, it also attenuates the fibrinolytic pathway[83]. Currently, specific inhibitors against factors XIIa and XIa are being studied as potential therapeutic targets for prevention of thrombosis[23,84,85]. Factor XII deficient humans have a normal hemostatic capacity, while patients lacking factor XI only have a

mild trauma-induced bleeding disorder. Contrary to their role in normal hemostasis, animal models revealed a more important role of FXIIa-driven coagulation in arterial thrombosis[86], and furthermore, factor XIIa contributes to inflammation through the activation of the inflammatory bradykinin-producing kallikrein-kinin system[22,23]. Besides attenuation of coagulation, factor XI deprivation has also been shown to slow down atherogenesis in apoE/factor XI double knockout mice[21]. Thus, pharmacological inhibition of factors XI(a) and XII(a) interferes with both thrombosis and inflammation, and targeting the FXIIa-driven contact system may eventually be a safe therapeutic strategy, potentially with additional beneficial anti-inflammatory and anti-atherogenic effects.

Although the link between inflammation and atherosclerosis is known for several decades[2,3], only very recently clinical data demonstrating a direct benefit of targeting inflammation in patients with atherosclerotic disease have become available. Results of the CANTOS trial showed that in patients with prior myocardial infarction with elevated biomarkers of inflammation (hsCRP), anti-inflammatory therapy with canacinumab (an IL-1β blocker) reduced the incidence of recurrent cardiovascular events[87]. However, due to safety concerns (more fatal infections) and costs, it is currently unclear whether canakinumab or other anti-inflammatory therapies will ultimately be used in high-risk patients with atherosclerotic disease.

ISTH Berlin report

Lind and colleagues report that incident VTE was associated with progression of existing carotid atherosclerotic lesions, but not with new plaques in an analysis from the Tromsø study[88]. Importantly, this effect was not mediated by hs-CRP, suggesting that it is probably the hypercoagulability associated with VTE that drives atherosclerosis, which could also explain the increased risk of arterial thrombotic events following incident VTE[89].

Novel preclinical data were reported by Owens and colleagues showing that PAR-2 is a relevant mediator of proatherogenic effects; PAR-2 deficient mice had smaller aortic plaques, possibly due to combined effects on cholesterol efflux from macrophages and attenuation of VSMC activity[90]. While coagulation proteases like factors VIIa and Xa may be important ligands in PAR-2 mediated effects, in the context of atherosclerosis this link needs further exploration. Finally, van Gorp et al investigated warfarin as compared to dabigatran in effects on atherogenesis, showing that only dabigatran

attenuated atherogenesis[91]. Posthuma and colleagues showed that rivaroxaban not only inhibited atherogenesis but even diminished existing lesions, the mechanisms of which need further study[92].

Data on hemostasis biomarkers of cardiovascular disease were reported by Komarov et al, showing that addition of D-dimer to well-known scoring systems in patients after elective PCI could improve the diagnostic capacity to predict MACE in these patients[93]. Soluble thrombomodulin (sTM) was studied as a biomarker of endothelial injury in CAD patients by Komanasin and colleagues, reporting that raised plasma sTM was associated with increasing coronary artery stenosis[94]. Finally, Eggebrecht and colleagues studied the effects of DOACs compared to VKAs on several biomarkers of cardiovascular structure and function in individuals with documented cardiac dysfunction. Although this analysis demonstrated differential relations between these biomarkers in individuals with DOACs versus VKA's, fibrinogen, the only hemostasis biomarker included, did not appear to be different between treatment groups[95].

Conclusion

Contrary to what was previously thought, the coagulation system plays an important role in the development of thrombotic complications in patients with established atherosclerotic disease. Coagulation activation and generation of thrombin not only promotes fibrin production and platelet activation, but seems to be involved in atherogenesis, mainly through PAR-activation. This has important implications for treatment of patients with atherosclerotic disease, like CAD and PAD, who have high residual risk of atherothrombotic events, despite classical management with antiplatelet therapy. The results of the COMPASS trial provide clinical evidence of the concept that combined antiplatelet and anticoagulant treatment provides net benefit in secondary prevention of cardiovascular complications. Future studies will have to explore further opportunities of attenuating thrombo-inflammation and optimizing vascular protection in atherosclerotic disease.

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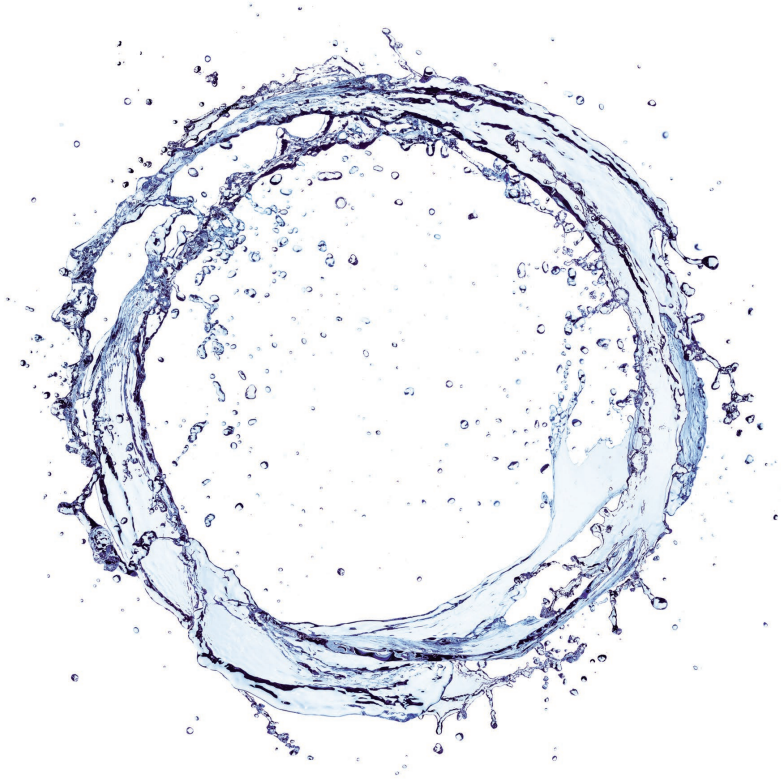

PART I

HIGH RISK PATIENTS WITH CORONARY ARTERY DISEASE

Chapter 3

Antithrombotic therapy in high-risk patients after percutaneous coronary intervention; study design, cohort profile and incidence of adverse events

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Abstract

Background

Patients with multiple clinical risk factors are a complex group in whom both bleeding and recurrent ischemic events often occur during treatment with dual/triple antithrombotic therapy after percutaneous coronary intervention. Decisions on optimal antithrombotic treatment in these patients are challenging and not supported by clear guideline recommendations. A prospective observational cohort study was set up to evaluate patient-related factors, platelet reactivity, genetics, and a broad spectrum of biomarkers in predicting adverse events in these high-risk patients. Aim of the current paper is to present the study design, with a detailed description of the cohort as a whole, and evaluation of bleeding and ischemic outcomes during follow-up, thereby facilitating future research questions focusing on specific data provided by the cohort.

Methods

We included patients with >3 predefined risk factors who were treated with dual/triple antithrombotic therapy following PCI. We performed a wide range of haemostatic tests, and collected all ischemic and bleeding events during 6-12 months follow-up.

Results

We included 524 high-risk patients who underwent PCI within the previous 1-2 months. All patients used a P2Y12-inhibitor (clopidogrel n=388, prasugrel n=61, ticagrelor n=75) in combination with aspirin (n=397) and/or anticoagulants (n=160). Bleeding events were reported by 254 patients (48.5%), necessitating intervention or hospital admission in 92 patients (17.5%). Major adverse cardiovascular events (myocardial infarction, stroke, death) occurred in 69 patients (13.2%).

Conclusion

The high risk for both bleeding and ischemic events in this cohort of patients with multiple clinical risk factors illustrates the challenges that the cardiologist faces to make a balanced decision on the optimal treatment strategy. This cohort will serve to answer several future research questions about the optimal management of these patients on dual/triple antithrombotic therapy, and the possible value of a wide range of laboratory tests to guide these decisions.

Introduction

Percutaneous coronary intervention (PCI) is the treatment of choice in most patients with acute coronary syndrome (ACS) and frequently performed in patients with chronic coronary artery syndrome[1]. As results with PCI have improved due to better stents and antithrombotic treatment, increasingly complex patient populations are treated. International guidelines recommend a period of 6-12 months of dual antiplatelet therapy (DAPT) after PCI, sometimes in combination with oral anticoagulation if other comorbidities (e.g., atrial fibrillation) demand to do so[1,2]. Thus, cardiologists are more and more challenged in treating complex, high risk-patients with dual or triple antithrombotic therapy. With the introduction of the more potent P2Y12 inhibitors prasugrel and ticagrelor next to clopidogrel[3,4], and the widespread availability of direct oral anticoagulants (DOAC) next to vitamin K antagonists (VKA), physicians are enabled to select different and individualized treatment regimens. Although for most patients evidence on optimal treatment exists, the "high-risk" patients with multiple clinical risk factors (in whom both bleeding complications and recurrent ischemic events occur more often), remain a challenging group. However, these patients are frequently excluded from or underrepresented in the large clinical trials, and although several bleeding risk scores have been developed, these scores have not been specifically validated in high-risk subjects[5].

This cohort study was designed to provide evidence on predictors, safety and outcome in a relevant subgroup of high-risk patients, and is part of an ongoing clinical care pathway. Patients are managed based on current international guidelines during the 6-12 month period of combined antithrombotic treatment following PCI (either with ACS indication or elective procedure). The clinical care pathway involves the assessment of the risk balance between thrombosis and bleeding prevention by identification and, if possible, removing such risk enhancing factors. In this study, we aim to evaluate patient-related factors, on-treatment platelet reactivity, biomarkers and bleeding questionnaires in predicting adverse events in high-risk patients. Future goals are to optimize the therapeutic windows of platelet functions tests (PFTs) for this specific group and to validate and/or develop risk estimation tools for prediction of bleeding complications in a population with multiple clinical risk factors.

The aim of the current cohort profile paper is to present a detailed description of the cohort as a whole, with evaluation of bleeding and ischemic outcomes during follow-up, thereby facilitating future research questions focusing on specific data provided by the cohort.

Methods

This prospective observational cohort study is conducted at the Thrombosis Expertise Centre in the Maastricht University Medical Centre (MUMC+) in the Netherlands. The medical ethical committee of the MUMC+ approved this study as an evaluation of patient care analysis (NL38767.068.11, METC number 11-2-096), and all patients provided written informed consent.

Study population

Patients treated with PCI or coronary thrombolysis between May 2014 and May 2019 were screened for the presence of 3 or more predefined risk factors (Table 3.1) by one dedicated interventional cardiologist. These patients, all being treated with either DAPT or a combination of antiplatelet therapy with oral anticoagulants, were referred to a specialized outpatient clinic within the Thrombosis Expertise Centre for assessment of their bleeding risks and ischemic risks. After informed consent was obtained, data on patient history, medication and comorbidities were collected, and blood was drawn for extensive haemostatic and genetic testing. Treatment decisions and following medication switches were not part of the study, and initiated on the treating physician's discretion, although all this information on medication switches was collected in the dataset.

Clinical care pathway

The clinical care pathway is illustrated in Figure 3.1. At the first visit (1-2 months after PCI) information on medical history, medication and compliance was collected. A thorough history on both previous and current minor and major bleedings was taken, using the International Society on Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT)[6]. During all three study visits, bleeding events were recorded using the definition of the Bleeding Academic Research Consortium (BARC), which contains unified and validated bleeding criteria[7,8]. Finally, blood was drawn for extensive testing, including PFTs as described below. At the second visit 6 months post-PCI, we collected information on ischemic and bleeding events, checked the medication, compliance and side effects. Standard laboratory evaluation during this second visit was performed in the first 200 included patients, and in further patients additional testing was only performed if indicated by clinical clues. If the P2Y12 inhibitor was prescribed for more than 6 months, information on bleeding and ischemic events was collected during an additional telephone call at 12 months. Thus, depending on duration of combination therapy, the total follow-up time was 6 to 12 months.

Table 3.1 Inclusion and exclusion criteria.

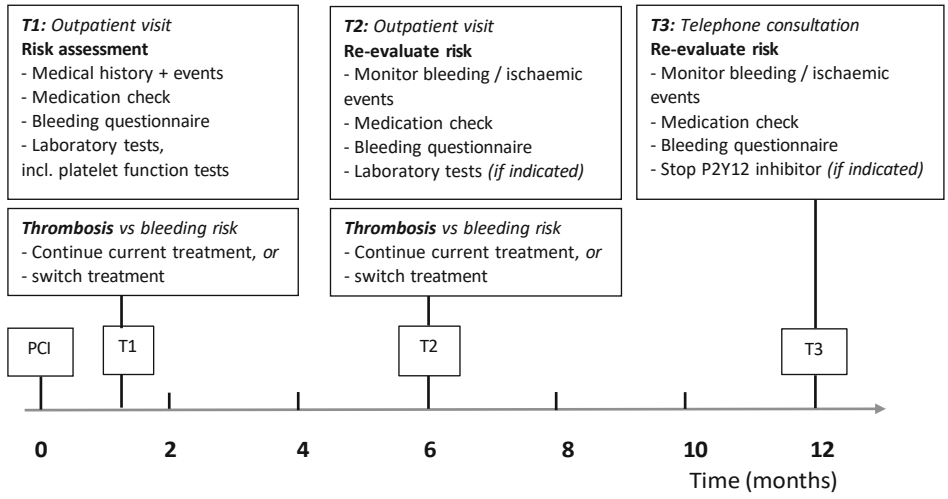
Inclusion criteria	Definition
PCI 30-90 days before study inclusion	Elective or emergency procedure
Dual / triple antithrombotic therapy	Including a P2Y12 inhibitor
Classified as 'vulnerable' by ≥3 predefined risk factors:	Age ≥75 years Female gender Renal dysfunction (MDRD-eGFR ≤60 ml/min) Body weight ≤60 kg Hypertension (previously diagnosed, or on medication) Diabetes mellitus Anaemia (Hb <8.2 mmol/l for men, <7.3 mmol/l for women) Previous stroke Previous major bleeding Liver dysfunction (known hepatitis or transplant) History of gastric/duodenal ulcers Daily use of NSAIDs or SSRIs Triple antithrombotic therapy (DAPT + oral anticoagulants) Previous in-stent thrombosis or high risk coronary stent (≥3 lesions treated, total stent length >60 mm, last remaining vessel, or left main coronary artery stenting)
Exclusion criteria	**Definition**
Known platelet function disorders	Previously diagnosed platelet function disorders
Recent coronary intervention	PCI or CABG ≤7 days
Recent new ischemic event	ACS or stroke ≤7 days
Signs of active infection	Fever, antibiotic treatment or hospital admission during laboratory assessment of platelet function
Medication non-compliance	Confirmed non-compliance in antithrombotic medication by patient interview or pharmacy dispensing

Abbreviations: PCI, percutaneous coronary intervention; MDRD-eGFR, Modification of Diet in Renal Disease – estimated glomerular filtration rate; Hb, Haemoglobin; NSAIDs, non-steroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors; DAPT, dual antiplatelet therapy; CABG, coronary artery bypass graft; ACS, acute coronary syndrome.

Laboratory evaluation

Information on blood collection and detailed description of all performed laboratory tests is described in the Supplemental data. In short, laboratory evaluation consisted of total blood count, renal function, routine haemostatic parameters, rotational thromboelastometry and thrombin generation assays, and DOAC levels if applicable. On-treatment platelet reactivity was measured using three different platelet function tests with multiple agonists; VerifyNow, Multiple Electrode Impedance Aggregometry by Multiplate and Light Transmission Aggregometry (LTA). Finally, samples were stored to measure coagulation factors, markers of fibrinolysis, and to perform additional genetic testing (e.g., CYP2C19 polymorphisms).

Figure 3.1 Timeline of the clinical care pathway.



Endpoints

The primary endpoint was defined as any bleeding (≥BARC type 1) according to the Bleeding Academic Research Consortium criteria[7,8]. The primary ischemic endpoint was defined as a composite of myocardial infarction[9], ischemic stroke (including transient ischemic attack), and all-cause death. Other ischemic endpoints include coronary revascularization, peripheral artery disease revascularization and venous thromboembolism.

Statistical analysis

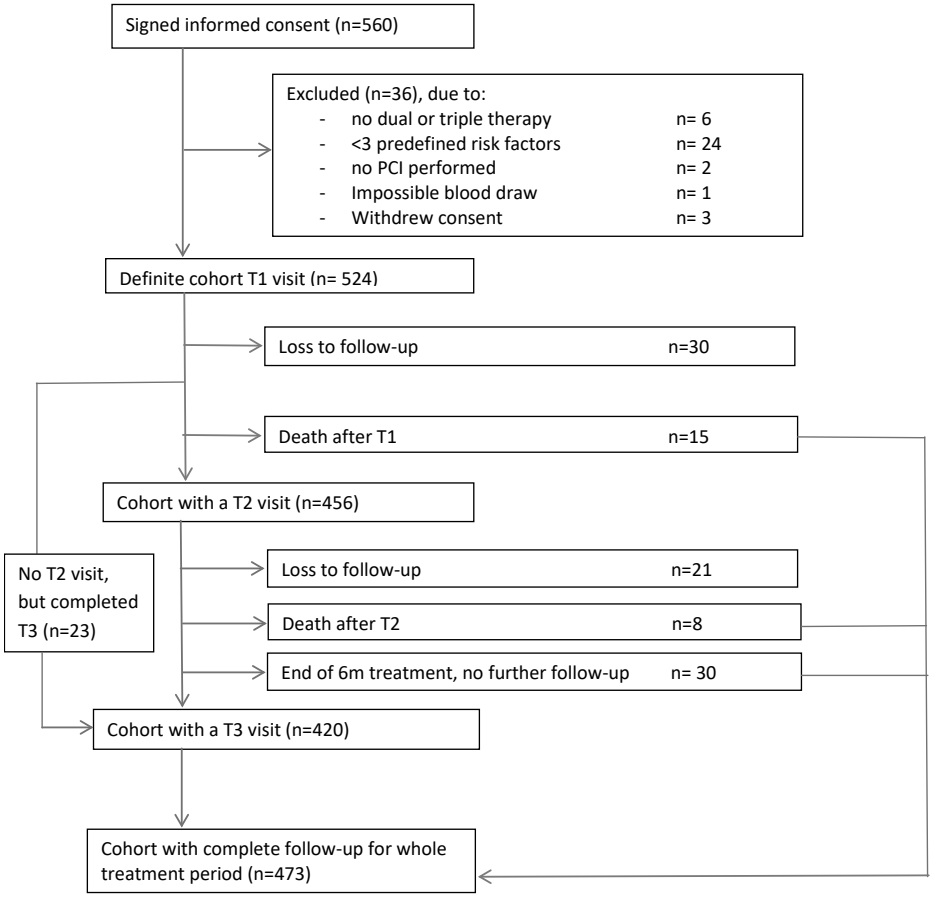
Continuous variables are expressed as either mean ± standard deviation for normally distributed traits or median with interquartile range [IQR] otherwise. Categorical variables are expressed as counts and percentages. Statistical analyses were performed with IBM SPSS statistics version 25.0.

Results

Initially 560 patients were included in the study and informed consent was obtained. However, subsequently 36 patients had to be excluded for various reasons, and therefore, the final study population consisted of 524 high-risk patients (Figure 3.2). Baseline characteristics of the study population are shown in Table 3.2. Mean age is 74.7 ± 8.7 years and patients have a median number of 4 [IQR 3-5] predefined clinical

risk factors. At the first study visit (T1), 46 [37-59] days post-PCI, all patients used a P2Y12 inhibitor (clopidogrel n=388, prasugrel n=61, ticagrelor n=75) according to the inclusion criteria, in combination with aspirin (n=392) and/or anticoagulants (n=160). In most patients (n=364, 69.4%) the antithrombotic strategy consisted of dual antiplatelet therapy, whereas 17.0% (n=89) used a P2Y12 inhibitor in combination with anticoagulants, and 13.5% (n=71) had a strategy with triple therapy for at least one month.

Figure 3.2 Flowchart of study inclusion and follow-up.



Follow-up

The second and third study visit took place after a median of 201 days [187-217] and 369 [358-381] days post-PCI, respectively. As shown in the flow diagram of study

inclusion and follow-up (Figure 3.2), the cohort of patients with total follow-up for the entire treatment period, or until death as endpoint, consisted of 473 patients (90.3% of the initial cohort).

Bleeding events

Approximately 1.5 month after PCI (T1), 147 patients (28.1%) reported a total number of 188 bleeding events, 26% of which were BARC type 2 or 3 bleedings (Table 3.3). Although the prevalence of bleeding symptoms had decreased to 19.5% in the period between T2 and T3 (compared to 28.1% and 29.6% between PCI and T1, and T1 and T2 respectively), the percentage of BARC type 2 or 3 amongst these bleedings remained stable (29.5% out of 95 bleeding events) as compared to T1.

After 12 months, 254 patients (48.5%) had reported one or more BARC type 1-3 bleedings. Most patients (30.9%) had only reported mild bleeding (BARC type 1), for which no consultation or interventions were necessary. However, still 92 patients (17.5%) had experienced a BARC type 2 or 3 bleeding at any time point, necessitating consultation, diagnostic tests, interventions, blood transfusions and/or hospital admittance.

Ischemic events

During one year follow-up, 69 patients (13.2%) had a major adverse cardiovascular event (Table 3.3, Figure 3.3); 36 patients with myocardial infarction, 8 patients with confirmed stent thrombosis, 13 patients with stroke and 23 of them had died during follow-up, of whom 6 patients with confirmed cardiovascular death.

Medication switch

The type, dosage or duration of P2Y12 inhibitor had to be adjusted in 78 patients (14.9%) during 1-year follow-up due to (a combination of) bleeding episodes, recurrent ischemic events, risk assessment, PFT results or side effects. In another 33 patients (6.3%) an unplanned change in anticoagulants and/or aspirin was necessary during follow-up (Supplementary Table S3.1).

Table 3.2 Baseline characteristics of the full cohort (n=524).

Variable	*N* (%), *or* mean ± SD
Age, years	74.7 ± 8.7
Male	302 (57.6)
Body mass index, kg/m^2 ^	27.4 ± 4.6
Current smoking#	72 (13.7)
Alcohol consumption ≥ 7 drinks/week*	99 (18.9)
PPI use at inclusion	441 (84.2)
Predefined risk factors	
Number of predefined risk factors, *median [min-max]*	4 [3-9]
Age ≥ 75 years	318 (60.7)
Women	222 (42.4)
Weight ≤ 60 kg	60 (11.5)
Diabetes mellitus	186 (35.5)
Hypertension	448 (85.5)
Anaemia	204 (38.9)
Renal dysfunction (MDRD-eGFR <60)	313 (59.7)
Liver failure	2 (0.4)
History of gastric/duodenal ulcers	61 (11.6)
Previous major bleeding	65 (12.4)
Previous stroke	138 (26.3)
Use of NSAIDs	21 (4.0)
Use of SSRIs	31 (5.9)
Triple antithrombotic therapy	71 (13.5)
High-risk PCI	47 (9.0)
Index PCI	
Acute coronary syndrome	333 (63.5)
Elective procedure	191 (36.5)
Radial access	232 (44.3)
Number of stents	
0 (DEB, POBA, thrombolysis)	29 (5.5)
1	352 (67.2)
2	98 (18.7)
3	45 (8.6)
Type of stent/procedure	
DES	490 (93.5)
BMS	4 (0.8)
Absorb	1 (0.2)
Drug eluting balloon	12 (2.3)
POBA +/- thrombus aspiration	14 (2.7)
Thrombolysis	3 (0.6)
Cardiovascular history	
Prior PCI	197 (37.6)
Prior CABG	106 (20.2)
Prior Stroke	138 (26.3)
Atrial fibrillation	138 (26.3)
Peripheral artery disease	76 (14.5)
Prior venous thromboembolism	39 (7.4)
Previous history	
Active malignancy	24 (4.6)
Peptic ulcer disease	61 (11.6)

Table 3.2 (continued)

Variable	(%),*N* (%), *or* mean ± SD
Treatment at first study visit	
P2Y12 inhibitor	524 (100.0)
Clopidogrel	388 (74.0)
Prasugrel	61 (11.6)
Ticagrelor	75 (14.3)
Aspirin	392 (74.8)
Vitamin K antagonist	91 (17.3)
DOAC	68 (13.0)
Apixaban	20 (3.8)
Rivaroxaban	34 (6.5)
Edoxaban	4 (0.8)
Dabigatran	10 (1.9)
LMWH	1 (0.2)
Dipyridamol	2 (0.4)
Combination strategies	
Dual antiplatelet treatment (DAPT)	364 (69.4)
For 6 months	62 (11.8)
For 12 months	302 (57.6)
P2Y12 inhibitor with VKA/DOAC/LMWH	89 (17.0)
Initial triple therapy$^{\$}$	71 (13.5)
For 1 month	64 (12.2)
For 3-6 months	7 (1.4)
Laboratory test (reference range)	
Haemoglobin	
Male (8.2-11.0 mmol/l)	8.4 ± 1.1
Female (7.3-9.7 mmol/l)	8.0 ± 0.9
Haematocrit	
Male (0.42-0.52 L/l)	0.41 ± 0.05
Female (0.36-0.48 L/l)	0.39 ± 0.04
MCV (80-100 fL)	91.7 ± 5.8
Platelet count, (150-350 10^9/L)	261 ± 78
MPV (80-100 fL)	10.3 ± 0.9
PT (9.9-11.5 sec) §	10.7 ± 0.5
APTT (23-32 sec) §	26.2 ± 2.1
Fibrinogen (1.7-4.0 g/l)	3.7 ± 0.9
Creatinine (50-100 µmol/L)	116.6 ± 74.9
MDRD-eGFR (ml/min/1.73m^2)	57.1 ± 21.0
Platelet function test (cutoff values for LPR and HPR »)	
Multiplate ADP (19-46 AU)~	47.7 ± 23.2
LTA ADP (20-59 % max aggr) $^{\Delta}$	41.4 ± 16.5
VerifyNow P2Y12 (85-208 PRU)^	136.9 ± 84.7

*^ missing in 6 patients, # missing in 3 patients, * missing in 11 patients, ~ missing in 8 patients, $^{\Delta}$missing in 9 patients; $^{\$}$ Triple therapy consists of a P2Y12 inhibitor plus aspirin plus anticoagulants (VKA, DOAC, LWMH); § in 364 patients not on anticoagulants (VKA, DOAC, LMWH) » cutoff values according to consensus documents Abbreviations: PPI, proton pump inhibitor; MDRD-eGFR, Modification of Diet in Renal Disease – estimated Glomerular Filtration Rate; NSAIDs, non-steroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors; PCI, percutaneous coronary intervention; DEB, drug eluting balloon; POBA, plain old balloon angioplasty; DES, drug eluting stent; BMS, bare metal stent; CABG, coronary artery bypass graft; DOAC, direct oral anticoagulants; VKA, vitamin K antagonist; LMWH, low molecular weight heparin;*

Table 3.3 Bleeding events and ischemic events during follow-up.

Bleeding endpoint	Cumulative (n=524)	T1 visit (n=524)	T2 visit* (n=456)	T3 visit* (n=420)
Any bleeding	254 (48.5)	147 (28.1)	135 (29.6)	82 (19.5)
Most severe bleeding				
BARC type 1	162 (30.9)	102 (19.5)	105 (23.0)	54 (12.9)
BARC type 2	63 (12.0)	34 (6.5)	19 (4.2)	19 (4.5)
BARC type 3	29 (5.5)	11 (2.1)	11 (2.4)	9 (2.1)
Total number of bleeding events^	442	188	159	95
BARC type 1	332 (75.1)	139 (73.9)	126 (79.2)	67 (70.5)
BARC type 2	75 (16.9)	36 (19.1)	20 (12.6)	19 (20.0)
BARC type 3	35 (7.9)	13 (6.9)	13 (8.2)	9 (9.5)

Ischemic event	Cohort (n=524)
No ischemic events	416 (79.2)
Major adverse cardiovascular event (myocardial infarction, stroke or all cause death)	69 (13.2)
Myocardial infarction	36 (6.9)
Stent thrombosis	8 (1.5)
Stroke	13 (2.5)
Death, all cause	23 (4.4)
Confirmed cardiovascular death#	6 (1.1)
Death (non-cardiovascular, unknown)	17 (3.2)
Coronary revascularisation	37 (7.1)
PAD with revascularisation	17 (3.2)
Venous thromboembolism	3 (0.6)

*^ in patients reporting any bleeding symptoms (one patient can report more than one bleeding event at the same visit); * Bleeding events since last study visit; # Confirmed cardiovascular death is defined as death due to acute myocardial infarction, death due to stroke, or in-hospital cardiac arrest. Abbreviations: BARC, Bleeding Academic Research Consortium; PAD, peripheral artery disease*

Publications about the cohort to date

In a first publication, the agreement between different platelet function tests, as well as the factors influencing this agreement in vulnerable patients were assessed[10]. Results suggest that the agreement is only slight to moderate, and that PFTs are not interchangeable when determining the response to antiplatelet therapy. More recently, a small study was done focusing on possible strategies to optimize the agreement between the Multiplate and VerifyNow assay[11]. A study on the relationship between genetics (CYP2C19 metabolism) and results of PFTs in clopidogrel-treated patients was presented at the annual meeting of the European Society of Cardiology[12] and the full manuscript is currently in preparation, as well as manuscripts on the value of thrombin generation assays[13] and rotational thromboelastometry. Finally, an interim analysis presented at the Eurothrombosis Congress of the ESC Working Group on Thrombosis showed that using the previously proposed cut-off levels[14-16], PFTs performed at

1 month after PCI were not able to accurately predict bleeding complications in our high-risk population during a 1-year follow-up period[17].

Discussion

In this paper we present our well characterized cohort of high-risk patients on dual or triple antithrombotic therapy after PCI. This cohort will serve to answer several future research questions about predictors, safety and outcome of patients with multiple clinical risk factors on dual or triple antithrombotic therapy. The high incidence of both bleeding and ischemic events, as well as the frequent need for medication adjustment during follow-up, indicates the need for strict monitoring of this patient group and illustrates challenges in optimal antithrombotic management.

In the past decade, several studies have shown that tailoring antiplatelet therapy based on PFTs does not prevent ischemic and bleeding outcomes in the general PCI population[18-20]. With the recent advances in stent technology and broader use of potent P2Y12 inhibitors, thrombotic events have dramatically decreased, and consequently, prevention of bleeding complications has become a major goal[21-23]. Thus, as was also suggested in the recent expert consensus statement on platelet function testing for guiding P2Y12 inhibitor treatment, platelet function testing may play a more important role in a bleeding reduction strategy[22]. Indeed, randomized trials incorporating PFT results to deescalate DAPT have shown promising results[24,25]. Reflecting these results, recent guidelines included a Class IIb recommendation for de-escalation of P2Y12 inhibition treatment guided by PFTs to be considered as an alternative DAPT strategy, especially for ACS patients deemed unsuitable for 12-month potent platelet inhibition[1]. Building on this, such a risk assessment strategy might be even more beneficial when results of PFTs would be combined with other variables in an algorithm[26]. This cohort can serve to optimize such risk assessment strategies.

Future directions

To further optimize the applicability of PFTs, adjustment of cut-off levels in various conditions (e.g., type of P2Y12 inhibitor, comorbidities) might be necessary, as the predictive capacity is currently limited. Our data could serve to adjust these cut-off levels for the different PFTs in specific, high-risk patient groups. Furthermore, the descriptive data, in combination with laboratory assays, genetics and bleeding questionnaires could be used for the construction of a multimarker risk prediction model. Current risk prediction models[27-30] are generally developed for the average PCI

population, whereas a risk prediction model specifically developed for a high bleeding risk population currently does not exist[5]. At a later stage, such model could be used in intervention studies stratifying therapy to high-risk patients. Collaboration with other research groups with comparable data is welcomed, and would be beneficial to further the prediction modelling plans. Besides optimization of the combination and treatment duration of antithrombotic therapy in high-risk patients, new treatment options for high-risk patient populations are on the way. These recent advances not only involve new antithrombotic strategies (e.g., dual pathway inhibition[31]), but also anti-inflammatory drugs (e.g., canakinumab[32] or colchicine[33,34]. These new therapies could be implemented and evaluated when continuing data collection on future cohorts of comparable high-risk patients in our centre.

Strengths and limitations

Strengths of this study are that it comprises a large prospective clinical cohort with detailed data and extensive laboratory testing. Particularly valuable is the comparison of three different PFTs with multiple agonists in a large cohort of high-risk patients. Another strength of our study is the detailed information on minimal bleedings (BARC type 1), which were collected during the whole follow-up, although retrospectively from PCI until the first study visit. These minimal bleedings often have impact on patients' daily life, but as most studies only collect the bleeding events retrospectively, these BARC type 1 bleedings could often not be reported. A limitation of our study is that due to rapid developments in stent technology, stronger platelet inhibition and guideline updates, the relatively long inclusion time of 5 years may have caused heterogeneity within the cohort. Moreover, due to the observational nature of the study, some patients decided to refrain from further hospital visits, chose to visit their regional cardiologist or general practitioner instead, or could not be contacted for study visits, leading to loss to follow-up in 9.7%. Another limitation might be that the identification of risk factors for selection of high-risk patients was based on literature and expert consensus when initiating the study in 2014. Only recently, a consensus document from the Academic Research Consortium for High Bleeding Risk (ARC-HBR) was published, presenting a consensus definition of patients at high bleeding risk[5]. Our risk factors show substantial overlap with this consensus definition; all minor criteria were included and out of the major criteria only recent or non-deferrable major surgery was not counted as a risk factor for inclusion in this cohort study. However, these data are retrievable when needed for analysis. Data on thrombocytopenia, active malignancy and chronic bleeding diathesis were structurally collected but not counted as a predefined risk factor in our cohort. In fact, due to concurrent research on platelet function and clotting factors, thrombocyte count <100 and known coagulation

disorders were exclusion criteria in our study. However, the most important and reliable predictor of bleeding in patients with bleeding diatheses is a personal history of bleeding, which can be assessed with a bleeding questionnaire[5,35], and this valuable information was collected in our study.

Conclusion

In this well characterized cohort of patients with multiple clinical risk factors treated with dual or triple antithrombotic therapy after PCI, we showed the high risk for both bleeding and ischemic events. This challenges the treating physician to make a balanced decision on the optimal, individualized antithrombotic treatment strategy. Future results of this cohort study will serve to further expand the knowledge on the optimal treatment of these high-risk patients, and the implementation of patient characteristics and a wide range of laboratory tests to guide treatment decisions.

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Supplemental data

Laboratory evaluation

Patients were asked to avoid fat-rich food 4 hours prior to blood withdrawal. Venous blood was collected by antecubital venepuncture on the same day as the first outpatient visit. Standard laboratory evaluation consisted of total blood count (Sysmex, XN-9000), renal function (Cobas-8000; Roche reagents), and routine haemostatic parameters (Sysmex CS-2100; Siemens reagents). In patients using direct oral anticoagulants (DOAC), the DOAC levels were measured using specific anti-Xa levels for direct factor Xa-inhibitors (Biophen, daXa), or diluted thrombin time (dTT) for patients using dabigatran (Biophen, Hemoclot®). On-treatment platelet reactivity was measured using three different platelet function tests (PFTs), as described below. Additionally, thrombin generation assays (Calibrated Automated Thrombography, CAT) and rotational thromboelastometry (Werfen, ROTEM® delta) were performed. Finally, samples were stored to measure coagulation factors, von Willebrand factor (vWF), markers of fibrinolysis, and to perform additional genetic testing (e.g., CYP2C19 polymorphisms). Platelet function measurements were started within one hour of blood withdrawal, which was done simultaneously for the following three assays.

Multiple electrode impedance Aggregometry by Multiplate

Blood was collected in Hirudin Blood Tubes (3 mL, Double Wall; Verum Diagnostica GmbH, Munich). Agonist-induced platelet aggregation was measured by the Multiplate Analyser (Dynabyte, Munich, Germany), according to manufacturer's instructions. The Multiplate analyser is a multiple electrode impedance aggregometer that measures platelet aggregation in whole blood. Agonists used were adenosine diphosphate (ADP) (6.4 µmol/L; Roche), Collagen (3.2 ug/mL), thrombin receptor-activating peptide (TRAP) (32 µmol/L; Roche) and arachidonic acid (AA) (0.5mM; Roche). Results are expressed as arbitrary aggregation units (AU). For evaluation of P2Y12 receptor blockers, the ADP-induced platelet aggregation was used. According to a previous consensus document by Tantry et. al, low on-treatment platelet reactivity is defined as AU <19 and high on-treatment platelet reactivity as AU >46[1].

VerifyNow

Blood was collected in 3.2% sodium citrate Vacuette partial-fill tubes (2 mL; Greiner Bio-One, GmbH, Kremsmuntster, Austria). VerifyNow ASPI and VerifyNow P2Y12 assay (Accumetrics Inc, San Diego, CA, USA) were performed according to manufacturer's instructions. VerifyNow is a turbidimetric-based optical detection system, in which whole blood is added to a device using fibrinogen-coated microbeads and, after

addition of an agonist, the increase in light transmittance is detected to measure platelet reactivity. Agonists used were AA (1 mmol/L; Accumetrics), or, for evaluation of P2Y12 inhibitors, a combination of ADP (20 µmol/L) and prostaglandin E1 (22nmol/L). Results are expressed in P2Y12 reaction units (PRU), with low on-treatment platelet reactivity being defined as PRU <85 and high on-treatment platelet reactivity as PRU >208[1].

Light Transmission Aggregometry (LTA)

Blood was collected in 3.2% sodium citrate Vacuette tubes (9mL; Greiner Bio-One). For preparation of platelet-rich plasma, blood was centrifuged at 170 g for 10 min at 18°C. For preparation of platelet-poor plasma citrated-blood was centrifuged at 2500 g for 5 min and then at 10000 g for 10 min at 18°C. Platelet count in platelet-rich plasma was adjusted with autologous platelet-poor plasma to 250 x 10^9 platelets/L. In vitro platelet aggregation was measured in response to AA (1 mM; Bio Date Corporation (Emergo)), ADP (20 µmol/L; Chrono-Par, CH 384), and TRAP (15 µmol/L; Boom H8105) at 37°C (Chrono-log 490-4D; Chrono-Log Corp.). For evaluation of P2Y12 inhibitors, the main result was the percentage of maximal platelet aggregation in response to ADP. Low on-treatment platelet reactivity was defined as maximal aggregation <20%[2] and high on-treatment platelet reactivity as maximal aggregation >59%[3].

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Figure S3.1 Event-free survival for major adverse cardiovascular events (a composite of myocardial infarction, ischemic stroke and all-cause death) during follow-up..

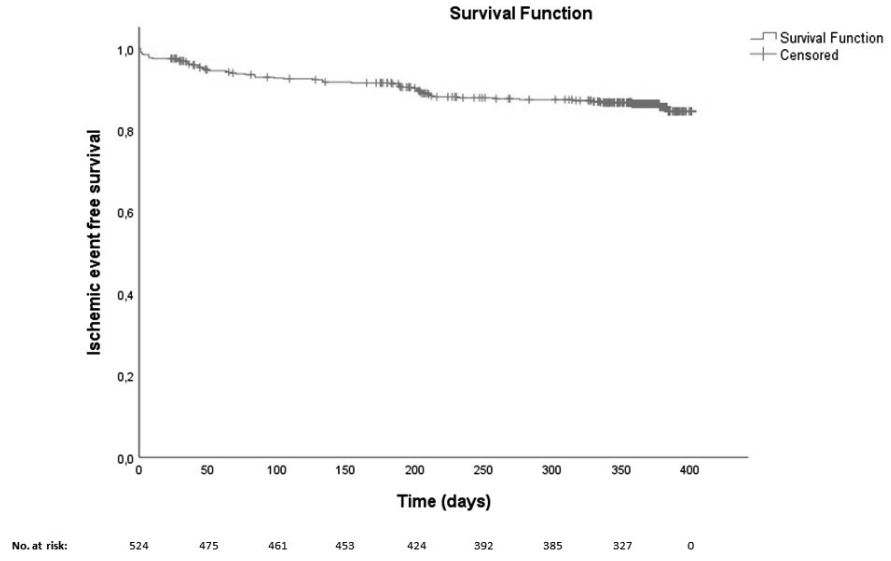

Table S3.1 Medication switches during follow-up in full cohort (n=524).

Treatment	Type of adjustment	(de-)escalation	Adjustment of therapy	N
P2Y12 inhibitor	Medication type	Escalation	Clopidogrel – Prasugrel	18
n=78	n=68	n=26	Clopidogrel – Ticagrelor	8
			Prasugrel 5 mg – 10 mg	0
		De-escalation	Ticagrelor – Clopidogrel	18
		n=37	Prasugrel – Clopidogrel	10
			Prasugrel 10 mg – 5 mg	9
		Other	Ticagrelor – Prasugrel	1
		n=5	Prasugrel – Ticagrelor	2
			Other #	2
	Duration		Shortened	9
	n=10		Prolonged	1
Aspirin	Start			3
n=10	Prematurely stopped			7
Oral anticoagulants	Type OAC		VKA - DOAC	1
n=23	n=6		DOAC - VKA	1
			DOAC - DOAC	4
	Dose adjustment		Escalation	1
	n=8		De-escalation	7
	Start			8
	Stop			1

 # Patients accidentally used ticagrelor once daily instead of twice daily. Abbreviations: OAC, oral anticoagulants; VKA, vitamin K antagonist; DOAC, direct-acting oral anticoagulants.

Chapter 4

Differential impact of cytochrome 2C19 allelic variants on three different platelet function tests in clopidogrel-treated patients

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Abstract

Aims

On-treatment platelet reactivity in clopidogrel-treated patients can be measured with several platelet function tests (PFTs). However, the agreement between different PFTs is only slight to moderate. Polymorphisms of the *CYP2C19* gene have an impact on the metabolization of clopidogrel and, thereby, have an impact on on-treatment platelet reactivity. The aim of the current study is to evaluate the differential effects of the *CYP2C19* genotype on three different PFTs.

Methods

From a prospective cohort study, we included patients treated with clopidogrel following percutaneous coronary intervention (PCI). One month after PCI, we simultaneously performed three different PFTs; light transmission aggregometry (LTA), VerifyNow P2Y12, and Multiplate. In whole EDTA blood, genotyping of the *CYP2C19* polymorphisms was performed.

Results

We included 308 patients treated with clopidogrel in combination with aspirin (69.5%) and/or anticoagulants (33.8%) and, based on *CYP2C19* genotyping, classified them as either extensive (36.4%), rapid (34.7%), intermediate (26.0%), or poor metabolizers (2.9%). On-treatment platelet reactivity as measured by LTA and VerifyNow is significantly affected by *CYP2C19* metabolizer status ($p<0.01$); as metabolizer status changes from rapid, via extensive and intermediate, to poor, the mean platelet reactivity increases accordingly ($p<0.01$). On the contrary, for Multiplate, no such ordering of metabolizer groups was found ($p=0.10$).

Conclusions

For VerifyNow and LTA, the on-treatment platelet reactivity in clopidogrel-treated patients correlates well with the underlying *CYP2C19* polymorphism. For Multiplate, no major effect of genetic background could be shown, and effects of other (patient-related) variables prevail. Thus, besides differences in test principles and the influence of patient-related factors, the disagreement between PFTs is partly explained by differential effects of the *CYP2C19* genotype.

Introduction

Dual antiplatelet therapy with aspirin and a P2Y12 receptor blocker (clopidogrel, prasugrel, or ticagrelor) is the cornerstone of antithrombotic therapy preventing recurrent cardiovascular events in patients undergoing percutaneous coronary intervention (PCI)[1]. Although guidelines favour more potent next-generation P2Y12 inhibitors, such as ticagrelor and prasugrel, in acute coronary syndrome (ACS) patients[1,2], clopidogrel remains the most widely prescribed P2Y12 inhibitor, partly due to clinical and economic factors[3]. There is substantial variability in pharmacodynamic action of clopidogrel, which translates into variation in clopidogrel effectiveness after PCI[4,5]. Besides several contributing clinical factors, this variability is partly explained by genetic polymorphisms encoding cytochrome P450 (CYP) 2C19, the hepatic enzyme involved in biotransformation of the prodrug clopidogrel to its active metabolite[6]. The hepatic conversion of clopidogrel to its active metabolite is a two-step biotransformation process. Because 85% of the prodrug clopidogrel is inactivated by esterases after intestinal absorption, only 15% is available for transformation to the active metabolite, for which the hepatic *CYP2C19* enzyme is a key determinant. The gene that codes the *CYP2C19* enzyme is highly polymorphic, and over 30 gene alleles have been identified[7]. The *CYP2C19*1* allele is the most prevalent and represents a normal activity allele. *CYP2C19*2* and *CYP2C19*3* alleles are the most frequently observed polymorphisms leading to a complete loss of enzyme activity, with reduced clopidogrel conversion. This results in higher residual platelet reactivity[5,8-10] and is associated with a higher incidence of major adverse cardiovascular and cerebrovascular events[4-6,9-11]. On the other hand, the *CYP2C19*17* allelic variant represents a gain-of-function mutation that leads to increased catalytic activity and increased production of active metabolites, which might result in more pronounced platelet inhibition, higher bleeding risk, and lower risk for ischemic events with clopidogrel[12-15]. However, other studies have reported no significant association between the **17* allele and ischemic and bleeding outcomes after accounting for the **2* allele, so its clinical relevance is still controversial[16,17]. In contrast to clopidogrel, the *CYP2C19* genotype does not impact the clinical effects of prasugrel or ticagrelor, for which clinical trials have shown the superiority over clopidogrel in reducing ischemic events, although accompanied by higher bleeding risks[18,19].

Especially in patients with multiple clinical risk factors, clinicians will have to weigh the bleeding and ischemic risks to individualize treatment decisions following PCI[20]. Platelet function tests (PFTs) could guide such decisions; however, although personalized treatment strategies based on PFTs have been evaluated, all have failed to show significant clinical benefit[21-23]. Moreover, multiple PFTs using different techniques are

available, but previous studies have shown that the agreement between different PFTs is only slight to moderate, leading to conflicting results[24-28]. More recently, studies evaluating pharmacogenomic-based tailoring of P2Y12 inhibitors seemed to be more promising[29-32].

The aim of the current study is to evaluate the differential effects of *CYP2C19* genotypes on three different platelet function tests. Understanding the relationship between PFTs and genetic polymorphisms is important in the interpretation of the disagreement between these PFTs and determination of optimal antiplatelet therapy in high-risk patients, and it could help to explain the disappointing PFT-tailoring studies in comparison to the more promising pharmacogenomic approach.

Materials and methods

This prospective cohort study is conducted at the Thrombosis Expertise Centre in the Maastricht University Medical Center (MUMC+) in the Netherlands. The study was reviewed and approved by the medical ethical committee of the MUMC+ (NL38767.068.11, METC number 11-2-096), and all patients provided written informed consent.

Study population

Patients were selected from a cohort of high-risk patients with dual or triple antithrombotic therapy after PCI. This prospective cohort study is extensively described elsewhere[33]. In brief, all patients underwent PCI (either elective or following ACS) and were classified as high-risk patients by the presence of ≥3 predefined risk factors (Table S4.1*).* Patients either had dual antithrombotic therapy (a P2Y12 inhibitor plus aspirin or anticoagulants) or triple therapy (a combination of a P2Y12 inhibitor, aspirin, and anticoagulants) in the case of concomitant atrial fibrillation. For the current research question, we only selected the patients on clopidogrel for whom *CYP2C19* analysis had been performed by June 2018. Exclusion criteria were previously diagnosed platelet function disorders, a new ischemic event or coronary revascularization procedure ≤7 days, confirmed noncompliance, or signs of active infection.

Laboratory measurements

On-treatment platelet reactivity was measured using three different PFTs. Blood for all these PFTs was drawn simultaneously 1–2 months after PCI, together with the sample needed for genotyping of the *CYP2C19* polymorphisms. Furthermore, we measured blood count and renal function.

Multiple electrode impedance aggregometry by multiplate

Blood was collected in Hirudin Blood Tubes (3 mL, Double Wall; Verum Diagnostica GmbH, Munich, Germany). Adenosine diphosphate (ADP, 6.4 uM)-induced platelet aggregation was measured by the Multiplate analyzer (Dynabyte, Munich, Germany), according to the manufacturer's instructions. The Multiplate analyzer is a multiple electrode impedance aggregometer that measures platelet aggregation in whole blood. Results are expressed as arbitrary aggregation units (AU).

VerifyNow

Blood was collected in 3.2% sodium citrate Vacuette partial-fill tubes (2 mL; Greiner Bio-One, GmbH, Kremsmuntster, Austria). VerifyNow P2Y12 assay (Accumetrics Inc, San Diego, CA, USA) was performed according to the manufacturer's instructions. This is a turbidimetric-based optical detection system, in which whole blood is added to a device using fibrinogen-coated microbeats. After addition of an agonist (20 µmol/L ADP–22 nmol/L prostaglandin E1), the increase in light transmittance is detected to measure platelet agglutination, and results are expressed in P2Y12 reaction units (PRU).

Light Transmission Aggregometry (LTA)

Blood was collected in 3.2% sodium citrate Vacuette tubes (9 mL; Greiner Bio-One). For preparation of platelet-rich plasma, blood was centrifuged at 170× *g* for 10 min at 18°C. For preparation of platelet-poor plasma, citrated blood was centrifuged at 2500× *g* for 5 min and then at 10,000× *g* for 10 min at 18°C. Platelet count in platelet-rich plasma was adjusted with autologous platelet-poor plasma to 250 × 10^9 platelets/L. ADP (20 µmol/L, Chrono-Par, CH 384) was added at 37°C. The main result was the percentage of maximal platelet aggregation.

CYP2C19 analysis

From whole EDTA blood, genomic DNA for genetic analysis was isolated with the MagNa Pure 96 DNA isolation system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Using the LightCycler® (Roche Diagnostics), genotyping of the *CYP2C19* polymorphism was performed using a FRET LightMix® assay (TIB MOLBIOL, Berlin, Germany). Genotypes were determined blinded without knowledge of platelet aggregation values. CYP variants were grouped according to the guideline of the Clinical Pharmacogenetic Implementation Consortium (CPIC)[8,34]; *CYP2C19 *1/*1* is referred to as normal or extensive metabolizer (EM), genotypes with one loss-of function allele (*CYP2C19 *1/*2, *1/*3* or **2/*17*) were assembled under the term 'intermediate metabolizer' (IM), and the genotype with two loss-of-function alleles (*CYP2C19 *2/*2*) is referred to as 'poor metabolizer' (PM). The

last group consists of the genotypes *CYP2C19 *1/*17* and **17/*17*, and is referred to as 'rapid metabolizers' (RM).

Statistical analysis

Continuous variables are expressed as either mean with standard deviation (SD) for normally distributed traits or median with interquartile range (IQR) otherwise. Categorical variables are expressed as counts and percentages. The distribution of the data was estimated using visual inspection of histograms and confirmed using the Shapiro–Wilk test. To compare categorical variables between the metabolizer groups, the Chi-square test was used, with Fisher's Exact Test when applicable. Normally distributed continuous variables were compared between metabolizer groups using ANOVA. The Jonckheere–Terpstra test for ordered alternatives was used to test for statistically significant ordinal trends between the metabolizer groups and for pairwise comparison of the different metabolizer groups compared to extensive metabolizers (reference category), with Bonferroni post-test to correct for multiple testing. Multivariable linear regression analysis was performed to adjust for other factors influencing platelet reactivity. Variables were included in the model based on existing literature or if univariate analysis indicated the variable to be associated with at least one of the platelet function tests at $p<0.20$. Presence of multicollinearity was checked using variation inflation factors. Simple correlation between the three platelet function tests was assessed with Pearson correlation coefficients (ρ). A value of $p<0.05$ was considered to be statistically significant. Statistical analyses were performed with IBM SPSS statistics version 25.0 and GraphPad Prism version 5.

Results

Baseline characteristics

From the total cohort of 524 patients[33], 308 patients met the inclusion criteria and were included in this analysis. Baseline characteristics of the study population are shown in Table 4.1. Mean age is 75.2 (8.5) years, with 41.2% being female. All patients were treated with clopidogrel (100%) in combination with aspirin (69.5%) and/or anticoagulants (33.8%).

Table 4.1 Baseline characteristics for the different metabolizer groups.

	All patients (*n*=308)	RM (*n*=107)	EM (*n*=112)	IM (*n*=80)	PM (*n*=9)	*p* ^
Age, years	75.2 (8.5)	74.9 (9.6)	74.7 (7.9)	76.5 (7.4)	74.3 (9.4)	0.478
Female	127 (41.2)	47 (43.9)	43 (38.4)	34 (42.5)	3 (33.3)	0.807
BMI, kg/m^2	27.4 (4.5)	27.8 (4.5)	27.2 (4.6)	27.3 (4.6)	25.6 (3.9)	0.477
Current smoking	41 (13.3)	12 (11.2)	18 (16.5)	11 (14.5)	0 (0.0)	0.429
Index PCI–ACS	172 (55.8)	59 55.1	60 (53.6)	47 (58.8)	6 (66.7)	0.809
Index PCI–Elective	136 (44.2)	48 (44.9)	52 (46.4)	33 (41.2)	3 (33.3)	
Medication						
Clopidogrel	308 (100.0)	107 (100.0)	112(100.0)	80(100.0)	9 (100.0)	1.000
Aspirin	214 (69.5)	71 (66.4)	77 (68.8)	57 (71.3)	9 (100.0)	0.204
VKA	72 (23.4)	28 (26.2)	27 (24.1)	17 (21.3)	0 (0.0)	0.328
DOAC	32 (10.4)	10 (9.3)	12 (10.7)	10 (12.5)	0 (0.0)	0.668
(es-)omeprazole use	28 (9.1)	9 (8.4)	13 (11.6)	5 (6.3%)	1 (11.1)	0.646
Risk factors						
Age ≥75 years	193 (62.7)	65 (60.7)	67 (59.8)	56 (70.0)	5 (55.6)	0.459
Women	127 (41.2)	47 (43.9)	43 (38.3)	34 (43.)	3 (33.3)	0.807
Weight <60 kg	28 (9.1)	11 (10.3)	10 (8.9)	7 (8.8)	0 (0.0)	0.778
Diabetes mellitus	110 (35.7)	35 (32.7)	39 (34.8)	29 (36.3)	7 (77.8)	0.060
Hypertension	258 (83.8)	94 (87.9)	92 (82.1)	66 (82.5)	6 (66.7)	0.313
Anemia	107 (34.7)	37 (34.6)	39 (34.8)	27 (33.8)	4 (44.4)	0.938
Renal dysfunction	178 (57.8)	52 (48.6)	67 (59.8)	53 (66.3)	6 (66.7)	0.088
Liver failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	-
Peptic ulcer disease	42 (13.6)	12 (11.2)	15 (13.4)	13 (16.3)	2 (22.2)	0.667
Prior major bleeding	47 (15.3)	21 (19.6)	13 (11.6)	11 (13.8)	2 (22.2)	0.360
Previous stroke	89 (28.9)	31 (29.0)	32 (28.8)	24 (30.0)	2 (22.2)	0.971
Use of NSAIDs	14 (4.5)	4 (3.7)	6 (5.4)	4 (5.0)	0 (0.0)	0.850
Use of SSRIs	15 (4.9)	4 (3.7)	7 (6.3)	4 (5.0)	0 (0.0)	0.748
Triple therapy	34 (11.0)	11 (10.3)	15 (13.4)	7 (8.8)	1 (11.1)	0.772
High-risk PCI	17 (5.5)	9 (8.4)	5 (4.5)	3 (3.8)	0 (0.0)	0.398
Previous history						
Prior PCI	113 (36.7)	31 (29.0)	42 (37.5)	35 (43.8)	5 (55.6)	0.118
Prior CABG	69 (22.4)	22 (20.6)	30 (26.8)	16 (20.0)	1 (11.1)	0.520
Atrial fibrillation	90 (29.2)	33 (30.8)	30 (26.8)	27 (33.8)	0 (0.0)	0.174
Active malignancy	16 (5.2)	4 (3.7)	6 f(5.4)	5 (6.3)	1 (11.1)	0.732
Laboratory test						
Hemoglobin, mmol/L	8.2 (1.1)	8.3 (1.1)	8.2 (1.0)	8.1 (1.1)	7.9 (1.0)	0.582
Platelet count, 1×10^9/L	257 (76)	269 (85)	256 (73)	246 (66)	218 (45)	0.080
Creatinine, µmol/L	117 (67)	104 (48)	126 (85)	120 (55)	131 (85)	0.102
MDRD-eGFR, mL/min/1.73 m^2	56.1 (20.1)	59.9 (18.6)	54.9 (21.6)	52.(19.1)	55.4 (23.1)	0.082

Continuous variables are expressed as mean (standard deviation). Categorical variables are expressed as counts (percentages). ^ p-value calculated using either Chi-square test for categorical variables or ANOVA for continuous variables. Abbreviations: RM, rapid metabolizers; EM, extensive metabolizers; IM, intermediate metabolizers; PM, poor metabolizers; BMI, body mass index; PCI, percutaneous coronary intervention; VKA, vitamin K antagonist; DOAC, direct oral anticoagulant; CABG, coronary artery bypass graft; NSAIDs, nonsteroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors; MDRD-eGFR, Modification of Diet in Renal Disease–estimated glomerular filtration rate.

Table 4.2 shows the distribution of *CYP2C19* polymorphisms in our population, with 36.4% extensive (normal) metabolizers *(*1/*1*). About 34.7% of our population was classified as rapid metabolizers *(*1/*17* or **17/*17*), whereas 26.0% was intermediate metabolizers *(*1/*2*, **2/*17* or **1/*3*), and 9 patients (2.9%) turned out to be poor metabolizers *(*2/*2*).

Table 4.2 Distribution of *CYP2C19* polymorphisms in study population.

Metabolism	CYP2C19 Alleles	Frequency *n* (%), *n*=308	Group Total *n* (%)
Rapid metabolizer (RM)	*1/*17	91 (29.5)	107 (34.7)
	*17/*17	16 (5.2)	
Extensive metabolizer (EM)	*1/*1	112 (36.4)	112 (36.4)
Intermediate metabolizer (IM)	*1/*2	67 (21.8)	80 (26.0)
	*2/*17	12 (3.9)	
	*1/*3	1 (0.3)	
Poor metabolizer (PM)	*2/*2	9 (2.9)	9 (2.9)

Agreement between platelet function tests

All 3 platelet function tests were measured simultaneously after a median of 46 (37-59) days post-PCI. Simple correlation between platelet reactivity values was moderate for the 3 platelet function tests: ρ=0.566 (p<0.0001) for the correlation between VerifyNow and LTA, ρ=0.493 (p<0.0001) for VerifyNow and Multiplate, and ρ=0.423 (p<0.0001) for LTA and Multiplate.

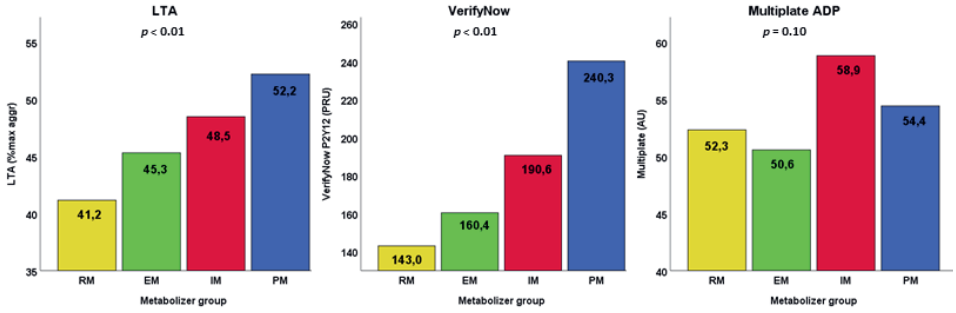
Residual platelet reactivity per group of CYP2C19 metabolism

Mean values of residual platelet reactivity per group of *CYP2C19* metabolism as measured by LTA VerifyNow and Multiplate are shown in Figure 4.1. For LTA, the intermediate and poor metabolizers have higher residual platelet reactivity as compared to extensive metabolizers, whereas the rapid metabolizers have the lowest on-treatment platelet reactivity. The residual platelet reactivity as measured by the LTA is significantly affected by *CYP2C19* metabolizer status (p<0.01). A Jonckheere–Terpstra test for ordered alternatives showed that there was a statistically significant trend of higher platelet reactivity with consecutive metabolizer groups; as metabolizer status changes from rapid, via extensive and intermediate, to poor, the mean LTA value increases accordingly (p<0.01) (Table 4.3).

The same applies to the VerifyNow test; residual platelet reactivity is significantly affected by *CYP2C19* metabolizer status (p<0.01), with a statistically significant trend of higher platelet reactivity when metabolizer status changes from rapid, extensive, intermediate to poor metabolizer (p<0.01).

Contrary to the LTA and VerifyNow, for the Multiplate no such trend can be found; mean residual platelet reactivity is not significantly different (p=0.10) between the metabolizer groups. Additionally, the Jonckheere–Terpstra test showed no statistically significant ordering of the metabolizer groups (p=0.10).

Figure 4.1 **Mean platelet reactivity as measured by three platelet function tests in the clopidogrel group expressed per group of *CYP2C19* metabolism.**



*Differences in platelet reactivity per group of CYP2C19 metabolism were measured using ANOVA. Poor metabolizers (PM) have genotype CYP2C19*2/*2, intermediate metabolizers (IM) CYP2C19*1/*2, *1/*3, or *2/*17, extensive metabolizers (EM) CYP2C19*1/*1, and rapid metabolizers (RM) CYP2C19*1/*17 or *17/*17.*

Table 4.3 **Mean platelet reactivity as measured by the three platelet function tests, expressed per group of *CYP2C19* metabolism**.

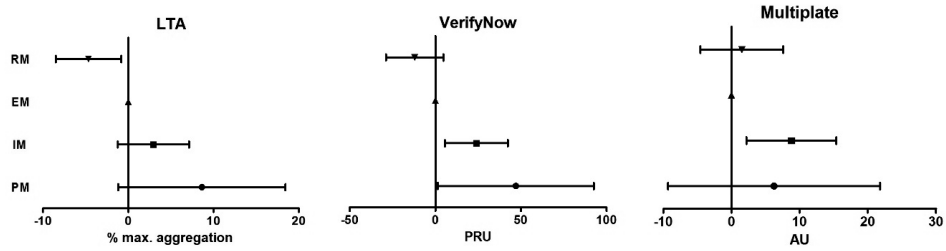
Platelet function test	CYP2C19 metabolism*	Patients, n(%) total n=308	Mean +/- SD	Jonckheere - Terpstra test ^
LTA	Metabolizer status			<0.0001
(n=300),	Rapid metabolizer	103 (34.3%)	41.2 ± 16.0	0.02
% max aggr	Extensive metabolizer	110 (36.7%)	45.3 ± 15.5	*Ref.*
	Intermediate metabolizer	78 (26.0%)	48.5 ± 12.0	0.03
	Poor metabolizer	9 (3.0%)	52.2 ± 13.8	0.08
VerifyNow	Metabolizer status			<0.0001
(n=304),	Rapid metabolizer	106 (34.9%)	143.0 ± 73.2	0.06
PRU	Extensive metabolizer	110 (36.2%)	160.4 ± 74.9	*Ref.*
	Intermediate metabolizer	80 (26.3%)	190.6 ± 62.3	<0.01
	Poor metabolizer	8 (2.6%)	240.3 ± 49.9	<0.01
Multiplate	Metabolizer status			0.10
(n=305),	Rapid metabolizer	105 (34.4%)	52.3 ± 22.6	*n/a*
AU	Extensive metabolizer	111 (36.3%)	50.6 ± 23.8	*Ref.*
	Intermediate metabolizer	80 (26.3%)	58.9 ± 24.8	*n/a*
	Poor metabolizer	9 (3.0%)	54.4 ± 15.7	*n/a*

*Values are expressed as median with interquartile range (IQR). Categorical data are expressed as absolute numbers and percentages (n (%)). n/a; not applicable (pairwise comparisons are not performed because the overall test does not show significant differences across groups). * Poor metabolizers have genotype CYP2C19*2/*2, intermediate metabolizers CYP2C19*1/*2, *1/*3, or *2/*17, extensive metabolizers CYP2C19*1/*1, and rapid metabolizers CYP2C19*1/*17 or *17/*17. ^ Jonckheere–Terpstra test for ordered alternatives, with pairwise comparisons for the different metabolizer groups compared to extensive metabolizers (reference category).*

Effect of metabolizer status on platelet reactivity

Figure 4.2 shows the effect of the different metabolizer groups on the residual platelet reactivity as measured by LTA, VerifyNow, and Multiplate. Platelet reactivity in this multivariable model was adjusted for age, body weight, diabetes, renal insufficiency, previous stroke, current smoking, concomitant use of (es-)omeprazole, hemoglobin, platelet count, and use of aspirin and/or anticoagulants, based on results of the univariate analysis *(*Table S4.2*).* In this adjusted model, poor metabolizer status is associated with a nonsignificant increase of 8.8% in maximal aggregation in the LTA (Beta: 8.8; 95% CI: -1.0-18.6; *p*=0.08) as compared to the EM group, whereas a rapid metabolizer status will lead to a decrease of 4.6% (Beta: -4.6; 95% CI: -8.5--0.8; *p*=0.02) in maximum aggregation of LTA. Compared to the wild type (EM), poor and intermediate metabolizer status is associated with an increase of 48.3 PRU (Beta: 48.3; 95% CI: 2.4-94.3; *p*=0.04) and 22.5 PRU (Beta: 22.5; 95% CI: 4.0-40.9; *p*=0.02) in VerifyNow, respectively (Table S4.2). As can be appreciated from Figure 4.2, the metabolizer status affects platelet reactivity as measured by both LTA and VerifyNow following an ordinal order; PM and IM status is associated with a (numerical) platelet reactivity increase and RM status with a (numerical) decrease. However, no such ordinal order could be found for the association of metabolizer status with platelet reactivity as measured by Multiplate.

Figure 4.2 Different metabolizer groups as predictor of residual platelet reactivity in multivariate linear regression analysis.



Partial regression coefficients (B) with 95% confidence interval for metabolizer group as predictor of residual platelet reactivity as measured by the 3 platelet function tests. Extensive metabolizers (EM) are the reference group. Abbreviations: PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; RM, rapid metabolize. Max. aggregation, percentage of maximum aggregation; PRU, P2Y12 reaction units; AU, aggregation units.

Relative importance of metabolizer status on platelet reactivity

Besides this effect of metabolizer status on differences in platelet reactivity between the PFTs, these differences are also explained by several clinical risk factors. Because the regression coefficients of all variables in the multivariable model are expressed in different measurement units, direct comparison of variables is difficult. Therefore, we calculated the standardized regression coefficients (beta weights) so that the effect of both patient-related factors and the metabolizer status on the platelet reactivity could be compared, giving a crude indication of the relative importance of the different variables (Table 4.4). This indicates that the variables with the strongest effect on platelet reactivity measured by LTA are metabolizer group (beta weight: 0.227), hemoglobin (beta weight: 0.155), and aspirin use (beta weight: -0.139). For VerifyNow, the variables with the strongest effect on platelet reactivity are hemoglobin (beta weight: -0.337), platelet count (beta weight: -0.268), and metabolizer group (beta weight: 0.212), respectively. However, for platelet reactivity as measured by Multiplate, the metabolizer group status (beta weight: 0.110) is not among the most important variables, which are platelet count (beta weight: 0.265), previous stroke (beta weight: 0.163), and concomitant use of (es-) omeprazole (beta weight 0.156).

Table 4.4 **Standardized regression coefficients (beta weights) of the multivariable model indicating the relative importance of the different variables on platelet reactivity as measured by the platelet function tests**.

Variables	LTA Beta Weight	VerifyNow Beta Weight	Multiplate Beta Weight
Age >75 years	0.119	0.077	0.079
Body weight <60 kg	−0.099	−0.119	−0.068
Diabetes	0.003	0.049	0.054
Renal dysfunction	−0.029	0.053	0.090
Previous stroke	0.112	0.111	0.163*
Current smoking	−0.054	−0.073	−0.002
(es-) omeprazole use	0.035	0.156	0.156*
Aspirin use	−0.139*	0.005	−0.013
Anticoagulant use	0.073	−0.033	0.049
Hemoglobin	0.155*	−0.337*	−0.046
Platelet count	−0.072	−0.268*	0.265*
Metabolizer group	0.227*	0.212*	0.110

** Indicates the 3 variables with the highest beta weights per platelet function test.*

Discussion

In this study, we investigated the differential impact of *CYP2C19* allelic variants on ADP-induced platelet aggregation as measured by three different platelet function tests in

high-risk patients on clopidogrel. According to the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines, patients in our study were divided into four metabolizer groups: poor, intermediate, extensive, and rapid metabolizers. The distribution of patients within these metabolizer groups is in line with previous reports in a European population[8]. We showed that residual platelet reactivity as measured by VerifyNow and LTA follows an ordinal trend for the different metabolizer groups, whereas for the Multiplate test, no such trend could be shown, suggesting that the genetic background has little effect on residual platelet reactivity as measured by the Multiplate assay.

Genetic variation in *CYP2C19* is certainly not the only factor that determines the response to clopidogrel. Previous studies have revealed that the *CYP2C19*2* polymorphism accounted for only 5-12% of clopidogrel variability in platelet reactivity[6,35]. Other factors, such as body weight, age, health conditions, and concomitant medication, also influence patients' response to clopidogrel[35]. Although the *CYP2C19*2* loss-of-function polymorphism was the strongest predictor of high on-treatment platelet reactivity in the study by Hochholzer et al., the *CYP2C9*2* carrier status, together with demographic and clinical predictors for high on-clopidogrel platelet reactivity, could only explain 11.5% of residual platelet reactivity in this study[35]. This indicates that there are still some relevant factors interfering with clopidogrel response that are yet unknown. These known and unknown specific patient-related factors have a differential influence on the results of ex vivo PFTs[36]. Our data suggest that in the Multiplate test, these patient-related factors might prevail and that genetic background only plays a minor role. Comparing the beta weights of the different variables, we showed indeed that the influence of factors such as platelet count, previous stroke, and concomitant medication is more pronounced in the Multiplate test, whereas metabolizer status had less effect on platelet reactivity as measured by this assay. In a previous study, we already showed that the agreement between various PFTs is only slight to moderate and that PFTs may be affected by different factors to a variable degree[24]. The current study adds the underlying *CYP2C19* allelic variation as one of these factors that might further explain this disagreement between platelet function tests.

Another factor to consider when interpreting our findings is the differences between test principles. Multiplate and VerifyNow are whole-blood tests, whereas in LTA, platelet aggregation is assessed in platelet-rich plasma. Moreover, the assays differ in anticoagulant used in the test tubes. Multiplate is performed in hirudin-anticoagulated blood, compared to citrated blood for LTA and VerifyNow. The low calcium

environment in citrated blood compared to hirudin blood could attenuate platelet aggregation[37]. Furthermore, the VerifyNow test includes prostaglandin E1 to suppress the platelet activation contribution of ADP binding to the P2Y1 receptor (which is unaffected by clopidogrel administration), and thereby, the assay selectively measures the ADP-P2Y12 pathway[38,39]. VerifyNow is an aggregation-based test using fibrinogen-coated beads, whereas LTA depends completely on the aggregation of platelets in the plasma environment. Lastly, the Multiplate test is based on the measurement of the increase in electrical impedance when platelets adhere and aggregate on two silver-coated copper wires. Thus, aggregation in Multiplate and VerifyNow takes place on surfaces, whereas in LTA, aggregation occurs more or less in a liquid phase[40].

Our findings are in concordance with Harmsze et al., who evaluated the impact of genotypes on on-treatment platelet reactivity as measured by LTA and VerifyNow[41]. Similar to our results, they found for both assays a decreasing trend in residual platelet reactivity comparing PM, IM, EM, and RM groups. However, in this study, the Multiplate assay was not evaluated. Another study that found differences in correlation with genetic background between two different platelet function assays studied the effect of *CYP2C19*17* in 598 ACS patients after loading dose and observed a significant impact of the **17* carriage on clopidogrel responsiveness when measured with vasodilator-stimulated phosphoprotein (VASP) assay, but not with LTA measurements[42]. Platelet response in LTA was assessed using 10 µmol ADP compared to 20 µmol ADP in our assay, and, contrary to our study, they did not evaluate the loss-of-function allelic variants and did not compare different metabolizer groups. Finally, Gremmel et al. investigated the influence of CYP2C9 allelic variants on ADP-induced platelet aggregation was determined by five different PFTs, including LTA, Multiplate, and VerifyNow. Although investigating the differential impact of CYP2C9 allelic variants instead of *CYP2C19*, their findings were comparable to ours; a significantly higher platelet reactivity was found for patients with loss-of-function status compared to the normal-function genotype using the VerifyNow assay or LTA, while results did not differ for the Multiplate assay[43].

Recently, several studies evaluated pharmacogenomic testing as an approach of personalized antiplatelet drug administration by either escalation or de-escalation of P2Y12 inhibitor therapy based on *CYP2C19* allelic variants[29,30,44,45]. The POPular Genetics trial demonstrated that a personalized approach using genetic testing to de-escalate to clopidogrel was noninferior to standard treatment with either ticagrelor or prasugrel in terms of the primary composite outcome net clinical benefit (consisting of all-cause death, recurrent MI, definite stent thrombosis, stroke, and PLATO major bleeding),

while it was superior in reducing combined major and minor bleedings[29]. A meta-analysis of eight randomized controlled trials, including the recently published TAILOR-PCI trial[30], confirmed that in patients with *CYP2C19*, loss-of function allele prescription of ticagrelor and prasugrel compared to clopidogrel resulted in a significant reduction in ischemic events (RR: 0.70; 95% CI: 0.59–0.83) but not in noncarriers of these alleles (RR: 1.0; 95% CI: 0.80–1.25)[32].

Current guidelines recommend risk stratification for tailoring individual treatment strategies[46,47]. Both platelet function testing and genotyping can provide useful prognostic insights, but trials evaluating treatment strategies have produced mixed results. Pharmacogenomic testing could be an attractive approach because treatment decisions can be made before the start of antiplatelet therapy, unlike with PFT, and the genotype does not change over time, unlike the phenotype of platelet reactivity[48,49]. One of the major limitations of PFTs is the great variability of the results, as is also shown in our study, and this could be overcome by genetic testing. On the other hand, the information derived from genotyping cannot be taken as a surrogate for PFT to assess antiplatelet drug responses, as genetic variants are just one influential factor affecting clopidogrel activity, and numerous epigenetic factors such as comorbidities, gastrointestinal absorption, drug interactions, and adherence are also important determinants. Thus, insight into the relationship between PFT results and genetics, as provided by this study, remains useful in further optimization of antiplatelet strategies. Furthermore, our findings could be of value to laboratories without the opportunity of performing genetic tests or that have to make a choice in the multitude of PFTs.

Important strengths of our study include the careful evaluation of included patients and the concomitant comparison of three different PFTs. Tests were performed 1-2 months after PCI, when a stable situation had been reached, not only in the inflammatory response to stent placement but also in individual clopidogrel response, and an influence of time from clopidogrel loading to platelet function testing could be excluded. A limitation of this study is that we did not assess plasma levels of the active metabolite of clopidogrel, which could have provided more mechanistic insight into the observed platelet response and correlation with the genetic background.

Conclusions and future remarks

In the near future, studies will keep focusing on the role of platelet function testing and genotyping to guide decision making in (high-risk) patients on antiplatelet therapy. In

this study, we have shown that the disagreement between PFTs is partly explained by differences in correlation with genetic background. For Multiplate, no major effect of genetic background could be shown, whereas for VerifyNow and LTA, the residual platelet reactivity in patients treated with clopidogrel correlates well with the underlying *CYP2C19* polymorphism. Understanding the (dis-) agreement between these different PFTs and how this relates to genetic variation in *CYP2C19* will help us in the interpretation of these future clinical trials focusing on personalizing antiplatelet therapy.

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Appendix 4

Table S4.1 Inclusion and exclusion criteria.

Inclusion criteria	Definition
PCI 30–90 days before study inclusion	Elective or emergency procedure
Dual/triple antithrombotic therapy	Including clopidogrel as P2Y12 inhibitor
Classified as 'vulnerable' by ≥ 3 predefined risk factors:	Age ≥75 years
	Female gender
	Renal dysfunction (MDRD-eGFR ≤ 60 mL/min)
	Body weight ≤60 kg
	Hypertension (previously diagnosed, or on medication)
	Diabetes mellitus
	Anemia (Hb <8.2 mmol/L for men, <7.3 mmol/L for women)
	Previous stroke
	Previous major bleeding
	Liver dysfunction (known hepatitis or transplant)
	History of gastric/duodenal ulcers
	Daily use of NSAIDs or SSRIs
	Triple antithrombotic therapy (DAPT + oral anticoagulants)
	Previous in-stent thrombosis or high-risk coronary stent (e.g., last remaining vessel or left main coronary artery)
CYP2C19 polymorphism available	Analysis of *CYP2C19* polymorphism performed by June 2018

Exclusion criteria	Definition
Known platelet function disorders	Previously diagnosed platelet function disorders
Recent coronary intervention	PCI or CABG ≤ 7 days
Recent new ischemic event	ACS or stroke ≤ 7 days
Signs of active infection	Fever, antibiotic treatment or hospital admission during laboratory assessment of platelet function
Medication noncompliance	Confirmed noncompliance in antithrombotic medication by patient interview or pharmacy dispensing

Abbreviations: PCI, percutaneous coronary intervention; MDRD-eGFR, Modification of Diet in Renal Disease–estimated glomerular filtration rate; Hb, hemoglobin; NSAIDs, nonsteroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors; DAPT, dual antiplatelet therapy; CABG, coronary artery bypass graft; ACS, acute coronary syndrome.

Table S4.2 Partial regression coefficients of variables for LTA, VerifyNow, and Multiplate in univariate and multivariate analysis.

Variables	LTA ADP20 (n=300)						VerifyNow P2Y12 (n=304)						Multiplate (n=305)					
	Univariate			Multivariate*			Univariate			Multivariate*			Univariate			Multivariate*		
	B	95% CI	p	B	95% CI	p	B		p	B	95% CI	p	B	95% CI	p	B	95% CI	p
Age ≥75 years	3.456	-0.072 – 6.983	0.055	3.746	0.27 – 7.23	0.035	28.013	11.050 – 44.976	0.001	11.381	-3.88 – 26.64	0.143	3.121	-2.373 – 8.615	0.264	3.493	-1.99 – 8.97	0.211
Gender, female	-1.292	-4.793 – 2.209	0.468				3.973	-12.950 – 20.986	0.644				2.306	-3.097 – 7.709	0.402			
Weight<60 kg	-10.356	-16.454 – -4.259	0.001	-5.386	-11.64 – 0.87	0.091	-51.291	-79.540 – -23.042	0.000	-30.905	-56.79 – -5.02	0.019	-3.585	-13.115 – 5.944	0.460	-6.025	-15.64 – 3.59	0.218
Diabetes Mellitus	0.033	-3.537 – 3.602	0.986	-0.028	-3.45 – 3.40	0.987	13.874	-3.480 – 31.229	0.117	7.221	-7.83 – 22.27	0.346	4.480	-1.044 – 10.003	0.112	2.753	-2.64 – 8.15	0.316
Hypertension	-1.171	-5.851 – 3.510	0.623				4.893	-17.597 – 27.383	0.669				-1.923	-9.114 – 5.268	0.599			
Renal dysfunction	-1.263	-4.734 – 2.209	0.475	-0.878	-4.31 – 2.56	0.615	25.743	9.140 – 42.346	0.002	8.096	-7.01 – 23.20	0.292	4.920	-0.449 – 10.288	0.072	4.474	-0.96 – 9.91	0.106
History of bleeding	-0.288	-5.095 – 4.520	0.906				8.777	-14.269 – 31.824	0.454				4.724	-2.634 – 12.082	0.207			
Stroke in history #	4.733	1.013 – 8.453	0.013	3.699	0.11– 7.29	0.043	17.203	-0.919 – 35.325	0.063	17.793	2.02 – 33.57	0.027	8.439	2.641 – 14.236	0.004	8.390	2.70 – 14.08	0.004
Current smoking #	-3.147	-8.176 – 1.883	0.219	-2.370	-7.23 – 2.49	0.338	-33.738	-58.169 – -9.308	0.007	-15.310	-36.84 – 6.22	0.163	-0.760	-8.635 – 7.116	0.850	-0.019	-7.65 – 7.62	0.996
(Es-) omeprazole	1.490	-3.505 – 6.484	0.558	1.644	-3.97 –7.26	0.565	24.248	0.236 – 48.259	0.048	39.726	14.96 – 64.49	0.002	8.702	1.036 – 16.369	0.026	13.40	4.49 – 22.32	0.003
Aspirin	-6.837	-10.478 – -3.197	0.000	-4.471	-11.64 – 2.70	0.220	6.074	-12.011 – 24.160	0.509	-0.286	-31.20 – 30.63	0.986	-2.491	-8.305 – 3.324	0.400	-0.979	-12.12 – 10.17	0.863
Anticoagulant	6.134	2.587 – 9.681	0.001	2.343	-4.61 – 9.30	0.508	-3.139	-20.674 – 14.396	0.725	-4.957	-34.91 – 24.99	0.745	2.204	-3.422 – 7.831	0.441	2.064	-8.70 – 12.83	0.706
Hemoglobin (mmol/l)	2.347	0.774 – 3.920	0.004	2.145	0.50 – 3.79	0.011	-20.674	-28.101 – -13.248	0.000	-22.703	-30.01 – -15.40	0.000	-3.396	-5.864 – -0.928	0.007	-1.021	-3.64 – 1.60	0.444
Platelets (10^9/L)	-0.035	-0.056 – -0.014	0.001	-0.013	-0.04 – 0.01	0.246	-0.223	-0.324 – -0.122	0.000	-0.243	-0.34 – -0.15	0.000	0.070	0.038 – 0.103	0.000	0.076	0.04 – 0.11	0.000
Metabolizer status																		
PM	6.895	-3.222 – 17.012	0.181	8.644	-1.16 – 18.44	0.084	79.859	28.945 – 130.773	0.002	47.029	1.49 – 92.56	0.043	3.859	-12.154 – 19.872	0.636	6.243	-9.30 – 21.78	0.430
IM	3.173	-1.147 – 7.492	0.149	2.958	-1.23 – 7.14	0.165	30.234	9.804 – 50.664	0.004	23.981	5.64 – 42.32	0.011	8.264	1.488 – 15.040	0.017	8.792	2.21 – 15.37	0.009
EM	ref	ref	-	ref	ref	-	ref		-	ref	ref	-	ref		-	ref	ref	-
RM	-4.153	-8.154 – -0.151	0.042	-4.648	-8.49 – -0.81	0.018	-17.381	-36.306 – 1.543	0.072	-12.047	-28.87 – 4.77	0.160	1.757	-4.533 – 8.047	0.583	1.516	-4.55 – 7.58	0.623

** Based on existing literature or if univariate analysis indicated the variable to be associated with at least one of the platelet function tests at p<0.20, this variable was included in the multivariate model. The multivariate model is adjusted for age, weight, diabetes, renal dysfunction, previous stroke, current smoking, use of (es-)omeprazole, aspirin or anticoagulant use, Hb, platelet count and metabolizer status; # missing data; current smoking missing n=7 and stroke in history missing n=1. Abbreviations: PM; poor metabolizer, IM; intermediate metabolizer, EM; extensive metabolizer, RM; rapid metabolizer.*

Chapter 5

Thrombin generation as a method to identify the risk of bleeding in high clinical-risk patients using dual antiplatelet therapy

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Abstract

Background

Patients using dual antiplatelet therapy after percutaneous coronary intervention are at risk for bleeding. It is currently unknown whether thrombin generation can be used to identify patients receiving dual antiplatelet therapy with increased bleeding risk.

Objectives

To investigate whether thrombin generation measurement in plasma provides additional insight in the assessment of bleeding risk for high clinical-risk patients using dual antiplatelet therapy.

Methods

Coagulation factors and thrombin generation in platelet-poor plasma were measured in 93 high clinical-risk frail patients using dual antiplatelet therapy after percutaneous coronary intervention. During 12-month follow-up, clinically relevant bleedings were reported. Thrombin generation at 1 and 6 months after percutaneous coronary intervention was compared between patients with and without bleeding events.

Results

One month after percutaneous coronary intervention the parameters of thrombin generation, endogenous thrombin potential, peak height and velocity index, were significantly lower in patients with bleeding in the following months compared to patients without bleeding. At 6 months follow-up, endogenous thrombin potential, peak height and velocity index were still (significantly) decreased in the bleeding group as compared to non-bleeders. Thrombin generation in the patients' plasma was strongly dependent on factor II-, V- and VIII- activity and fibrinogen.

Conclusion

High clinical-risk patients using dual antiplatelet therapy with clinically relevant bleeding during follow-up show reduced and delayed thrombin generation in platelet poor plasma, possibly due to variation in coagulation factors. Thus, impaired thrombin-generating potential may be a 'second hit' on top of dual antiplatelet therapy, increasing the bleeding risk in high clinical-risk patients. Thrombin generation has potential to improve the identification of patients using dual antiplatelet therapy at increased risk of bleeding.

Introduction

Each year in Europe about 3.6 million patients receive dual antiplatelet therapy (DAPT) during 6 to 12 months after a percutaneous coronary intervention (PCI)[1]. This patient population has an increased risk for both bleeding- and ischemic events. Risk factors for bleeding- and ischemic events are multifactorial and it remains difficult to predict the individual bleeding versus ischemic risk. Known risk factors for the occurrence of bleeding complications are, among others, low on-treatment platelet reactivity, the use of chronic oral anticoagulation therapy and older age[2,3]. On the other hand, ischemic events after PCI are associated with initial presentation with acute coronary syndrome (ACS), the stent itself (in-stent thrombosis), older age, diabetes and chronic kidney disease[4]. Identification of patients who are at higher risk of bleeding or ischemic events could lead to better personalized treatment and reduce the burden of adverse events.

While DAPT substantially reduces the risk of ischemic events, it comes with an impairment of primary hemostasis[5,6]. However, the overall hemostatic potential is not only determined by platelet reactivity, but also by other factors like endothelial cell barrier integrity and the coagulation system including multiple coagulation factors[7]. Variation in coagulant activity therefore impacts the bleeding risk in patients with a platelet function impairment inflicted by DAPT. This “second hit” of coinciding coagulation impairment can be due to variation in specific plasma components, like the level of factor VIII that shows substantial variation in the population[8]; or DAPT-dependent reduction in the availability of the platelet procoagulant surface for efficient assembly of coagulation factor complexes promoting the formation of thrombin[9-11]. Both mechanisms can be addressed in thrombin generation (TG) measurements using the ‘calibrated automated thrombogram’(CAT)[12]. Thus far, a number of studies have demonstrated TG as a valid tool to detect a bleeding tendency in patients with hemophilia A and B, von Willebrand disease, factor XI deficiency, platelet disorders and vitamin K antagonist (VKA) treatment[13-17].

One would expect that a reduction of platelet activation and aggregation by antiplatelet therapy leads to a diminished TG, measured in platelet rich plasma (PRP) or whole blood (WB). Indeed, studies confirmed that treatment with aspirin, clopidogrel or both reduced TG parameters in PRP and WB[18-23]. The relationship between TG and the risk of bleeding in case of DAPT is rather underexposed. Therefore, in the present study we investigated the potential use of the TG assay for the assessment of bleeding risk in high clinical-risk patients using DAPT. Besides, we were interested whether bleeding risk is influenced by the level of coagulation factors[24]. TG in this study was measured in PPP, since this is by far the most used and standardized method of TG

measurement. Hence, we hypothesized that patients who suffer from clinically relevant bleedings during DAPT show lower TG-parameters compared to patients without bleeding events.

Materials and methods

Study population and design

The current study is a sub-study of a prospective cohort study in the Maastricht University Medical Center+ (MUMC+), monitoring high clinical-risk patients on dual/triple antithrombotic therapy after PCI, as previously described[25]. DAPT includes a combination of low-dose aspirin with either clopidogrel, ticagrelor or prasugrel. DAPT duration was either 6 or 12 months, dependent on the PCI indication: elective versus acute (ACS), respectively. In this exploratory study, patients were included via the outpatient clinic of the Thrombosis Expertise Center in the MUMC+, during a period of 2 years.

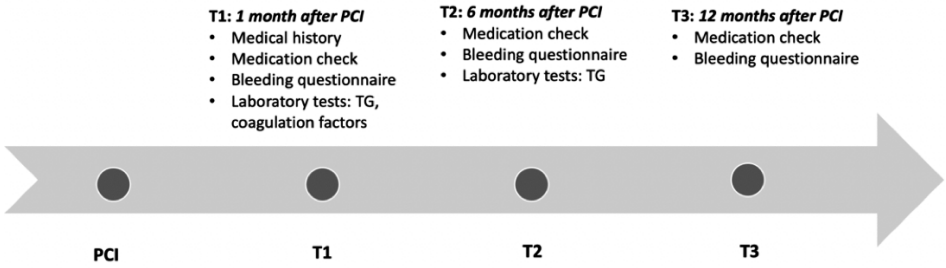
All PCI patients were screened by one dedicated cardiologist, and, after informed consent was obtained, high clinical-risk patients were included within one month after PCI. High clinical-risk patients in the prospective cohort study were defined as patients who had ≥3 risk factors for bleeding or ischemic events, which include: female gender, hypertension, anemia at time of PCI (<13.2 g/dL for men, <11.8 g/dL for women), age ≥75 year, previous stroke, previous major bleeding, renal dysfunction (estimated Glomerular Filtration Ratio (eGFR) <60 mL/min), known hepatitis or liver transplant, triple antithrombotic therapy, previous gastric ulcers, diabetes mellitus, low body weight (<60 kg), use of non-steroidal anti-inflammatory drugs or selective serotonin reuptake inhibitors, previous in-stent thrombosis, and/or high-risk stenting (multivessel PCI or main coronary artery stenting). The cohort population included patients who met the criteria named above, had been fasting for 4 hours before blood withdrawal and had a PCI in the past 1-2 months. Patients were excluded in case of non-compliance. The latter was checked during the visit by interview and by contacting the pharmacy to confirm dispensing of the drugs. According to the study protocol, TG was measured in the first group of 200 patients included in the prospective cohort study. Specific inclusion criteria for this sub-study were usage of a combination of low-dose aspirin (80-100 mg) and a P2Y12 inhibitor (clopidogrel 75 mg, prasugrel 5-10 mg or ticagrelor 90 mg) (DAPT) for a planned duration of ≥6 months, and patients for whom TG measurements were available. An additional exclusion criterion for this sub-study was the use of vitamin K agonists, direct oral anticoagulants or low molecular weight heparins. All patients provided written consent. Ethical approval was obtained from the

medical ethical committee of the MUMC+ ((METC aZM/UM), approval number: 11-2-096).

Follow-up and study endpoints

After referral, consultation at the thrombosis expertise center and study visits were planned at 1 month (T1) and 6 months (T2) after PCI (Figure 5.1). Twelve months after PCI (T3) patients using DAPT >6 months were contacted by phone for final follow-up. Blood was drawn at T1 and once again at T2 in case DAPT was continued for a total treatment duration of 9-12 months. During all three contact moments a thorough history on minor and major bleeding was taken using the International Society on Thrombosis and Haemostasis (ISTH) Bleeding Assessment Tool (ISTH-AT). Using these structured questionnaires, bleeding events were recorded and categorized according to the definition of the Bleeding Academic Research Consortium (BARC), which contains unified and validated bleeding criteria[26-28]. The primary endpoint of this study was clinically relevant bleeding during DAPT treatment, defined as BARC type 2, 3 or 5. Bleeding events presented at T1 include bleedings from PCI until T1, at T2 from T1 until T2 and at T3 from T2 until T3 (Figure 5.1). To investigate whether TG could identify patients with future clinically relevant bleedings, all bleeding events recorded at T2 and T3 were included in the analysis for TG measured at T1, and bleeding events recorded at T3 for TG measured at T2.

Figure 5.1 Timeline of the study, showing the three contact moments: TI, T2, and T3, at respectively, 1, 6, and 12 months after PCI.



The collected information and performed tests at each consultation is described. TG, Thrombin generation; PCI, percutaneous coronary intervention.

In addition, during the follow-up visits medical history, recurrent ischemic events, concomitant medication, intoxications and compliance were recorded. To minimize recall bias, this information was collected in conversation with the patient, and cross-checked with the hospital's electronic medical records.

Blood samples

During the first and second study visit, venous blood was collected through separate antecubital venipuncture in Vacuette® tubes (Greiner Bio-One, GmbH, Kremsmünster, Austria) containing sodium citrate (3.2%). PPP was prepared by two centrifugation steps at 2,000g for 10 min and aliquots were stored at -80°C. TG was measured in all samples, simultaneously.

Reagents

Innovin (Dade-Behring, Marburg, Germany) was used as a source of recombinant tissue factor (TF). Synthetic phospholipids (PL), consisting of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (1:1:3, mol:mol:mol), were from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Z-Gly-Gly-Arg-aminomethylcoumarine (ZGGR-AMC) was purchased from Bachem (Basel, Switzerland). The calibrator, α2-macroglobulin-thrombin complex, was prepared as described by Hemker et al.[12] Hepes buffers containing 5 mg/ml or 60 mg/ml bovine serum albumin were prepared as described previously[29].

Thrombin generation

TG in PPP was measured by calibrated automated thrombogram (CAT) in triplicate, as described[30]. In short, the reaction was initiated with 20 µl of stimulus (tissue factor (TF)), containing a final concentration of 1 pM TF and 4 µM phospholipids (PL). Next, 80 µl of plasma was added to the well. The calibration wells contained 20 µl calibrator, with a final thrombin concentration of 100 nM. The reaction was started by adding 20 µl of FluCa (416.7 µM ZGGR-AMC and 16.7 mM $CaCl_2$ in BSA60 buffer). To minimize variation, samples of normal pool plasma were measured on each plate. Data were analyzed using software from Thrombinoscope version 5.0 (Maastricht, the Netherlands).

Low TF concentration was used to include the contribution of the intrinsic pathway factors VIII, IX, and XI[31,32]. The analyzed TG parameters for all samples were: lag time, Endogenous Thrombin Potential (ETP), velocity index, peak level of thrombin formation and time-to-peak.

Additional analyses

Measurements of coagulation factors were performed using the STA-R Evolution analyzer (Stago, Asnières sur Seine, France). Factor (F)II, V, VII, VIII, IX, X, XI and XII activity levels were assessed with clotting assays triggered by either a thromboplastin-based reagent (FII, -V, -VII and -X) or a kaolin-based reagent (FVIII, -XI, -IX and -XII).

Fibrinogen levels were measured using the Clauss method, and both fibrinogen and von Willebrand factor (vWF) were measured with the CS2100i analyser from Sysmex[33].

Statistical analysis

Statistical analysis was performed using SPSS software (version 24). Patients with missing data (other than TG) were not excluded from the analysis. Normal distribution of all continuous variables was calculated using the Kolmogorov-Smirnov and Shapiro-Wilk Test of Normality combined with evaluation of the histograms. For normally distributed continuous variables, differences between groups were tested by Student's t-test and represented by means and standard deviations (SD). For not-normally distributed continuous variables, differences between groups were tested by Mann-Whitney U test and represented by medians and interquartile range (IQR) or range (when subgroups where too small for an IQR)). Categorical variables were tested by Chi-square test and represented as numbers and percentages.

A multiple regression analysis was done to investigate coagulation factors as determinants of thrombin generation. For each model, the adjusted R^2 and the standardized regression coefficients (beta) of the independent variables (coagulation factors) were calculated. A two-sided p-value of ≤0.05 was considered statistically significant.

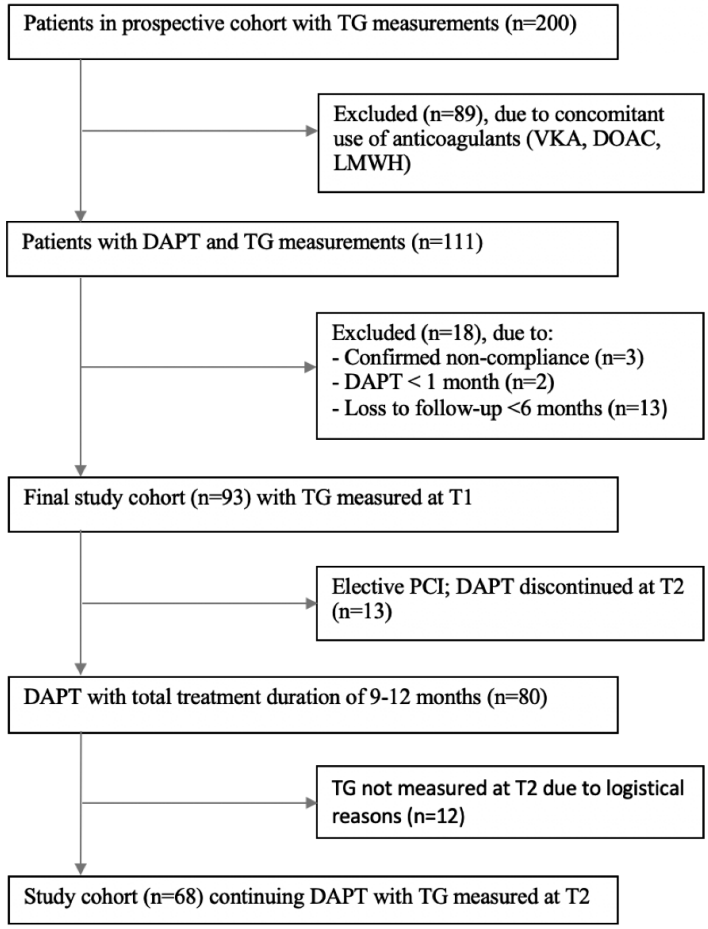
Results

Study population

In total, 111 patients were eligible for this sub-study, of whom 18 patients had to be excluded for several reasons: 3 patients were not compliant, 2 patients used DAPT <1 month, and 13 patients were lost to follow-up within 6 months (Figure 5.2). Thus, the final study population consisted of 93 patients, of whom 13 patients with an elective PCI could finalize their DAPT at 6 months. Out of 80 patients continuing DAPT with a total treatment duration of 9-12 months, blood was drawn again at the second study visit (T2, 6 months) in 68 patients. Unfortunately, no TG could be measured in the other 12 patients due to logistical reasons. Baseline characteristics of patients lost to follow-up or with missing TG measurements at T2 did not differ from the final study population or the T2 population, respectively (Supplementary Table S5.2 and Table S5.3). During the treatment period, 14 patients (15,1%) suffered from clinically relevant bleeding (BARC score ≥2), of which 7 bleedings were documented at T1, 3 bleedings at

T2 and 5 bleedings at T3 (Supplementary Table S5.1). No patient had more than 1 clinically relevant bleeding event during follow-up.

Figure 5.2 Flow diagram of study inclusion and follow-up.



TG, thrombin generation; VKA, vitamin K antagonist; DOAC, direct-acting oral anticoagulants; LMWH, low molecular weight heparin; DAFT, dual antiplatelet therapy; PCI, percutaneous coronary intervention.

Baseline characteristics of the 93 included patients are listed in Table 5.1. Significant differences in prevalence of anemia and TIA/CVA in medical history were found between patients with and without clinically relevant bleeding. No significant differences were found in amount of days from PCI to T1 between both groups. Most of the patients (n=80, 86.0%) used DAPT for 9-12 months after PCI of which 12 patients (15.0%) suffered from clinically relevant bleeding during treatment. Thirteen patients (14.0%) used DAPT for 6 months according to prescription after elective PCI, of whom 3 (20.0%) had a clinically relevant bleeding.

Table 5.1 Baseline characteristics.

	All patients (N=93), Mean (±SD), number (%)	Patients with clinically relevant bleeding (N=15) Mean (±SD), number (%)	Patients without clinically relevant bleeding (N=78) Mean (±SD), number (%)	Significance (p-value)
Inclusion criteria				
Female gender	49 (52.7%)	7 (46.7%)	42 (53.8%)	0.610
Age >75 years	49 (52.7%)	8 (53.3%)	41 (52.6%)	0.956
Age (years)	72,4 (9.0)	70.5 (8.6)	72.8 (9.1)	0.359
Weight <60 kg	6 (6.5%)	2 (13.3%)	4 (5.1%)	0.236
Hypertension	86 (92.5%)	12 (80,0%)	74 (86.9%)	0.080
Anemia	22 (23.7%)	8 (53.3%)	14 (17.9%)	0.003*
eGFR <60 ml/min	51 (54.8%)	9 (60.0%)	42 (53.8%)	0.661
Renal function (eGFR ml/min)	59,6 (20.1)	55.5 (20.7)	60.4 (20.0)	0.390
Known hepatitis or liver transplant	0 (0%)	0	0	-
TIA/CVA in medical history	24 (25.8%)	7 (46.7%)	17 (21.8%)	0.044*
Bleeding in medical history	8 (8.6%)	2 (13.3%)	6 (7.7%)	0.475
Diabetes mellitus	32 (34.4%)	4 (26.7%)	28 (35.9%)	0.491
NSAID usage	8 (8.6%)	1 (6.7%)	7 (9.0%)	0.770
SSRI usage	5 (5.4%)	0 (0.0%)	5 (6.4%)	0.313
Gastric ulcer /bleeding in medical history	15 (16.1%)	5 (33.3%)	10 (12.8%)	0.062
High risk referral	14 (15.1%)	1 (6.7%)	13 (16.7%)	0.680
Laboratory characteristics				
Hemoglobin(g/dL)	13.2 (1.0)	12.9 (1.1)	13.4(1,0)	0.245
Hematocrit (L/L)	0.40 (0.04)	0.39 (0.05)	0,40 (0,04)	0.356
Thrombocytes ($x10^9$/L)	252.0 (72.7)	252.9 (66.6)	251.8 (74.2)	0.958
Leukocytes ($x10^9$/L)	7.5 (2.05)	6.9 (2.0)	7.6 (2.1)	0.217
PT (sec)	10.5 (0.5)	10.7 (0.6)	10.5 (0.5)	0.113
aPTT (sec)	26.3 (2.0)	26.2 (1.5)	26.3 (2.1)	0.821
Patient characteristics				
Smoking	15 (16.1%)	1 (6.7%)	14 (17.9%)	0.314
≥8 glasses alcohol per week**	10 (10.8%)	2 (13.3%)	8 (10.3%)	0.346
Dyslipidemia	35 (37.6%)	7 (46.7%)	28 (35.9%)	0.430
Malignancy (active)	7 (7.5%)	3 (20.0%)	4 (5.1%)	0.080
Atrial fibrillation	4 (4.3%)	0 (0.0%)	4 (5.1%)	1.000
PPI usage	75 (80.6%)	14 (93.3%)	61 (78.2%)	0.174
ACS as PCI indication	59 (63.4%)	11 (73.3%)	48 (61.5%)	0.385
Interval PCI to T1 in days	54.7 (31.2)	65.9 (47.2)	52.5 (27.01)	0.128

Table 5.1 (continued)

	All patients (N=93), Mean (±SD), number (%)	Patients with clinically relevant bleeding (N=15) Mean (±SD), number (%)	Patients without clinically relevant bleeding (N=78) Mean (±SD), number (%)	Significance (p-value)
Antithrombotics				
Aspirin	93 (100%)	15 (100%)	78 (100%)	-
Clopidogrel	69 (74.2%)	11 (73.3%)	58 (74.3%)	0.934
Ticagrelor	5 (5.4%)	1 (6.7%)	4 (5.1%)	0.809
Prasugrel	19 (20.4%)	3 (20.0%)	16 (20.5%)	0.964
Treatment period				
DAPT duration 6 months	13 (14.0%)	3 (20.0%)	10 (12.8%)	
DAPT duration 9 to 12 months	80 (86.0%)	12 (80.0%)	68 (87.2%)	0.463

** a p value <0.05 was considered significant; ** A cut-off value of ≥8 glasses alcohol per week was chosen based upon the HASBLED bleeding score[46]. N=number of patients, SD=Standard Deviations, BARC=Bleeding Academic Research Consortium, eGFR=estimated Glomerular Filtration Rate, TIA=Transient Ischemic Attack, CVA=cerebral vascular attack, NSAID=Nonsteroidal Anti-Inflammatory Drug, SSRI=Selective Serotonin Reuptake Inhibitor, PT=Prothrombin Time, aPTT=Activated Partial Thromboplastin Time, PPI=Proton Pump Inhibitor, ACS=Acute Coronary Syndrome, PCI=Percutaneous Coronary Intervention, DAPT=Dual Antiplatelet Therapy.*

Thrombin generation and clinically relevant bleeding during follow-up

TG measured one month after PCI (T1) was significantly lower with respect to ETP (p<0,001), peak height (p=0,004) and velocity index (p=0,016) in plasma from patients with bleeding during follow-up (n=8) compared to patients without bleeding complications (n=85) (bleedings recorded at T2 and T3) (Figure 5.3). Moreover, patients with bleeding had a significantly longer time-to-peak (p=0.007) and lag time (p=0.036) compared to patients without bleeding (Figure 5.3).

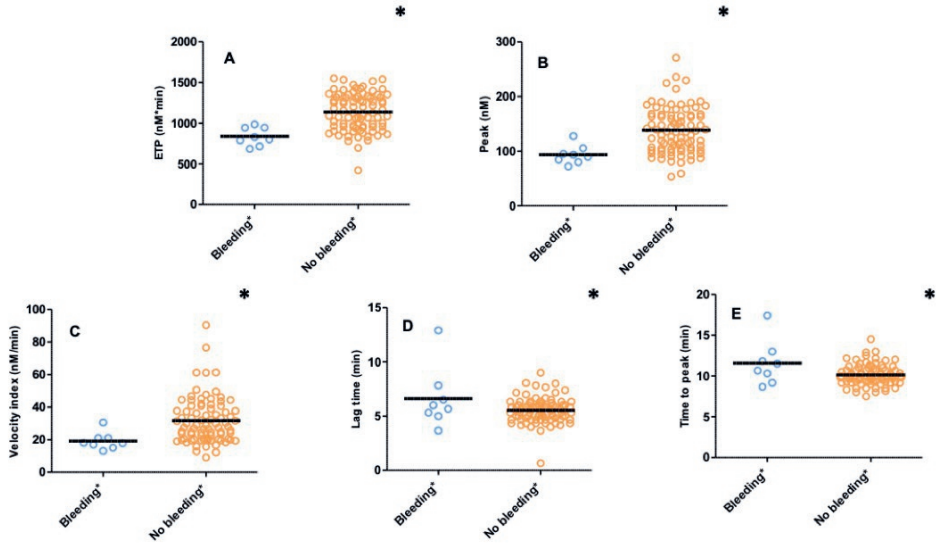
Similarly, at 6 months after PCI (T2, n=68), significantly lower peak level (p=0.039), velocity index (p=0.031), and a trend toward a lower ETP (p=0.072) were detected in plasma from patients who experienced bleeding during follow-up (n=5) (bleedings recorded at T3) (Table 5.2).

Coagulation factors as determinants of TG in PPP

At T1, ETP was significantly determined by FII activity level (Beta 8.675 (95% CI 1.336-16.014), p=0.021), as expected, while the peak level was significantly determined by the FV activity level (Beta -0.663 (95% CI -1.244- -0.083), p=0.026). ETP, peak level and velocity index were all significantly affected by FVIII activity (Beta respectively 2.128 (95% CI 0.045-4.210), 0.516 (95% CI 0.156-0.877) and 0.171 (95% CI 0.052-0.291) (p-value respectively 0.045, 0.006 and 0.006). Lag time and time-to-peak were mainly determined by the plasma fibrinogen concentration (Beta respectively 0.688 (95% CI

0.119-1.258) and 0.788 (95% CI 0.270) (p-value respectively 0.019 and 0.004) (Table 5.3).

Figure 5.3 **Thrombin generation (TG) in PPP patients with (*n*=8) and without (*n*=85) clinically relevant bleeding(*N*=93) during follow-up (bleedings recorded at T2 and T3). (A) ETP, (B) Peak, (C) Velocity index, (D) Lag time, and (E) Time to peak.**



*Significant lower ETP levels (p≤0.001), peak height (p=0.004), and velocity index (p=0.016) in patients with clinically relevant bleeding during follow-up. Significant longer time to peak(p=0.007) and lag time (p=0.036) in patients with clinically relevant bleeding. Means are indicated by lines. *p<0.05. ETP, Endogenous Thrombin Potential; PPP, Platelet Poor Plasma; N, number of patients.*

Table 5.2 **Thrombin generation 6 months after PCI in PPP for patients with and without clinically relevant bleeding during follow-up (recorded at T3).**

	Clinically relevant bleeding Medians (IQR)	Non-clinically relevant bleeding Medians (IQR)	p-value
Thrombin generation in PPP 1pM (N=68) (Bleeding N=5, Non-bleeding N=63)			
ETP	807.18 (608.09 – 1006.28)	1030.15 (911.22 – 1149.08)	0.072
Peak	88.88 (63.79 –113.97)	127.50 (104.24 –150.76)	0.039*
Velocity index	18.61 (13.30 – 23.92)	27.24 (21.51 – 32.97)	0.031*
Lag time	5.34 (4.63 – 6.05)	5.33 (4.55 –6.05)	0.734
Time to peak	10.17 (9.42 – 10.92)	10.17 (9.25 –11.09)	0.575

** a p value <0.05 was considered significant. PPP=Platelet Poor Plasma, pM=Picomolar, BARC=Bleeding Academic Research Consortium, IQR=Interquartile range, N=Number of patients, ETP=Endogenous Thrombin Potential.*

Table 5.3 Coagulation factors as determinants of thrombin generation - multiple regression analysis.

	ETP	Peak	Velocity index	Lag time	Time to peak
Beta (95% CI)					
(Adjusted R^2)	0.104	0.255	0.273	0.265	0.161
Fibrinogen	49.344	5.050	2.339	0.788*	0.688*
	(-38.385-137.073)	(-10.132-20.233)	(-2.700-7.379)	(0.270-1.370)	(0.119-0.1258)
vWF activity	-0.174	-0.017	0.006	0.005	0.004
	(-1.930-1.582)	(-0.320-0.287)	(-0.095-0.107)	(-0.005-0.015)	(-0.008-0.015)
Factor II activity	8.675*	0.666	0.099	-0.025	-0.007
	(1.336-16.014)	(-0.604-1.936)	(-0.323-0.520)	(-0.068-0.019)	(-0.055-0.040)
Factor V activity	-2.220	-0.663*	-0.181	0.018	0.021
	(-5.574-1.134)	(-1.244- -0.083)	(-0.374-0.011)	(-0.002-0.038)	(0.000-0.043)
Factor VII activity	-0.977	0.031	0.030	-0.008	-0.011
	(-4.454-2.500)	(-0.571-0.632)	(-0.169-0.230)	(-0.029-0.120)	(-0.033-0.012)
Factor VIII activity	2.128*	0.516*	0.171*	-0.003	-0.008
	(0.045-4.210)	(0.156-0.877)	(0.052-0.291)	(-0.015-0.009)	(-0.022-0.005)
Factor IX activity	-1.120	0.012	0.013	-0.003	-0.002
	(-5.125-2.885)	(-0.681-0.705)	(-0.218-0.243)	(-0.027-0.020)	(-0.028-0.024)
Factor X activity	-1.629	0.051	0.115	0.026	0.011
	(-6.824-3.567)	(-0.849-0.950)	(-0.184-0.413)	(-0.005-0.057)	(-0.023-0.045)
Factor XI activity	-1.665	-0.219	-0.059	0.009	0.008
	(-3.876-0.546)	(-0.601-0.164)	(-0.186-0.068)	(-0.004-0.022)	(-0.006-0.022)
Factor XII activity	1.149	0.083	-0.030	-0.007	-0.005
	(-1.631-3.928)	(-0.398-0.564)	(-0.190-0.129)	(-0.024-0.009)	(-0.023-0.013)

**indicates a p value <0.05, considered as significant. CI=Confidence Interval, ETP=Endogenous Thrombin Potential, vWF=von Willebrand factor.*

Discussion

The main aim of our study was to investigate whether TG might have potential to identify patients with an increased bleeding risk during the following DAPT treatment period of 6-12 months, by comparing the TG parameters between 'bleeders' and 'non-bleeders'. TG parameters measured in PPP, at 1 month and 6 months after PCI, showed (significant) lower ETP, peak and velocity index levels in patients with a clinically relevant bleeding episode compared to patients without bleeding during follow-up.

Since thrombin generation was performed in the absence of platelets, the observed differences between patients with and without bleeding might be explained by variation in coagulation factors. Following this theory, impaired thrombin generation resulting from one or several coagulation factors levels in the low-normal range, on top of DAPT, could ultimately contribute to an increased risk of bleeding. Mean values of all coagulation factors measured in this study were within normal range. We showed in this study that ETP was mostly determined by prothrombin levels, and peak height by

FV level, as confirmed by previous studies[24,34-36]. ETP, peak height and velocity index were all significantly determined by the level of factor VIII. This positive relation can be explained by the strong feedback loop between thrombin and factor VIII[36,37]. Higher ETP in case of higher factor II (prothrombin) levels was expected, since factor II is the precursor of thrombin, and has been confirmed in previous research[24,35,38]. We found a negative association between FV activity and peak height. Interestingly, this inverse relationship was also found in healthy individuals in a previous publication by Dielis et al.[35]. This negative relation might be attributed to the fact that factor V has both procoagulant and anticoagulant potential; activated FV functions in the procoagulant pathways, but in its inactivated form FV acts as a cofactor for activated protein C (APC) in the regulation of FVIIIa[39]. Increased lag time and time-to-peak were both associated with higher levels of fibrinogen. Based on other studies investigating TG in normal and defibrinated plasma, this can be explained by the ability of fibrinogen/fibrin to bind thrombin, and subsequently inhibit thrombin-mediated FVIII activation[24,35,40,41]. In the presence of DAPT, such effect may obviously increase the risk of bleeding too. Besides coagulation factors, circulating cell-derived microparticles also play a role in thrombogenesis[42]. However, in this study we did not focus on the contribution of these microparticles to TG potential. In this study we have shown that TG potential is dependent on multiple coagulation factors and that lower activity of various coagulation factors can lead to impaired TG. This indicates that TG could help in the identification of patients with a high bleeding risk, along with the assessment of other risk factors that are associated with bleeding such as presented in Table 5.1.

A key strength of the present study is the use of state-of-the-art TG in a real-life setting with prospective documentation of bleeding complications, according to internationally accepted criteria (BARC 2 and 3 bleedings), an important patient-centric outcome with impact on the patients' quality of life. Moreover, we studied high-risk patients receiving DAPT, representing a challenging and complex patient group in whom both bleeding complications and recurrent ischemic events frequently occur. However, several limitations to this study also need to be acknowledged. First, the study population was small, especially in certain subgroups. Nevertheless, the sample size was large enough to find clinically relevant and significant differences between groups. Another limitation is the lost to follow-up of 13 patients within 6 months and missing TG data of 12 patients at T2. However, selection bias is considered unlikely since no relevant differences in baseline characteristics were found between these patients and the included study population (supplementary tables 2 and 3) and no patients were lost to follow-up because of bleeding or death (as checked with their general practitioner and the hospital's electronic medical records). Next, baseline characteristics of the study

population showed (significant) differences in prevalence of anemia, TIA/CVA and malignancy between patients with and without clinically relevant bleeding. Unfortunately, subgroups were too small to perform a multivariate analysis to adjust for these possible confounders. Although anemia at time of PCI was found more frequently in the bleeding group, hemoglobin levels at time of TG measurements were comparable between groups (12.9 g/dL and 13.4 g/dL for bleeders and non-bleeders, respectively, p=0.245). Therefore, it seems unlikely that the hemoglobin-levels have affected the differences in TG parameters between groups. TIA/CVA and malignancy are, amongst others, well-known risk-factors for increased bleeding risk after PCI, as was also stated in the recent Academic Research Consortium for High Bleeding Risk (ARC-HBR) consensus document[43]. Thus, differences between bleeders and non-bleeders in incidence of these factors were to be expected. Previous studies have shown both negative and positive associations between TIA/CVA and TG parameters, making it difficult to speculate about the possible impact on TG parameters of baseline differences in previous stroke[44,45]. Lastly, it would have been interesting to investigate the effect of DAPT on TG in PRP or whole blood as well. Nevertheless, we have deliberately measured TG in PPP, since this is by far the most used and standardized method of TG measurement and offers the opportunity to rule out the possible role of platelets in this setting.

Conclusion

High clinical-risk patients with DAPT with clinically relevant bleeding during follow-up have (significantly) lower ETP levels, peak height and velocity index in PPP compared to patients without bleeding, both at 1 and at 6 months after PCI. We have shown that this might at least be partly explained by variation in coagulation factors. Relatively low thrombin generation in these patients acts as a 'second hit', on top of DAPT, thus increasing the bleeding risk. TG performed in PPP may have potential to aid in the identification of patients with an increased bleeding risk during DAPT. However, more research, with a larger sample size, would be needed to determine cut-off values for TG to be able to adequately identify these patient groups. Furthermore, future research could investigate the capability of other TG based assays to identify patients with increased bleeding risk during DAPT, e.g. the potential benefit of PRP and/or additional measurement of microparticles.

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Appendix 5

Table S5.1 Most severe bleeding event during treatment period at T1, T2 and T3

	T1, number (%)	T2, number (%)	T3, number (%)
No bleeding	74 (79.6%)	71 (76.3%)	53 (77.9%)
BARC 1	12 (12.9%)	19 (20.4%)	11 (16.2%)
BARC 2	6 (6.5%)	3 (3.2%)	4 (5.9%)
BARC 3	1 (1.1%)	0 (0%)	1 (1.5%)
Total (N)	93	93	68

T1=consultation 1 month after PCI, T2=consultation 6 months after PCI, T3=consultation 12 months after PCI, PCI=Percutaneous Coronary Intervention, N=number of patients, BARC=Bleeding Academic Research Consortium.

Table S5.2 Baseline characteristics from patients included in this study (N=93) compared with patients lost to follow-up within 6 months (N=13).

	Patients included in study (N=93) Mean (±SD), number (%)	Patients lost to follow-up <6months (N=13) Mean (±SD), number (%)	Significance (p-value)
Inclusion criteria			
Female gender	49 (52.7%)	6 (46.2%)	0.660
Age >75 years	49 (52.7%)	9 (69.2%)	0.264
Age (years)	72,4 (9.0)	76.4 (11.5)	0.155
Weight <60 kg	6 (6.5%)	1 (7.7%)	0.867
Hypertension	86 (92.5%)	9 (69.2%)	0.010*
Anemia	22 (23.7%)	2 (15.4%)	0.506
eGFR <60 ml/min	51 (54.8%)	3 (23.1%)	0.033*
Renal function (eGFR ml/min)	59.6 (20.1)	68.3 (18.7)	0.145
Known hepatitis or liver transplant	0 (0.0%)	0 (0.0%)	1.000
TIA/CVA in medical history	24 (25.8%)	4 (30.8%)	0.705
Bleeding in medical history	8 (8.6%)	1 (7.7%)	0.913
Diabetes mellitus	32 (34.4%)	7 (53.8%)	0.175
NSAID usage	8 (8.6%)	1 (7.7%)	0.913
SSRI usage	5 (5.4%)	2 (15.4%)	0.176
Gastric ulcer /bleeding in medical history	15 (16.1%)	0 (0%)	0.120
High risk referral	14 (15.1%)	0 (0%)	0.136
Laboratory characteristics			
Hemoglobin(g/dL)	13.2 (1.61)	13.99(1.68)	0.128
Hematocrit (L/L)	0.40 (0.04)	0,42 (0.05)	0.090
Thrombocytes ($x10^9$/L)	252.0 (72.7)	251.1 (41.3)	0.967
Leukocytes ($x10^9$/L)	7.5 (2.05)	7.8 (1.9)	0.631
PT (sec)	10.5 (0.5)	10.6 (0.5)	0.826
aPTT (sec)	26.3 (2.0)	26.1 (2.6)	0.709
Patient characteristics			
Smoking	15 (16.1%)	4 (30.8%)	0.207
≥8 glasses alcohol per week**	10 (10.8%)	3 (23.1%)	0.133
Dyslipidemia	35 (37.6%)	3 (23.1%)	0.308
Malignancy (active)	7 (7.5%)	1 (7.7%)	0.983
Atrial fibrillation	4 (4.3%)	0 (0.0%)	0.448
PPI usage	75 (80.6%)	10 (76.9%)	0.837
ACS as PCI indication	59 (63.4%)	8 (61.5%)	0.895
Interval PCI to T1 in days	54.7 (31.2)	59.5 (39.2)	0.149
Antithrombotics			
Aspirin	93 (100%)	12 (100%)	-
Clopidogrel	69 (74.2%)	12 (92.3%)	0.152
Ticagrelor	5 (5.4%)	1 (7.7%)	0.736
Prasugrel	19 (20.4%)	0 (0.0%)	0.073

** a p value <0.05 was considered significant; ** A cut-off value of ≥8 glasses alcohol per week was chosen based upon the HASBLED bleeding score[46]. N=number of patients, SD=Standard Deviations, BARC=Bleeding Academic Research Consortium, eGFR=estimated Glomerular Filtration Rate, TIA=Transient Ischemic Attack, CVA=cerebral vascular attack, NSAID=Nonsteroidal Anti-Inflammatory Drug, SSRI=Selective Serotonin Reuptake Inhibitor, PT=Prothrombin Time, aPTT=Activated Partial Thromboplastin Time, PPI=Proton Pump Inhibitor, ACS=Acute Coronary Syndrome, PCI=Percutaneous Coronary Intervention, DAPT=Dual Antiplatelet Therapy.*

Table S5.3 Baseline characteristics from patients with TG measured at T1 and T2 compared with patients with missing TG data at T2, both using DAPT >6 months

	Patients with TG at T1 and T2 (N=68) Mean (±SD), number (%)	Patients with missing TG at T2 (N=12) Mean (±SD), number (%)	Significance (p-value)
Inclusion criteria			
Female gender	38 (55.9%)	7 (58.3%)	0.875
Age >75 years	31 (45.6%)	7 (58.3%)	0.418
Age (years)	71.7 (8.1)	71.4 (13.1)	0.910
Weight <60 kg	6 (8.8%)	0 (0.0%)	0.288
Hypertension	64 (94.1%)	12 (100%)	0.392
Anemia	14 (20.6%)	2 (16.7%)	0.756
eGFR <60 ml/min	38 (55.9%)	6 (50.0%)	0.707
Renal function (eGFR ml/min)	61.1 (19.5)	52.8 (24.6)	0.192
Known hepatitis or liver transplant	0 (0.0%)	0 (0.0%)	1.000
TIA/CVA in medical history	15 (22.1%)	4 (33.3%)	0.400
Bleeding in medical history	6 (8.8%)	2 (16.7%)	0.407
Diabetes mellitus	25 (36.8%)	3 (33.3%)	0.821
NSAID usage	6 (8.8%)	1 (8.3%)	0.956
SSRI usage	4 (5.9%)	0 (0.0%)	0.392
Gastric ulcer /bleeding in medical history	13 (19.1%)	1 (8.3%)	0.368
High risk referral	10 (14.7%)	3 (25%)	0.376
Laboratory characteristics			
Hemoglobin(g/dL)	13.36 (1.69)	12.9 (1.42)	0.486
Hematocrit (L/L)	0.40 (0.05)	0,39 (0.04)	0.550
Thrombocytes (x10^9/L)	244.2 (68.2)	293.8 (103.4)	0.134
Leukocytes (x10^9/L)	7.2 (1.8)	8.2 (2.1)	0.121
PT (sec)	10.5 (0.5)	10.7 (0.5)	0.186
aPTT (sec)	26.2 (2.1)	26.5 (1.7)	0.716
Patient characteristics			
Smoking	13 (19.1%)	1 (8.3%)	0.115
≥8 glasses alcohol per week**	6 (8.8%)	1 (8.3%)	0.988
Dyslipidemia	28 (41.2%)	3 (25.0%)	0.292
Malignancy (active)	5 (7.4%)	1 (8.3%)	0.906
Atrial fibrillation	4 (5.9%)	0 (0.0%)	0.392
PPI usage	53 (77.9%)	11 (91.7%)	0.276
ACS as PCI indication	45 (66.2%)	5 (41.7%)	0.108
Interval PCI to T1 in days	51.5 (24.6)	52.3 (26.9)	0.915
Antithrombotics			
Aspirin	68 (100%)	12 (100%)	-
Clopidogrel	50 (73.5%)	7 (58.3%)	0.287
Ticagrelor	4 (5.9%)	1 (8.3%)	0.748
Prasugrel	14 (20.6%)	4 (33.3%)	0.333

** a p value <0.05 was considered significant; ** A cut-off value of ≥8 glasses alcohol per week was chosen based upon the HASBLED bleeding score[44]. N=number of patients, SD=Standard Deviations, BARC=Bleeding Academic Research Consortium, eGFR=estimated Glomerular Filtration Rate, TIA=Transient Ischemic Attack, CVA=cerebral vascular attack, NSAID=Nonsteroidal Anti-Inflammatory Drug, SSRI=Selective Serotonin Reuptake Inhibitor, PT=Prothrombin Time, aPTT=Activated Partial Thromboplastin Time, PPI=Proton Pump Inhibitor, ACS=Acute Coronary Syndrome, PCI=Percutaneous Coronary Intervention, DAPT=Dual Antiplatelet Therapy.*

Chapter 6

Rotational thromboelastometry in high-risk patients on dual antithrombotic therapy after percutaneous coronary intervention

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Abstract

Aims

Patients using antithrombotic drugs after percutaneous coronary intervention (PCI) are at risk for bleeding and recurrent ischemia. We aimed to explore routine and tissue plasminogen activated (tPA) ROTEM results in a post-PCI population on dual antithrombotic treatment.

Methods and results

In this prospective cohort, 440 patients treated with double antithrombotic therapy after recent PCI and with ≥3 risk factors for either ischemic or bleeding complications were included and compared with a control group (n=95) consisting of perioperative patients not using antithrombotic medication. Laboratory assessment, including (tPA) ROTEM, was performed one month post-PCI and bleeding/ischemic complications were collected over a five-month follow-up. Patients were stratified by antithrombotic regimen consisting of a P2Y12 inhibitor with either aspirin (dual antiplatelet therapy; DAPT, n=323), a vitamin K antagonist (VKA, n=69) or a direct oral anticoagulant (DOAC, n=48). All post-PCI patients had elevated ROTEM clot stiffness values, but only the DAPT group additionally presented with a decreased fibrinolytic potential as measured with tPA ROTEM. Patients receiving anticoagulants had prolonged clotting times (CT) when compared to the control and DAPT group; EXTEM and FIBTEM CT could best discriminate between patients (not) using anticoagulants (AUC>0.97). Furthermore, EXTEM CT was significantly prolonged in DAPT patients with bleeding complications during follow-up (68 [62-70] vs. 62 [57-68], p=0.030).

Conclusion

ROTEM CT has high potential for identifying anticoagulants and tPA ROTEM could detect a diminished fibrinolytic potential in patients using DAPT. Furthermore, the ability of EXTEM CT to identify patients at risk for bleeding may be promising and warrants further research.

Introduction

Patients with coronary artery disease (CAD) undergoing percutaneous coronary intervention (PCI) are generally prescribed dual antiplatelet therapy (DAPT), consisting of aspirin and a P2Y12 inhibitor (P2Y12i), for 6-12 months to prevent recurrent atherothrombotic events[1,2]. In patients with comorbidities, such as atrial fibrillation or a mechanic valve, the P2Y12 inhibitor is often combined with an anticoagulant[3,4]. A delicate balance between limiting ischemic risk while preventing bleeding emerges in patients on antithrombotic treatment. Nowadays, physicians can choose from multiple antithrombotic treatment regimens including the more potent P2Y12 inhibitors prasugrel[5] and ticagrelor[6] next to clopidogrel, and the widespread availability of direct oral anticoagulants (DOACs) in addition to vitamin K antagonists (VKA). However, patients with comorbidities, or with recurring ischemic or bleeding events remain a challenging group that often require individualized treatment strategies. Risk factors for recurrent ischemic and bleeding events show considerable overlap, further complicating prediction and subsequently prevention of these adverse events. International guidelines therefore recommend individual assessment of benefit/risk ratios in these high-risk patients[2,7]. Individual benefit/risk evaluation in the form of monitoring multiple antithrombotic drugs and identifying patients at risk for ischemic and/or bleeding events remains a major challenge in clinical practice. Hemostasis tests could potentially characterize patients with either hemostatic abnormalities predisposing for bleeding events, or with a more prothrombotic phenotype leading to recurrent ischemic events despite antithrombotic therapy.

Hemostasis tests and platelet function tests (PFTs) have multiple limitations when monitoring patients on antithrombotic therapy. First, most laboratory assays developed to monitor antithrombotic drugs assess one specific hemostasis pathway. Clear examples are the direct thrombin inhibitors (dabigatran) and factor (F)Xa-inhibitors (rivaroxaban, apixaban, edoxaban), which concentrations are monitored by using the diluted thrombin time and the anti-Xa assay, respectively[8]. Alternatively, residual platelet reactivity in patients on antiplatelet drugs (e.g. P2Y12 inhibitor) can be measured using platelet function assays such as Light Transmission Aggregometry (LTA), Platelet Function Analyzer (PFA), VerifyNow and Multiplate[9]. Though high and low on-treatment platelet reactivity are associated with recurrent ischemia and bleeding, respectively, clinical risk prediction and subsequent treatment modification in a real-life setting remains suboptimal[10,11]. Second, PFTs and routine hemostatic assays do not evaluate the fibrinolytic properties in a patient. Reduced susceptibility to fibrinolysis was recognized as an independent risk factor for recurrent ischemia in patients recovering from acute CAD[12,13]. Hence, a whole blood assay to quickly identify

patients with diminished fibrinolytic potential might be of interest for more accurate risk assessment in patients post-PCI.

No global assays of hemostasis are currently implemented in drug monitoring guidelines to evaluate the overall effect of antithrombotic treatment strategies and potential additional hemostatic or fibrinolytic abnormalities. Rotational thromboelastometry (ROTEM) is a whole blood point-of-care (POC) viscoelastic assay that provides a quick overview of global hemostatic parameters. It is currently recommended to guide blood transfusion in cardiovascular surgery, trauma care and post-partum hemorrhage[14]. In addition to its applicability in transfusion management, new areas of interest are explored including characterizing septic coagulopathy and detecting the presence of antithrombotic drugs[15,16]. An additional tissue plasminogen activator (tPA) based ROTEM assay was recently developed to assess the dynamic properties of fibrinolysis after clot formation[17]. These characteristics highlight the potential of ROTEM for hemostasis (and fibrinolysis) monitoring in post-PCI patients on antithrombotic drugs.

The primary aims of the current paper are 1) to evaluate (tPA) ROTEM results in vulnerable post-PCI patients on dual antithrombotic treatment, including the potential value of (tPA) ROTEM to identify the presence of anticoagulants and antiplatelet drugs, and 2) to evaluate the prognostic value of (tPA) ROTEM regarding the identification of post-PCI patients on DAPT at risk for bleeding or recurrent ischemic events.

Methods

Study population

Antiplatelet therapy outpatient clinic

The primary cohort study design has been described more extensively elsewhere[18]. High clinical-risk patients using dual antithrombotic medication after elective or emergency PCI between April 2014 and January 2019 at Maastricht University Medical Centre+ (MUMC+) were included in this study. Vulnerable patients were referred to a specialized outpatient clinic within the Thrombosis Expertise Centre by one dedicated interventional cardiologist for assessment of on-treatment bleeding and ischemic risks. High clinical-risk patients were defined as patients who had ≥3 risk factors for bleeding or ischemic events, which include: old age (≥75 years), female gender, renal dysfunction (estimated Glomerular Filtration Ratio (eGFR) <60 mL/min), anemia at the time of PCI (<13.2 g/dL for men and <11.8 g/dL for women), low body weight (<60 kg),

hypertension, diabetes mellitus, previous stroke, previous major bleeding, liver dysfunction, history of gastric/duodenal ulcers, previous in-stent thrombosis, high-risk stenting (multivessel PCI or main coronary artery thrombosis), daily use of non-steroidal anti-inflammatory drugs (NSAIDs) and/or use of serotonin reuptake inhibitors (SSRIs). Exclusion criteria for the general cohort were known platelet function disorders, coronary intervention or new ischemic event within 7 days before inclusion, signs of active infection during the visit to the outpatient department, non-compliance and withdrawal of informed consent. Additional exclusion criteria for the current analyses were no dual antithrombotic treatment at the time of ROTEM measurement and no ROTEM performed.

Dual antithrombotic treatment consisted of a P2Y12 inhibitor (clopidogrel, prasugrel, ticagrelor) in combination with either aspirin (DAPT) or an anticoagulant (VKA or DOAC). At the first outpatient visit approximately 1 month post PCI (T1) the clinician recollected medical history, bleeding/ischemic events and performed a medication check. Blood was drawn 3-8 hours after last drug intake for laboratory assessment, including ROTEM. Patients were stratified in different antithrombotic treatment groups based on the treatment regimen at T1. At a second outpatient visit 6 months post PCI (T2) the occurrence of ischemic and/or bleeding events was re-evaluated. This study was approved by the medical ethical committee of the MUMC+ (NL38767.068.11, METC number 11-2-096) and written informed consent was obtained from all patients.

Control group

The control group consists of a random sample of preoperative patients (n=95) not using antithrombotic medication. The control group consisted of a subgroup from the study by Vries et al. defined as ‘patients not reporting bleeding symptoms’ (19). Here, preoperative patients admitted at the MUMC+ for any elective surgery were included between September 2013 and January 2016. Exclusion criteria were age ≤18, known bleeding disorders, antithrombotic drug use, NSAID use, platelet count <100.000/mL, anemia, pregnancy or a positive anesthesiology bleeding questionnaire. Blood was withdrawn during the post-operative study visit where (tPA) ROTEM, complete blood count and renal function were determined among other things.

Laboratory

Blood was collected in a 3.2% sodium-citrate tube and all laboratory tests were performed within 4 hours after blood collection. Platelet poor plasma (PPP) was obtained by centrifugation at 2500 g for 5 minutes, followed by centrifugation at 10000 g for 10 minutes at 18°C.

Rotational thromboelastometry

ROTEM is a whole blood assay that measures changes in viscoelastic properties during clot formation. In the current study, EXTEM, INTEM, FIBTEM and tPA ROTEM assays were performed on a ROTEM delta (Werfen; Barcelona, Spain). EXTEM and FIBTEM clot formation is triggered by tissue factor (extrinsic coagulation pathway), with FIBTEM containing additional cytocholasin D to eliminate platelet function. The INTEM assay is activated by kaolin and illustrates the intrinsic coagulation pathway. The following standard ROTEM parameters were analyzed: CT (clotting time in seconds), A5 (amplitude at 5 minutes in mm), A10 (amplitude at 10 min in mm), CFT (clot formation time in seconds), MCF (maximum clot firmness in mm), alpha angle (the angle between the middle axis and the tangent to the clotting curve through the 2 mm amplitude point), Ly30 (percentage lysis of MCF at 30 minutes in %), Ly45 (percentage lysis of MCF at 45 minutes in %) and Ly60 (percentage lysis of MCF at 60 minutes in %).

TPA ROTEM is an EXTEM-like assay where recombinant tissue plasminogen activator (r-tPA) is added to evaluate the fibrinolytic properties of a clot. The assay was previously validated by Kuiper et al. (20). In short, 125 ng/mL tPA was added in addition to 35 pM TF to induce clot formation and breakdown simultaneously. Additional ROTEM parameters specific to the tPA assay include LOT (lysis onset time; time from CT until a 15% drop in MCF) and LT (lysis time; time from CT until a 90% drop in MCF). If LOT and/or LT were not reached within 2 hours due to limited clot breakdown, results were capped at 7200 seconds.

Other laboratory parameters

Fibrinogen level (Clauss method, Thrombin reagent, Siemens), PT (Innovin Pt, Siemens) and activated partial thromboplastin time (aPTT; Actine FSL, Siemens) were determined in PPP on a Sysmex CS2100i.

Clinical outcomes

We aimed to explore whether ROTEM parameters at T1 could identify patients on DAPT at risk for developing clinically relevant bleeding or major adverse cardiovascular events (MACE) until the 6-month follow-up (T2) and compared them with routine hemostasis assays (PT, aPTT, fibrinogen and platelet count). The VKA+P2Y12i and DOAC+P2Y12i groups were not analyzed for clinical outcomes due to the limited number of patients available. Bleeding events were recorded using the Bleeding Academic Research Consortium (BARC) criteria, which contains unified and validated bleeding criteria[21,22]. Clinically relevant bleeding was defined as BARC type ≥2. In addition, recurrent ischemic events were recorded during the follow-up visits and

MACE was defined as myocardial infarction, stroke, and all-cause mortality. Both bleeding and ischemic endpoints were assessed at 1 month (T1) and 6 months (T2) after PCI. The analysis was performed in patients treated with DAPT for ≥6 months. Exclusion criteria were clinically relevant bleeding (BARC type ≥2) or MACE prior to ROTEM measurement at T1, switch to an oral anticoagulant (OAC) before T2 and no T2 follow-up.

Statistical analysis

The statistical analyses were performed in IBM SPSS Statistics 25.0 for Windows and figures were plotted in GraphPad Prism for Windows unless stated otherwise. Normality was determined by visual assessment and the Shapiro-Wilk test. Normally distributed continuous variables are presented as mean with standard deviation (SD) and non-parametric data are presented as median with interquartile range (IQR). At baseline, statistical significance was determined between the control vs post-PCI group and within the different medication groups using Mann-Whitney U, Kruskal-Wallis and chi-squared test as appropriate. The Kruskal-Wallis test was applied to evaluate differences in ROTEM parameters between antithrombotic treatment groups. When significant ($p<0.05$), follow-up Mann-Whitney U tests were used for pairwise comparisons. To adjust for multiple testing, a Bonferroni correction was applied and effects are reported at a (0.05/6=) 0.008 level of significance.

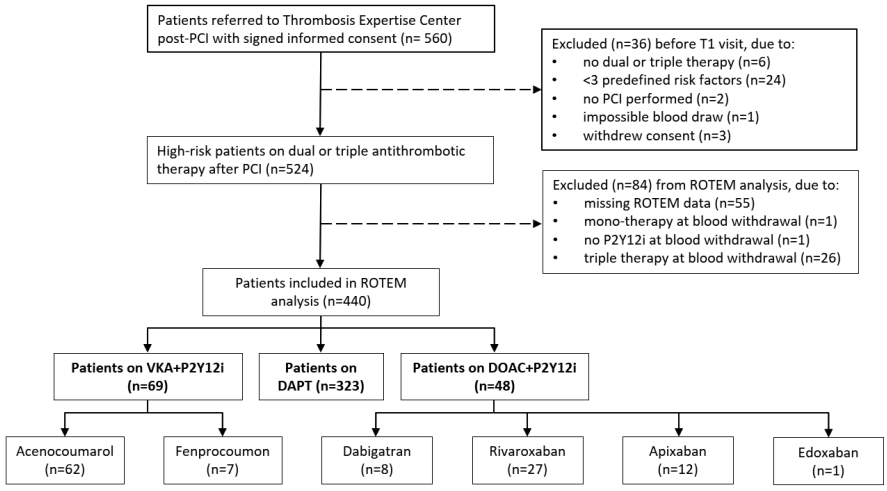
The discriminating ability of ROTEM parameters and routine hemostasis assays (PT and aPTT) to identify patients on anticoagulant treatment (VKA + DOAC, VKA separately, and DOAC separately) was assessed using receiver operating characteristics (ROC). Both the control group and patients on DAPT were included as patients not receiving anticoagulant treatment. The Youden index was calculated (J = sensitivity + specificity - 1) to determine the cut-off value for maximized sensitivity and specificity. The area under the curve (AUC) was compared using DeLong test in R. In an attempt to evaluate the prognostic value of ROTEM, the Mann-Whitney U test was used for comparison of T1 ROTEM parameters between patients with and without clinically relevant bleeding or MACE until T2. In cases where significant differences were achieved ($p<0.05$), discriminative performance was further assessed by ROC analysis.

Results

Study population

The initial cohort consisted of 560 high-risk patients undergoing PCI at the MUMC+ between May 2014 and May 2019[18]. As illustrated in Figure 6.1, 440 patients were suitable for analysis. Demographics, baseline characteristics and routine laboratory values of the antithrombotic treatment and control groups are presented in Table 6.1. In general, the control group appears younger and with fewer comorbidities (e.g. diabetes) compared to the post-PCI population. Patients on anticoagulants often had a medical history with atrial fibrillation and both PT and aPTT were generally prolonged in patients receiving a DOAC or VKA. Most patients received DAPT (n=323, 73.4%), followed by VKA in combination with a P2Y12i (VKA+P2Y12i; n=69, 15.7%) and DOAC in combination with a P2Y12i (DOAC+P2Y12i; n=48, 11.1%). In the VKA+P2Y12i and DOAC+P2Y12i group 12 (17.4%) and 14 (28.6%) patients started with triple antithrombotic therapy, respectively, which was converted to dual antithrombotic treatment prior to the first outpatient clinic visit (T1). Patients were seen at T1 after a median [IQR] of 45 [36-56] days post-PCI.

Figure 6.1 Flow diagram of study inclusion and stratification in treatment groups.



DAPT: dual antiplatelet therapy consisting of aspirin and a P2Y12-inhibitor (P2Y12i), DOAC: direct oral anticoagulant, PCI: percutaneous coronary intervention, ROTEM: rotational thromboelastometry, VKA: vitamin K antagonist.

Table 6.1 Demographics, baseline characteristics and routine laboratory values of the control, DAPT, VKA+P2Y12i and DOAC+P2Y12i groups.

Mean (st. dev.) Median [IQR] n (%)	Control n=95	DAPT n=323	VKA + P2Y12i n=69	DOAC + P2Y12i n=48	p-value: control vs. post-PCI patients	p-value: medication groups
Patient characteristics						
Age (years)	57 [47-67]	76 [69-81]	78 [72-80]	75 [71-80.75]	0.000	0.552
Gender (man)	53 (55.8)	165 (51.1)	50 (72.5)	32 (66.7)	0.951	0.002
BMI$^{\phi}$	26.0 [24.1-28.4]	26.6 [24.1-30.0]	27.6 [24.7-30.3]	26.9 [23.8-30.0]	0.065	0.758
Smoking (yes)$^{\phi}$	22 (23.2)	45 (14.0)	7 (10.3)	7 (14.6)	0.018	0.697
Diabetes (yes)	5 (5.3)	120 (37.2)	23 (33.3)	15 (31.3)	0.000	0.648
Hypertension (yes)	ND	274 (84.8)	58 (84.1)	41 (85.4)		0.978
Previous stroke (CVA and/or TIA)$^{\phi}$	ND	80 (24.8)	22 (31.9)	15 (31.3)		0.364
Previous PCI	ND	121 (37.5)	26 (37.7)	20 (41.7)		0.854
Atrial fibrillation in medical history	ND	7 (2.2)	52 (75.4)	45 (93.8)		0.000
Laboratory values						
Platelets (10^9/L)	259 [226-299]	252 [214-296]	245 [195-303]	248.0 [212.75-311.5]	0.286	0.542
Leukocytes (10^9/L)	6.9 [5.6-8.1]	7.6 [6.4-9.3]	7.3 [6.7-8.6]	7.50 [6.3-8.4]	0.000	0.595
Hematocrit (L/L)	0.43 [0.41-0.45]	0.40 [0.37-0.43]	0.41 [0.37-0.44]	0.41 [0.38-0.44]	0.000	0.426
Hemoglobin (mmol/L)	9.0 (0.8)	8.2 (1.0)	8.3 (1.1)	8.2 (1.1)	0.000	0.737
PT (s)$^{\phi}$	10.3 [10.1-10.5]	10.6 [10.3-11.0]	25.5 [21.4-32.0]	12.7 [11.4-14.4]	0.000	0.000
aPTT (s)$^{\phi}$	26 [25-27]	26.0 [25-27]	36 [34-38]	33.0 [29-36.75]	0.000	0.000
Fibrinogen (g/L)$^{\phi}$	3.2 [2.8-3.6]	3.7 [3.1-4.2]	3.8 [3.3-4.2]	3.5 [3.0-4.0]	0.000	0.453
P2Y12 inhibitor						
Clopidogrel		218 (67.5)	68 (98.6)	43 (89.6)		0.000
Prasugrel		53 (16.4)	1 (1.4)	1 (2.1)		
Ticagrelor		52 (16.1)	0 (0)	4 (8.3)		
Other antithrombotic medication						
Acenocoumarol			62 (89.9)			
Fenprocoumon			7 (10.1)			
Rivaroxaban				27 (56.3)		
Dabigatran				8 (16.7)		
Apixaban				12 (25.0)		
Edoxaban				1 (2.1)		
Index PCI						
Acute		219 (67.8)	34 (49.3)	31 (64.6)		0.014
Elective		104 (32.2)	35 (50.7)	17 (35.4)		
Days between ROTEM and PCI		46 (36-56)	47 [36-60]	44 [37.25-53.75]		0.824

Patient characteristics and laboratory values as presented at the outpatient department of vascular medicine one month post-PCI visit (T1; DAPT, VKA + P2Y12i, DOAC + P2Y12i groups) and the post-operative study visit (control group). PCI: percutaneous coronary intervention, BMI: body mass index, SD: standard deviation, IQR: interquartile range, PT: prothrombin time, aPTT: activated partial thromboplastin time, ND: not determined. $^{\phi}$ *Data are missing for BMI (DAPT n=6), smoking (DAPT n=2, VKA+P2Y12i n=1), previous stroke (CVA and/or TIA) (DAPT n=1), PT/aPTT/Fibrinogen (control n=1, DAPT n=2).*

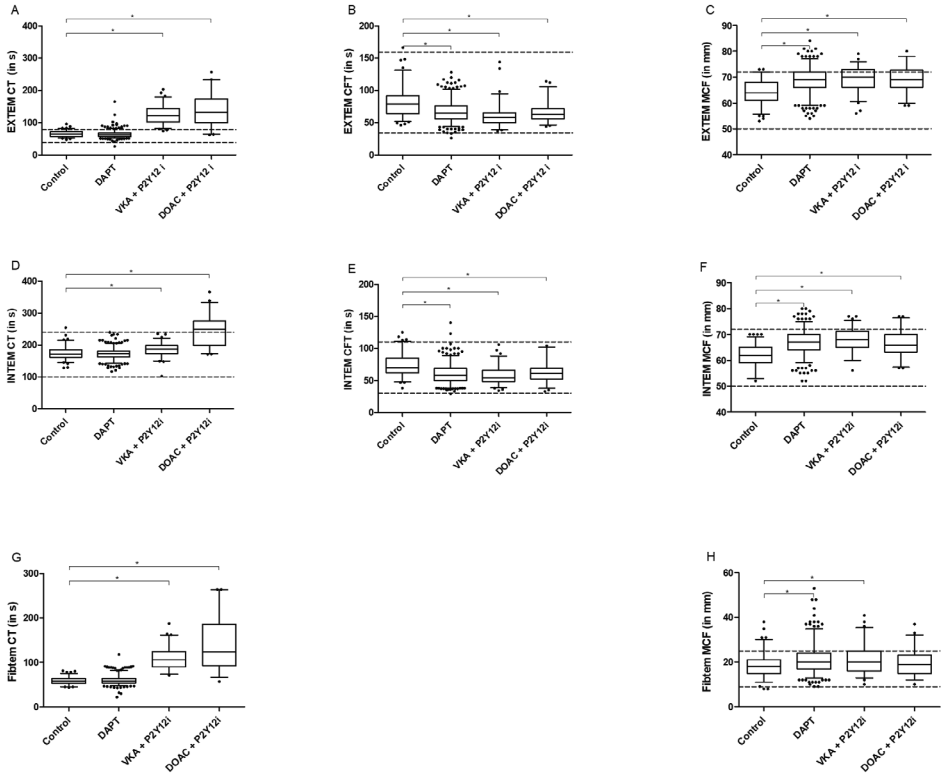
Rotational thromboelastometry

All ROTEM assays showed significantly prolonged CT in patients receiving anticoagulants (VKA+P2Y12i and DOAC+P2Y12i) when compared to the control group and patients receiving DAPT (Figure 6.2-A/D/G, Supplementary Table S6.1). Almost all patients on anticoagulants exceeded the manufacturer's reference range for EXTEM CT. In general, CFT was shorter and A5, A10, and MCF were increased in post-PCI patients compared to the control group, irrespective of antithrombotic treatment in both the EXTEM and INTEM ROTEM assays (Figure 6.2-C/F). The effect was less pronounced for the FIBTEM assay, as clot stiffness parameters were not consistently increased in all post-PCI antithrombotic treatment groups when compared to control (Supplementary Table S6.1). Furthermore, fibrinogen was elevated and PT was prolonged in post-PCI patients, though the DAPT group showed limited PT prolongation compared to control (10.6 [10.3-11.0] vs. 10.3 [10.1-10.5]; $p<0.001$, Supplementary Figure S6.1A). Fibrinolysis parameters (LOT and LT) were significantly prolonged in DAPT patients compared to the control population. However, VKA and DOAC +P2Y12i did not show a similar profile and the previously determined reference ranges for LOT and LT were generally not exceeded in any group (Figure 6.3-B/C).

ROC-analysis revealed that EXTEM and FIBTEM CT had good discriminating capacity to detect the presence of anticoagulants with an AUC of 0.979 and 0.978, respectively (Table 6.2). However, both assays did not perform significantly better than the routine PT. INTEM and tPA CT had poorer discriminating ability compared to PT, evidenced by the significantly lower AUC ($p<0.001$). When analyzed separately, PT outperformed all ROTEM CT assays in the detection of patients on VKA+P2Y12i treatment (Supplementary Table S6.2). Furthermore, the discriminating capacity to detect the presence of DOACs was similar for PT and EXTEM CT with an AUC of 0.929 and 0.963, respectively (Supplementary Table S6.3). However, EXTEM CT in the DOAC+P2Y12i group prolonged relatively more compared to control (Figure 6.2A) than the PT (Supplementary Figure 6.1A).

In summary, ROTEM CT was significantly prolonged in patients using anticoagulants and, though not outperforming PT, EXTEM CT had good discriminating ability to detect the presence of both VKAs and DOACs. Additionally, ROTEM clot firmness was generally increased in all post-PCI patients and ROTEM LOT and LT demonstrated diminished fibrinolysis in the DAPT group only.

Figure 6.2 EXTEM, INTEM and FIBTEM results for clotting time (CT), clot formation time (CFT) and maximum clot firmness (MCF).



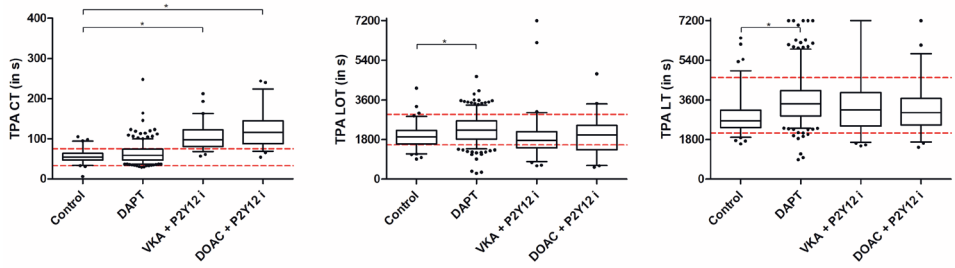
Presented are median, IQR and 5-95 percentile whiskers. Dashed lines illustrate reference ranges according to the manufacturer. Significant differences (p<0.008) compared to the control group are reported with an asterisk.

Table 6.2 ROC analysis for anticoagulant presence.

	AUC	Youden's index	Sensitivity	Specificity	p-value
PT*	0.971	11.35	92.3%	90.8%	Reference
aPTT*	0.965	28.5	92.3%	89.2%	0.48
EXTEM CT	0.979	80.5	94.9%	94.5%	0.39
INTEM CT	0.791	180.5	71.8%	73.2%	<0.001
FIBTEM CT	0.978	74.5	90.6%	92.1%	0.47
tPA CT ᵗ	0.916	78.5	82.1%	83.2%	<0.001

**PT and aPTT missing for 3 patients who did not receive anticoagulant treatment; ᵗ tPA CT missing for 1 patient who did not receive anticoagulant treatment.*

Figure 6.3 Clotting time (CT), lysis onset time (LOT) and lysis time (LT) tissue plasminogen activator (tPA) ROTEM.



Presented are median, IQR and 5-95 percentile whiskers. Dashed lines illustrate tPA ROTEM reference range as determined by Kuiper et al.[20] Significant differences (p<0.008) compared to the control group are reported with an asterisk.

Clinical outcomes in patients using DAPT

The current analysis was performed on patients using DAPT only (n=323). Patients were excluded from the current analysis due to clinically relevant bleeding or MACE prior to ROTEM measurement at T1 (n=28), switch to an oral anticoagulant before T2 (n=3), DAPT<6 months (n=32), and no T2 follow-up (n=33) (several patients presented with >1 exclusion criteria). Thus, 252 patients (78%) were eligible for follow-up analysis of clinically relevant bleeding or MACE. The second outpatient visit (T2) took place 154 [146-168] days after T1. Thirteen patients (5.2%) developed MACE during T1-T2 follow-up (non-STEMI; n=4, CVA/TIA; n=2, all-cause death; n=7) . Neither ROTEM parameters nor routine hemostasis assays at T1 significantly differed between patients with and without MACE. Thirteen patients (5.2%) developed clinically relevant bleeding between T1 and T2 (BARC 2; n=10, BARC 3; n=3). Statistical significance between patients with and without clinically relevant bleeding was achieved for EXTEM CT (68 [62-70] vs. 62 [57-68], p=0.030). Routine hemostasis assays (PT, aPTT, fibrinogen and platelet count) were unable to discern patients at risk for clinically relevant bleeding. ROC analysis revealed a moderate discriminative ability of EXTEM CT to detect clinically relevant bleeding during T1-T2 follow-up, with an AUC of 0.679 (p=0.030). ROTEM values and routine hemostasis assays stratified by bleeding and MACE are presented in Supplementary Table S6.4 and S6.5, respectively.

Discussion

The current study evaluated routine and tPA ROTEM results in a vulnerable post-PCI population on dual antithrombotic treatment and yielded four main findings: 1) EXTEM

CT had good discriminating capacity for the detection of anticoagulants, though not outperforming PT. 2) All post-PCI patients, irrespective of antithrombotic treatment, had increased clot formation as evidenced by shortened CFT and increased MCF one month after intervention. 3) Fibrinolysis was diminished in patients on DAPT treatment, but not in the anticoagulant treatment groups. 4) EXTEM CT could discriminate between patients with and without clinically relevant bleeding (BARC≥2) over a five-month follow-up in the DAPT treatment group.

We assessed whether ROTEM could identify patients using antithrombotic treatment. Currently, there is an unmet clinical need for a global screening assay, such as ROTEM, to identify the presence of antithrombotic medication in emergency situations, such as severe trauma, massive hemorrhage, stroke, and urgent surgery. An unknown unconscious or delusional patient, unavailable for anamnestic evaluation, could present with severe (i.e. intracranial) bleeding or could be awaiting thrombolytic therapy in stroke whilst under antithrombotic treatment. It is essential to screen for the presence of these drugs to provide appropriate hemostatic interventions when required. The ideal global screening assay should be able to detect clinically relevant levels of antithrombotic drugs, have a short turn-around time, and be available on-demand. Our results showed clear ROTEM CT prolongation in the presence of anticoagulants, specifically in the EXTEM, FIBTEM and tPA assays. Contrarily, ROTEM CT was similar between the control group and patients receiving antiplatelet medication only in the DAPT group. In line with our observation that ROTEM is unable to identify antiplatelet drugs, the general insensitivity of ROTEM to detect platelet function was previously reported[17]. Another study in patients receiving DAPT (n=78) did show a significant EXTEM and INTEM CT prolongation when compared to healthy controls[23]. However, there was evident overlap between the DAPT and control group in CT values and, thus, ROTEM would still be considered unsuitable to identify patients using antiplatelet drugs. Similarly, no differences in ROTEM parameters were observed between patients using 75mg vs. 150 mg clopidogrel one month after PCI[24]. A novel ROTEM assay, ROTEM platelet impedance aggregometry (ROTEM-PLT), has recently become available to evaluate antiplatelet therapy in patients[25]. However, this novel ROTEM platelet assay was not evaluated in the current study. As previously acknowledged, the effect of anticoagulants was clearly evidenced by the CT prolongation in all ROTEM assays and subsequent ROC analysis revealed that EXTEM and FIBTEM CT had excellent discriminative ability between patients that do (not) receive anticoagulants. In vitro spiking studies demonstrated a DOAC dose-response effect in ROTEM CT, which was most pronounced for the EXTEM assay[26]. Similar effects were observed in vivo in patients treated with a DOAC or VKA[23,27-30], hinting that ROTEM may be suitable to

monitor anticoagulant drug concentrations. Although EXTEM CT had almost perfect discriminatory ability in the current setting, the observed AUC (0.979) did not outperform the PT assay (Table 6.2). However, ROTEM's whole blood, on-demand availability and other POC characteristics make it more suitable in emergency situations. Therefore, ROTEM analysis might be convenient in an unknown, unconscious patient to quickly determine the presence of anticoagulants. Specifically for the detection of DOACs in acute situations, as no POC-assay is currently available for this purpose.

Post-PCI patients generally presented with increased clot stiffness one month after the intervention irrespective of antithrombotic treatment, illustrated by the shorter CFT and higher MCF values compared to control. This suggests a more universal pathology underlying increased clot stiffness in patients requiring PCI rather than an effect of antithrombotic treatment. For correct interpretation of these results, it is essential to realize that ROTEM clot stiffness (partly) depends on fibrinogen concentration. The fibrinogen concentration was elevated in all post-PCI treatment groups (Supplementary Figure S6.1), thus possibly explaining the rise in MCF. However, the lack of consistent FIBTEM clot stiffness increase, which is most dependent on fibrinogen due to cytochalasin D platelet inhibition, may point to an alternative mechanism. In recent years the association between clot architecture pathophysiology and clinical phenotypes has been reviewed in literature. It was established that patients with (recurrent) thrombosis, such as CAD and stroke, demonstrate abnormal clot architecture[31-33]. Specifically, patients with thrombotic complications form dense fibrin networks with increased stiffness and limited permeability. It has been suggested that ROTEM could detect such subtle differences in clot architecture. Two studies that evaluated ROTEM clot stiffness in plasma with different fibrinogen entities and a fibrinogen cross-linking polymer support that ROTEM is susceptible to changes in clot architecture[34,35]. The rise in clot stiffness in post-PCI patients compared to controls may therefore originate from both the elevated fibrinogen concentration and the abnormal clot architecture associated with CAD.

The described clot abnormalities in patients with thrombosis, higher density and reduced permeability, predispose to limited clot breakdown as the formed fibrin fibers are less accessible for tPA-mediated fibrinolysis[36,37]. Additionally, patients with thrombosis show increased levels of plasminogen activator inhibitor-1 (PAI-1)[31,38]. Over the last two decades strong connections between abnormal fibrin structures, fibrinolysis and thrombotic complications were identified[31-33,36,39]. Farag et al. demonstrated that POC measurement of fibrinolysis (Global Thrombosis Test) could aid

in identifying STEMI patients undergoing PCI at risk for recurrent thrombosis[12]. Similarly, Sumaya et al. evaluated fibrinolysis in acute coronary syndrome patients with a turbidimetric assay and found that diminished fibrinolysis was associated with myocardial infarction, cardiovascular death, and all-cause death[13]. Our tPA ROTEM did not illustrate a similar predictive ability for MACE and no significant difference in fibrinolysis parameters between the control, VKA+P2Y12i and DOAC+P2Y12i groups was observed. However, the DAPT patient group had significantly prolonged LOT and LT reflecting a decreased fibrinolytic potential. Of note, patients included in the studies of Farag and Sumaya et al. received no anticoagulants at blood withdrawal. Therefore, the lack of LOT and LT prolongation in our anticoagulant treatment groups may arise from the effect of DOACs and VKA on fibrinolysis. Indeed, DOACs enhance fibrinolysis by (indirectly) inhibiting thrombin and, consequently, thrombin-activatable fibrinolysis inhibitor (TAFI)[40-43]. Additionally, the FXa inhibitors apixaban and rivaroxaban enable the tPA cofactor function of a pro-fibrinolytic FXa breakdown product[44]. Finally, all anticoagulants limit thrombin activity, increase clot permeability and, thereby, make fibrin fibers more available for degradation[45,46]. Taken together, the similar tPA ROTEM LOT and LT values in the anticoagulant therapy groups (DOAC+P2Y12i and VKA+P2Y12i) as in the control patients might be explained by the described anticoagulant-mediated pro-fibrinolytic mechanism that overrules any potential natural hypofibrinolytic phenotype in these patients. A limitation to our study is that no sample prior to the treatment start was obtained to distinguish between the native hemostasis profile and the effect of the antithrombotic drugs on an individual level.

To the best of our knowledge, there are no studies available that evaluated the long-term potential of ROTEM to predict bleeding. We found a predictive ability of EXTEM CT for the development of clinically relevant bleeding over five months in post-PCI patients on DAPT treatment. EXTEM CT was significantly prolonged in patients with clinically relevant bleeding when compared to non-bleeders. Furthermore, ROC analysis illustrated a moderate discriminative ability with an AUC of 0.679. FIBTEM and tPA CT showed a similar trend, suggesting that use of an extrinsic activator (tissue factor) reveals the bleeding phenotype in these patients. However, since routine hemostasis assays, including tissue factor activated PT, did not differ between bleeders and non-bleeders, this aspect seems unique to ROTEM assays. This might introduce a novel application of viscoelastic testing to better identify patients at risk for bleeding in addition to the 'classic' risk factors. However, results should be interpreted with care as only 13 patients developed clinically relevant bleeding. Additionally, VKA+P2Y12i and DOAC+P2Y12i medication groups were not evaluated due to the limited number of

patients. Larger studies evaluating the ability of ROTEM to predict long-term bleeding complications in patients on antithrombotic medication are therefore required.

Our study has several limitations. First, a control group of similar age and comorbidities without any antithrombotic medication was unavailable for analysis. Second, though patient compliance was checked verbally and with the pharmacy, correct intake of antithrombotic drugs prior to the blood withdrawal cannot be confirmed. Third, patients received their antithrombotic treatment strategy based on clinical risk factors, e.g. patients with atrial fibrillation receive an oral anticoagulant, introducing classification bias inherent to the real-world setting. Fourth, patients who developed bleeding complications and/or MACE in the first month prior to T1 could not be included in the follow-up analysis. Since most events occurred during the first month post-PCI earlier ROTEM assessment may yield valuable results in future studies. Furthermore, a longer follow-up (≥1 year) and inclusion of lower clinical risk patients would be of interest in future studies. Nonetheless, this is the first ROTEM study performed in a large post-PCI high-risk cohort with long-term follow-up of ischemic and bleeding complications.

In conclusion, ROTEM has high potential for identifying the presence of anticoagulants in acute situations. Furthermore, tPA ROTEM did illustrate diminished fibrinolysis in CAD patients receiving DAPT, but no association with clinical outcomes was observed. We did observe a moderate predictive ability of EXTEM CT to identify patients at risk for clinically relevant bleeding, which may be of interest to help guide bleeding risk assessment in patients post-PCI. These findings could serve as a stepping stone for further exploration of ROTEM in emergency situations and long-term risk assessment.

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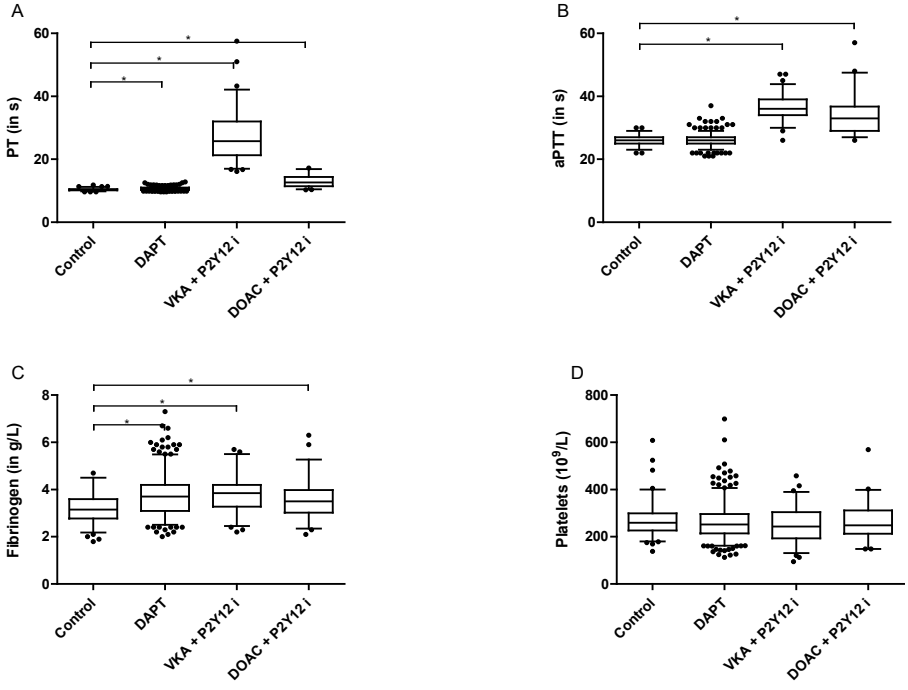
Supplementary material

Supplementary figure

Figure S6.1 Prothrombin time (PT; A), activated partial thromboplastin time (aPTT; B), fibrinogen (C) and platelet count (D).



Presented are median, IQR and 5-95 percentile whiskers. Significant differences (p<0.008) compared to the control group are reported with an asterisk.

Supplementary Tables

Table S6.1 EXTEM, INTEM, FIBTEM and tPA ROTEM parameters in the control, DAPT, P2Y12i+VKA and P2Y12i+DOAC groups.

Median [IQR]	Control group (n=95)*	DAPT (n=323)**	P2Y12i + VKA (n=69)ϕ	P2Y12i + DOAC (n=48)ϕϕ
EXTEM				
CT	65 [58-71]	63 [57-68]	122 [103-144] [a,b]	132 [100-173] [a,b]
A5	47 [43-52]	53 [49-58] [a]	56 [52-60] [a,b]	55 [51-59] [a]
A10	57 [54-61]	62 [59-67] [a]	65 [60-68] [a]	64 [60.25-67.75] [a]
CFT	79 [64-92]	65 [56-76] [a]	58 [50-65] [a,b]	63 [56-71.75] [a]
MCF	64 [61-68]	69 [66-72] [a]	70 [66-73] [a]	69 [66-72.25] [a]
Alpha angle	75 [71-77]	77 [75-79] [a]	78 [77-80] [a,b]	77.5 [76-79] [a]
Li30	100 [99-100]	100 [100-100] [a]	100 [100-100] [a]	100 [100-100]
Li45	96 [94-98]	98 [96-99] [a]	98 [97-99] [a]	97 [95.25-99]
Li60	92 [90-94]	94 [92-96] [a]	94 [92-96] [a]	93.5 [92-96] [a]
INTEM				
CT	170 [161-184]	172 [162-181]	187 [173-197] [a,b]	249 [197.75-275.75] [a,b,c]
A5	47 [43-51]	52 [48-56] [a]	54 [50-59] [a]	53 [48-55.75] [a]
A10	57 [53-60]	61 [58-65] [a]	63 [59-67] [a]	61.5 [57-64.75] [a]
CFT	70 [62-84.25]	58 [50-69] [a]	54 [49-66] [a]	62 [52-69.5] [a]
MCF	62 [59-65]	67 [64-70] [a]	68 [65-71] [a]	66.5 [63.25-70] [a]
Alpha angle	76 [73-77]	78 [76-80] [a]	79 [76-80] [a]	77.5 [75-79] [a]
Li30	99 [98-100]	100 [99-100] [a]	100 [100-100] [a]	100 [99-100] [a, c]
Li45	94 [92-96]	97 [95-98] [a]	98 [96-98] [a]	96 [94-98] [a]
Li60	91 [88-93]	94 [91-96] [a]	94 [93-96] [a]	93 [90-96] [a]
FIBTEM				
CT	57 [53-64]	59 [54-64]	106 [90-125] [a,b]	124 [93-185.25] [a,b]
A5	16 [13-19]	18 [15-21] [a]	17 [14-22]	16 [12.25-19] [b]
A10	17 [15-20]	19 [16-23] [a]	19 [15-23]	17.5 [14-20.75]
MCF	18 [15-21]	20 [17-24] [a]	20 [16-25] [a]	19 [15-23]
Li30	100 [99-100]	100 [100-100] [a]	100 [100-100] [a]	100 [100-100]
Li45	100 [97-100]	100 [99-100] [a]	100 [100-100] [a]	100 [100-100] [a]
Li60	100 [95-100]	100 [98-100] [a]	100 [100-100] [a]	100 [99-100] [a]
TPA				
CT	54 [47-63]	59 [47-74]	97 [81-119] [a,b]	116 [88-145.25] [a,b]
A5	46 [39-51]	53 [46-57] [a]	53 [47-58] [a]	52.5 [41.25-57] [a]
A10	54 [47-59]	60 [54-65] [a]	60 [53-65] [a]	58 [48-65]
CFT	70 [58-95]	59 [51-77] [a]	63 [56-79]	66.5 [58.25-102.5] [b]
MCF	58 [50-62]	64 [58-69] [a]	61 [54-67]	61 [48.5-67]
Alpha angle	76 [72-78]	78 [75-79] [a]	77 [74-79] [b]	76.5 [69.5-78.75] [b]
Li30	90 [68-96]	96 [86-99] [a]	83 [52-95] [b]	92 [53-97.75] [b]
Li45	7 [1-50.25]	60 [20-83] [a]	29 [4-64]	33 [4.25-75]
Li60	1 [0-3.25]	6 [2-35) [a]	3 [1-18]	2.5 [1-19]
LOT	1918 [1606-2219]	2227 [1823-2651][a]	1755 [1429-2133]b	2004 [1328-2435][b]
LT	2649 [2345-3117]	3414 [2867-4025][a]	3133 [2405-3928]	3019 [2459-3669][b]

*[a]. control vs treatment group p<0.008; [b]. DAPT vs anticoagulant + P2Y12i p<0.008; c. VKA + P2Y12i vs DOAC + P2Y12i p<0.008; *Values missing in the control group were INTEM CFT (n=1); tPA Li45, Li60 and LT (n=1); **Values missing in the DAPT patient group were EXTEM Li60 (n=1), INTEM Li60 (n=1), tPA CT, A5, A10, CFT, MCF, Alpha angle, Li30, Li45 (n=1), Li60 (n=2), LOT (n=7), LT (n=11); ϕ Values missing in the VKA + P2Y12i patient group tPA Li60 and LT (n=1); ϕϕ Values missing in the DOAC + P2Y12 patient group were FIBTEM MCF, Lit30, Li45 and Li60 (n=1); TPA LOT and LT (n=1).*

Table S6.2 ROC analysis for VKA presence.

	AUC	Youden's index	Sensitivity	Specificity	p-value
PT*	1.000	14.45	1.000	1.000	ref
aPTT*	0.988	29.5	1.971	0.959	0.10
EXTEM CT	0.990	82.5	0.957	0.957	0.013
INTEM CT	0.697	178.5	0.652	0.682	<0.001
FIBTEM CT	0.986	75.5	0.929	0.928	<0.001
tPA CT‡	0.903	76.5	0.800	0.797	<0.001

**PT and aPTT missing for 3 patients who did not receive VKA treatment; ‡ tPA CT missing for 1 patient who did not receive VKA treatment.*

Table S6.3 ROC analysis for DOAC presence.

	AUC	Youden's index	Sensitivity	Specificity	p-value
PT*	0.929	11.15	0.854	0.843	Ref
aPTT*	0.933	28.5	0.833	0.892	0.76
EXTEM CT	0.963	80.5	0.938	0.945	0.13
INTEM CT	0.926	186.5	0.813	0.816	0.89
FIBTEM CT	0.967	69.5	0.938	0.873	0.095
tPA CT‡	0.933	84.5	0.833	0.890	0.89

**PT and aPTT missing for 3 patients who did not receive DOAC treatment; ‡ tPA CT missing for 1 patient who did not receive DOAC treatment.*

Table S6.4 ROTEM parameters of patients with and without clinically relevant bleeding (BARC≥2). P-values>0.1 were noted as not significant (Ns).

	Patients without clinically relevant bleeding (n=239)	Patients with clinically relevant bleeding(n=13)	P-value
EXTEM			
CT (in s)	62 [57-68]	68 [62-70]	0.030
A5 (in mm)	53 [49-57]	55 [52-58]	Ns
A10 (in mm)	62 [59-66]	64 [61-66]	Ns
CFT (in s)	65 [56-76]	64 [54-68]	Ns
MCF (in mm)	68 [66-72]	69 [67-72]	Ns
Alpha angle (in °)	77 [75-79]	77 [76-79]	Ns
Li30 (in %)	100 [100-100]	100 [100-100]	Ns
Li45 (in %)	98 [96-99]	97 [96-98]	Ns
Li60 (in %)	94 [92-96]*	94 [91-96]	Ns
INTEM			
CT (in s)	171 [162-182]	177 [163-190]	Ns
A5 (in mm)	52 [48-56]	53 [50-56]	Ns
A10 (in mm)	61 [58-65]	62 [60-65]	Ns
CFT (in s)	58 [50-69]	60 [48-65]	Ns
MCF (in mm)	67 [64-70]	67 [66-69]	Ns
Alpha angle (in °)	78 [76-80]	78 [77-80]	Ns
Li30 (in %)	100 [99-100]	100 [99-100]	Ns
Li45 (in %)	97 [95-98]	97 [95-98]	Ns
Li60 (in %)	94 [91-95]*	93 [92-96]	Ns

Table S6.4 (continued)

	Patients without clinically relevant bleeding (n=239)	Patients with clinically relevant bleeding(n=13)	P-value
FIBTEM			
CT (in s)	59 [54-63]	62 [57-71]	0.058
A5 (in mm)	18 [15-21]	19 [17-21]	Ns
A10 (in mm)	19 [16-23]	21 [19-23]	Ns
MCF (in mm)	20 [17-24]	22 [20-24]	Ns
Li30 (in %)	100 [100-100]	100 [100-100]	Ns
Li45 (in %)	100 [100-100]	100 [99-100]	Ns
Li60 (in %)	100 [99-100]	100 [97-100]	Ns
TPA			
CT (in s)	57 [46-73]*	74 [54-86]	0.063
A5 (in mm)	52 [47-57]*	50 [45-55]	Ns
A10 (in mm)	60 [55-64]*	58 [53-62]	Ns
CFT (in s)	59 [51-76]*	59 [55-82]	Ns
MCF (in mm)	64 [59-69]*	64 [55-66]	Ns
Alpha angle (in °)	78 [75-79]*	78 [73-79]	Ns
Li30 (in %)	97 [86-99]*	94 [92-98]	Ns
Li45 (in %)	61 [20-83]*	53 [19-71]	Ns
Li60 (in %)	6 [2-30]**	4 [2-15]	Ns
LOT (in s)	2273 [1825-2651]$^{\phi}$	2080 [1966-2385]	Ns
LT (in s)	3379 [2868-3977]$^{\phi\phi}$	3530 [3083-4074]	Ns
Routine hemostasis assays			
Fibrinogen (in g/L)	3.6 [3.0-4.2]**	3.4 [3.2-4.5]	Ns
PT (in s)	10.6 [10.3-10.9]**	10.7 [10.4-10.9]	Ns
aPTT (in s)	26 [25-27]**	26 [26-29]	Ns
Platelets (in 10^9/L)	248 [212-291]	280 [244-287]	Ns

**Missing value for 1 patient; ** missing values for 2 patients; $^{\phi}$ missing values for 7 patients; $^{\phi\phi}$ missing values for 10 patients; CT: Clotting Time, CFT: Clot Formation Time, AX: Amplitude at X minutes, MCF: Maximum Clot Firmness, LiX: Lysis index at X minutes, LOT: Lysis Onset Time, LT: Lysis Time, PT: prothrombin time, aPTT: activated partial thromboplastin time.*

Table S6.5 **ROTEM parameters of patients with and without major cardiovascular events (MACE).** P-values>0.1 were noted as not significant (Ns).

	Patients without MACE (n=239)	Patients with MACE (n=13)	P-value
EXTEM			
CT (in s)	63 [57-68]	58 [55-61]	Ns
A5 (in mm)	53 [49-58]	53 [50-54]	Ns
A10 (in mm)	62 [59-67]	62 [59-63]	Ns
CFT (in s)	65 [56-76]	68 [63-76]	Ns
MCF (in mm)	68 [66-72]	68 [66-70]	Ns
Alpha angle (in °)	77 [75-79]	77 [75-77]	Ns
Li30 (in %)	100 [100-100]	100 [100-100]	Ns
Li45 (in %)	98 [96-99]	97 [95-98]	Ns
Li60 (in %)	94 [92-96]*	93 [91-95]	Ns

Table S6.5 (continued)

	Patients without MACE (n=239)	Patients with MACE (n=13)	P-value
INTEM			
CT (in s)	172 [162-184]	172 [164-179]	Ns
A5 (in mm)	52 [48-56]	52 [50-52]	Ns
A10 (in mm)	61 [58-65]	61 [59-61]	Ns
CFT (in s)	59 [50-69]	58 [50-64]	Ns
MCF (in mm)	67 [64-70]	66 [65-70]	Ns
Alpha angle (in °)	78 [76-80]	78 [77-79]	Ns
Li30 (in %)	100 [99-100]	100 [99-100]	Ns
Li45 (in %)	97 [95-98]	96 [95-96]	Ns
Li60 (in %)	94 [91-96]*	92 [91-94]	Ns
FIBTEM			
CT (in s)	59 [54-64]	58 [53-67]	Ns
A5 (in mm)	18 [15-21]	18 [17-20]	Ns
A10 (in mm)	19 [16-23]	19 [18-22]	Ns
MCF (in mm)	20 [17-24]	20 [19-24]	Ns
Li30 (in %)	100 [100-100]	100 [100-100]	Ns
Li45 (in %)	100 [100-100]	100 [100-100]	Ns
Li60 (in %)	100 [99-100]	100 [100-100]	Ns
TPA			
CT (in s)	58 [47-74]*	57 [47-62]	Ns
A5 (in mm)	52 [47-57]*	54 [50-55]	Ns
A10 (in mm)	60 [54-65]*	61 [60-64]	Ns
CFT (in s)	60 [51-76]*	55 [52-67]	Ns
MCF (in mm)	64 [58-69]*	64 [61-67]	Ns
Alpha angle (in °)	78 [75-79]*	79 [76-79]	Ns
Li30 (in %)	97 [86-99]*	96 [83-98]	Ns
Li45 (in %)	61 [20-83]*	55 [26-72]	Ns
Li60 (in %)	7 [2-31]**	2 [1-6]	Ns
LOT (in s)	2261 [1836-2643]$^{\phi}$	2188 [1759-2418]	Ns
LT (in s)	3433 [2868-4005]$^{\phi\phi}$	3266 [2763-3411]*	Ns
Routine hemostasis assays			
Fibrinogen (in g/L)	3.6 [3.0-4.2]*	3.6 [3.1-4.0]*	Ns
PT (in s)	10.6 [10.3-10.9]*	10.6 [10.3-11.8]*	Ns
aPTT (in s)	26 [25-27]*	27 [25-29]*	Ns
Platelets (in 10^9/L)	250 [214-292]	232 [189-260]	0.078

**Missing value for 1 patient; ** missing values for 2 patients; $^{\phi}$ missing values for 7 patients; $^{\phi\phi}$ missing values for 9 patients; CT: Clotting Time, CFT: Clot Formation Time, AX: Amplitude at X minutes, MCF: Maximum Clot Firmness, LiX: Lysis index at X minutes, LOT: Lysis Onset Time, LT: Lysis Time.*

PART II

ANTITHROMBOTIC TREATMENT STRATEGIES

Chapter 7

Dual versus triple antithrombotic therapy after percutaneous coronary intervention: the prospective multicenter WOEST 2 Study

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Abstract

Background

For patients with oral anticoagulants (OAC) undergoing percutaneous coronary intervention (PCI), European guidelines have recently changed their recommendations to dual antithrombotic therapy (DAT; $P2Y_{12}$ inhibitor and OAC) without aspirin.

Aims

The prospective WOEST2 registry was designed to obtain contemporary real-world data on antithrombotic regimens and related outcomes after PCI in patients with an indication for OAC.

Methods

In this analysis, we compare DAT ($P2Y_{12}$ inhibitor and OAC) to triple antithrombotic therapy (TAT; aspirin, $P2Y_{12}$ inhibitor, and OAC) on thrombotic and bleeding outcomes after one year. Clinically relevant bleeding was defined as Bleeding Academic Research Consortium classification (BARC) grade 2, 3, or 5; major bleeding as BARC grade 3 or 5. Major adverse cardiac and cerebrovascular events (MACCE) was defined as a composite of all-cause mortality, myocardial infarction, stent thrombosis, ischemic stroke, and transient ischemic attack.

Results

A total of 1075 patients was included between 2014 and 2021. Patients used OAC for atrial fibrillation (93.6%) or mechanical heart valve prosthesis (4.7%). Non-vitamin K oral anticoagulant (NOAC) was prescribed in 53.1% and vitamin K antagonist in 46.9% of patients. At discharge 60.9% received DAT, and 39.1% TAT. DAT was associated with less clinically relevant and similar major bleeding (16.8% vs. 23.4%, $p<0.01$, and 7.6% vs. 7.7%, not significant) compared to TAT. MACCE was not statistically significant different (12.4% vs. 9.7%, $p=0.17$). Multivariable adjustment and propensity score matching confirmed these results.

Conclusions

Dual antithrombotic therapy is associated with substantially lower risk of clinically relevant bleeding without a statistically significant penalty in ischemic events.

Introduction

Patients with acute coronary syndrome or who are undergoing percutaneous coronary intervention (PCI), require dual antiplatelet therapy (DAPT) with aspirin and a $P2Y_{12}$ inhibitor to prevent them from myocardial infarction (MI) and stent thrombosis (ST)[1]. Most patients with atrial fibrillation (AF) require oral anticoagulant (OAC) therapy to prevent them from ischemic stroke and systemic embolism[2]. Patients with mechanical heart valves require OAC to prevent valve thrombosis[3]. Coronary artery disease (CAD) frequently co-occurs in patients with AF[4], and/or a mechanical heart valve prosthesis[5], warranting both DAPT and OAC. Although this combination therapy is effective, it is also accompanied with a high risk of bleeding complications[6], which are associated with mortality[7,8]. Thus, this combination therapy should be carefully considered[9,10].

The WOEST trial (2013) found an important reduction in bleeding complications for PCI patients with OAC when treated without aspirin, thus treated with dual antithrombotic therapy (DAT, consisting of OAC plus a $P2Y_{12}$ inhibitor, also referred to as double antithrombotic therapy; not to be confused with DAPT) when compared to triple antithrombotic therapy (TAT: combination therapy of OAC, $P2Y_{12}$ inhibitor and aspirin)[11]. Since then, several randomized controlled trials (RCTs) and their meta-analyses confirmed this result[12-17]. However, dropping aspirin might come at the cost of reduced antithrombotic efficacy as MI and ST tend to increase with this strategy, albeit at a lower incidence and effect size as the bleeding reduction[16,17]. Following these RCT results, international guidelines have shifted their recommendations from one full year of TAT to a personalized risk-guided approach with the aspirin duration limited to hospital admission for most patients, and up to one month in high ischemic risk patients[9]. Reliable and contemporary real-world data is required to further support the current default strategy of DAT recommended by European and American guidelines[9,18].

Within this context, the prospective, international, multicentre WOEST 2 registry was conducted, to obtain real-world data on prescription trends, thrombotic outcomes, and bleeding complications, with dual or triple antithrombotic therapy after PCI in patients with an indication for OAC.

Methods

Study population

The "What is the Optimal antiplatElet and anticoagulant therapy in patients with oral anticoagulation undergoing revasculariSaTion 2" (WOEST 2) registry is an international, multi-centre, prospective, non-interventional cohort study designed to evaluate the use, safety and efficacy of combined use of OAC and antiplatelet drugs after PCI in a real-world population (clinicaltrials.gov identifier NCT02635230). The study was performed in 10 academic and non-academic PCI centres in the Netherlands and Belgium. Patients were included from 2014 to 2021.

Adult patients who underwent successful PCI with an indication for long-term OAC use (vitamin K antagonist [VKA] or non-vitamin K oral anticoagulant [NOAC]) for AF or a mechanical heart valve prosthesis were eligible for inclusion. Patients with both elective PCI and patients undergoing PCI for acute coronary syndrome (ACS) were included. Patients with a new indication for OAC (e.g. AF de novo) were eligible for inclusion when OAC was started within 72 hours after PCI. Patients with a life expectancy less than one year or a contra-indication for $P2Y_{12}$ inhibitor use were not included. Written informed consent was obtained for all participants.

Data collection

Subject data regarding demographics, comorbidities, PCI procedure, and antithrombotic therapy were collected and directly entered into an online case report form (REDCap[19]). Follow-up data was collected at 1, 3, 6 and 12 months by medical file review and/or telephone interview. The data were stored, handled and secured according to national and local safety regulations. The study was performed in accordance with the provisions of the Declaration of Helsinki and with the International Conference on Harmonization Good Clinical Practices.

Treatment groups

Patients were classified as receiving DAT if their discharge medication consisted of a $P2Y_{12}$ inhibitor (clopidogrel, prasugrel, or ticagrelor) and OAC (acenocoumarol, phenprocoumon, apixaban, dabigatran, edoxaban, or rivaroxaban), without aspirin. Patients were classified as receiving TAT if their discharge medication consisted of a $P2Y_{12}$ inhibitor, OAC, and aspirin.

All decisions regarding the choice and duration of antithrombotic therapy after PCI were solely up to the treating physician.

Outcomes

The primary safety endpoint was clinically relevant bleeding defined as type 2, 3, or 5 bleeding according to the Bleeding Academic Research Consortium (BARC) criteria[20]. The primary efficacy endpoint was a composite of major adverse cardiac and cerebrovascular events (MACCE), containing all-cause mortality, MI, definite ST, ischemic stroke, and transient ischemic attack (TIA). Secondary endpoints included major bleeding (BARC type 3 or 5), a composite of MI and ST, and the separate components of the primary safety and efficacy endpoints. All outcomes were independently adjudicated.

Statistical analysis

For this analysis, all outcomes were assessed from discharge to one year post-discharge. In-hospital bleedings were not included in this analysis since the treatment groups were defined at discharge and recommended in-hospital treatment contains aspirin for both groups. Data for patients who were lost to follow-up were censored at the time of their last known status.

To examine temporal trends in antithrombotic strategy, the proportion of patients prescribed DAT or TAT by year of procedure was tested for trend using the Cochran-Armitage test. We then examined the proportion of patients prescribed DAT or TAT across the different study centres. Baseline characteristics of patients who were prescribed DAT or TAT were compared using descriptive statistics.

The time from discharge to the first occurrence of the primary endpoints was analysed using Cox proportional-hazards models with the treatment group as covariate to obtain a point estimate of the hazard ratio (HR) with two-sided 95% confidence interval (CI). Unadjusted cumulative event rates were estimated at 365 days post-discharge and visualized in a Kaplan-Meier plot. P values were calculated using the two-sided log-rank test. A landmark analysis at 30 days was performed to evaluate differences in outcomes during the true aspirin treatment in the TAT arm.

Given the observational nature of the study, we used multivariable Cox proportional hazard models and propensity matching to account for clinical differences causing potential confounding bias between patients receiving DAT or TAT. Based on contemporary literature, the following patient characteristics were considered as potential confounders: age, sex, body-mass index, CHA_2DS_2-VASc, HAS-BLED, indication for PCI (elective, unstable angina, non-STEMI, or STEMI), medical history of atrial fibrillation, mechanical heart valve prosthesis, myocardial infarction, congestive heart

failure, peripheral artery disease, chronic kidney disease, clinically relevant bleeding, or discharge on potent P2Y12 inhibitors (ticagrelor or prasugrel). Cases with missing values for potential confounders were left out of the adjusted analyses. To prevent the multivariable Cox proportional-hazard models from overfitting, backward stepwise selection was used. Only confounders that contributed statistically significant to the model were retained in the models to obtain the adjusted point estimates. The propensity score consisted of all above-mentioned potential confounders. Patients on DAT were matched to patients on TAT in a 1:1 ratio, using exact matching for the indication of PCI within nearest neighbour matching, with calliper 0.25 and random matching order. The quality of the propensity model was evaluated by obtaining the C-statistic of the propensity score in the entire and matched dataset, in which a C-statistic of 0.5 in the matched dataset would represent perfect matching for the entered confounders.

Analyses for the subgroups were performed for the primary outcomes with time to first event with the use of the Cox proportional-hazards model to evaluate treatment by subgroup interaction.

P values of less than 0.05 were considered to be statistically significant. All statistics analysis were performed using R 3.6.3 and Rstudio 1.3 (The R Foundation for Statistical Computing), utilizing the packages *survival* and *MatchIt* for survival analysis and propensity score matching.

Results

Study population and prescription patterns

Between 2014 and 2021, a total of 1075 patients were included in the WOEST2 registry. After exclusion of 17 patients that were not treated with DAT or TAT at discharge, a total of 1058 patients, included in 10 centres in the Netherlands and Belgium, were analysed. At 1 year, 10 patients were lost to follow-up. Baseline characteristics according to treatment are summarized in Table 7.1.

Table 7.1 Baseline characteristics of patients treated with triple and dual antithrombotic therapy.

		Antithrombotic strategy		
		Triple therapy n=415	Dual therapy n=644	p value
Demographics				
Age (mean (SD))		73.53 (8.11)	73.85 (8.21)	0.52
Female (%)		101 (24.3)	160 (24.8)	0.91
Body-mass index (mean (SD))		28.16 (5.03)	27.54 (4.39)	**0.04**
Caucasian ethnicity (%)		325 (94.2)	527 (95.1)	0.65
Indication for OAC				
Atrial fibrillation (%)		379 (91.3)	613 (95.2)	**0.02**
de novo (%)		28 (7.9)	54 (9.7)	0.42
Mechanical heart valve prosthesis (%)		22 (5.3)	27 (4.2)	0.50
Not specified (%)		20 (4.8)	20 (3.1)	0.21
Medical history				
CHA2DS2-VASc (mean (SD))		3.83 (1.57)	4.19 (1.60)	**<0.001**
CHA2DS2-VASc ≥5 (%)		142 (34.2)	265 (41.1)	**0.03**
HAS-BLED (mean (SD))		3.08 (1.11)	3.03 (1.01)	0.46
HAS-BLED ≥3 (%)		293 (70.8)	444 (69.2)	0.63
Myocardial infarction (%)		105 (25.3)	177 (27.5)	0.47
PCI (%)		143 (34.5)	243 (37.8)	0.30
CABG (%)		84 (20.2)	129 (20.1)	1.00
Congestive heart failure (%)		71 (17.1)	169 (26.3)	**0.001**
Stroke (%)		58 (14.0)	122 (19.0)	**0.04**
Peripheral artery disease (%)		61 (14.7)	103 (16.0)	0.63
Chronic kidney disease (%)		151 (36.4)	249 (38.7)	0.48
Bleed requiring medical attention (%)		42 (10.1)	92 (14.3)	0.06
Active malignancy (%)		8 (1.9)	19 (3.0)	0.40
Diabetes Mellitus (%)		112 (27.0)	186 (29.0)	0.52
Hypertension (%)		282 (68.0)	498 (77.8)	**<0.001**
Hypercholesterolemia (%)		262 (63.7)	431 (67.4)	0.24
Smoking (%)		57 (14.0)	87 (13.9)	1.00
Admission and procedural characteristics				
Indication for PCI (%)	elective	237 (58.4)	424 (68.1)	**0.001**
	unstable angina	30 (7.4)	51 (8.2)	
	non-STEMI	118 (29.1)	113 (18.1)	
	STEMI	21 (5.2)	35 (5.6)	
Prior OAC use (%)		357 (86.0)	577 (89.6)	0.10
Interruption of OAC (%)		154 (45.6)	134 (24.8)	**<0.001**
Femoral access (%)		159 (38.3)	241 (37.4)	0.82
UFH use during PCI (%)		297 (80.9)	533 (94.3)	**<0.001**
dose (IU/kg, mean (SD))		88.92 (35.90)	94.96 (30.47)	**0.02**
GPI use during PCI (%)		34 (8.2)	39 (6.1)	0.22
Treated vessel (%)	LAD	213 (51.3)	298 (46.6)	0.15
	LCx	128 (30.8)	204 (31.9)	0.78
	RCA	132 (31.8)	217 (33.9)	0.52
	graft	19 (4.6)	32 (5.0)	0.84
Complex PCI (any criterion, %)		70 (16.9)	129 (20.0)	0.23
3 vessels treated (%)		5 (1.2)	6 (0.9)	0.92
≥3 lesions treated (%)		22 (5.3)	28 (4.4)	0.59
≥3 stents implanted (%)		54 (13.1)	85 (13.3)	1.00
bifurcation with 2 stents implanted (%)		11 (5.2)	20 (4.1)	0.66
total stent length > 60mm (%)		37 (13.8)	64 (11.7)	0.46
chronic total occlusion (%)		9 (4.3)	35 (7.2)	0.20

Table 7.1 (continued)

		Antithrombotic strategy		
		Triple therapy n=415	**Dual therapy n=644**	**p value**
Discharge medication				
Vitamin K Antagonist (%)		182 (43.9)	314 (48.8)	0.13
NOAC	apixaban (%)	76 (18.3)	111 (17.2)	0.71
	5 mg	45 (13.0)	83 (15.0)	0.48
	2.5 mg	21 (6.1)	18 (3.2)	0.06
	dabigatran (%)	39 (9.4)	46 (7.1)	0.23
	150 mg	16 (4.6)	14 (2.5)	0.13
	110 mg	16 (4.6)	30 (5.4)	0.72
	edoxaban (%)	24 (5.8)	19 (3.0)	**0.03**
	60 mg	11 (3.2)	14 (2.5)	0.71
	30 mg	9 (2.6)	5 (0.9)	0.08
	rivaroxaban (%)	93 (22.4)	155 (24.1)	0.58
	20 mg	44 (12.8)	96 (17.3)	0.08
	15 mg	38 (11.0)	35 (6.3)	**0.02**
P2Y12 inhibitor	clopidogrel (%)	391 (94.2)	604 (93.8)	0.88
	ticagrelor (%)	19 (4.6)	40 (6.2)	0.32
	prasugrel (%)	5 (1.2)	0 (0.0)	**0.02**

CABG: coronary artery bypass grafting; GPI: glycoprotein IIb/IIIa inhibitors; LAD: left anterior descending artery; LCx: left circumflex artery; NOAC: non-vitamin K oral anticoagulants; OAC: oral anticoagulant; PCI: percutaneous coronary intervention; RCA: right coronary artery; SD: standard deviation; UFH: unfractionated heparin.

The mean age in this cohort was 74 (standard deviation [SD] 8) years and 24.4% were female. About one third (34.7%) of the patients underwent PCI in the setting of ACS. Of the included patients, 87.7% reported prior OAC use.

In 46.9% of the patients, a VKA was prescribed at discharge. In patients receiving NOAC (53.1%), rivaroxaban was most frequently prescribed, followed by apixaban, dabigatran and edoxaban (43.8%, 33.3%, 15.3%, and 7.7%, respectively). Patients with prior OAC use were seldomly switched from VKA to NOAC or vice versa at the time of PCI (2.9% and 3.2%, respectively; Table 7.2). Patients who were OAC naïve more often received NOAC than VKA (70.4% vs. 29.6%).

The indication for OAC use at discharge was mainly driven by atrial fibrillation (93.6%), and in 4.7% the OAC was for a mechanical heart valve prosthesis. Of the patients with AF without a mechanical heart valve prosthesis, 44.7% were prescribed VKA and 55.3% a NOAC. In patients with a mechanical heart valve prosthesis, 98% was prescribed VKA, and one patient (2%) received NOAC.

At discharge, DAT was prescribed in 644 patients (60.9%) and TAT in 414 patients (39.1%). Median aspirin duration with TAT was 30 days (IQR 29-57 days). In the majority

of patients (93.6%) clopidogrel was the $P2Y_{12}$ inhibitor of choice. The prescription of DAT differed widely between study sites, varying from 7.4% DAT and 92.6% TAT to 85.7% DAT and 14.3% TAT (Supplementary Table S7.1). A significant temporal trend towards increased DAT prescription and decreased TAT prescription was found from 2014 through 2021 (Cochran-Armitage $p_{trend} < 0.001$, Supplementary Table S7.2).

Table 7.2 Antithrombotic medication at discharge, according to prior medication use.

	Discharge medication			
	VKA		NOAC	
	DAT	TAT	DAT	TAT
Prior VKA	241/372	120/372	5/372	6/372
	64.8%	32.3%	1.3%	1.6%
Prior NOAC	4/429	10/429	254/429	161/429
	0.9%	2.3%	59.2%	37.5%
No prior OAC	19/125	18/125	48/125	40/125
	15.2%	14.4%	38.4%	32.0%

DAT: double antithrombotic therapy; NOAC: non-vitamin K oral anticoagulant; OAC: oral anticoagulant; TAT: triple antithrombotic therapy; VKA: vitamin K antagonist.

Patient characteristics with dual and triple antithrombotic therapy

Patients receiving DAT tended to have more comorbidities as compared to patients receiving TAT, including a history of hypertension (77.8% vs. 67.9%), previous stroke (19.0% vs. 14.0%), congestive heart failure (26.3% vs. 17.1%) and atrial fibrillation (95% vs. 91%, p<0.05 for all). In line with this, the thrombotic risk predicted by the CHA_2DS_2-VASc was significantly higher in patients treated with DAT than with TAT (score ≥5 in 41% vs. 34% of patients, p=0.03). Patients treated with TAT, on the other hand, had a higher body-mass index (28.1 vs. 27.5, p=0.04), and were more likely to have had PCI for ACS instead of elective PCI (41% vs. 31%, p<0.01). The risk of bleeding predicted by the HAS-BLED score did not differ between the groups, although patients prescribed DAT were slightly more likely to have had a history of bleeding (14% vs. 10%, p=0.06). There were no meaningful differences in other baseline characteristics (Table 7.1).

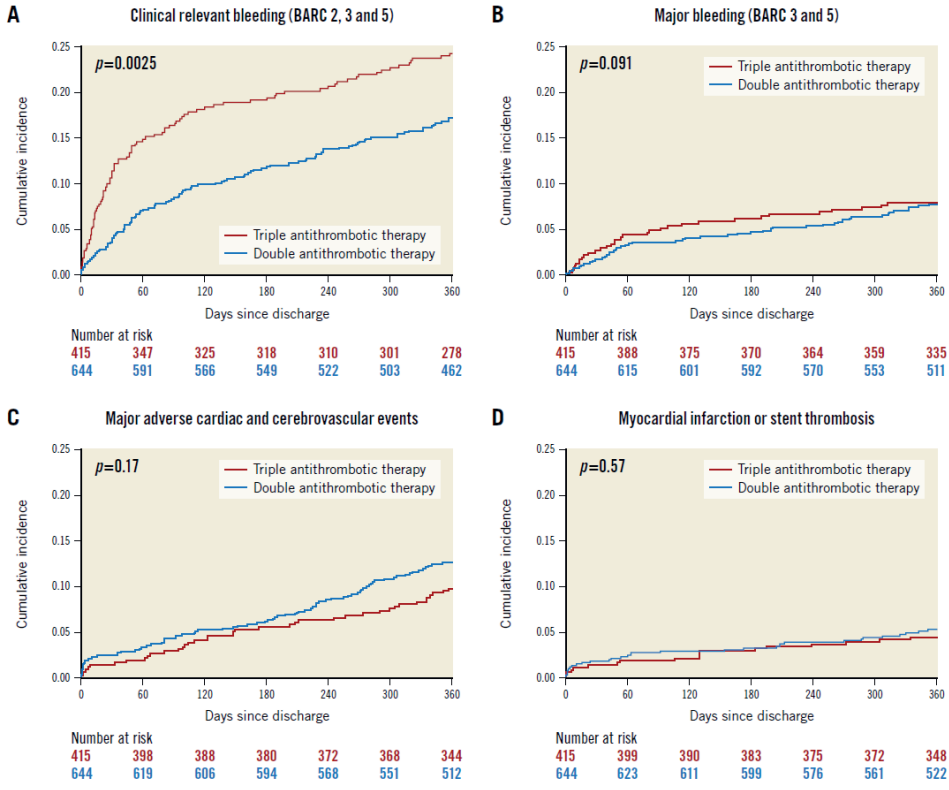
Outcomes with dual and triple therapy

The use of DAT after discharge was associated with a significant decrease of 6.6% in clinically relevant bleeding (BARC type 2, 3, or 5) at 1 year, compared to patients treated with TAT (16.8% vs. 23.6%, p=0.003, Central Illustration A). However, no significant differences were found in major bleeding (7.6% vs. 7.7%, p=0.97, Central Illustration B) or haemorrhagic stroke (0.3% vs. 0.7%, p=0.35; Table 7.2). The use of DAT was associated with a numerically higher rate of MACCE (12.4% vs. 9.6%, p=0.17, Central Illustration C). The numerical increase of 2.8% in MACCE with DAT compared to

TAT consisted of myocardial infarction (5.0% vs. 4.3%) (Central illustration D), stent thrombosis (1.7% vs. 1.0%), and all-cause death (6.2% vs. 5.8%), however, statistical significance between DAT and TAT for any of the separate endpoints was not reached.

Landmark analysis at 30 days showed no statistically significant differences in outcomes beyond this time point (Supplementary Figure S7.1).

Central illustration: Incidence curves of outcomes with double or triple antithrombotic therapy



A) Outcomes for clinically relevant bleeding; B) major bleeding; C) major adverse cardiac and cerebrovascular events; and D) myocardial infarction or stent thrombosis. BARC: Bleeding Academic Research Consortium classification.

Adjusted outcome analysis

Unadjusted and adjusted outcomes by multivariable Cox regression and propensity score matching are summarized in Table 7.3.

Table 7.3 Unadjusted and adjusted clinical outcomes.

			Full cohort				Propensity score matched cohort	
	Dual therapy	Triple therapy	Unadjusted HR		Adjusted HR		HR	
	n (%)	n (%)	(95% CI)	p value	95% CI	p value	95% CI	p value
Bleeding events								
Clinically relevant bleeding	108 (16.8%)	98 (23.6%)	**0.66** (0.50-0.87)	0.003	**0.67** (0.50-0.89)	0.006	0.**55** (0.37-0.81)	0.002
Major bleeding	49 (7.6%)	32 (7.7%)	**0.97** (0.62-1.52)	0.91	0.**96** (0.60-1.54)	0.86	**0.49** (0.24-0.97)	0.04
Haemorrhagic stroke	2 (0.3%)	3 (0.7%)	**0.43** (0.07-2.56)	0.35	**0.37** (0.06-2.32)	0.29	**0.51** (0.05-5.62)	0.58
According to BARC classification								
BARC 2	76 (11.8%)	75 (18.1%)	**0.61** (0.44-0.84)	0.002	**0.61** (0.43-0.85)	0.004	**0.60** (0.39-0.93)	0.02
BARC 3	48 (7.5%)	30 (7.2%)	**1.02** (0.65-1.61)	0.94	**1.02** (0.63-1.67)	0.92	**0.53** (0.26-1.11)	0.08
BARC 5	1 (0.2%)	3 (0.7%)	**0.21** (0.02-2.04)	0.18	**0.13** (0.01-1.40)	0.09	-	-
Ischaemic events								
MACCE	80 (12.4%)	40 (9.6%)	**1.30** (0.89-1.9)	0.17	**1.34** (0.89-2.01)	0.17	**1.38** (0.83-2.40)	0.20
All cause death	42 (7.5%)	23 (6.6%)	**1.12** (0.67-1.86)	0.64	**1.07** (0.62-1.84)	0.82	1.**30** (0.65-2.63)	0.49
Cardiovascular death	28 (5.0%)	16 (4.6%)	**1.08** (0.58-1.99)	0.82	**1.05** (0.53-2.05)	0.90	**1.24** (0.53-2.97)	0.68
Ischemic stroke or TIA	12 (1.9%)	5 (1.2%)	**1.54** (0.54-4.39)	0.41	**1.45** (0.49-4.29)	0.50	**3.98** (0.45-36.0)	0.21
Myocardial infarction	32 (5.0%)	18 (4.3%)	**1.15** (0.64-2.04)	0.64	**1.38** (0.74-2.56)	0.32	**1.43** (0.56-3.43)	0.48
Stent thrombosis	11 (1.7%)	4 (1.0%)	**1.77** (0.56-5.55)	0.33	**1.92** (0.60-6.13)	0.27	**1.49** (0.25-8.87)	0.65
Target vessel revascularization	35 (6.2%)	20 (5.8%)	**1.07** (0.62-1.85)	0.81	**1.24** (0.68-2.25)	0.48	**1.24** (0.54-2.69)	0.66

BARC: Bleeding academic research consortium; HR: hazard ratio; MACCE: major adverse cardiac and cerebrovascular events; TIA: transient ischemic attack.

After multivariate adjusted Cox regression, clinically relevant bleeding remained significantly decreased with DAT as compared to TAT (HR 0.67, 95% CI 0.50–0.89), while major bleeding remained similar (HR 0.96 95% CI 0.60–1.54). Neither MACCE (HR 1.34 95% CI 0.89–2.01) nor any of its separate components showed a significant association with TAT or DAT (all-cause death: HR 1.07, 95% CI 0.62–1.84; MI: HR 1.38, 95% CI 0.74-2.56; ST: HR 1.92, 95% CI 0.60–6.13).

The propensity score showed near-perfect matching with a C-statistic of decreasing from 0.63 in the unmatched cohort to 0.51 in the matched cohort. The matched cohort consisted of 302/519 patients treated with DAT matched to 302/321 patients on TAT. Its baseline characteristics are summarized in Supplementary Table S7.3. After propensity score matching, clinically relevant bleeding remained significantly decreased with DAT (HR 0.55, 95% CI 0.37–0.81). In contrast to the unadjusted and multivariate Cox regression adjusted analyses, major bleeding was reduced with DAT in the propensity score matched cohort. MACCE remained indifferent between the groups (HR 1.38, 95%CI 0.83-2.40). The separate components all-cause death (HR 1.30, 95% CI 0.65–2.63), MI (HR 1.43, 95% CI 0.56–3.43), and ST (HR 1.49, 95% CI 0.25–8.87) remained indifferent as well.

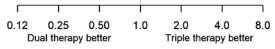
Subgroup analyses

Analyses of the primary endpoints were performed in several subgroups (mentioned in the methods section) at risk of bleeding or thrombosis. The results were generally consistent with those in the whole cohort (Figure 7.1 and Figure 7.2).

Especially, for ACS patients, a similar decrease in bleeding (HR 0.60, 95% CI 0.38-0.94) and no increase of MACCE (HR 1.4, 95% CI 0.83–2.4) and MI (HR 0.91, 95% CI 0.44–1.9) or ST (HR 0.84, 95% CI 0.21–3.4) was seen with the use of DAT compared to TAT. Even so, separately evaluating patients with AF, thus leaving out patients with mechanical heart valve prosthesis, decreased bleeding (HR 0.68, 95% CI 0.50–0.90) and no increase of MACCE (HR 1.34, 95% CI 0.90–2.00) and MI (HR 1.5, 95% CI 0.76–2.8) or ST (HR 2.3, 95% CI 0.64–8.2) was found. Similarly, for patients with a mechanical heart valve prosthesis a non-significant decrease in bleeding (HR 0.59, 95 CI 0.24–1.44) and no increase of MACCE (HR 0.94, 95% CI 0.25–3.49) and MI (HR 0.19, 95% CI 0.02–1.7) or ischemic stroke or TIA (HR 0.76, 95% CI 0.05–12) was seen.

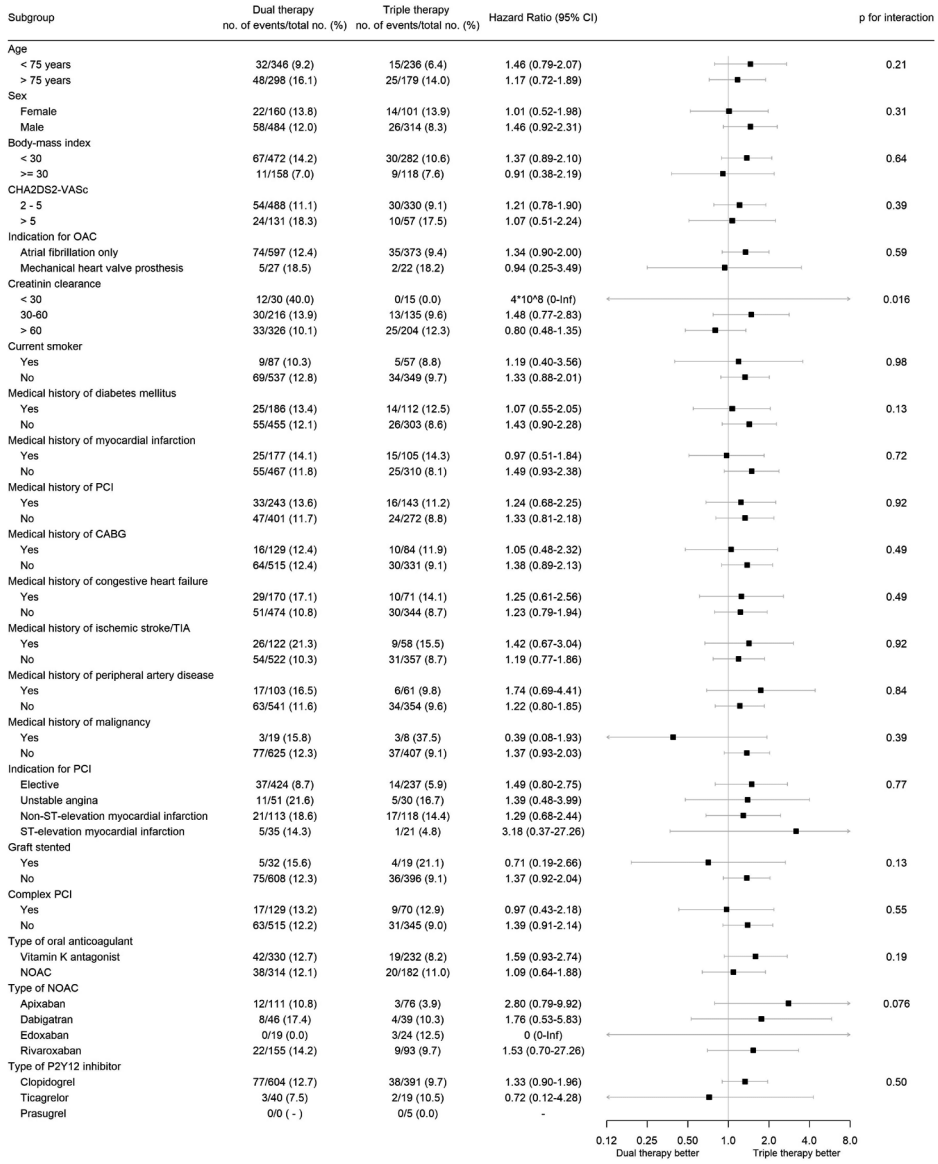
Figure 7.1 Subgroup analyses for clinically relevant bleeding.

Subgroup	Dual therapy no. of events/total no. (%)	Triple therapy no. of events/total no. (%)	Hazard Ratio (95% CI)	p for interaction
Age				
< 75 years	56/346 (16.2)	56/236 (23.7)	0.63 (0.43-0.91)	0.71
> 75 years	52/298 (17.4)	42/179 (23.5)	0.69 (0.46-1.04)	
Sex				
Female	19/160 (11.9)	21/101 (20.8)	0.54 (0.29-1.00)	0.46
Male	89/484 (18.4)	77/314 (24.5)	0.69 (0.51-0.94)	
Body-mass index				
< 20	1/11 (9.1)	3/9 (33.3)	0.22 (0.02-2.16)	0.30
>= 20	104/619 (16.8)	91/391 (23.3)	0.67 (0.51-0.89)	
HAS-BLED				
Low (0 - 1)	3/21 (14.3)	4/14 (28.6)	0.47 (0.11-2.10)	0.65
Moderate (2)	28/177 (15.8)	26/107 (24.3)	0.57 (0.34-0.98)	
High (3 - 5)	73/435 (16.8)	63/280 (22.5)	0.70 (0.50-0.98)	
Very high (> 5)	4/9 (44.4)	5/13 (38.5)	1.32 (0.35-4.93)	
Indication for OAC				
Atrial fibrillation only	124/597 (20.8)	96/373 (25.7)	0.68 (0.50-0.90)	0.77
Mechanical heart valve prosthesis	10/27 (37.0)	11/22 (50.0)	0.59 (0.24-1.44)	
Medical history of bleeding				
Yes	24/92 (26.1)	12/42 (28.6)	0.88 (0.44-1.76)	0.34
No	84/552 (15.2)	86/373 (23.1)	0.61 (0.45-0.82)	
Medical history of ischemic stroke/TIA				
Yes	21/122 (17.2)	13/58 (22.4)	0.71 (0.36-1.42)	0.82
No	87/522 (16.7)	85/357 (23.8)	0.65 (0.48-0.87)	
Creatinin clearance				
< 30	10/30 (33.3)	4/15 (26.7)	1.42 (0.44-4.55)	0.29
30 - 60	42/216 (19.4)	35/135 (25.9)	0.69 (0.44-1.09)	
> 60	44/326 (13.5)	46/204 (22.5)	0.53 (0.35-0.80)	
Indication for PCI				
Elective	70/424 (16.5)	52/237 (21.9)	0.71 (0.49-1.01)	0.58
Unstable angina	8/51 (15.7)	11/30 (36.7)	0.38 (0.15-0.95)	
Non-ST-elevation myocardial infarction	18/113 (15.9)	28/118 (23.7)	0.62 (0.34-1.12)	
ST-elevation myocardial infarction	8/35 (22.9)	5/21 (23.8)	0.89 (0.29-2.71)	
Access site				
Femoral access	42/241 (17.4)	32/159 (20.1)	0.84 (0.53-1.32)	0.20
Radial access	66/403 (16.4)	66/256 (25.8)	0.57 (0.41-0.81)	
Type of oral anticoagulant				
Vitamin K antagonist	60/330 (18.2)	55/232 (23.7)	0.71 (0.49-1.03)	0.54
NOAC	48/314 (15.3)	43/182 (23.6)	0.60 (0.40-0.91)	
Type of NOAC				
Apixaban	15/111 (13.5)	13/76 (17.1)	0.74 (0.35-1.55)	0.92
Dabigatran	4/46 (8.7)	6/39 (15.4)	0.55 (0.15-1.94)	
Edoxaban	5/19 (26.3)	6/24 (25.0)	0.93 (0.28-3.06)	
Rivaroxaban	36/155 (23.2)	30/93 (32.3)	0.65 (0.40-1.06)	
Type of P2Y12 inhibitor				
Clopidogrel	101/604 (16.7)	89/391 (22.8)	0.68 (0.52-0.91)	0.36
Ticagrelor	7/40 (17.5)	7/19 (36.8)	0.40 (0.14-1.16)	
Prasugrel	0/0 (-)	2/5 (40.0)	-	



NOAC: non-vitamin K oral anticoagulant; OAC: oral anticoagulant; PCI: percutaneous coronary intervention; TIA: transient ischemic attack;

Figure 7.2 Subgroup analyses for major adverse cardiac and cerebrovascular events.



CABG: coronary artery bypass grafting; NOAC: non-vitamin K oral anticoagulant; OAC: oral anticoagulant; PCI: percutaneous coronary intervention; TIA: transient ischemic attack.

Discussion

In this analysis of the WOEST 2 registry in patients with OAC undergoing PCI between 2014 and 2021, we found that there was a wide variety in prescription of DAT or TAT across centres, with an increased preference for DAT over the years. In our primary, unadjusted analysis at one year post-discharge, we found that dual antithrombotic therapy was associated with significantly less clinically relevant post-discharge bleeding complications without a statistically significant increase in MACCE. We conclude that major bleeding seemed not to differ between patients treated with DAT or TAT since only the propensity-score matched analysis but not the unadjusted and adjusted Cox regression analysis showed a difference. Thus the difference between the two groups was primarily driven by BARC type 2 bleeding. These results were also consistent for patients with complex PCI or high bleeding risk, as shown in the subgroup analyses. These findings support the clinical benefit of DAT as a strategy to prevent bleeding complications without an apparent increase of thrombotic events such as myocardial infarction for most patients on OAC undergoing PCI. It also indicates that high-risk patients may be adequately identified and accordingly treated.

Comparison to existing literature

Both ischemic events and bleeding complications in patients using antithrombotic therapy after PCI are strongly associated with adverse outcomes[8]. In the past decade, several RCTs have focussed on the safety and efficacy of DAT and TAT in patients with oral anticoagulants undergoing PCI, with the primary aim to reduce bleeding complications of antithrombotic therapy. The WOEST[11], PIONEER AF-PCI[12], RE-DUAL PCI[13], AUGUSTUS[14], and ENTRUST-AF PCI[15] found reduced bleeding risk with DAT as compared to TAT, with generally no differences in MACCE. However, none of these trials were completed with statistical power to detect differences in MACCE, whilst meta-analyses of these trials found increased risk of myocardial infarction and stent thrombosis[16,17]. Nonetheless, international guidelines and consensus papers have shifted their recommendations over the years during this registry, towards an approach of post-discharge DAT for its clear benefit with regards to bleeding complications, with TAT reserved for those patients with high thrombotic risk and favourable bleeding risk[9,18,21]. Still, in a recent survey, the majority of interventional cardiologists indicated that they would prescribe TAT to a PCI patient discharged on OAC[22]. Thus, prospective real-world studies with reliable and high-quality data on this subject, like the present study, might be needed to convince medical specialists of the efficacy of DAT.

Our study adds to the literature, providing a prospective registry with reliable insight in real-world data of a heterogeneous, contemporary cohort of patients treated with DAT or TAT. Two other prospective real-world cohorts comparing the safety and efficacy of DAT and TAT in patients with AF undergoing PCI, the MUSICA[23] and AFCAS[24] studies, included patients before randomized controlled trials of DAT vs. TAT were published. They found only 8-11% of patients to be treated with DAT and 69-74% with TAT, the remainder being treated with dual antiplatelet therapy. In contrast with the results of this present study and the aforementioned RCTs, the AFCAS registry found no statistically difference in bleeding outcomes between DAT and TAT, whereas the MUSICA study found less minor bleeding with DAT as compared to TAT. Ischemic outcomes did not differ between the groups in either study. Also, more recently, numerous retrospective cohort studies, including large nationwide registries[6,25,26], generally found a decreased risk of bleeding with DAT, without significant differences in thrombotic outcomes. This is in line with our observations. As the current study prospectively enrolled patients in a contemporary cohort, the portion of patients receiving DAT was much higher (60.9%). In our view, this allowed for a better estimation of (un-)adjusted bleeding and thrombotic outcomes in relation to antithrombotic regimen.

The RCTs on DAT and TAT included a predominantly (around 75%) male study population. Also, patients with other indications for OAC than AF were excluded in most RCTs, except for the WOEST trial. Patients with severe renal insufficiency were also not included. The observational nature of this study, without stringent in- and exclusion criteria allowed us to report on a broader patient population. Our study confirms the imbalance of sexes in this population, which has also been found in other recent real-world cohorts[27]. We also found consistent results within the whole cohort amongst patients with a mechanical heart valve prosthesis. In all major RCTs on this subject, patients with AF and a mechanical heart valve were excluded[12-15].

Limitations

Some limitations have to be addressed for this study. First, inherent to the design and goals of the study, the study was non-randomized. Still, we believe the results of our observational cohort are convincing, given the all-comers design of the study and the in-depth description of baseline and procedural characteristics. Moreover, the consistency of the study results in multivariable Cox regression, propensity-score matching with exact matching for PCI indication, and extensive subgroup analyses showed consistent results. Second, due to slow enrolment, the study was underpowered to draw conclusions for thrombotic outcomes. However, we found

similar effect sizes as meta-analyses of the trials[16,17], which might support the reliability of our results. For example, in the meta-analyses, a significant increase of MI and ST was found with point estimates for HR of 1.2 and 1.5, respectively. Our study found similar point estimates for the HR: 1.1 and 1.8, respectively, however these were non-significant due to smaller sample size. Third, we did not record the motivation of the cardiologist to prescribe DAT or TAT. This is mostly covered by recording patient's characteristics including risk scores, however, we cannot fully explain why patients with a high CHA_2DS_2-VASc were more likely to receive DAT, whereas HAS-BLED did not differ between groups. Despite this appearance of not being guided by risk scores, cardiologists seem to choose the right regimen as no significant differences in thrombotic outcomes were found. Also, this could be another example of the treatment-risk paradox in which patients at the highest risk tend to be treated less intensively[28]. Finally, since patients using (N)OAC for pulmonary embolism, deep venous thrombosis, or intra-cardiac thrombus were not included (unless AF or a mechanical heart valve prosthesis was present), we cannot generalize this result to those populations.

Future perspectives

Over the past decades, the antithrombotic treatment after PCI in patients with an indication for (N)OAC has improved vastly by limiting aspirin duration from 1 year to a maximum of one month, which reduces bleeding risk. During the first month, some benefit may still be obtained from intensified antithrombotic treatment[29]. It may be reasonable to select high-risk patients eligible for initial TAT by a recently proposed risk score, based on clinical parameters including extent of vascular and coronary disease, platelet count, left ventricular ejection fraction, and renal function[32]. Since TAT is accompanied with an unacceptable high bleeding risk for most patients, current studies are focusing on alternative antithrombotic strategies to optimise treatment for this population. The ZON-HR RE-DUAL PCI (clinicaltrials.gov: NCT04688723) investigates DAT with ticagrelor as a more potent option compared to DAT with clopidogrel. The WOEST3 trial (clinicaltrials.gov: NCT04436978) will compare dual antiplatelet therapy without OAC in the first month after ACS or PCI to standard therapy consisting of NOAC and $P2Y_{12}$ inhibitor, and aspirin only limited to high-risk cases. Also, genetic testing to select the most effective $P2Y_{12}$ inhibitor may be a reasonable option[30], however, it has not yet been studied in this population[31].

Conclusion

Our prospective real-world cohort study comparing DAT to TAT in patients with AF or a mechanical heart valve prosthesis undergoing PCI, found a substantially lower risk of clinically relevant bleeding without a statistically significant penalty in ischemic events. Major bleeding did not significantly differ between the groups.

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Supplemental material

Table S7.1 Inclusions and treatment per study site.

		% of total inclusions	Triple antithrombotic therapy			Dual antithrombotic therapy		
			n	N	%	n	N	%
St. Antonius Hospital, Nieuwegein	NL	34,3%	51	363	14,0%	312	363	86,0%
Maastricht UMC+, Maastricht	NL	15,1%	70	160	43,8%	90	160	56,3%
OLVG Hospital, Amsterdam	NL	14,9%	63	158	39,9%	95	158	60,1%
Ziekenhuis Oost-Limburg, Genk	BE	10,8%	68	114	59,6%	46	114	40,4%
Amphia Hospital, Breda	NL	7,9%	36	84	42,9%	48	84	57,1%
University Hospital Leuven, Leuven	BE	5,1%	23	54	42,6%	31	54	57,4%
University Hospital Antwerp, Antwerp	BE	5,0%	43	53	81,1%	10	53	18,9%
Imelda Hospital, Bonnheiden	BE	2,6%	26	28	92,9%	2	28	7,1%
OLV Hospital, Aalst	BE	2,4%	19	25	76,0%	6	25	24,0%
Elisabeth-TweeSteden Hospital, Tilburg	NL	1,9%	16	20	80,0%	4	20	20,0%

Table S7.2 Inclusions and treatment per year.

	Number of patients included			Triple antithrombotic therapy			Dual antithrombotic therapy		
	n	N	%	n	N	%	n	N	%
2014	58	1058	5,5%	9	58	15,5%	49	58	84%
2015	104	1058	9,8%	20	104	19,2%	84	104	81%
2016	305	1058	28,8%	193	305	63,3%	112	305	37%
2017	221	1058	20,9%	107	221	48,4%	114	221	52%
2018	174	1058	16,4%	52	174	29,9%	122	174	70%
2019	166	1058	15,7%	21	166	12,7%	145	166	87%
2020	29	1058	2,7%	11	29	37,9%	18	29	62%
2021	2	1058	0,1%	2	2	100,0%	0	2	0%

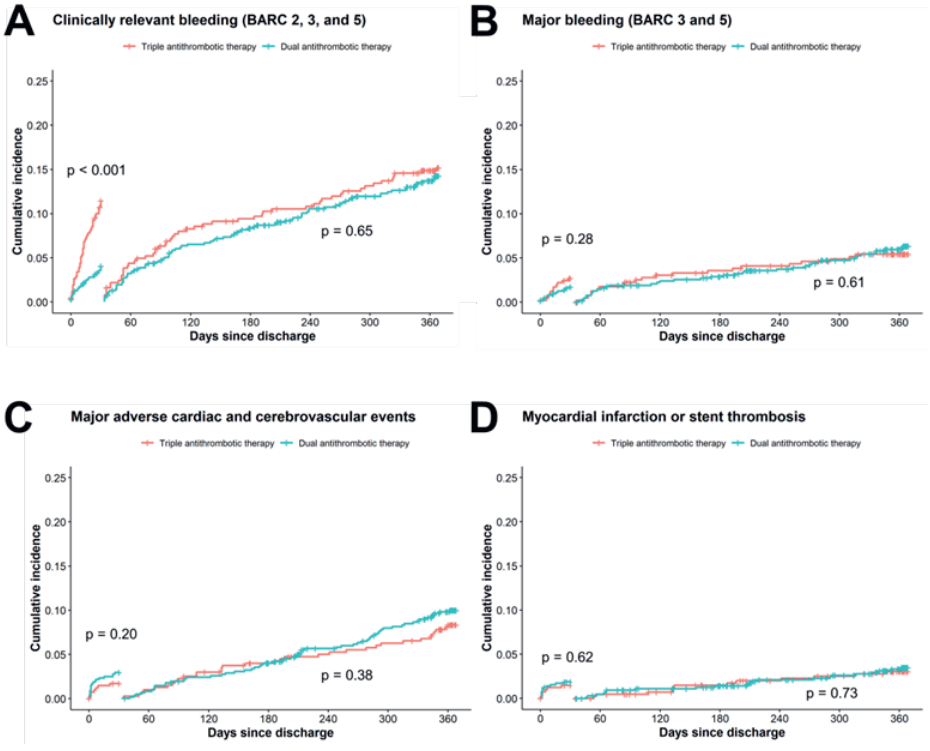
p for trend (Cochran-Armitage) <0.001

Table S7.3 Baseline characteristics of the propensity-score matched cohort.

	Antithrombotic strategy		p
	Triple therapy n=302	Dual therapy n=302	
Demographics			
Age (mean (SD))	73.35 (8.23)	72.47 (8.21)	0.187
Female (%)	70 (23.2)	67 (22.2)	0.846
Body-mass index (mean (SD))	28.11 (4.89)	28.20 (4.52)	0.817
Caucasian ethnicity (%)	284 (94.0)	291 (96.4)	0.253
Indication for OAC			
Atrial fibrillation (%)	286 (94.7)	285 (94.4)	1.000
Mechanical heart valve prosthesis (%)	13 (4.3)	11 (3.6)	0.835
Medical history			
CHA2DS2-VASc (mean (SD))	3.70 (1.55)	3.69 (1.45)	0.892
CHA2DS2-VASc ≥ 5 (%)	92 (30.5)	84 (27.8)	0.531
HAS-BLED (mean (SD))	2.86 (0.99)	2.78 (0.92)	0.289
HAS-BLED ≥ 3 (%)	197 (65.2)	183 (60.6)	0.273
Myocardial infarction (%)	84 (27.8)	87 (28.8)	0.857
PCI (%)	101 (33.4)	111 (36.8)	0.443
CABG (%)	57 (18.9)	60 (19.9)	0.837
Congestive heart failure (%)	54 (17.9)	50 (16.6)	0.746
Stroke (%)	35 (11.6)	38 (12.6)	0.803
Peripheral artery disease (%)	46 (15.2)	53 (17.5)	0.510
Chronic kidney disease (%)	100 (33.1)	98 (32.5)	0.931
Bleed requiring medical attention (%)	26 (8.6)	25 (8.3)	1.000
Active malignancy (%)	3 (1.0)	10 (3.3)	0.093
Diabetes Mellitus (%)	77 (25.5)	85 (28.3)	0.488
Hypertension (%)	196 (64.9)	224 (74.7)	0.012
Hypercholesterolemia (%)	198 (66.2)	216 (72.0)	0.149
Smoking (%)	41 (13.9)	41 (14.2)	1.000
Admission characteristics			
Acute coronary syndrome (%)	99 (32.8)	99 (32.8)	1.000
Stable coronary artery disease (%)	203 (67.2)	203 (67.2)	1.000
Prior OAC use (%)	264 (87.4)	270 (89.4)	0.525
Haemoglobin (mean (SD))	8.47 (1.08)	8.62 (1.02)	0.128
Creatinin clearance (mean (SD))	65.15 (19.63)	64.98 (20.14)	0.923
Discharge medication			
Vitamin K Antagonist (%)	122 (40.4)	139 (46.0)	0.189
NOAC (%)	180 (59.6)	164 (54.3)	0.218
apixaban (%)	63 (20.9)	56 (18.5)	0.539
dabigatran (%)	27 (8.9)	25 (8.3)	0.885
edoxaban (%)	17 (5.6)	13 (4.3)	0.574
rivaroxaban (%)	73 (24.2)	70 (23.2)	0.848
P2Y12 inhibitor			
clopidogrel (%)	286 (94.7)	286 (94.7)	1.000
ticagrelor (%)	15 (5.0)	16 (5.3)	1.000
prasugrel (%)	1 (0.3)	0 (0.0)	1.000

CABG: coronary artery bypass grafting; GPI: glycoprotein IIb/IIIa inhibitors; LAD: left anterior descending artery; LCx: left circumflex artery; NOAC: non-vitamin K oral anticoagulants; OAC: oral anticoagulant; PCI: percutaneous coronary intervention; RCA: right coronary artery; SD: standard deviation; UFH: unfractionated heparin.

Figure S7.1 Landmark analysis for 30-day timepoint.



Chapter 8

Effects of the PAR-1 antagonist vorapaxar on platelet activation and coagulation biomarkers in patients with stable coronary artery disease

Letter to the editor

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On top of standard antiplatelet therapy, vorapaxar reduces the risk of ischemic events or cardiovascular death in patients with stable coronary artery disease (CAD)[1]. Vorapaxar is a selective antagonist of protease-activated receptor-1 (PAR-1), thereby blocking thrombin-mediated platelet activation[2,3]. However, other actions of thrombin, such as fibrin formation and protein-C activation, are not inhibited by blocking PAR-1. Although vorapaxar probably does not affect the coagulation process directly, platelet inhibition leading to reduced availability of a procoagulant surface for the assembly of coagulation factors, might lower the rate of thrombin formation indirectly. Therefore, aim of this study is to assess whether the beneficial effect of vorapaxar on top of standard antiplatelet therapy in stable CAD patients can only be ascribed to more potent platelet inhibition or to an additional effect on thrombin generation.

To study whether vorapaxar reduces thrombin generation indirectly, we selected two upstream biomarkers of coagulation activity, factor IXa-antithrombin (FIXa-AT) and factor Xa-antithrombin (FXa-AT). As marker of downstream coagulation activity, we measured thrombin-antithrombin (TAT) complexes. Finally, to study the additional effect on platelet activity, soluble P-selectin was assessed as a plasma biomarker of α-granule release induced by platelet activation[4-6].

The study was performed on plasma samples obtained from patients from three Norwegian centers participating in the Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events – Thrombolysis in Myocardial Infarction 50-trial (TRA2°P-TIMI-50)[1]. The institutional review board approved the study protocol and all patients gave written informed consent. Patients with a previous history of myocardial infarction were randomly assigned to receive either vorapaxar (2.5 mg daily) or placebo in a blinded fashion, on top of standard antiplatelet agents, as managed by the treating physicians according to standards of care[1,7]. Patients using anticoagulant medication during follow-up were excluded.

A total of 135 patients with stable CAD were randomized to vorapaxar (n=73) or placebo (n=62). Baseline characteristics including age, comorbidity, and co-medication were well balanced between both groups (Table 8.1). To study the effects of long-term treatment, blood samples were taken after a mean study drug exposure of 904 (±149) days. The use of concomitant antiplatelet agents was comparable between both groups; at the time of blood sampling, 92.6% and 22.2% were treated with aspirin and clopidogrel, respectively.

Table 8.1 Patient characteristics.

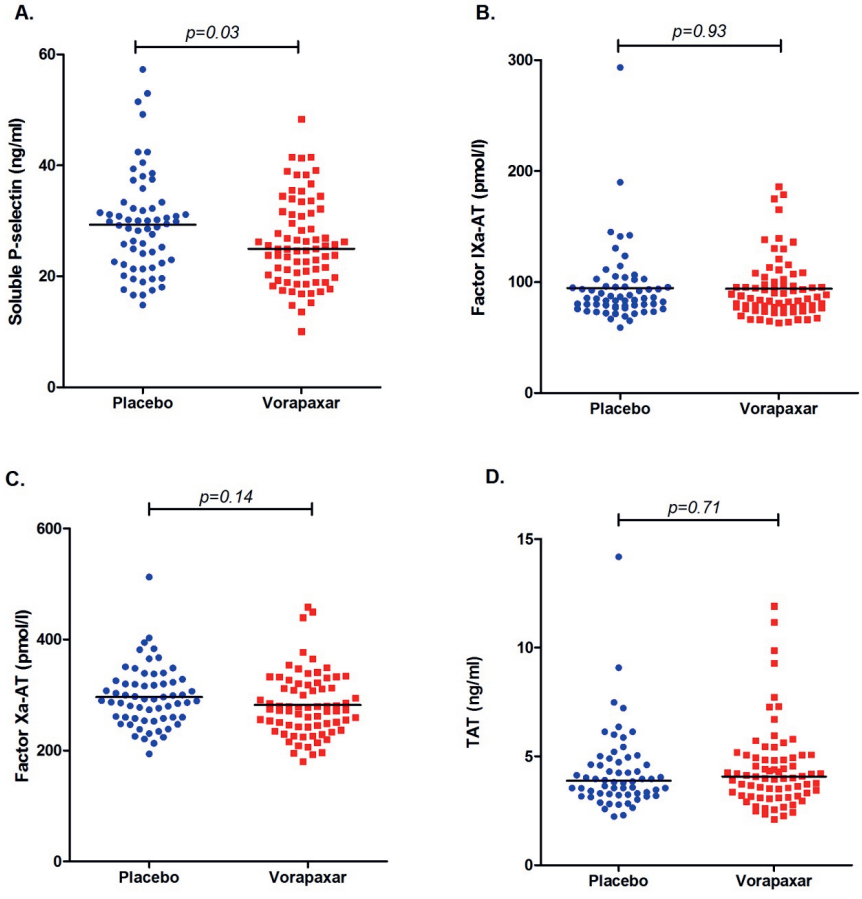
	Vorapaxar (n=73) mean ± SD, or n (%)	Placebo (n=62) mean ± SD, or n (%)	p-value
Patient characteristics			
Age (years)	61.3 ± 11.8	60.0 ± 10.3	0.48
Male	58 (79.5)	53 (85.5)	0.36
BMI (kg/m^2)	27.6 ± 4.6	27.7 ± 3.7	0.85
eGFR (ml/min)	92.8 ± 29.1	94.1 ± 22.7	0.78
Diabetes mellitus	7 (9.6)	4 (6.5)	0.51
Hypertension	31 (42.5)	19 (30.6)	0.16
Hyperlipidemia	32 (43.8)	20 (32.3)	0.17
Treatment exposure (days)	898.5 ± 155.1	910.6 ± 143.0	0.64
Co-medication at sampling			
Aspirin	67 (91.8)	58 (93.5)	1.00
Clopidogrel	18 (24.7)	12 (19.4)	0.53
Dual antiplatelet therapy	17 (23.3)	11 (17.7)	0.39
Laboratory characteristics			
Hemoglobin (g/dL)	14.0 ± 1.1	14.3 ± 1.2	0.10
Platelet count (10^9/L)	223.9 ± 53.3	225.6 ± 57.1	0.86
MPV (fL)	10.6 ± 0.9	10.9 ± 0.8	0.18

Values expressed as either mean ± standard deviation (SD) or as counts and percentages. Continuous variables were compared using Student's t-test. Categorical variables were compared using the χ^2 – test or Fisher's exact test when frequencies were <5. Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; MPV, mean platelet volume.

According to soluble P-selectin levels, platelet activation was reduced in vorapaxar-treated patients compared to the placebo group (24.9 ng/mL [interquartile range, IQR: 20.0–31.5] vs. 29.3 [IQR: 22.3–32.5]; p=0.027). No difference was found in FIXa-AT levels (94.0±27.1 vs. 94.5±34.0; p=0.93), FXa-AT levels (282.0±57.4 vs. 296.4±54.6; p=0.14) and TAT levels (4.0 [IQR: 3.1–5.0] vs. 3.8 [IQR: 3.0–4.9]; p=0.71) between the vorapaxar-group and placebo-group (Figure 8.1). No differences in platelet or coagulation biomarker levels were found when comparing patients on single or dual antiplatelet therapy for both treatment groups (Appendix 8).

Our data suggest that, on top of aspirin and/or clopidogrel, vorapaxar further reduces platelet activation in patients with stable CAD. This fits with the observed reduction in atherothrombotic events in these patients in the TRA2°P-TIMI-50-trial[1]. However, we found no additional effect on markers of coagulation activation, indicating that vorapaxar does not further reduce thrombin generation via intensified platelet inhibition.

Figure 8.1 Results of soluble P-selectin, FIXa-AT, FXa-AT and TAT complexes in vorapaxar-treated patients and placebo group.



Compared with the placebo group, vorapaxar-treated patients had significantly lower levels of soluble P-selectin (p=0.03) ***(A)****. No significant differences were found in factor IXa-antithrombin (FIXa-AT)* ***(B)****, factor Xa-antithrombin (FXa-AT)* ***(C)*** *and thrombin-antithrombin (TAT)* ***(D)*** *complexes between vorapaxar-treated patients and the placebo group.*

Although P-selectin can also be secreted from endothelial cells, the increase in soluble P-selectin in (pre-)thrombotic conditions is assumed to be mainly derived from activated platelets[4]. Biomarkers derived from platelets have been shown to correlate with antiplatelet therapy utilization[5,8,9]. Thus, the significant reduction of soluble P-selectin levels in vorapaxar-treated patients indicates a further reduction in platelet activation on top of standard antiplatelet therapy. Our study in chronic CAD patients shows that this reduction might only occur over the course of treatment, as Storey et

al. have previously shown that P-selectin and sCD40-ligand were comparable between vorapaxar-treated patients and control patients in the acute phase after ACS[8].

Intrinsic tenase and prothrombinase complexes assemble on the procoagulant surface of activated platelets. Using our assays, the direct end products of these complexes, factor Xa and thrombin, were determined. If vorapaxar indeed reduces thrombin generation via intensified platelet inhibition, lower factor activity levels can be expected.

The finding that no difference is observed in factor IXa-AT, factor Xa-AT and TAT complexes between the vorapaxar and placebo group might be explained by the concomitant use of aspirin and clopidogrel, which already reduces platelet-dependent thrombin generation to such an extent, that the impact of adding vorapaxar is only small. For clopidogrel, several studies have already shown an inhibitory effect on surface-generated thrombin and thrombin-induced clot formation[10,11]. Furthermore, in this study samples were taken from stable CAD patients, while the beneficial effect of vorapaxar in reducing thrombin generation might become more pronounced in conditions of acute plaque rupture, when levels of thrombin rise explosively. This is in line with the consistent reduction in the rate of type 1 (spontaneous) myocardial infarction in vorapaxar-treated patients[1]. Thrombin levels in stable conditions might be just too low to show a detectable reduction via platelet inhibition.

Finally, it is possible that vorapaxar has indeed no interference with the coagulation process, in accordance with the absence of effect on parameters of thromboelastography in a previous study[12]. Theoretically, part of the beneficial effect of vorapaxar on the risk of ischemic events might also be attributable to effects of vorapaxar on the vascular endothelium. Since PAR-1 on endothelial cells and vascular smooth muscle cells mediates mitogenic effects, vorapaxar might be effective in reducing vascular remodeling and consecutive progression of atherosclerosis.

Strengths of our analysis include the simultaneous measurement of both coagulation and platelet biomarkers in a patient population on long-term treatment with stable CAD. Limitations are the relatively small sample size, the fact that we were only able to measure at a single time point, and that total platelet P-selectin was not assessed. Although we cannot fully prove that the detected decrease in soluble P-selectin is related to additional platelet inhibition, it is likely to be at least in part a reflection of total platelet P-selectin[4].

In conclusion, our data suggest that the beneficial effect of vorapaxar on top of standard antiplatelet therapy in stable CAD patients is likely due to further attenuation of platelet reactivity, without detectable reduction in thrombin generation.

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Appendix 8

Table S8.1 Comparison of platelet and coagulation biomarkers in patients with single versus dual antiplatelet therapy.

Treatment group	Biomarker	SAPT	DAPT	p-value
Vorapaxar	P-selectin	26.6 ± 8.0	24.6 ± 6.8	0.36
	FIXa-AT	94.4 ± 25.5	88.4 ± 28.6	0.42
	FXa-AT	285.4 ± 62.1	265.2 ± 41.2	0.21
	TAT	4.5 ± 2.1	4.0 ± 1.3	0.41
Placebo	P-selectin	28.9 ± 8.4	31.7 ± 12.1	0.37
	FIXa-AT	96.6 ± 36.9	87.2 ± 19.3	0.42
	FXa-AT	297.4 ± 56.4	286.0 ± 47.8	0.54
	TAT	5.2 ± 2.9	3.5 ± 1.3	0.26

Abbreviations: SAPT, single antiplatelet therapy; DAPT, dual antiplatelet therapy; FIXa-AT, Factor IXa – Antithrombin; FXa-AT, Factor Xa – Antithrombin; TAT, thrombin – antithrombin.

PART III

SUMMARY, DISCUSSION AND IMPACT

Chapter 9

General discussion and future prospects

General discussion and future prospects

Patient-tailored antithrombotic therapy following percutaneous coronary intervention

Dual antiplatelet therapy (DAPT), consisting of aspirin and a P2Y12 inhibitor, is the standard of care for patients with acute coronary syndrome (ACS) and chronic coronary syndrome (CCS) undergoing percutaneous coronary intervention (PCI) to prevent ischemic events. However, in the last decennium choosing the optimal antithrombotic strategy for each patient has become more and more challenging due to the broad spectrum of antithrombotic treatment options. Not only with the introduction of prasugrel and ticagrelor next to clopidogrel as P2Y12 inhibitors and of direct oral anticoagulants (DOACs) as alternative for vitamin K antagonists (VKA), but also with the introduction of several combination strategies of antiplatelet and anticoagulant therapy, the treating physician is faced with a complex panel of choices during antithrombotic treatment of coronary artery disease (CAD) patients. Besides, more frequently, complex patients with multiple comorbidities are being treated. Current guidelines recommend risk stratification for tailoring the antithrombotic treatment strategy[1,2]. Platelet function testing, genotyping and several risk scores have been introduced as risk stratification methods for the general PCI population, however, they have neither been sufficiently investigated nor validated in the specific group of high-risk patients[3]. This thesis focused on the optimization of antithrombotic therapy in these patients with multiple clinical risk factors, evaluating patient-related factors, platelet function testing, genetics and coagulation assays.

Prospective study cohort

In **chapter 3**, we describe the prospective cohort of 524 high-risk patients that was set up in the Maastricht University Medical Center. This observational cohort study was set up to evaluate patient-related factors, platelet reactivity, genetics, and a broad spectrum of biomarkers in predicting adverse events in high-risk patients on dual or triple antithrombotic therapy after PCI. Almost half of these patients (48.5%) reported at least one BARC type 1-3 bleeding event during 6-12 months follow-up. Although most patients reported mild bleeding (BARC type 1), still 92 patients (17.5%) had experienced a BARC type 2 or 3 bleeding at any time point, necessitating consultation, diagnostic tests, interventions, blood transfusions and/or hospitalization. Next to bleeding events, 13.2% had a major adverse cardiovascular event during follow-up[4]. These incidences of both bleeding and ischemic events are much higher than reported in RCTs in the CAD population, indicating the substantially increased risk in these patients with multiple clinical risk factors, and the need for optimal risk stratification methods.

Several diagnostic tests and treatment strategies have been evaluated to reduce both bleeding and ischemic risks in post-PCI patients. This thesis focused on some of these strategies for the group of patients with multiple clinical risk factors, or so-called 'high-risk patients', and we will discuss their applicability one-by-one, followed by an algorithm for the suggested approach of future high-risk patients.

Platelet function testing

We performed platelet function testing in this cohort, using 3 different platelet function tests: VerifyNow P2Y12, Multiplate ADP and Light Transmission Aggregometry (LTA) ADP20. As previous research in this cohort had already indicated, the agreement between these PFTs was only slight to moderate[5]. It is questionable whether previously proposed cut off levels can be applied in our selected high-risk population, as for example mean platelet reactivity as measured by Multiplate in our cohort is 47.7 AU and, according to the proposed cut off levels (19-46 AU), indicates high on-treatment platelet reactivity while bleeding complications in this cohort prevail. Based on a combination of clinical factors, bleeding questionnaires, side effects, laboratory results and/or platelet function testing we changed the type, dosage or duration of P2Y12 inhibitor in 15% of our patients in an attempt to reduce the risk of complications[4]. Therefore, we could not evaluate whether PFT results predicted adverse events in our population or which of the PFTs was most accurate in predicting bleeding. Platelet function testing can provide useful insights, but trials evaluating treatment strategies guided by these stratification methods have produced mixed results. In the general PCI population, several studies have shown that tailoring antiplatelet therapy solely based on PFT results does not prevent ischemic and bleeding outcomes[6-8]. Since then, due to advances in stent technology and broader use of potent P2Y12 inhibitors, thrombotic events have dramatically decreased, and consequently, prevention of bleeding complications has become a more important goal, especially in patients with multiple clinical risk factors. Thus, PFTs may play a more important role in a bleeding reduction strategy, and indeed, randomized trials incorporating PFT results to de-escalate DAPT have shown more promising results[9,10]. Current guidelines recommend de-escalation of P2Y12 inhibition treatment guided by PFTs to be considered as an alternative DAPT strategy, especially for these patients at high risk for bleeding, deemed unsuitable for 12-month potent platelet inhibition[11]. However, for optimal applicability of PFTs in high-risk patients, adjustment of cut-off levels in various conditions seems to be necessary. Furthermore, it remains a challenge to select the right PFT for the right situation. Besides, all PFTs are in vitro tests, that do not per se reflect the effect of antiplatelet therapy in vivo. Based on current knowledge, it is impossible to prefer one PFT over another, as the assays differ in test principle, feasibility, costs and availability,

and guidelines do not recommend on the selection of assays for tailoring antiplatelet therapy[2,11,12].

Genetics

Polymorphisms of the *CYP2C19* gene have an impact on the metabolization of clopidogrel and, thereby, have an impact on on-treatment platelet reactivity. In **chapter 4** we evaluated the differential effects of the *CYP2C19* genotype on three different PFTs in our cohort of high-risk patients. For the Multiplate assay, no major effect of genetic background could be shown; effects of other (patient-related) variables prevailed. Thus, besides differences in test principles and the influence of patient-related factors, the aforementioned disagreement between PFTs is partly explained by the differential effects of the *CYP2C219* genotype. Contrary to the Multiplate assay, for Verify Now and LTA, carriers of the loss-of-function alleles (*CYP2C19*2* and *CYP2C19*3*) have lower residual platelet reactivity, whereas carriers of the gain-of-function allele *CYP2C19*17* have higher residual platelet reactivity[13]. Our study was too small to correlate these differences in residual platelet reactivity to clinical endpoints such as major bleeding or major cardiovascular events. However, several previous larger studies have already shown that the higher residual platelet reactivity in these patients with *CYP2C19*2* or *CYP2C19*3* allelic variants is associated with a higher incidence of major adverse cardiovascular and cerebrovascular events[14]. Thus, genotyping might be of utility in risk stratification and tailoring of P2Y12 inhibitor therapy. Recently, several studies evaluated pharmacogenomic testing as an approach to personalize antiplatelet drug administration by either escalation or de-escalation of P2Y12 inhibitor therapy based on *CYP2C19* allelic variants. The TAILOR-PCI trial compared a genotype-guided escalation strategy (ticagrelor instead of clopidogrel in carriers of *CYP2C19* loss-of-function alleles) to standard treatment with clopidogrel in patients treated with PCI. In the genotype-guided group there were numerically, though not statistically significantly, fewer adverse cardiovascular events[15]. In a meta-analysis including the aforementioned TAILOR-PCI trial, prescription of ticagrelor and prasugrel in patients with *CYP2C19* loss-of-function alleles compared to clopidogrel resulted in a significant reduction of ischemic events, but not in non-carriers of these alleles[16]. In the POPular genetics trial, acute myocardial infarction patients undergoing primary PCI were randomized to genotype-guided de-escalation (ticagrelor in carriers and clopidogrel in non-carriers) or standard treatment with either ticagrelor or prasugrel. Genotype-guided P2Y12 de-escalation was non-inferior to standard treatment in terms of net clinical benefit, and there was a significant reduction in the primary bleeding outcome[17]. Taken together, there is more and more evidence supporting genotype-

guided P2Y12 inhibition, and it is currently finding its way towards implementation in clinical practice.

Genotyping versus platelet function testing

Both platelet function testing and genotyping can provide useful prognostic insights. Pharmacogenomic testing could be an attractive approach because treatment decisions can be made before the start of antiplatelet therapy, unlike with PFT, and furthermore, the individual genotype does not change over time, unlike the phenotype of platelet reactivity. Moreover, one of the major limitations of PFTs is the great variability of the results in the different assays, as was also shown in our cohort, and this could be overcome by genetic testing. On the other hand, genetic variants are just one influential factor affecting clopidogrel activity, and previous studies have revealed that *CYP2C19*2* carrier status accounted for only 5-12% of variability in platelet reactivity. Thus, the information derived from pharmacogenomic testing cannot be taken as a surrogate for PFT to assess antiplatelet drug responses; numerous epigenetic factors such as comorbidities, gastrointestinal absorption, drug interactions, and adherence are also important determinants of P2Y12 inhibitor activity[12] (Table 9.1).

Table 9.1 Advantages and drawbacks of platelet function testing and genotyping.

	Platelet function testing	Genotyping
Easy to use	√/x	√
Results rapidly available	√	√/x
Direct measure of response to therapy	√	x
Test possible before start of P2Y12 inhibitor	x	√
Agreement between different test assays	x	√
Results affected by patient-related factors	√	x
Stable intra-individual results over time	x	√
Associated with ischemic events	√	√
Associated with bleeding events	√	√
Benefits in tailoring strategy established in RCTs	√/x	√/x

Other coagulation assays

Instead of specific assays that evaluate only one specific hemostasis pathway (e.g. INR, DOAC level or PFTs), global assays of hemostasis could provide insight into prediction of adverse events in patients on antithrombotic agents. As explained in **chapter 2** atherothrombosis is an interplay between platelets and coagulation[18]. Such global coagulation assays could potentially identify patients at risk either due to low residual platelet reactivity, or due to the 'second hit hypothesis'. Following this second hit hypothesis, patients with bleeding events while on antiplatelet therapy might have an (undetected) mild coagulation disorder, e.g., due to variation in specific plasma

components like the level of factor VIII. Starting (dual) antiplatelet therapy in these patients is the last straw that breaks the camel's back, and eventually results in bleeding events, while platelet reactivity might be within range. In our cohort, we assessed two additional coagulation assays: thrombin generation (TG) **(chapter 5)** and ROTEM **(chapter 6)**. Thrombin generation was performed in the absence of platelets (in PPP; platelet poor plasma), so the observed differences between patients with and without bleeding while on DAPT, are more likely attributable to plasma components of coagulation. TG was performed in a subgroup of patients with DAPT, so all patients with concomitant use of anticoagulants were excluded. In patients with clinically relevant bleeding during follow-up, the parameters of thrombin generation (endogenous thrombin potential, peak height and velocity index) were significantly lower compared to patients without bleeding. These results suggest that TG performed in PPP may have the potential to aid in the identification of patients with an increased bleeding risk during DAPT[19]. However, to adequately identify these patients, cut off values for TG would be needed. Furthermore, assessment of TG in platelet rich plasma (PRP) or whole blood could be of even more interest, as these assays might give insight in the interplay between the platelets and the plasma components of coagulation. In **chapter 6** we evaluated routine and tissue plasminogen activated (tPA) ROTEM results in the high-risk patients in our cohort, either treated with DAPT, or with P2Y12 inhibitors plus VKA/DOAC. We showed that all post-PCI patients, irrespective of antithrombotic treatment combination, had increased clot formation as evidenced by shortened clot formation time (CFT) and increased maximum clot formation (MCF) one month after intervention as compared to control patients without PCI and antithrombotic treatment. This suggests a more universal pathology underlying increased clot stiffness in patients requiring PCI rather than an effect of antithrombotic treatment. Only the DAPT group additionally presented with a decreased fibrinolytic potential as measured with tPA ROTEM compared to the control group without coronary artery disease. This again might reflect abnormal clot architecture in patients with thrombosis; the higher density and reduced permeability of the clot predispose to limited clot breakdown as the formed fibrin fibers are less accessible for tPA-mediated thrombosis. In patient groups treated with P2Y12 inhibitors plus DOAC or VKA, this hypofibrinolytic state might have been overruled by anticoagulant-mediated pro-fibrinolytic mechanisms. In contrary to clear ROTEM clotting time (CT) prolongation for VKA or DOAC, ROTEM is unable to identify antiplatelet drugs. Interestingly, we showed that EXTEM CT did have predictive ability for the development of clinically relevant bleeding (BARC type 2 or 3) during follow-up in patients on DAPT treatment[20]; this may be of interest for guiding bleeding risk assessment in such patients. Due to limited number of patients, we were

not able to correlate ROTEM parameters with clinical outcomes in patients on anticoagulants (VKA/DOAC).

Other measures to optimize the antithrombotic strategy

High risk patients with triple therapy

Between 5% and 10% of patients with atrial fibrillation are eventually referred for PCI[21]. Vice versa, it is estimated that atrial fibrillation develops in up to 20% of patients with acute coronary syndromes[22]. Consequently, a substantial group of patients will have an indication for both (dual) antiplatelet therapy and oral anticoagulants. In observational studies, patients treated with triple therapy (aspirin, a P2Y12 inhibitor, and an oral anticoagulant) were at high risk for bleeding. The WOEST (What is the Optimal Antiplatelet and Anticoagulant Therapy in Patients with Oral Anticoagulation and Coronary Stenting) trial compared DAPT and VKA with clopidogrel and VKA[23]. The trial suggested but did not prove because of a small sample size, that treatment with a P2Y12 inhibitor and anticoagulant might be an effective strategy that balances the anti-ischemic benefit against the risk of bleeding. Since then, several trials have now documented a reduction in bleeding when a DOAC and a P2Y12 inhibitor are used together, as compared with warfarin-based triple therapy[24-27]. International guidelines have shifted their recommendations over the years towards an approach of post-discharge dual antithrombotic therapy (DAT), with triple antithrombotic therapy (TAT) only reserved for those patients with high thrombotic risk and favorable bleeding risk[2]. In **chapter 7** we included 160 high-risk patients from our cohort-study in the WOEST2 study, a large prospective real-world cohort study comparing DAT to TAT in 1075 patients with AF undergoing PCI. We found a substantially lower risk of clinically relevant bleeding without a significant penalty in ischemic events. This supports the clinical benefit of DAT as a strategy to prevent bleeding complications and indicates that high-risk patients may be adequately identified by their cardiologist and accordingly treated.

Risk scores

Risk scores favored by the ESC guidelines are the PREdicting bleeding Complications In patients undergoing Stent implantations and subsequent Dual AntiPlatelet Therapy (PRECISE-DAPT) and the Academic Research Consortium High Bleeding Risk (ARC-HBR) score. The PRECISE-DAPT score consists of the variables hemoglobin, age, creatinin clearance, white blood cell count and previous spontaneous bleeding. In patients with a high PRECISE DAPT score ($\geq$25), standard 12-month DAPT was associated with no reduction in ischemic events, but with a strong increase in bleeding[28]. According to the ESC guidelines, these high bleeding risk patients should receive shorter DAPT ($\leq$6

months)[2]. Patients with a PRECISE-DAPT score <25 should be treated with standard 12-month DAPT. The ARC-HBR score was not developed to tailor DAPT duration, but as a consensus definition of patients at high bleeding risk, defined as a BARC type 3 or 5 bleeding risk of ≥4% and/or risk of intracranial hemorrhage ≥1% within 1 year after PCI[3]. Twenty clinical criteria are classified as major or minor, and patients are considered to be at high bleeding risk if at least 1 major or 2 minor criteria are met. The selection criteria for high-risk patients in our own cohort shows considerable overlap with these ARC-HBR criteria[4]. Finally, for ACS-patients that have tolerated 12-month DAPT and did not suffer thrombotic or bleeding events, the DAPT-score could be used to select patients (score ≥2) that may benefit from extended DAPT beyond 12 months[29]. In patients with a DAPT score <2 DAPT should not be extended, as this was associated with an increase in bleeding events, without reductions in ischemic events.

Shortening or prolonging duration of APT

Historically, DAPT was recommended for 12 months after PCI because of concerns over late stent thrombosis. However, with the introduction of new generation DES and the introduction of more potent P2Y12 inhibitors, the rates of stent thrombosis have decreased considerably. Following this trend, several RCTs have studied short course DAPT (3-6 months) as compared to 12 months DAPT in patients with relatively low risk of thrombotic events and with increased bleeding risk, showing a reduction in bleeding complications without a signal of increased ischemic events. Other RCTs have investigated the efficacy and safety of aspirin discontinuation after a short course of DAPT (1-3 months). Although some of these trials were underpowered, meta-analyses concluded that P2Y12 monotherapy preceded by short DAPT is associated with a lower incidence of clinically relevant bleeding compared to standard DAPT without an increase in cardiovascular events after 1 year, and could thus be considered as an alternative in patients without high ischemic risk[30,31].

Alternative antithrombotic strategies

Dual pathway inhibition

As discussed in **chapter 2**, atherothrombosis is the result of inflammatory processes involved in atherogenesis and the interplay between thrombin and platelets. Dual pathway inhibition (DPI), the addition of (low-dose) anticoagulants on top of platelet inhibitors, has the potential to modulate a number of inflammatory pathways (via PAR-receptors) and to inhibit the formation of thrombin, which plays a crucial role in both coagulation and platelet activation[18]. The concept of targeting both platelets and coagulation proteases is attractive, because platelets are key players in atherosclerosis and atherothrombosis, while preclinical studies indicate the importance of coagulation

proteases on atherosclerotic plaque formation and plaque phenotype. The combination of antiplatelet and anticoagulant activities is likely to raise the potency of anti-thrombotic therapy aimed at preventing atherothrombosis and thromboinflammation, because the combination not only offers a more intense antithrombotic effect, but also enhances vascular protection via the impact on inflammation and vascular endothelium[32,33]. The COMPASS trial showed that low-dose rivaroxaban on top of aspirin was associated with reduced risk of cardiovascular death, MI or stroke in patients with chronic coronary disease and PAD, at the price of more major (but not fatal) bleeding events (34). Current guidelines now recommend such a DPI strategy for long-term secondary prevention in CAD and PAD patients with high ischemic risk and low bleeding risk[2].

Vorapaxar

In **chapter 8** we investigated another agent that affects PAR-signaling; the platelet inhibitor vorapaxar. Vorapaxar is a selective antagonist of protease-activated receptor-1 (PAR-1), thereby blocking thrombin-mediated platelet activation. Other actions of thrombin, such as fibrin formation and protein-C activation, are not inhibited by blocking PAR-1. We studied whether the beneficial effect of vorapaxar on top of antiplatelet therapy in stable CAD patients could only be ascribed to more potent platelet inhibition or to an additional effect on thrombin generation due to a reduced availability of activated platelets as a procoagulant surface for the assembly of coagulation factors. We did not find evidence for such a dual pathway inhibition effect of vorapaxar, as levels of factor IXa-antithrombin, factor Xa-antithrombin and thrombin-antithrombin complexes were comparable between patients treated with vorapaxar and controls[35]. However, PAR-1 is not only the thrombin receptor expressed on platelets, but is also expressed in endothelial cells, vascular smooth muscle cells and immune cells. Therefore, PAR-1 signaling is central in mediating thrombo-inflammation. More recent studies suggest that also the non-hemostatic, platelet-independent, pleiotropic effects of PAR-1 inhibition with vorapaxar could be responsible for the reduction of thrombo-inflammation in patients with atherosclerosis[36,37]. Although vorapaxar is EMA and FDA approved and has proven to reduce cardiovascular events in stable CAD patients, it is currently not part of international guidelines, due to an increased association with bleeding[38].

Future prospects; an algorithm

Physicians need to weigh all these clinical and laboratory aspects together with input from risk scores, and in selected patients from PFT or genotyping, before choosing an antithrombotic strategy. Furthermore, as a patient's bleeding and ischemic risk may

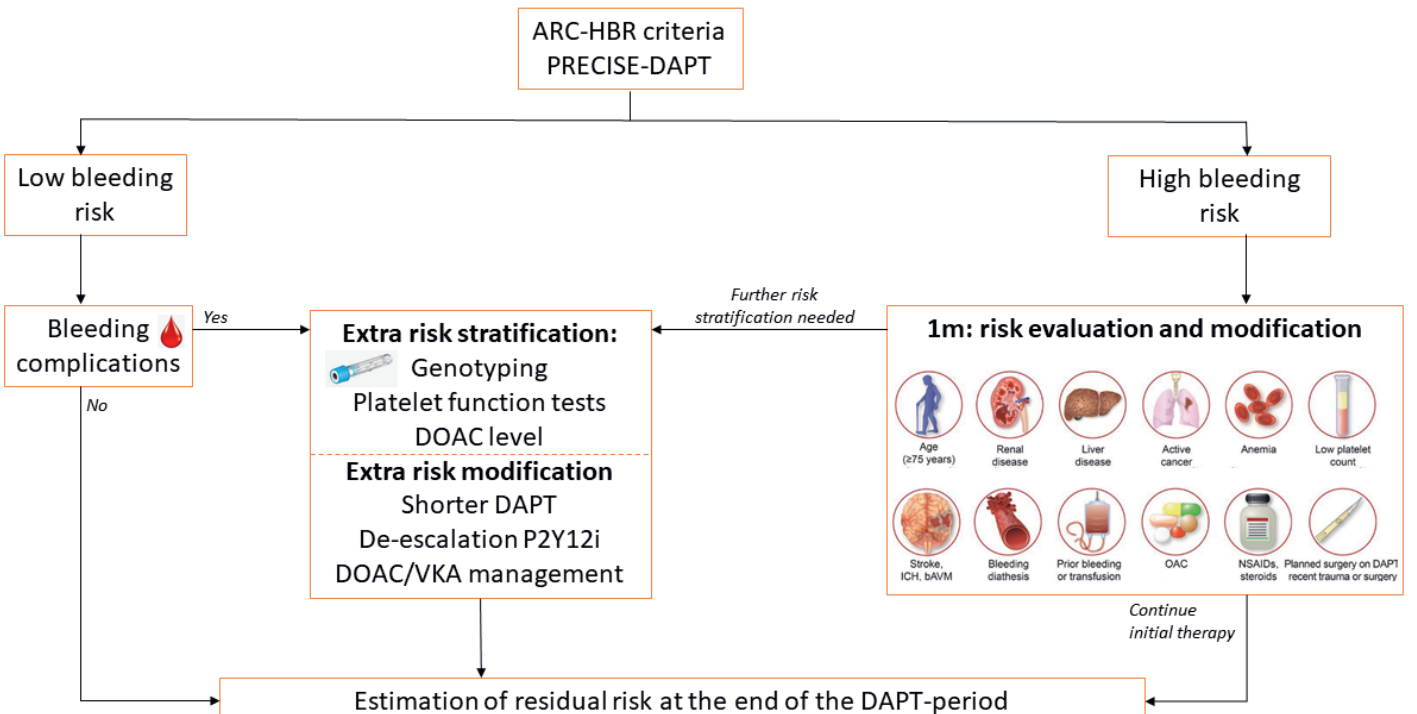
change over time, this strategy should be evaluated regularly, especially in high-risk patients or patients with bleeding or ischemic events while on therapy. The high incidence of both bleeding and ischemic events in our cohort, as well as the frequent need for medication adjustment during follow-up, indicates the need for strict monitoring of this patient group and illustrates challenges in optimization of antithrombotic management. This supports the necessity for a multidisciplinary outpatient clinic, as was set up in the Maastricht University Medical Center and presented in this thesis.

Combining all the aspects described in this thesis in an algorithm would hypothetically not only lead to a more reliable identification of high-risk patients, but also to a more balanced approach in personalization and optimization of antithrombotic therapy. Based on the novel insights from this thesis, the current guidelines, recent trials and our own experience, we propose the following algorithm for risk stratification and management of future patients.

In this algorithm (Figure 9.1), risk stratification of the heterogeneous group of PCI patients should start at discharge post-PCI using the ARC-HBR criteria. Patients are considered high (HBR) or low (LBR) bleeding risk depending on the definition of ARC-HBR. Patients at LBR, in combination with a PRECISE-DAPT score < 25 at discharge post-PCI, do not need to be referred for additional evaluation, and can be treated with standard DAPT for 6 (CCS) or 12 (ACS) months. Depending on local availability, pharmacogenomic testing could be used prior to PCI to select P2Y12 inhibitor therapy, or to promote early de-escalation.

For the group of patients that meet the criteria for HBR (at least 1 major or 2 minor criteria) we propose a risk evaluation 1 month post-PCI at a dedicated outpatient clinic. During this visit, factors associated with an increased bleeding risk can be identified and modifiable risk factors (i.e. co-medication, renal disease) should be managed to reduce bleeding risk. In our years of experience at the Thrombosis Expertise Center, we frequently encountered medical problems, such as progressive renal insufficiency or newly diagnosed iron deficiency anemia. Furthermore, during medication review, we frequently added a proton pump inhibitor in patients at high risk for gastric ulcers, stopped co-medication like NSAIDs, or corrected medication errors, either caused by the doctor (wrong prescription) or the patient. Following this risk evaluation and modification visit, during which also the PRECISE-DAPT score at discharge should be evaluated to determine treatment duration, this patient group can be divided in patients that can continue the initial therapy for the rest of their 6-12 month treatment period, or patients in whom further risk stratification is needed.

Figure 9.1 Algorithm for risk stratification of PCI patients.



Abbreviations: ARC-HBR; Academic Research Consortium for High Bleeding Risk, DOAC; direct oral anticoagulants, DAPT; dual antiplatelet therapy, VKA; vitamin K antagonist, ICH; intracranial hemorrhage, bAVM; brain arteriovenous malformation, OAC; oral anticoagulants, NSAIDs; nonsteroidal anti-inflammatory drugs. (Figure adapted from Urban et al., Circulation 2019 (3))

In the latter group, genotyping, platelet function testing and/or DOAC level monitoring could guide further personalized antithrombotic treatment. In these HBR patients, the bleeding risk often outweighs the ischemic risk, favoring shorter (3-6m) DAPT strategies or de-escalation of P2Y12 inhibitors to reduce the bleeding risk. ROTEM and TG are not part of standard of care, but could be implemented for research purposes.

When patients that initially qualified as low-bleeding risk with standard DAPT duration of 12 months develop bleeding complications during treatment, they should be referred to the dedicated outpatient clinic for risk evaluation and stratification of antithrombotic treatment comparable to the high bleeding risk group. In these patients, PFTs could play a role to monitor compliance, to determine individual response to therapy taking into account all other patient-related factors, and to de-escalate P2Y12 inhibitor therapy. Treatment adjustments should be made on an individual basis with structural follow-up.

Finally, for all patients, an 'end of DAPT' evaluation should take place, with estimation of the residual risk. After 12 months of uneventful DAPT, the DAPT score can be used to identify patients with high ischemic risk without increased bleeding risk. For these patients, antithrombotic treatment options are either extended DAPT or DPI. Furthermore, besides optimization of antithrombotic treatment, other measures to reduce residual atherothrombotic risk, such as anti-inflammatory drugs (e.g. colchicine), should also be taken into account.

Future prospects

Patients qualifying for intensified follow-up should form the basis for our future research cohort. Our next research focus in this field will be on optimization of PFT cut-off levels for patients with multiple clinical risk factors, further evaluation of ROTEM and TG to predict and prevent bleeding complications, and evaluation of flow-based assays in this setting. Furthermore, the descriptive data, in combination with laboratory assays, genetics, and bleeding questionnaires could be used for the construction of a multimarker risk prediction model specifically developed for high-risk patients. Besides optimization of the combination and duration of antithrombotic therapy in high-risk patients with atherothrombotic events, the role of anti-inflammatory drugs such as colchicine should be explored in this future cohort of comparable high-risk patients.

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Chapter 10

Impact paragraph

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In this thesis, we explored a number of scientific questions related to clinical problems that are encountered in everyday management of high-risk patients with antithrombotic therapy after percutaneous coronary intervention (PCI). We aimed to explore ways to identify patients at highest risk for bleeding while on dual/triple antithrombotic therapy. In this chapter, the relevance of the results described in this thesis and their scientific and societal impact will be discussed.

Prescribing the optimal combination of antithrombotic agents for each individual patient is a growing challenge for physicians. Not only do we treat more complex patients with more comorbidities, also the spectrum of antithrombotic drugs has been expanded during the last decennia, making the best selection of (a combination of) these treatment options a complex decision. Common characteristic for all antithrombotic drugs is that they reduce the risk for (recurrent) thrombosis, however, always at the price of increased bleeding risk. Indeed, with current generations of very potent antithrombotic medication, bleeding is the main adverse effect, particularly given its association with mortality[1]. Balancing these risks and benefits is the cornerstone for successful antithrombotic treatment.

With this in mind, an outpatient clinic for high-risk patients was initiated within the Thrombosis Expertise Center in the Maastricht University Medical Center in 2014 with the purpose of: 1) close monitoring of the high-risk patients during their 6-12 month treatment period on dual antiplatelet therapy 2) personalizing treatment regimens with the goal of optimizing efficacy and safety outcome in the individual patient, and 3) increasing the knowledge on the value of platelet function testing (PFT) and other laboratory assays to predict bleeding and to guide treatment decisions. Close collaboration between the department of Internal Medicine, department of Cardiology, Central Diagnostic Laboratory and the Cardiovascular Research Institute Maastricht (CARIM) has led to a well-organized outpatient clinic with affiliated scientific research that formed the basis for this thesis.

Bleeding complications have major impact on patients' health and well-being, since they can cause additional monitoring or hospital visits, extra diagnostic procedures, blood transfusion, hospitalization, or even death. But also, the minimal bleedings (BARC type 1), which are often not monitored nor reported in large clinical trials, have impact on patients' quality of life[2]. These minimal bleedings are often neglected and thought to be 'part of the game', however with an incidence of 30.9% within our cohort, we

believe that they do not only have impact on the quality of life, but also affect adherence, and might be a prediction of future clinically relevant bleeding[3]. Identifying these patients at risk for bleeding is a first important step towards prevention of bleeding. When these patients have been identified, they can be monitored closely with adjustment of therapy when indicated. Besides close monitoring, extra laboratory tests could help to predict bleeding and identify patients at highest risk.

For patients, we believe that this close monitoring could lead to safer prescription of dual/triple antithrombotic therapy, although no comparative cohort is available to serve as a control group for the cohort described in this thesis. A safer treatment will result in a reduction in healthcare costs and an increase in quality of life. Reduction in bleeding episodes could eventually lead to decreased hospitalization, decreased use of transfusion products, with accompanied economic and social benefits. However, such an economic evaluation of cost-effectiveness on our data has not been performed yet. Moreover, the close monitoring of these patients learned us that during follow-up they encounter several other issues that need to be addressed, such as evaluation of (iron-deficiency) anaemia, reduced kidney function, and drug-drug interactions. Unpublished data from our cohort, beyond the focus of this thesis, indicate that in ± 25% of our patients there is potential gain in improving safety by tackling these additional risk factors. We take these experiences with us when modifying the outpatient clinic for the whole spectrum of high-risk patients on antithrombotic medication in the near future.

This thesis also evaluated the use of established and new laboratory tests regarding the identification of patients at risk for bleeding. Current guidelines recommend risk stratification for tailoring individual treatment in high-risk patients post-PCI[4,5]. Both platelet function testing (PFT) and genotyping can provide useful prognostic insights, but previous trials evaluating treatment strategies based on these tests have produced mixed results[6]. Moreover, a multitude of PFTs is available and correlation of these tests is only slight to moderate[7]. We showed that the correlation to genetic background of the patient partly explains the poor correlation of these PFTs[8]. This is relevant for local laboratories without the opportunity of genetic testing, or that need to decide which test from the range of available PFTs to implement. Pharmacogenomic testing is expected to become more common soon. Recent studies have already shown that pharmacogenomic-based prescription of P2Y12 inhibitors is a cost-effective strategy and improves quality of life[9,10]. This thesis also proposed several new laboratory tests regarding the identification of patients at risk for bleeding. The results of chapter 5 and 6 indicated that it is of interest to measure thrombin generation and (tPA-) ROTEM to predict future bleeding episodes in patients on dual antithrombotic therapy[11,12].

However, substantial overlap in data between patients with and without bleeding make it still difficult to position any of these assays in the routine laboratory workup. Finally, in chapter 7 we provided real-world data on patients with atrial fibrillation undergoing PCI[13]. Our findings suggest that physicians did not use a 'one size fits all' strategy, but were capable to choose the optimal combination of antithrombotic drugs for different patients, which underlines the importance of monitoring high-risk patients and tailoring individual therapy.

All studies described in this thesis have been published in international peer review journals. The findings of this thesis were presented at the scientific meetings of the International Society of Thrombosis and Haemostasis (ISTH) and the European Society of Cardiology (ESC), and at several national congresses. In this way, our results are shared with a broad audience of both basic researchers in this field, and physicians (cardiologists, internists) treating patients on antithrombotic therapy. Together, we hope that we can further improve the care for high-risk post-PCI patients, so that the future care for these patients goes beyond prescribing 'standard' antithrombotic therapy.

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Chapter 11

Summary

Nederlandse samenvatting

Summary

This thesis focused on aspects in clinical care for high-risk patients with coronary artery disease on dual or triple antithrombotic therapy. In particular, I assessed the role of several laboratory tests in prediction and prevention of bleeding events in this patient group.

Chapter one provides an overview of relevant issues of atherothrombosis, and in particular coronary artery disease. Furthermore, it includes a description of the laboratory tests used in this thesis and gives an overview of cohort studies and a randomized trial that provided the data for studies included in this thesis.

In **chapter two** a review of the literature concerning the role of the coagulation system in atherothrombosis is provided with emphasis on therapeutic strategies in atherothrombotic disease.

Part one of this thesis (chapters 3 ,4, 5 and 6) is based on the Maastricht cohort of 524 high-risk patients on dual or triple antithrombotic therapy after percutaneous coronary intervention (PCI). In **chapter three** I describe the study design of this observational cohort study, including the different laboratory tests that were performed. Moreover, in this chapter baseline characteristics of the entire cohort are presented, as in each of the following chapters (4, 5, 6 and 7) a selection of this cohort was used to answer separate research questions.

After 12 months follow-up, 254 patients (48.5%) had reported one or more BARC type 1-3 bleeding events. Most patients (30.9%) reported mild bleeding symptoms (BARC type 1) for which no consultation or intervention was necessary. However, during 6-12 months follow-up, 17.5% of patients had experienced a BARC type 2 or 3 bleeding, necessitating diagnostic tests, (extra) consultation, interventions, blood transfusions and/or hospitalisation. An ischemic event (a composite of myocardial infarction, stroke or death) was recorded in 13.2% of patients during follow-up. Due to (a combination of) bleeding symptoms, ischemic events, risk assessment, results of laboratory testing, or side effects, we changed the P2Y12 inhibitor therapy in 14.9% and the anticoagulant therapy in 6.3% of patients, respectively. We concluded that the high incidence of both bleeding and ischemic events, as well as the frequent need for medication adjustment during follow-up indicates the need for strict monitoring of this group and illustrates the challenges in optimal antithrombotic management the patient and treating physician are faced with.

In **chapter four** we assessed the effects of the *CYP2C19* genotype on the results of platelet function tests (PFTs) in the subgroup of clopidogrel-treated patients in this cohort. Three different PFTs were used: VerifyNow P2Y12, Multiplate ADP and Light Transmission Aggregometry (LTA) APD20. Previous research in this cohort had revealed that the agreement between these PFTs was only slight to moderate and the current research question was whether this disagreement could be explained by the differential effects of the *CYP2C19* genotype. We found that for VerifyNow and LTA, carriers of the loss-of-function alleles had lower residual platelet reactivity, whereas carriers of the gain-of function allele had higher residual platelet reactivity. For the Multiplate assay, no major effect of genetic background could be shown, while effects of other (patient-related) variables on platelet reactivity prevailed. Thus, I concluded that besides differences in test principles and the influence of patient-related factors, the disagreement between PFTs can partly be explained by differential effects of the *CYP2C19* genotype.

In **chapter five** I showed that patients on dual antiplatelet therapy (DAPT) and not using any anticoagulants who had clinically relevant bleeding during follow-up had reduced and delayed thrombin generation (TG) both 1 and 6 months after PCI, as compared to non-bleeders. Since thrombin generation was performed in platelet poor plasma (PPP), the observed differences between patients with and without bleeding might be explained by variation in coagulation factors. The relatively low thrombin generation in these patients acts as a 'second hit', on top of DAPT, thus increasing the bleeding risk. I concluded that TG performed in PPP may have the potential to aid in the identification of patients with an increased bleeding risk during DAPT.

In the study described in **chapter six** rotational thromboelastometry (ROTEM) was performed in 440 cohort patients on several combinations of dual antithrombotic therapy, and the results were compared to a control group of perioperative (non-PCI) patients not using antithrombotic medication. The data showed that all post-PCI patients (independent of their combination of antithrombotic therapy) had elevated ROTEM clot stiffness values as compared to control patients without previous PCI and antithrombotic treatment. This might point to a more universal pathology underlying increased clot stiffness in coronary artery disease patients rather than an effect of antithrombotic treatment. Furthermore, we found that the group of patients using DAPT presented with a decreased fibrinolytic potential as measured with tissue plasminogen activated (tPA) ROTEM. Again, this might point to abnormal clot architecture in coronary artery disease patients. This hypofibrinolytic state shown in the DAPT group might have been overruled by the pro-fibrinolytic effect of oral

anticoagulants in the other groups of post-PCI patients. Interestingly, in DAPT patients that developed bleeding complications during follow-up, the EXTEM CT measured one month after PCI was prolonged as compared to non-bleeders. Thus, I concluded that the ability of ROTEM to identify patients at risk for bleeding may be promising and warrants further research.

Chapter seven reports on the WOEST2 study, a large prospective real-world cohort study comparing dual antithrombotic therapy (DAT) to triple antithrombotic therapy (TAT) in 1075 patients with atrial fibrillation undergoing PCI. One hundred sixty patients were selected from the Maastricht post-PCI cohort with concomitant atrial fibrillation and included in the WOEST2 study. DAT was associated with significantly less clinically relevant bleeding complications compared to TAT (16.8% vs. 23.6%; p=0.003) without a statistically significant increase in major adverse cardiac and cerebrovascular events (12.4% vs. 9.6%; p=0.17). These results should support clinicians to limit the use of TAT only to those at very high ischaemic risk and with limited bleeding risk.

In **chapter eight** the impact of another antiplatelet agent, the oral protease-activated receptor (PAR)-1 antagonist vorapaxar, on coagulation biomarkers was studied in 135 patients with stable coronary artery disease. Patients were randomized to receive vorapaxar or placebo on top of standard antiplatelet therapy (with aspirin and/or clopidogrel). In blood samples taken after a mean drug exposure of 904 days, a further reduction in soluble P-selectin levels (a marker of platelet activation) in vorapaxar-treated patients was shown, but no differences in the coagulation biomarkers. Therefore, I concluded that the beneficial effect of vorapaxar on top of standard antiplatelet therapy was likely due to further attenuation of platelet reactivity, without detectable reduction in thrombin generation.

Finally, in **chapter nine** the key findings of this present thesis and their clinical implications are discussed. In addition, an algorithm for risk stratification of PCI patients is proposed, and directions for future research are addressed.

Nederlandse samenvatting

De focus van dit proefschrift ligt op verschillende aspecten van de klinische zorg voor hoog-risico patiënten met coronairlijden die duale of triple antitrombotische behandeling krijgen. Daarbij heb ik voornamelijk de rol van verschillende laboratoriumtesten onderzocht bij het voorspellen en voorkomen van bloedings-complicaties bij deze groep hoog-risico patiënten.

Hoofdstuk één is de inleiding van dit proefschrift. Hierin wordt een overzicht gegeven van diverse relevante aspecten van trombose in de arteriële circulatie, ook wel atherotrombose genoemd. Een voorbeeld van atherotrombose is bijvoorbeeld een acute afsluiting van een kransslagader (hartinfarct), hetgeen in dit proefschrift coronairlijden wordt genoemd. In de inleiding worden de diverse antitrombotische middelen kort besproken, en in het bijzonder de behandeling van patiënten met coronairlijden die recent een percutane coronair interventie (PCI, dotterprocedure) hebben gehad. Deze patiënten worden traditioneel behandeld met aspirine, een remmer van de bloedplaatjes. Daarbij wordt vaak ook een tweede bloedplaatjesremmer voorgeschreven, een zogenoemde P2Y12-remmer, waarvan clopidogrel, prasugrel en ticagrelor voorbeelden zijn. De combinatie van twee bloedplaatjesremmers wordt duale antiplaatjestherapie (DAPT) genoemd. Daarnaast bestaan er medicijnen die de bloedstolling remmen, zoals vitamine K antagonisten (VKA) of directe orale anticoagulantia (DOACs). Deze middelen worden voorgeschreven aan patiënten met bijvoorbeeld boezemfibrilleren, een trombosebeen, of een kunsthartklep. Soms gebruikt een patiënt naast DAPT ook een van deze antistollingsmiddelen, dan spreken we van triple antitrombotische therapie. De inleiding geeft ook een beschrijving van de verschillende laboratoriumtesten die gebruikt zijn in dit proefschrift. Tenslotte geeft dit hoofdstuk een overzicht van de cohortstudies en een gerandomiseerd onderzoek waaruit de onderzoeksdata van de studies in dit proefschrift afkomstig zijn.

Hoofdstuk twee bevat een overzicht van de literatuur die beschikbaar is omtrent de rol van het bloedstollingssysteem bij het optreden van atherotrombose, waarbij de nadruk ligt op de verschillende behandelingsstrategieën bij patiënten met atherotrombotische aandoeningen.

Het eerste deel van dit proefschrift is gebaseerd op een Maastrichts cohort van 524 hoog-risico patiënten die duale of triple antitrombotische therapie gebruiken na een PCI. Hoog-risico patiënten in dit cohort zijn patiënten die minimaal drie risicofactoren

hebben om een bloedingscomplicatie te krijgen. In **hoofdstuk drie** wordt de onderzoeksopzet van deze cohortstudie beschreven. Daarnaast staat in dit hoofdstuk een overzicht van alle patiëntkenmerken van de geïncludeerde patiënten. In elk van de volgende hoofdstukken (4, 5, 6 en 7) wordt steeds een selectie van deze patiënten gebruikt om verschillende onderzoeksvragen te kunnen beantwoorden. De patiënten in het Maastrichtse cohort zijn 12 maanden opgevolgd na hun PCI. In die periode hebben 254 patiënten (48.5%) één of meerdere bloedingssymptomen gehad. Deze worden geclassificeerd volgens de BARC classificatie, waarin BARC type 1 een minimale bloeding is waarbij geen doktersbezoek nodig is, BARC type 2 een bloeding waarbij extra controle, onderzoek of interventie nodig is en BARC type 3 een ernstige bloeding waarbij bijvoorbeeld ziekenhuisopname of bloedtransfusie nodig is. De meeste patiënten (30.9%) hadden milde bloedingssymptomen; BARC type 1. Echter, tijdens de 6-12 maanden follow-up heeft ook 17.5% van de patiënten een ernstigere bloeding gehad, BARC type 2 of 3. Daar tegenover staat dat ook bij 13.2% van de patiënten een hartinfarct, beroerte of overlijden is opgetreden tijdens de follow-up periode. Op basis van de bloedingssymptomen, een recidief hartinfarct, de risico-inschatting op het spreekuur, uitslagen van laboratoriumonderzoeken, of bijwerkingen werd bij 14.9% van de patiënten besloten om een andere P2Y12-remmer voor te schrijven, en is bij 6.3% van de patiënten een andere wijziging in de antitrombotische medicatie gedaan. Gebaseerd op zowel de frequentie van bloedingscomplicaties en nieuwe ischemische gebeurtenissen, alsook de frequente noodzaak van aanpassingen in medicatie concluderen we dat er een behoefte is om deze patiënten strikt te monitoren. Ook laat dit zien met welke uitdagingen in de antitrombotische behandeling de patiënt en de behandelaar geconfronteerd worden.

In **hoofdstuk vier** hebben we onderzocht wat het effect is van genetische variaties in de metabolisatie van clopidogrel op de uitkomst van plaatjesfunctietesten bij patiënten uit het cohort die met clopidogrel behandeld werden. We hebben daarbij drie verschillende bloedplaatjesfunctietesten gebruikt: de VerifyNow, Muliplate en lichttransmissie-aggregometrie (LTA). Uit eerder onderzoek was al bekend dat de uitkomsten van deze drie testen binnen één patiënt vaak slecht overeenkomen, en bij dit onderzoek wilden we graag weten of deze slechte overeenkomst mogelijk te wijten zou kunnen zijn aan een verschillend effect van genetische variatie op het testresultaat. Het onderzoek liet zien dat bij de VerifyNow en LTA dit effect van genetica duidelijk weerspiegeld werd in het testresultaat, terwijl dit bij de Multiplate test niet het geval was. De conclusie van dit onderzoek was dan ook dat, naast verschillen in testprincipe en de invloed van verschillende patiëntgerelateerde factoren, de genetische variaties in

clopidogrel-metabolisme gedeeltelijk verantwoordelijk zijn voor de matige overeenkomst in plaatjesfunctietesten.
In **hoofdstuk vijf** hebben we laten zien dat patiënten met DAPT die bloedingscomplicaties kregen tijdens de follow-up een verminderde trombinegeneratie hebben in vergelijking met mensen zonder bloedingscomplicaties. Deze trombinegeneratietest was uitgevoerd met bloedplasma waarin geen bloedplaatjes meer aanwezig zijn. De verschillen in trombinegeneratie kunnen dus niet verklaard worden door het effect van DAPT, maar zouden verklaard kunnen worden door variatie in de stollingsfactoren. De verminderde trombinegeneratie bij deze patiënten is dan, bovenop het gebruik van DAPT, de spreekwoordelijke druppel waardoor zij bloedingscomplicaties krijgen en patiënten met normale trombinegeneratie niet. Mogelijk zou de trombinegeneratietest een rol kunnen krijgen in het identificeren van patiënten met een verhoogd bloedingsrisico tijdens behandeling met DAPT.

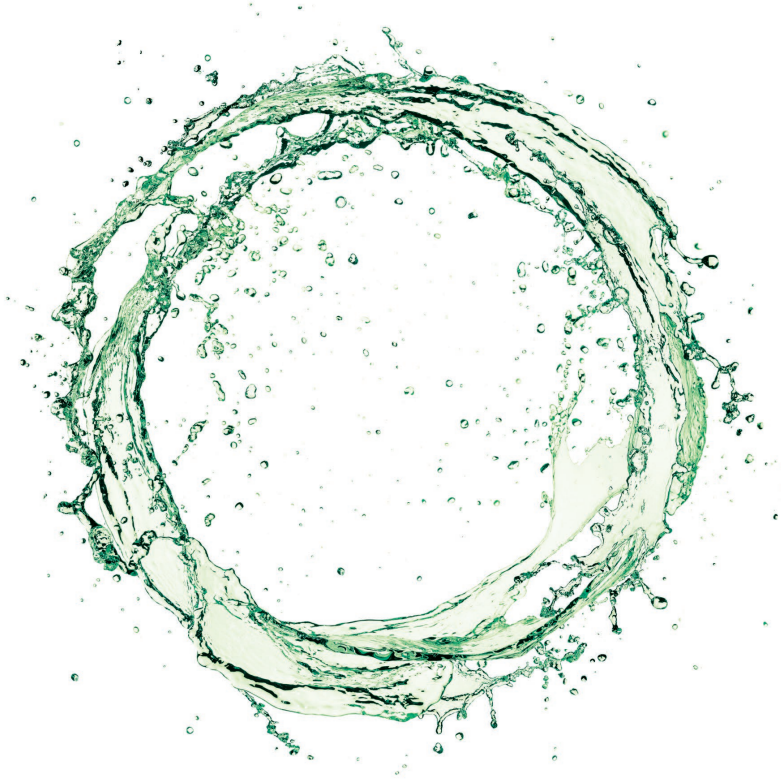
In de studie die in **hoofdstuk zes** wordt beschreven hebben we een andere algemene stollingstest, de rotational thromboelastometry (ROTEM), onderzocht bij 440 patiënten uit het Maastrichtse cohort. Deze patiënten gebruikten verschillende combinaties van antitrombotische medicatie: DAPT, P2Y12-remmer met VKA, of P2Y12-remmer met DOAC. Zij werden vergeleken met een groep controle-patiënten die géén antitrombotische medicatie gebruikten, en ook geen recente PCI hadden gehad. Het onderzoek liet zijn dat alle cohortpatiënten stevigere stolsels maakten vergeleken met de controle-patiënten. Dit heeft waarschijnlijk te maken met hun onderliggende coronairlijden en aderverkalking en niet met de antitrombotische medicatie zelf. Ook zagen we dat de DAPT-patiënten stolsels maakten die minder gemakkelijk afgebroken konden worden dan de stolsels van de controle-patiënten. Tenslotte vonden we verlengde bloedstollingstijden bij DAPT-patiënten die een bloedingscomplicaties kregen vergeleken met DAPT-patiënten zonder bloedingscomplicaties. Met toekomstig onderzoek zal moeten worden bekeken of de ROTEM test in staat is om patiënten met verhoogd bloedingsrisico te identificeren.

Het tweede deel van dit proefschrift gaat over behandelingsstrategieën bij patiënten met coronairlijden. **Hoofdstuk zeven** beschrijft de resultaten van de WOEST2 studie. Dit is een groot prospectief cohortonderzoek dat uitgevoerd is in verschillende ziekenhuizen in Nederland en België, waarbij behandeling met duale of triple antitrombotische therapie vergeleken werd. Duizend-vijfenzeventig patiënten met boezemfibrilleren die een PCI ondergingen namen deel aan dit onderzoek, waarin ook 160 patiënten uit het Maastrichtse cohort werden geïncludeerd. Bij duale therapie traden significant minder bloedingscomplicaties op vergeleken met triple therapie,

zonder dat er meer cardiale of cerebrovasculaire complicaties optraden. Deze resultaten tonen aan dat triple therapie voorbehouden moet zijn aan patiënten met een heel hoog risico op ischemische complicaties en met slechts een beperkt bloedingsrisico.

In **hoofdstuk acht** werd de impact van een andere bloedplaatjesremmer, vorapaxar, op biomarkers van de bloedstolling bekeken in 135 patiënten met chronisch coronairlijden zonder recente PCI. Deze patiënten werden behandeld met vorapaxar of een placebo, bovenop de gebruikelijke plaatjesremmers aspirine en/of clopidogrel. Patiënten die langdurig vorapaxar hadden gebruikt bovenop (D)APT hadden lagere waardes van soluble P-selectin, een biomarker voor plaatjesactivatie, vergeleken met patiënten die alleen (D)APT hadden. Er werden geen verschillen gezien in biomarkers voor bloedstolling. De conclusie van dit onderzoek was dan ook dat het gunstige effect van vorapaxar op het verminderen van recidief ischemische events te wijten is aan extra remming van de bloedplaatjes, maar niet aan een (indirect) effect van vorapaxar op de bloedstolling.

In **hoofdstuk negen** worden de belangrijkste bevindingen van dit proefschrift samengevat en bediscussieerd. Daarnaast wordt een algoritme voorgesteld waarmee het antitrombotische beleid en de risicostratificatie van toekomstige PCI patiënten kan plaatsvinden.



PART IV

ADDENDUM

Curriculum vitae

Curriculum vitae

Renske Hendrike Olie werd geboren op 8 september 1982 in Alkmaar. In 2000 behaalde ze haar Gymnasiumdiploma aan het Jan Arentsz College in Alkmaar. Vervolgens startte ze met de studie Geneeskunde aan de Universiteit Maastricht (UM), waar ze in 2004 cum laude haar doctoraalexamen behaalde. Vervolgens volgde zij onderwijs aan de Monash University te Melbourne (Australië) en deed ze een aantal coschappen in het Pretoria Academic Hospital in Pretoria (Zuid-Afrika). Nadat ze in 2006 cum laude het artsexamen aflegde aan de Universiteit Maastricht startte ze met de opleiding Interne Geneeskunde in het Elisabeth Ziekenhuis in Tilburg (opleider: dr. P.L. Rensma). In 2010 zette ze deze opleiding voort in het Maastricht Universitair Medisch Centrum (MUMC) (opleider: Prof. Dr. C.D.A. Stehouwer), alwaar ze vervolgens ook het aandachtsgebied vasculaire geneeskunde deed (opleider Prof. Dr. A.A. Kroon). Na het afronden van de opleiding tot internist-vasculair geneeskundige in 2013 kreeg ze een aanstelling als staflid op de afdeling algemene interne geneeskunde, sectie vasculaire geneeskunde in het MUMC. In 2014 startte ze haar promotieonderzoek onder supervisie van Prof. Dr. H. ten Cate, Prof. Dr. J.M. ten Berg en Dr. P.E.J. van der Meijden. Dit klinisch onderzoek vond plaats binnen het Trombose Expertise Centrum en het Cardiovascular Research Institute Maastricht (CARIM). Gedurende het promotieonderzoek initieerde ze een polikliniek voor hoog-risico patiënten met een indicatie voor antitrombotische medicatie na een percutane coronair interventie. Daarnaast is ze lid van de trombosecommissie in het MUMC+ en is ze verantwoordelijk voor de ziekenhuisbrede protocollen met betrekking tot antistolling en trombosezorg. Naast patiëntenzorg en onderzoek is ze betrokken bij het onderwijs aan de ‘Faculty of Health, Medicine and Life Sciences’ van de UM, waarbij ze onder andere colleges geeft, mentor is van studenten in de masterfase, studenten superviseert tijdens hun wetenschapsparticipatiestage, en modulecoördinator is in de master Arts-Klinisch Onderzoeker (AKO). Samen met haar man Erik van den Eerenbeemt en hun dochters Lieke (2012) en Jorinde (2014) woont ze in Asten.

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Dankwoord

Dankwoord

“To begin; begin….”
Die stelling heb ik mezelf regelmatig voorgehouden om de volgende stap te zetten in het voltooien van dit proefschrift. En nu gebruik ik hem om de laatste hobbel te nemen; het schrijven van het dankwoord. Dankzij de hulp en steun van veel collegae, vrienden en familie is dit proefschrift tot stand gekomen. Maar bovenal was dit onderzoek er nooit gekomen zonder de bereidheid van vele patiënten om mee te werken aan mijn studies en vele buisjes bloed af te staan, hartelijk dank!

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