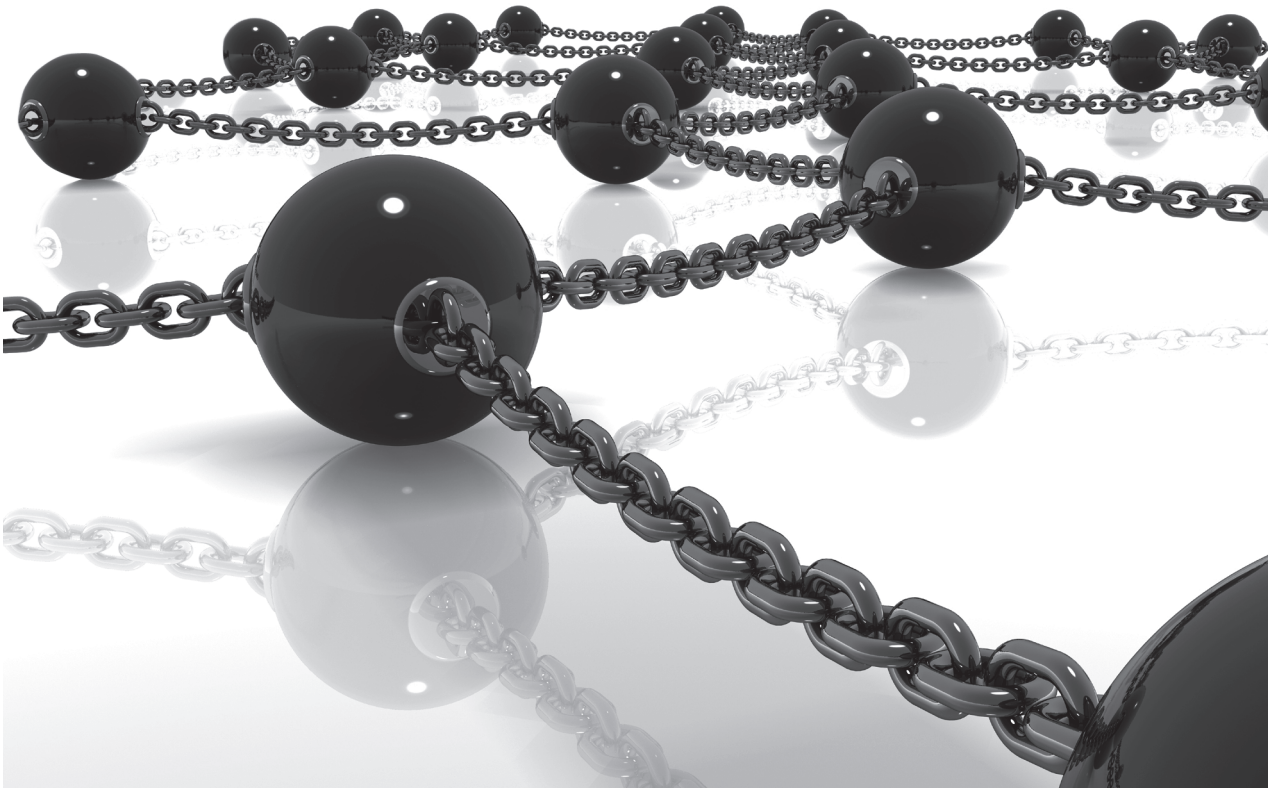
INFLUENCE OF GENETIC VARIANTS AND DRUG INTERACTIONS ON THE RESPONSE TO ANTIPLATELET DRUGS

Ankie Maxelante Harmsze



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INFLUENCE OF GENETIC VARIANTS AND DRUG INTERACTIONS ON THE RESPONSE TO ANTIPLATELET DRUGS

Invloed van genetische variaties en geneesmiddelinteracties op de respons op trombocytenaggregatieremmers
(*met een samenvatting in het Nederlands*)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 9 maart 2011 des middags te 2.30 uur

door

ANKIE MAXELANTE HARMSZE

geboren op 28 februari 1978 te Gouda

PROMOTOR:	Prof. dr. A. de Boer
CO-PROMOTOREN:	Dr. V.H.M. Deneer Dr. O.H. Klungel

Voor mijn ouders Tom en Maja

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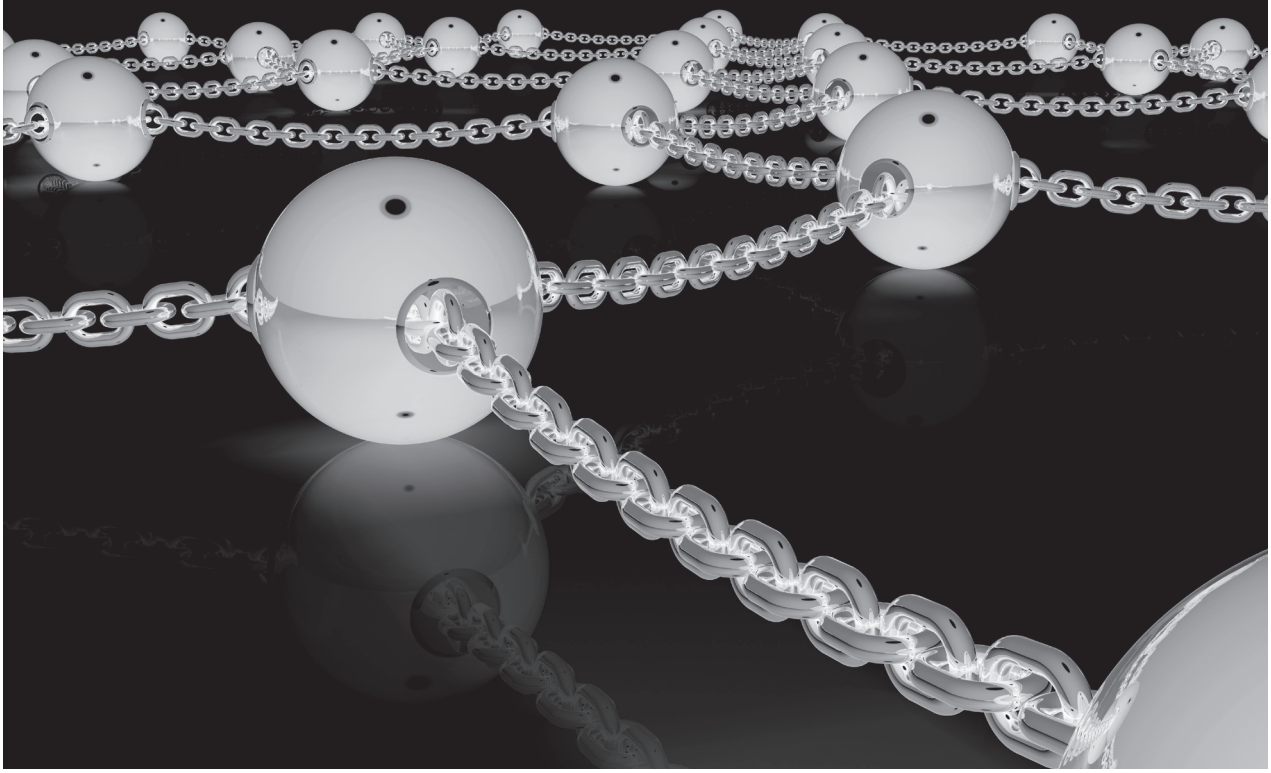
1 GENERAL INTRODUCTION



1.1

INTRODUCTION

INTRODUCTION

Antiplatelet therapy plays an important role in the treatment of cardiovascular disease. The combination of acetylsalicylic acid (ASA) and clopidogrel ('dual antiplatelet therapy') is routine care in patients with acute coronary syndromes (ACS) or undergoing percutaneous coronary interventions (PCI). Although the effectiveness of antiplatelet therapy is well established, studies have shown interindividual variability in the response to both antiplatelet drugs.[1, 2] This variability in response results in complications at both ends of the therapeutic spectrum (bleeding or thrombosis). In this thesis, the impact of genetic variations and co-prescribed drugs on the response to clopidogrel and ASA is investigated.

ATHEROTHROMBOTIC DISEASE AND THE ROLE OF PLATELETS

Although the pathophysiology of atherothrombosis is complex, the central role of platelets in the development of arterial thrombosis and cardiovascular disease is well established.[3] At rest, platelets circulate through the arterial vessels without interacting with any other cells. However, when a vessel is injured, platelets are immediately activated and are involved in three successive stages of arterial thrombus formation: platelet adhesion, activation and aggregation.

When the intima of a blood vessel is disrupted, subendothelial collagen and von Willebrand factor are exposed to circulating blood. Platelets in the blood adhere to subendothelial collagen and von Willebrand factor through their glycoprotein (GP) Ia/IIa and Ib/V/IX receptors.[4-6] Platelet adhesion stimulates platelet activation, which results in a change in their shape and the release of bound calcium within the platelet. The increased concentration of free ionic calcium in the platelet has several consequences. First, it induces a conformational change in the GP IIb/IIIa receptors on the surface of platelets, so that they can bind adhesive proteins in the circulation, such as fibrinogen. Second, it catalyses the release of active molecules (adenosine diphosphate (ADP) and others) from platelet granules into the circulation where they may bind to receptors on the surface of adjacent platelets and trigger their activation. Third, it promotes the action of phospholipase A_2 to produce arachidonic acid. Arachidonic acid in platelets is converted to thromboxane A_2 (TXA_2) in a reaction that is catalyzed by the enzymes cyclooxygenase 1 (to form prostaglandin G2/H2) and thromboxane synthase (to form TXA_2).[4-6] TXA_2 increases the expression of fibrinogen receptors on the platelet's membrane and is released into the circulation where it binds to thromboxane receptors on the surface of adjacent platelets to trigger their activation. TXA_2 also acts synergistically with other products released by activated platelets (such as ADP, fibrinogen, factor V) to increase platelet activation. Furthermore, TXA_2 is a potent vasoconstrictor.[4-6]

THE ANTIPLATELET DRUG CLOPIDOGREL

Efficacy and clinical application

The Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) study compared clopidogrel with ASA in a wide spectrum of patients at risk for atherothrombosis. Initial analysis revealed a statistically significant 8.7% (p=0.043) relative risk reduction in stroke,

myocardial infarction, or ischemic death in patients treated with clopidogrel.[7] Further research showed an even larger impact on high-risk populations such as patients with a previous coronary artery bypass graft, with a history of more than one ischemic event, diabetes or hypercholesterolemia.[8]

In various clinical trials, the addition of clopidogrel to ASA treatment was demonstrated to have statistically significant additive benefit in the prevention of adverse cardiovascular events over ASA monotherapy.[9, 10] The prospective Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) trial in patients with ACS, was the first to show that dual antiplatelet therapy with ASA and clopidogrel was superior to the combination of ASA and placebo in reducing long-term thrombotic events.[10] The subset of patients undergoing PCI in the CURE trial (PCI-CURE) achieved similar benefit with long-term clopidogrel and ASA. For the endpoint of myocardial infarction or cardiovascular death from the time of randomization to the end of follow-up, treatment with clopidogrel resulted in a 31% relative risk reduction (8.8% clopidogrel versus 12.6% placebo; p=0.002).[9]

Like the PCI-CURE trial, the Clopidogrel for the Reduction of Events During Observation (CREDO) trial aimed to determine the optimal duration of therapy, both before and after elective PCI.[11] The CREDO trial demonstrated a 26.9% relative risk reduction in cardiovascular events at one year after PCI. Clopidogrel pretreatment (a 300 mg loading dose) did not significantly reduce the combined risk of death, myocardial infarction or urgent target vessel revascularization at 28 days, compared to no pretreatment with clopidogrel. However, in a pre-specified subgroup analysis, patients who received the clopidogrel loading dose at least 6 hours before PCI experienced a relative risk reduction of 38.6% (p = 0.051) for this endpoint. This benefit was not observed when the loading dose was administered less than 6 hours before PCI.[11]

Metabolism

The thienopyridine clopidogrel is an inactive prodrug that needs to be converted in vivo to exhibit its antiplatelet effect. Following oral administration, the intestinal absorption of clopidogrel is limited by active luminal secretion via the adenosine triphosphate-binding cassette (ABC) efflux transporter P-glycoprotein (Pgp), encoded by the multidrug resistance gene MDR1 (ABCB1).[12] After absorption, the majority (85%) of clopidogrel is hydrolyzed predominantly by hepatic human carboxylesterase 1 (CES1) to an inactive carboxylic acid metabolite SR26334 (Figure 1).[13, 14] The remaining 15% of the absorbed clopidogrel is rapidly and extensively metabolized by the liver. Clopidogrel is converted to its active metabolite by the hepatic cytochrome P450 (CYP450) enzyme system in a two-step process. In the first step, the thiophene ring of clopidogrel is oxidized to 2-oxo-clopidogrel, which is subsequently hydrolyzed to a highly unstable active metabolite, R-130964 that has both carboxylic acid and thiol groups. This active metabolite covalently binds specifically and irreversibly to the P2Y12-receptor on the surface of platelets. The iso-enzymes CYP2C19, CYP1A2 and CYP2B6 are considered to be responsible for the first step whereas CYP2C19, CYP2C9, CYP2B6 and CYP3A are responsible for the second.[13-15]

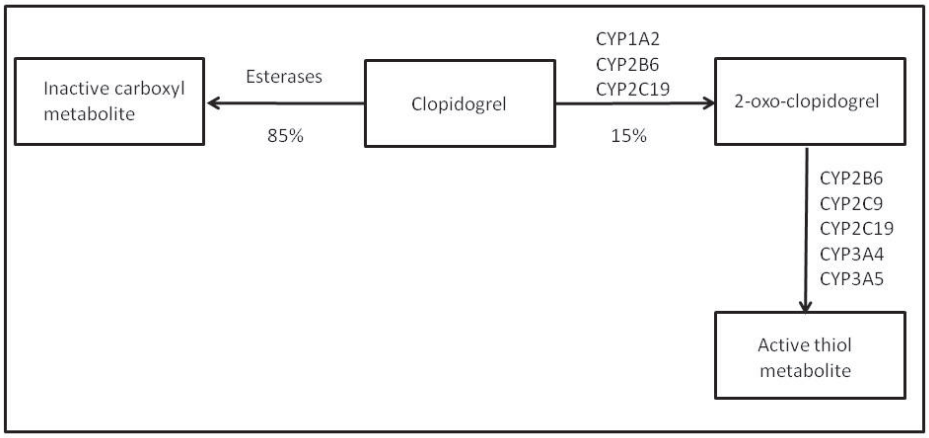


Figure 1: Biotransformation pathway of clopidogrel leading to its pharmacologically active thiol metabolite via 2-oxo-clopidogrel

Mechanism of action

As stated above, ADP is one of the most important mediators of both physiological hemostasis and thrombosis.[3] After platelet activation, ADP is released from its intracellular storage granules and further activates adjacent platelets, thereby amplifying this process. There are two main purinergic receptor types in the membrane: P2X1 and P2Y. P2X1 is a ligand-gated ion channel that utilizes adenosine triphosphate (ATP) as an agonist and mediates extracellular calcium influx leading to altered platelet shape. There are two known P2Y receptors, P2Y1 and P2Y12, both are (GTP) dependent G-protein coupled receptors which utilize ADP as agonist. ADP-mediated activation of the P2Y1 receptor leads to a series of signaling events that result in a weak and transient phase of platelet aggregation. In contrast, activation of P2Y12 receptor leads to a complex series of intracellular signaling events that yield in activation of the GP IIb/IIIa receptor, granule release, amplification of platelet aggregation and stabilization of the coagulated cells.[16] Clopidogrel selectively and irreversibly inhibits the P2Y12-receptor (Figure 2).[17] The reactive thiol group of the active metabolite forms a disulfide bridge between one or two cysteine residues (Cys17 and Cys270) of the P2Y12-receptor, resulting in its irreversible inhibition for the life span of the platelet because platelets are anucleate and cannot synthesize new protein.[18] In fact, platelet P2Y12 blockade prevents platelet degranulation and the release of prothrombotic and inflammatory mediators from the activated platelet, and also inhibits the transformation of the GP IIb/IIIa receptor that binds fibrinogen and links platelets.

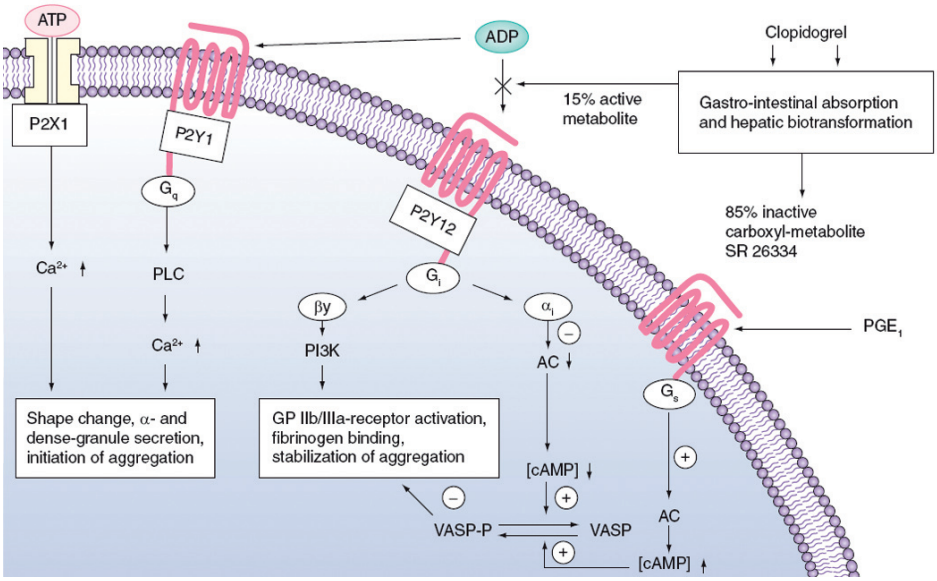


Figure 2: Mechanism of action clopidogrel.
The active metabolite selectively inhibits ADP-binding to its purinetic P2Y12-receptor. The P2Y12-receptor is coupled to the inhibitory G-protein G_i. After stimulation with ADP, activation of the P2Y12 receptor causes inhibition of adenylate cyclase (AC) with subsequently decreased cAMP-dependent protein kinase activity and thus diminished phosphorylation of vasodilator-stimulated phosphoprotein (VASP). After stimulation with PGE_1, activation of the G_s-coupled receptor causes activation of AC with subsequently an increased cAMP-dependent protein kinase activity and thus an increased phosphorylation of VASP. Phosphorylated VASP has an inhibitory effect on GP IIb/IIIa receptor activation.
AC: adenylate cyclase; GP: glycoprotein, PGE1: a platelet function inhibiting prostaglandin; PI3K: phosphatidylinositol-3-kinase; PLC: phospholipase C; VASP: vasodilator-stimulated phosphoprotein; VASP-P: phosphorylated vasodilator-stimulated phosphoprotein. Adapted, with permission, from Van Werkum *et al*.[19]

THE ANTIPLATELET DRUG ACETYLSALICYLIC ACID

Efficacy and clinical application

ASA still remains the most widely used and cost-effective drug in the prevention of platelet aggregation since the discovery of its effect over 40 years ago.[20] The role of ASA in the secondary prevention of cardiovascular disease is well established.[21] In 2002, the Antithrombotic Trialists' Collaboration performed a meta-analysis of over 100 studies and concluded that ASA therapy reduces the combined endpoint of serious vascular events by one quarter, nonfatal myocardial infarction (MI) by one third and vascular mortality by one sixth in high-risk patients with vascular disease.[22] In terms of primary prevention, ASA has been evaluated in six randomized controlled trials.[23-28] The Physicians Health Study showed a 44% risk reduction in first myocardial infarction among physicians treated with ASA.[27] The Thrombosis Prevention Trial (TPT) revealed the utility of ASA in men at high-risk for coronary disease, whereas the Hypertension Optimal Treatment (HOT) trial demonstrated a 36% reduction of MI in hypertensive patients treated with ASA.[24, 28] The Primary Prevention Project (PPP) extended the findings of these trials by

showing a 56% relative reduction in cardiovascular death in men and women with one or more major cardiovascular risk factor.[23] In a meta-analysis, which included five of these randomized controlled trials, ASA was found to be associated with a statistically significant 32% reduction in the risk of a first MI. However, the use of ASA did not result in a significant reduction of nonfatal stroke or vascular death.[29]

Metabolism

After oral administration, ASA is rapidly absorbed from the stomach and proximal small intestine. The gastric mucosa is permeable to the non-ionized form of ASA, which passes through the stomach wall by a passive diffusion process. After absorption, ASA is rapidly deacetylated to salicylic acid by human carboxylesterase 2 (CES2, Figure 3). The liver appears to be the principal site for salicylate metabolism. The three major metabolic products of salicylic acid are the phenolic, acyl and salicyluric acid glucuronides. A small fraction is converted to gentisic acid and other hydroxybenzoic acids. Besides CES2, the enzymes UGT1A6, ACSM2B and CYP2C9 are considered to play an important role in the metabolism of ASA (Figure 3).[30]

Mechanism of action

ASA inhibits the activation of platelets by irreversibly acetylating cyclooxygenase-1 (COX1) at the serine residue 529. COX1 catalyzes the conversion of arachidonic acid to prostaglandin H2 (Figure 4). This prostaglandin is further metabolized by isomerases to TXA_2, the main product of arachidonic acid metabolism and a strong platelet activator.[31] The inhibition of COX1 is rapid, irreversible and permanent for the entire life span of the platelet.[6, 21]

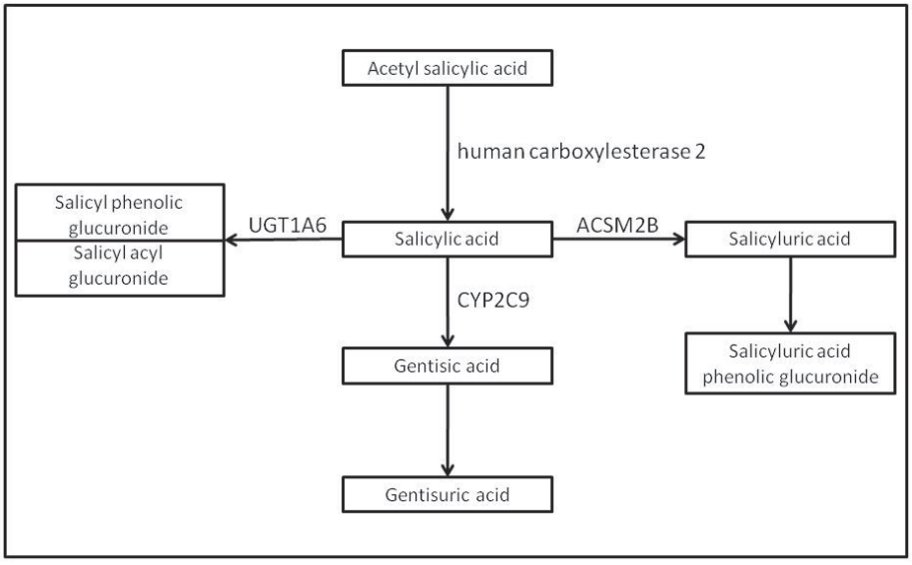


Figure 3: Metabolic pathways of acetylsalicylic acid

MONITORING ANTIPLATELET THERAPY

Platelet function tests are used for the quantification of *on-treatment platelet reactivity*, i.e. the magnitude of platelet reactivity that is measured in patients receiving antiplatelet therapy. Several platelet function tests have been developed over the years. Light transmission aggregometry (LTA) is the most commonly used method to evaluate platelet inhibition by clopidogrel and ASA and its relation to the risk of cardiovascular events.[33, 34] It measures the increase in light transmission through platelet-rich plasma (PRP) that occurs when platelets are aggregated upon binding to an agonist.

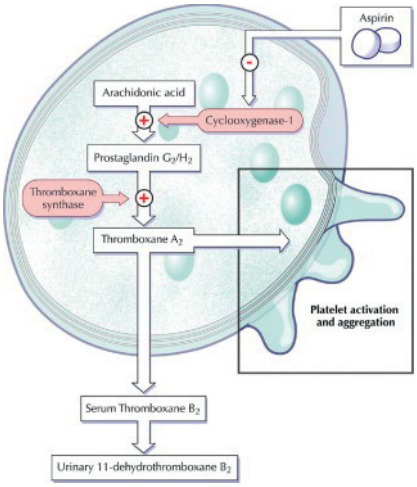


Figure 4: Inhibition of platelet thromboxane A2 pathways by low-dose ASA. With permission from Gasparyan *et al*. 2008[32]

Several platelet agonist ligands are used to stimulate platelet suspensions in a light transmission aggregometer. The most often used agonists include ADP (to determine the effectiveness of thienopyridines), arachidonic acid (to determine the effectiveness of ASA) and thrombin-receptor activating peptide stimulation (TRAP; to study the effectiveness of GP IIb/IIIa receptor antagonists). Upon addition of a platelet agonist, aggregation is reflected by increased light transmission through the cuvette.

For clopidogrel, the maximal amplitude of measured platelet aggregation in response to 5, 10, or 20 µmol/L ADP has been recorded in most studies. Multiple studies have shown an association between the level of on-treatment platelet reactivity and the occurrence of thrombotic events.[35] Two studies, in which the threshold for high on-treatment platelet reactivity was defined by ROC curve analysis, demonstrated that high on-treatment platelet reactivity was associated with a 2.1 to 3.8-fold increased risk of major adverse cardiovascular events at one to two years follow-up after elective PCI, respectively.[36, 37] In addition to maximum platelet aggregation, late aggregation measured 5 to 6 minutes after the addition of the agonist is also shown to be capable of identifying patients at risk for atherothrombotic events.[38, 39] With the LTA, large sample volumes and long processing times are required.[40] During the last decade,

many efforts have been made to develop point-of-care assays that are capable to easily provide instant information about platelet reactivity.
The point-of-care VerifyNow assay is a turbidimetric-based optical detection system that measures whole blood platelet aggregation. It is an automatic cartridge-based bed-side device that allows a rapid evaluation of platelet function in patients treated with either ASA, clopidogrel or GP IIb/IIIa receptor antagonists.[41-43] The VerifyNow P2Y12 assay is based upon the principle that activated platelets bind to fibrinogen-coated polystyrene beads that agglutinate in whole-blood in proportion to the number of unblocked ADP P2Y12-receptors. The rate of microbead agglutination is more pronounced and rapid if platelets are activated. Therefore, platelet stimulating reagents (TRAP, ADP and protstaglandin E1) are incorporated into the assay to induce platelet activation without fibrin formation.[40] Several studies have demonstrated a good correlation between the VerifyNow P2Y12 assay and the LTA and with plasma concentrations of clopidogrel's active thiol metabolite.[44, 45] Furthermore, it was demonstrated that patients with high on-treatment platelet reactivity as measured with the VerifyNow P2Y12 assay, have an approximately 2.5-fold increased risk on the occurrence of major adverse cardiovascular events at 1-year follow-up after PCI.[36, 46] The VASP assay, Plateletworks assay, the PFA-100, the Cone-and-Plate(let) analyzer and the thromboelastograph are other point-of-care platelet function tests.[40]

VARIABILITY IN RESPONSE TO ANTIPLATELET THERAPY

Consistent levels of platelet inhibition are required to deliver effective antiplatelet therapy. Adverse consequences of variable response are particularly apparent when antiplatelet drugs are used as an adjunct to PCI. PCI leads to micro-vascular damage to the endothelium, thereby exposing the subendothelial matrix to the circulating blood. In addition, the thrombogenic surface of metallic coronary stents also promotes the adhesion of platelets. Intensive periprocedural platelet inhibition minimizes morbidity and mortality, whereas the persistence of a prothrombotic environment necessitates chronic dual antiplatelet therapy. Failure to provide adequate platelet inhibition can result in adverse cardiovascular events such as myocardial infarction, stent thrombosis and death.[47]

Variability in the response to clopidogrel

There is substantial individual variability in response to clopidogrel. Platelet function studies have indicated that 5-40% of the patients exhibit high on-treatment platelet reactivity, while on treatment with clopidogrel.[38] Multiple studies have demonstrated a relationship between high on-treatment platelet reactivity measured by various platelet function assays and adverse ischemic cardiovascular events.[35, 36] In contrast, a substantial proportion of the patients on dual antiplatelet therapy exhibit an enhanced response to clopidogrel as measured with ADP-induced platelet function assays. Several studies have indicated that this phenomenon is associated with an increased risk of major bleeding events in clopidogrel-treated patients undergoing PCI.[48-50] There appears to be a therapeutic window for the inhibition of the P2Y12-receptor that is associated with both an optimal reduction in thrombotic events as well as a low rate of major bleeding.[35]

Variability in the response to ASA

The impact of incomplete COX inhibition by ASA on the occurrence of atherothrombotic events has been evaluated in several clinical studies with a variety of ASA sensitive assays.[51] In a cohort of high-risk cardiovascular patients on chronic ASA therapy it was found that subjects with high levels of urinary 11-dehydro thromboxane B2 (an inactive metabolite of TXA_2) had an 1.8-fold increased risk of myocardial infarction, cerebrovascular accident, or cardiovascular death.[52] In a large prospective observational study, it was demonstrated that patients with high platelet reactivity despite ASA therapy, as measured with LTA-ASA and the VerifyNow-ASA assay, had an 1.8 and 2.5-fold increased risk of the combined endpoint of all-cause death, myocardial infarction, stent thrombosis and ischemic stroke, respectively.[53]

Factors influencing variability in response to clopidogrel and ASA

The variability in response to clopidogrel is considered to be caused by several factors. Besides poor compliance, clinical factors such as diabetes mellitus, increased body mass index and acute coronary syndromes have been associated with a diminished antiplatelet response to clopidogrel.[54-56] The available evidence in literature suggests that variable active metabolite generation is the primary explanation for clopidogrel response.[57] Variable levels of active metabolite formation following clopidogrel administration could be caused by variable or limited P-glycoprotein-mediated intestinal absorption or by functional variability in the CYP P450 enzymes which are involved in the metabolism of clopidogrel. Stimulation of CYP3A4 activity by St. John's wort and CYP1A2 activity by tobacco smoking have both been shown to enhance platelet inhibition induced by clopidogrel.[58, 59] The effect of smoking on the antiplatelet effect of clopidogrel has been associated with clinical outcome.[60] In this context, pharmacokinetic drug-drug interactions are considered to play a role. Many drugs are substrates and/or inhibitors or inducers of CYP enzymes and combining these drugs with clopidogrel may result in ineffective plasma concentrations or concentrations associated with the development of side effects. Drugs may also induce or inhibit the transporter P-glycoprotein, resulting in a drug-drug interaction with clopidogrel, which is a substrate for this transporter.[12] Two drugs may also exert their effects via the same pathway. An example of a pharmacodynamic drug-drug interaction involves ASA and the NSAID ibuprofen.[61] The concomitant use of ibuprofen was found to antagonize platelet inhibition induced by ASA. ASA blocks the access of arachidonic acid to the catalytic site by irreversibly acetylating a serine residue at position 529 in platelet COX1 near but not within the catalytic site. Ibuprofen is a reversible, competitive inhibitor of the catalytic site. The use of ibuprofen results in the reversible inhibition of TXA_2 formation during the dosing interval. Prior occupancy of the catalytic site by ibuprofen prevents ASA from gaining access to its target serine.[61]

Furthermore, genetic variation in the DNA encoding proteins can result in a change in amino acid sequence in the protein or differences in transcription rates. These deviations may result in the increased or reduced effectiveness of drugs. A single nucleotide polymorphism (SNP) is a variation in nucleotide sequence within the DNA. SNPs in coding regions of the DNA (exons) may result in an alternative amino acid incorporated in the protein. These amino acid changes may result in decreased or increased activity of the protein. SNPs in the non-coding regions (introns) may result in changes in transcription rates and gene expression, resulting in higher or lower enzyme concentrations.[62] Genetic variations affect the activity of CYP enzymes, drug transporters and target receptors and may therefore explain a part of the variation in the response to clopidogrel and ASA.

OBJECTIVES AND OUTLINE OF THIS THESIS

The main objective of this thesis is to investigate whether genetic factors and drug-drug interactions are associated with the response to antiplatelet therapy.

Chapter 1.2 is a review of the use of the VerifyNow system to monitor antiplatelet therapy. Chapter 2 contains studies in which the influence of genetic variants and co-medication on on-treatment platelet reactivity and plasma concentrations of clopidogrel's active metabolite was investigated. Chapter 2.1 describes the effects of several genetic variants on on-treatment platelet reactivity. In the Chapters 2.2 and 2.3 the influences of concomitant use of calcium channel blockers and the proton pump inhibitor omeprazole on platelet function were assessed. Chapter 2.4 describes the drug-drug interaction between sulfonylureas and clopidogrel in a cohort of type 2 diabetes mellitus patients undergoing elective coronary stenting. In Chapter 2.5, the effects of the proton pump inhibitors esomeprazole and pantoprazole on plasma concentrations of clopidogrel's active metabolite were examined.

Chapter 3 contains studies in which the influence of genetic variations and co-medication on adverse cardiovascular events in patients on antiplatelet therapy was investigated. The first study (Chapter 3.1) examined the effects of genetic variants in clopidogrel's pharmacokinetic and –dynamic pathway of action on the occurrence of stent thrombosis. In Chapter 3.2 the combined impact of calcium channel blockers, proton pump inhibitors and *CYP2C19*2*-carriage was investigated in a large cohort of patients undergoing elective coronary stenting. Chapter 3.3 describes a nested case-control study investigating whether several variants in CES2, CYP2C9, UGT1A6, COX1 and COX2 modify the effect of ASA therapy in the prevention of a first myocardial infarction. In Chapter 3.4, the effect of *CYP2C19* genotypes on major bleeding risk was investigated within a cohort of patients undergoing elective coronary stenting.

Finally, in Chapter 4 the results of this thesis are summarized and put into the broader context of clinical practice and further research.

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THE USE OF THE VERIFYNOW SYSTEM TO MONITOR ANTIPLATELET THERAPY: A REVIEW OF THE CURRENT EVIDENCE

Jochem W. van Werkum
Ankie M. Harmsze
Ellen H.A.M. Elsenberg
Heleen J. Bouman
Jurriën M. ten Berg
Christian M. Hackeng

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ABSTRACT

Multiple studies have demonstrated the effectiveness of dual or triple antiplatelet therapy with aspirin, clopidogrel and glycoprotein (GP) IIb/IIIa therapy in patients with acute coronary syndromes as well as in patients undergoing coronary stent implantation. In the last few years, it is becoming clear that not all patients receive the full benefits with the current standard dosages of antiplatelet therapy. Specifically, numerous studies have revealed a wide interindividual variability in the response to these antiplatelet agents and, more importantly, both nonresponsiveness as well as a heightened residual platelet reactivity have been linked to the occurrence of adverse cardiovascular events. Therefore, assays that identify those patients with an impaired responsiveness or a heightened platelet reactivity despite dual antiplatelet therapy may contribute to better risk stratification and will probably improve clinical outcome when appropriate action is initiated.

Likewise, a considerable number of patients do not achieve the minimal inhibition of aggregation threshold with the current recommended weight-adjusted dosages of GP IIb/IIIa therapy. Identifying and optimizing the absolute degree of platelet inhibition in this subgroup of patients will probably improve clinical outcome.

The VerifyNow platform is one of the most user friendly point-of-care platelet function test systems because it produces rapid results at the patients bedside. The purpose of the present review is to give insight into the principal mechanisms of the VerifyNow system, to discuss its clinical utility for the monitoring of antiplatelet therapy and to discuss the proposed cut-off levels to segregate responders from non-responders for the different types of antiplatelet therapy.

INTRODUCTION

Multiple studies have demonstrated the effectiveness of dual antiplatelet therapy with aspirin and clopidogrel in patients with acute coronary syndromes as well as in patients undergoing coronary stent implantation.[1,2] Both antiplatelet drugs inhibit two distinct pathways that trigger platelet activation. Aspirin achieves its anti-thrombotic effect by the inactivation of cyclooxygenase-1 (COX1), a key enzyme in the arachidonic acid metabolism and subsequent thromboxane A2 (TXA_2) production.[3] Clopidogrel is a prodrug that is converted to its active metabolite by the hepatic cytochrome P450 enzyme system.[4] The active metabolite targets the adenosine-diphosphate pathway by irreversibly blocking the adenosine diphosphate (ADP)-sensitive P2Y12-receptor. As a result, the amplification of aggregation is prevented by the inactivation of the platelet fibrinogen receptor GP IIb/IIIa. It is important to note that a second ADP-receptor, the P2Y1 receptor, is not inhibited by the active metabolite of clopidogrel.[5] The P2Y1-receptor is responsible for the shape change of the platelet upon platelet stimulation and the initial wave of ADP-induced aggregation.

In the last few years, it is becoming clear that not all patients receive the full benefits with the conventional maintenance dosages of aspirin and clopidogrel therapy. Specifically, numerous studies have revealed a wide interindividual variability in the response to these antiplatelet agents[6] and, more importantly, both non-responsiveness as well as a heightened residual platelet reactivity have been linked to the occurrence of adverse cardiovascular events.[7-10] Therefore, assays that identify those patients with an impaired responsiveness or an augmented platelet reactivity despite dual antiplatelet therapy may contribute to better risk stratification and will probably improve clinical outcome when appropriate action is initiated.[11]
Randomized trials have demonstrated that glycoprotein (GP) IIb/IIIa inhibitors during percutaneous coronary intervention (PCI) reduce ischemic complications, death, and nonfatal myocardial infarction.[12,13] However, various studies have revealed significant variability in GP IIb/IIIa receptor occupancy and platelet inhibition after administration of any of the GP IIb/IIIa receptor inhibitor in the recommended doses.[14-16] Identifying and optimizing the absolute degree of platelet inhibition in this subgroup of patients will probably improve clinical outcome.
So far, the majority of studies have used light transmittance aggregometry (LTA) with either thrombin-receptor activating peptide stimulation (TRAP; to study the effectiveness of GP IIb/IIIa therapy), ADP stimulation (to determine the effectiveness of clopidogrel therapy) or arachidonic acid (AA) stimulation (to determine the effectiveness of aspirin therapy). However, although LTA is still considered as the “gold standard” assay, this method is not suitable for routine clinical practice because the results are not instantly available and the technique requires considerable technical expertise to perform. Therefore, a variety of so-called “point-of care” platelet function assays is currently available with the specific purpose to rapidly inform the clinician about the magnitude of platelet inhibition that is achieved with the individual antiplatelet regimen. The VerifyNow platform (formerly known as Ultegra Rapid Platelet Function Assay (RPFA)(Accumetrics, Inc., San Diego, CA) is one of the most user friendly point-of-care platelet function test systems because it produces rapid results at the patients bedside. The purpose of the present review is to give insight into the principal mechanisms of the VerifyNow system, to discuss its clinical utility for the monitoring of antiplatelet therapy and to discuss the proposed cut-off levels to segregate responders from non-responders for the different forms of antiplatelet therapy.

"POINT-OF-CARE"

The "gold standard" and most widely accepted platelet function tests (i.e. light transmittance aggregometry) are time-consuming, require intensive sample preparation and the results are not instant available.[11] Also, the reproducibility of these tests remains questionable. Therefore, many efforts have been made during the last decade to develop systems and instruments that are capable to provide instant information about the status of platelet reactivity to the risk of atherothrombotic disease near the patients bedside. Theoretically, bedside testing or "point-of-care" testing allows rapid decision making based on the instant information that is obtained. However, there is currently no available point-of care assay that is sufficiently validated to fulfil all the criteria for an ideal monitoring system, i.e. (1) testing can be performed with small amounts of whole-blood, (2) no pipetting or handling of the blood is required (3) results are reproducible as well as instantly available, and most importantly (4) results are predictive for the (re)-occurrence of atherothrombotic events.
Theoretically, the VerifyNow platform comes closest to the abovementioned criteria as compared to other platelet function assays such as the PFA-100 (Siemens)[17], the Cone-and-Plate(let) analyzer (Diamed)[18], the Plateletworks (Helena Laboratories)[19] or the thromboelastograph (TEG; Haemoscope)[20].

MAIN PRINCIPLE OF THE VERIFYNOW SYSTEM

The VerifyNow system is a whole-blood, point-of-care assay that consists of an instrument and disposable single-use cartridges that contains the biochemical reagents, agonists and fibrinogen coated beads required to perform the specific assay (Figure 1).[21] After the citrated tube is inserted into the cartridge, whole blood is mixed with the platelet agonists and the fibrinogen-coated beads by the movement of an electromagnetic driven steel ball. When the antiplatelet drug does not appropriately exhibit its inhibitory effect, the platelets become activated by the specific agonist. As a result, the activated platelets bind to fibrinogen-coated beads, cause agglutination and fall out of the solution. The VerifyNow instrument measures the light absorbance through the sample 16 times per second. Both the rate and extent of platelet-induced agglutination over a fixed period of time are measured and a proprietary algorithm is used to report the values in reaction units. The VerifyNow platform has currently three types of single-use, disposable cartridges that can be used to monitor different antiplatelet drugs: aspirin, clopidogrel and glycoprotein (GP) IIb/IIIa therapy.

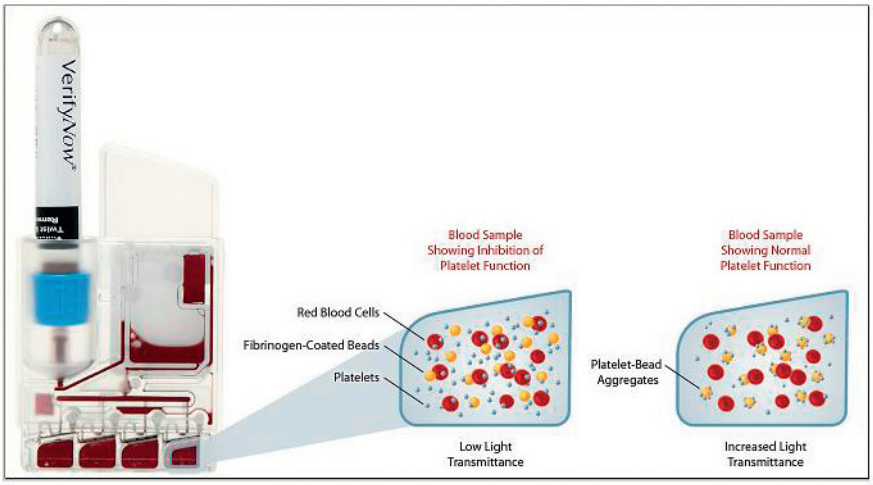

Figure 1: Illustration of the VerifyNow cartridge
The mixing chamber contains infrared dyed fibrinogen-coated beads as well as specific agonists. After the citrated tube is inserted into the cartridge, whole blood is mixed with the platelet agonists and the fibrinogen-coated beads by the movement of electromagnetic driven steel ball. When the antiplatelet drug does not appropriately exhibit its inhibitory effect, the platelets become activated by a specific agonist. As a result, the activated platelets bind to fibrinogen-coated beads, cause agglutination and fall out of the solution. Reprinted with permission from Accumetrics.

The VerifyNow IIb/IIIa assay

The VerifyNow IIb/IIIa cartridge was the first assay developed for use on the instrument. The GP IIb/IIIa cartridge contains fibrinogen-coated polystyrene beads and thrombin-receptor activating peptide (TRAP) as agonist.[22]
When the administered GP IIb/IIIa therapy does not completely block GP IIb/IIIa, platelets still agglutinate with the micro-beads upon TRAP stimulation. The platelet-bead complex fall out of the solution and the increase in light absorbance through the sample is reported as Platelet Aggregation units (PAU).

The VerifyNow IIb/IIIa assay: a laboratory evaluation and comparison with other well-established methods to evaluate the anti-platelet effects of aspirin
Multiple studies have demonstrated good agreement between the VerifyNow IIb/IIIa assay and conventional methodologies such as classical LTA and flowcytometry (Table 1).[23-27]

Current available evidence that PAU is a suitable marker to predict future atherothrombotic events in aspirin treated patients
The GOLD (AU-Assessing Ultegra) multicenter study has demonstrated that a platelet function inhibition of >95% at 10 minutes after the start of abciximab therapy was associated with a significant decrease in the incidence of MACE (odds ratio 0.46, 95% CI: 0.22 to 0.96, P=0.04) as compared to patients with a <95% inhibition of platelet function.[28]

The VerifyNow Aspirin assay

The second VerifyNow assay has been specifically designed for the monitoring of the inhibitory effects of aspirin therapy. The initial cartridge for this assay, the so-called RPFA-ASA, used to incorporate an agonist that contained 2 agents -metallic cations and propyl gallate (c-PG)- which activates platelets through the COX pathway.[29] However, the agonist in this cartridge was recently changed into (the more commonly used) agonist arachidonic acid (AA) in a final concentration of 1 mmol/L and the name was changed into the VerifyNow ASA assay.

AA is converted by the COX1 enzyme (the molecular target of aspirin therapy) into TXA_2. When aspirin does not completely inhibit COX1-activity, TXA_2 formation leads to platelet activation with subsequent microbead agglutination. The beads fall out of the solution and the increase in light transmittance through the sample is reported as Aspirin-Reaction Units (ARU).

The VerifyNow ASA assay: a laboratory evaluation and comparison with other well-established methods to evaluate the anti-platelet effects of aspirin

Various head-to-head comparisons between the VerifyNow ASA assay and other conventional methods to assess the individual response to aspirin have demonstrated good agreement in the majority of studies (Table 2)[29-35]. The manufacturer has designated an ARU value of ≥550 to segregate aspirin-responders from aspirin non-responders. This ≥550 ARU cut-off level has been initially derived from a correlation study with epinephrine-induced aggregation in platelet rich plasma. RPFA-ASA measurements and epinephrine induced aggregometry were performed in twenty-four patients to determine the most appropriate cut-off or threshold for the Ultegra RPFA-ASA assay. Results were evaluated visually at various thresholds and with a concordance Table. The threshold value of ≥550 ARU was then validated on a 144 patient sample population for whom baseline samples were evaluated. This was done by examination of the shape of the frequency distributions and the McNemar test for statistical equivalence between the 2 methods.[36] In 2003, the manufacturer changed the agonist in the assay into AA and a subsequent validation study was performed to verify whether the ≥550 ARU cut-off level was still appropriate. Venous whole blood samples in 3.2% sodium citrate were collected from 65 patients on chronic aspirin therapy (81 mg per day) and 71 patients before and after ingesting 325 mg of aspirin and tested in duplicate with the VerifyNow Aspirin Assay. The new VerifyNow Aspirin Assay results (duplicates) were evaluated in the presence and absence of aspirin, resulting in a specificity 100% and a sensitivity of 91.4% with the ≥550 ARU cut-off level.[37]

Table 1: Head-to-head comparisons between the VerifyNow IIb/IIIa assay and other conventional methods

Study, year	No.of patients	Patient population	Assays	Definition of low response	Results
Smith *et al*. 1999	54	50 Consecutive patients with CAD presenting for elective PCI and 4 healthy donors	The VerifyNow IIb/IIIa assay, LTA with ADP (20 µmol/L) as agonist and a GP IIb/IIIa receptor occupancy method using ^{125}I-abciximab	No definition was provided	The correlations between the VerifyNow IIb/IIIa assay and LTA was r^2=0.98 and that between VerifyNow IIb/IIIa and unblocked GPIIb/IIIa receptors was r^2=0.96
Storey *et al*. 1999	14	Increasing doses of fradafiban were added in vitro to whole blood obtained from 6 healthy volunteers. 8 patients with unstable angina were randomised to receive either oral lefradafiban or placebo on top of aspirin.	The VerifyNow IIb/IIIa assay, LTA with ADP (30 µmol/L) as agonist and a whole blood-single-platelet counting assay (WBSPC)	No definition was provided	The VerifyNow IIb/IIIa assay, LTA and WBSPC correlated well (r^2 ranged from 0.52 to 0.95)
Neumann *et al*. 2001	60	Aspirin and ticlopidine pretreated patients with CAD undergoing PCI	VerifyNow IIb/IIIa assay and LTA with ADP(20 µmol/L) and TRAP (50 µmol/L) as agonists	No definition was provided	Similar results between LTA and the VerifyNow GP IIb/IIIa were observed.
Wheeler *et al.* 2002	197	Aspirin treated patients with CAD undergoing PCI	VerifyNow IIb/IIIa assay, LTA using 20 µM ADP as agonist and a GP IIb/IIIa receptor occupancy method using ^{125}I-abciximab	No definition was provided	The correlations between the VerifyNow IIb/IIIa assay and LTA was r=0.89 and that between VerifyNow IIb/IIIa and unblocked GPIIb/IIIa receptors was r=0.89
Matzdorff *et al*. 2001	4	Increasing doses of abciximab, tirofiban, and eptifibatide were added in vitro to whole blood obtained from 4 healthy volunteers	VerifyNow IIb/IIIa assay, "classical" LTA, single platelet counting and flow Cytometry	No definition was provided	The correlation between aggregometry and VerifyNow IIb/IIIa assay results was linear for abciximab and eptifibatide. Tirofiban was a stronger inhibitor with the VerifyNow IIb/IIIa assay than with aggregometry.

ADP=adenosine-diphosphate; CAD=coronary artery disease; GP=glycoprotein; LTA=light-transmittance aggregometry; PCI=percutaneous coronary intervention; TRAP=thrombin-receptor activating peptide; WBSPC=whole blood-single-platelet counting.

Factors that could influence the results of the VerifyNow ASA cartridge
Several factors that affect the results obtained with the VerifyNow ASA assay have been reported in the literature.
1) The time between the sample collection and the assay performance should be at least 30 minutes. In addition, blood samples have to be tested within 4 hours of sample collection because of the decrease in platelet function. 2) In order to achieve reproducible results, the sample has to mixed gently before running the test. 3) Lee and co-workers identified in a large observational study that the results of the VerifyNow ASA assay are dependent on haemoglobin levels.[38] 4) Similarly, Wang and colleagues demonstrated in another multivariate analysis that the VerifyNow Aspirin results are influenced by the individual's platelet count and haematocrit levels.[39] The underlying mechanism for the association between haemoglobin levels, haematocrit levels and ARU is not clear, although it is possible that the light transmittance through the sample as well as the viscosity of the blood is influenced by these parameters. 5) Mirkhel *et al*. have recently revealed significant associations between ARU and smoking.[40]

Current available evidence that ARU is a suitable marker to predict future atherothrombotic events in aspirin treated patients
The most important question: "Is there a link between the in-vitro VerifyNow ASA assay measurements and the (re)-occurrence of atherothrombotic events?" has recently been addressed. Chen and co-workers used the VerifyNow ASA assay (with propyl gallate) to determine the responsiveness to aspirin (80-325 mg daily for ≥ 4 weeks) in 468 patients who were scheduled for elective PCI. Aspirin resistance was noted in 128 (27.4%) patients (ARU ≥ 550). After a mean follow-up of 379 ± 200 days, aspirin resistance was associated with an increased risk for the composite endpoint of cardiovascular death, MI, unstable angina pectoris requiring hospitalization, stroke and transient ischemic attack as compared to patients who were aspirin-sensitive (15.6% vs 5.3%, Hazard Ratio 3.2 95% CI 1.65 – 5.91 $p < 0.001$).[41]

Table 2: Head-to-head comparisons between the VerifyNow ASA assay and other conventional methods (part 1 of 2)

Study, year	No. of patients	Patient population	Assay	Definition of low response	Results
Coleman et al. 2004	422	422 patients with documented vascular disease or risk factors for vascular disease who used ASA ≥ 7 days before inclusion	VerifyNow Aspririn with propyl gallate as the agonist and LTA with epinephrine 5 µM as agonist	ARU ≥ 550 (VerifyNow) and platelet aggregation ≥55%	23% of the patients were aspirin non-responders (ARU ≥ 550 (VerifyNow)). VerifyNow Aspirin assay correlated (r = 0.902) with LTA-EPI.
Malanin et al. 2004	148	148 subjects with multiple risk factors for coronary artery disease before and after one dose of non-enteric coated aspirin (325 mg)	VerifyNow Aspirin with propyl gallate as the agonist and LTA using 5 µM epinephrine as agonist	ARU ≥ 550 (VerifyNow) and platelet aggregation ≥55%	A single dose of aspirin reduced platelet-rich plasma aggregation from 72 ± 21% to 25 ± 10% and diminished ARU from 647 ± 95 to 436 ± 69. The correlation between the two methods was 0.902.
Aleil et al. 2006	114	114 patients with vascular disease who had received aspirin for more than 1 week	VerifyNow Aspirin using arachidonic acid (AA) as agonist and LTA using 1mM AA as agonist	ARU ≥ 550 (VerifyNow) and platelet aggregation ≥ 40%	Results from the VerifyNow Aspirin assay were highly concordant with LTA-AA (κ = 0.90, p < 0.0001). The proportion of aspirin non-responders was 8.6% using the VerifyNow and 7.1% using AA-induced LTA.
Dichiara et al. 2007	110	110 patients with sTable coronary artery disease receiving ASA 81/162/325 mg/day	VerifyNow Aspirin using AA as agonist and LTA using 1, 2, 5 mM AA as agonist	ARU ≥ 550	A positive correlation was present between high platelet reactivity measured by the VerifyNow Aspirin assay and platelet reactivity as measured with 2mM AA and 5 mM AA-induced aggregation

Continuation Table 2: Head-to-head comparisons between the VerifyNow ASA assay and other conventional methods (part 2 of 2)

Study, year	No. of patients	Patient population	Assay	Definition of low response	Results
Paniccia et al. 2007	484	484 high-risk patients (ACS) with ischemic heart disease undergoing PCI receiving dual antiplatelet therapy	VerifyNow Aspirin with AA as agonist and LTA using 1 mM AA as agonist	ARU ≥ 550 (VerifyNow) and AA-induced platelet aggregation ≥ 20%	Aspirin resistance was detected in 30.0% of patients by LTA and in 14.3% by VerifyNow. Significant correlation was found (p<0.0001). Assuming LTA as the reference method, VerifyNow showed sensitivity of 39.3% and specificity of 96.4%.
Harrison et al. 2005	100	Patients receiving low-dose ASA therapy after transient ischemic attack (TIA) or ischemic stroke.	VerifyNow Aspirin, PFA-100 and LTA using 1 mg/mL AA as the agonist	An aspirin response was defined as <20% aggregation with 1 mg/mL AA and ARU ≥ 550	Aggregometry and the VerifyNow aspirin assay agreed in 78 samples (79%), with both tests indicating ASA responsiveness in 74 and both indicating nonresponsiveness in 4. However, the tests gave discordant results in 21 patients and overall agreement was not significantly greater than chance (κ=0.16, 95% CI: -0.08-0.39, P=0.11)
Lordkipanidzé *et al*. 2007	201	Patients with sTable CAD (diagnosis based on a positive stress test or angiographically documented coronary artery stenosis)	VerifyNow Aspirin with AA as agonist and LTA using 1.6 mM AA as agonist	ARU ≥ 550	Aspirin resistance was detected in 4.0% of patients by LTA and in 6.7% by VerifyNow. There was a poor correlation between the two tests (correlation coefficient: 0.133, p=ns)

AA=arachidonic acid; ACS=acute coronary syndrome; ARU=aspirin reaction units; ASA=aspirin; LTA=light-transmittance aggregometry; EPI=epinephrine

The VerifyNow P2Y12 assay

The third VerifyNow assay that was approved by the FDA for the specific purpose to monitor the inhibitory effects of thienopyridine-therapy is the VerifyNow P2Y12 assay. In contrast to the ASA cartridge and the GP IIb/IIIa cartridge, the P2Y12 cartridge contains two different chambers with agonists[42,43]:

1) The first chamber contains TRAP to determine a baseline (maximal) platelet function measurement because clinical logistics often do not allow pre-drug blood sampling. Iso-TRAP is a strong agonist for platelet activation and this can occur relatively independent of aspirin and clopidogrel therapy because its effects are only partly mediated by secreted ADP and TXA_2.

2) The second chamber contains both ADP (final concentration: 20 µmol/L) and prostaglandin E_1 (final concentration: 22 nmol/L). In an attempt to make the VerifyNow P2Y12 assay more sensitive for the specific ADP-P2Y12-pathway, PGE_1 is added to suppress the platelet activation contribution from ADP-binding to the P2Y1-receptor.

The VerifyNow P2Y12 assay reports results as P2Y12 reaction units (PRU), "percentage Inhibition" and BASE. Percentage inhibition of the P2Y12 receptor is calculated with the following equation: (1-(PRU/BASE))x100.

The VerifyNow P2Y12 assay: a laboratory evaluation and comparison with other well-established methods to evaluate the anti-platelet effects of clopidogrel

Various head-to-head comparisons between the VerifyNow P2Y12 assay and other conventional methods to assess the individual response to aspirin has demonstrated good agreement in the majority of studies (Table 3).[43-47]

Factors that could influence the results of the VerifyNow P2Y12 cartridge

Several factors that affect the results obtained with the VerifyNow P2Y12 assay have been reported by the manufacturer:

1) The time between the sample collection and the assay performance should be at least 10 minutes. In addition, blood samples have to be tested within 4 hours of sample collection because of the degeneration of platelet function.

2) The PRU and BASE results of the VerifyNow P2Y12 cartridge were not influenced by age, platelet count, hematocrit, fibrinogen, cholesterol, or triglycerides level in the initial preliminary validation studies of the VerifyNow P2Y12 cartridge.[48] However, in December 2006, Accumetrics sent a warning to all their customers because the assay reports an erroneous result instead of an error message in patients with a low hematocrit.[49] At present, this problem might have been overcome by adapted software.

Table 3: Head-to-head comparisons between the Verify*Now* P2Y12 assay and other conventional methods

Study, year	No. of patients	Patient population	Assay	Definition of low response	Results
von Beckerath *et al*. 2006	30	Consecutive patients with CAD were measured before and after different clopidogrel loading doses (300 mg, 600 mg or 900 mg)	VerifyNow P2Y12 assay and LTA with ADP (5 and 20 µM) as agonist	No definition was provided	The correlations of P2Y12 Reaction Units (PRU) with ADP(5 and 20 µM)-induced platelet aggregation were 0.86, p<0.0001 and 0.85, p<0.0001, respectively.
van Werkum *et al*. 2006	211	Aspirin and clopidogrel pretreated patients with CAD undergoing PCI	VerifyNow P2Y12 assay and LTA with ADP(20 µM) as agonist	No definition was provided	The correlations of P2Y12 Reaction Units (PRU) with "peak" and "late" platelet aggregation were 0.73, p<0.01 and 0.75, p<0.01, respectively.
Pannicia *et al*. 2006	1267	Aspirin and clopidogrel pretreated patients with CAD undergoing PCI	VerifyNow P2Y12 assay and LTA using 2 and 10 µM ADP as agonist	Patients who were in the highest quartile of the VerifyNow P2Y12 data. PRU>264	Results from the VerifyNow P2Y12 assay were concordant with LTA-ADP (r = 0.62, p < 0.0001 for 2 µM and r = 0.64, p < 0.0001). The proportion of clopidogrel low-responders was 24.7% using the >264 PRU cut-off.
Jakubowski *et al*. 2008	35	Healthy subjects on aspirin were administered a clopidogrel 600 mg loading dose (LD) followed by a 75 mg/d maintenance dose (MD) for 10 days. Subjects were then switched to a prasugrel 60 mg LD and then 10 mg/d MD for 10 days (n=16), or to a prasugrel 10 mg/d MD for 11 days (n=19).	VerifyNow P2Y12 assay and LTA 20 µM ADP as agonist	No definition was provided	In this study, multiple correlations were presented between various parameters obtained with LTA and the VerifyNow P2Y12 assay. Within the range of 20-70% aggregation, there was a good agreement between both tests. Another important observation in this study was that thienopyridine use altered the BASE levels. This can lead to an underestimation of the true percentage inhibition of aggregation

ADP=adenosine-diphophate; CAD=coronary artery disease; LTA=light-transmittance aggregometry; PCI=percutaneous coronary intervention; PRU=P2Y12-reaction units

Current available evidence that PRU is a suitable marker to predict future atherothrombotic events in clopidogrel treated patients

Only one study thus far has determined the possible relationship between the VerifyNow P2Y12 PRU results and the occurrence of atherothrombotic events. Price and co-workers measured post-treatment platelet reactivity with the VerifyNow P2Y12 assay in 380 patients undergoing PCI with DES implantation. Receiver Operating Characteristics (ROC) curve analysis was used to derive the optimal cut-off value (in PRU) for predicting 6-month cardiovascular death, non-fatal myocardial infarction and stent thrombosis. The optimal cut-off value for the combined endpoint was a PRU-value ≥235. Patients with post-treatment reactivity greater than the cut-off value had significantly higher rates of CV death (2.8% vs. 0%, p=0.04), stent thrombosis (4.6% vs. 0%, p=0.004) and the composite endpoint (6.5% vs. 1.0%, p=0.009). Post-treatment reactivity above the optimal cut-off was independently associated with the occurrence of the combined endpoint (odds ratio 7.9 [95% CI, 1.6 to 38.8, p=0.01).[50]

DISCUSSION

The multifactorial nature of arterial (coronary) thrombosis

Arterial thrombus formation is mediated by complex and multiple platelet signalling pathways. As a result, it cannot be expected that an antiplatelet drug that targets a single pathway is able to prevent the occurrence of all atherothrombotic events.

The same problem appears to be true with the currently available platelet function assays. As yet, there is no platelet function test that has the capability to cover the entire spectrum of platelet biology and function. Hence, the answer to the question: "What is currently the most suitable platelet function test?" is largely dependent upon the purpose of testing.

The VerifyNow system has been designed with the specific purpose to monitor different forms of antiplatelet therapy. As a consequence, these assays can be used to answer the question: "does the aspirin or clopidogrel therapy actually work in my patients?". However, the VerifyNow platelet function system has not been designed to screen the general platelet reactivity status (either with or without antiplatelet drug therapies) of a patient in order to stratify the risk for future atherothrombotic events. On the other hand, there is some evidence that the VerifyNow system is also capable to identify patients with a generalized hyperreactive phenotype.[32]

A critical appraisal to the VerifyNow platform

The VerifiyNow system has been shown to correlate well with the "gold standard" technique LTA with ADP or AA as the agonist. Nonetheless, important evidence that the ex vivo results of the different VerifyNow assays also correlate with clinical outcome is limited to very small studies with relatively low numbers of events. Furthermore, the cut-off levels to segregate responders from non-responders for the different VerifyNow assays are based on the results from relatively small patient studies and these thresholds should therefore be used with extreme caution. The major advantage of the VerifyNow system over other techniques for the monitoring of different forms of antiplatelet therapy is the fact that it produces rapid and reproducible results without any handling or preparation of the blood (Point-of-care). However, a major disadvantage includes the fact that VerifyNow platform is one of the most expensive platelet function assays. Our suggestion for the manufacturer: since many patients receive combined antiplatelet therapy with aspirin and clopidogrel, it might be useful to develop one assay that incorporates all the agonists for monitoring the individual response to both clopidogrel and aspirin therapy, this will reduce costs and testing time.

No case for routine platelet function evaluation at present

Very recently, a state-of-the-art review of platelet-function tests has been published by the lead experts in the field of monitoring antiplatelet therapy.[51] They state that before the ideal concept of individual dose-adjusting (based on a reliable, simple and quick point-of-care platelet function assay) can be put in daily clinical practice, several major issues need to be addressed:

1. Thorough evaluation and comparison of the currently available point-of-care platelet function tests in large prospective clinical trials that are based on clinical outcome.
2. Evidence-based thresholds that are based on clinical outcome need to be identified to segregate non-responders from responders for the different point-of-care assays.
3. We need evidence indicating that a change of therapy (e.g. increasing the dose of either or both aspirin and clopidogrel or switching to other antithrombotic therapies) will improve outcomes without any safety concern such as bleeding.
4. We need the support of at least some evidence that the individual monitoring of platelet function is cost-effective, especially in particular subgroups of patients in whom the risk for the reoccurrence of a atherothrombotic event is very low (e.g. stable angina pectoris patients).

CONCLUSION

In the last few years, an abundance of studies focussing on the relationship between responsiveness to antiplatelet therapy (particularly on aspirin and clopidogrel) and clinical outcome have been published. Furthermore, new techniques to study *in-vitro* platelet function have been introduced with the specific purpose to monitor the individual antiplatelet therapy or to stratify the future risk for the individual patient. The Verify*Now* system is currently the only point-of-care device that allows a rapid platelet evaluation without any required expertise. However, none of the currently available platelet function assays, including the Verify*Now* system, has been sufficiently validated and standardised to guide antiplatelet therapy to its optimal effect. Clinical trials of sufficient size are urgently needed to support or discourage the observations from small studies linking the magnitude of platelet inhibition to atherothrombotic events.

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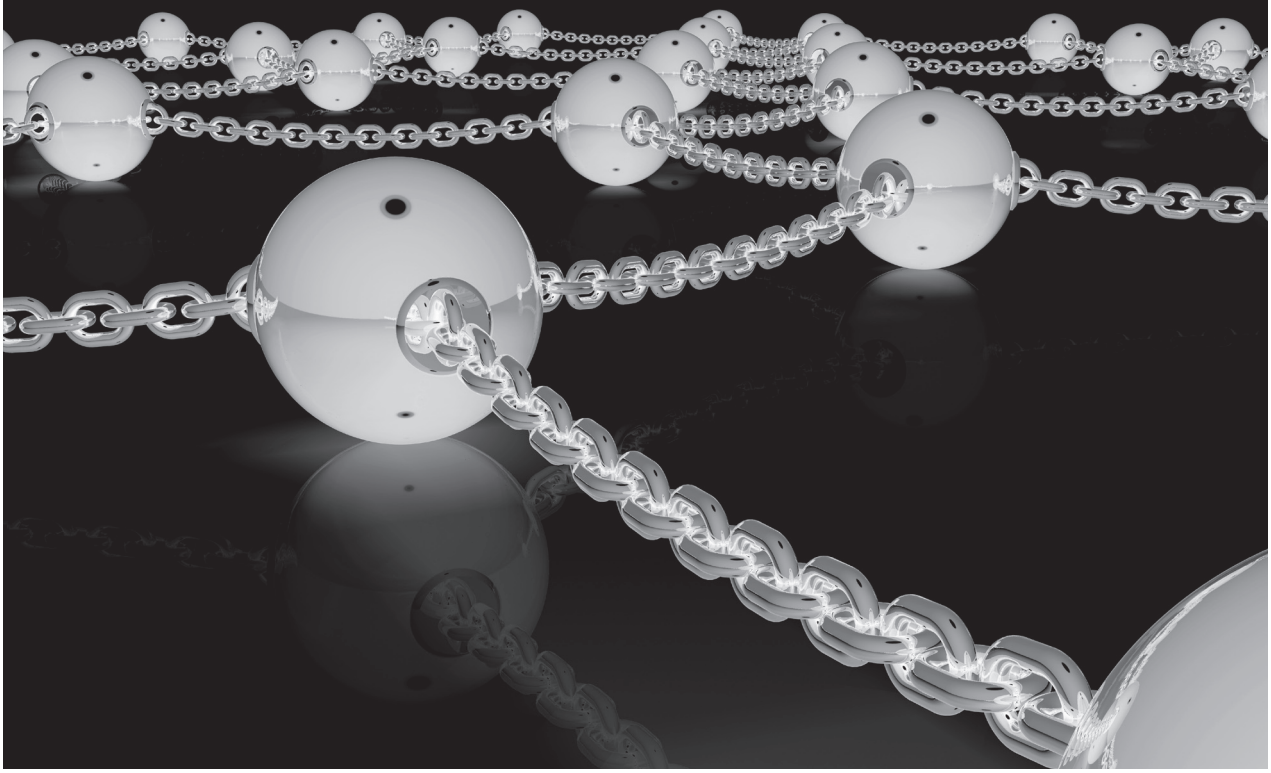
2

FACTORS INFLUENCING ON-TREATMENT PLATELET REACTIVITY AND CLOPIDOGREL'S ACTIVE METABOLITE PLASMA CONCENTRATIONS



2.1

BESIDES *CYP2C19*2*, THE VARIANT ALLELE *CYP2C9*3* IS ASSOCIATED WITH HIGHER ON-CLOPIDOGREL PLATELET REACTIVITY IN PATIENTS ON DUAL ANTIPLATELET THERAPY UNDERGOING ELECTIVE CORONARY STENT IMPLANTATION

Ankie M. Harmsze
Jochem W. van Werkum
Heleen J. Bouman
Hendrik J.T. Ruven
Nicoline J. Breet
Jurriën M. ten Berg
Christian M. Hackeng
Mathieu M. Tjoeng
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

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ABSTRACT

Introduction

The prodrug clopidogrel plays an important role in the prevention of thrombotic events in patients undergoing coronary stenting. However, a substantial number of atherothrombotic events still occur, which can partially be explained by heightened residual platelet reactivity. Several studies report that the genetic variation in *CYP2C19 (*2)* is associated with an impaired response to clopidogrel.

Objectives

To evaluate the effect of genetic variants affecting clopidogrel's absorption (*ABCB1 C1236T, G2677T/A, C3435T*), metabolism (*CYP2C9*2, *3, CYP2C19*3, CYP3A4*1B and CYP3A5*3*) and pharmacodynamics (*P2Y1 A1622G*) on top of the influence of *CYP2C19*2* on platelet reactivity in patients undergoing elective coronary stenting on dual antiplatelet therapy.

Methods

Platelet function was assessed by light transmittance aggregometry and VerifyNow P2Y12 assay® in 428 consecutive patients. Patients were either on chronic clopidogrel maintenance therapy (75 mg/day for ≥ 5 days before the intervention) or received a 300 mg clopidogrel loading dose (1-5 days before the intervention, followed by 75 mg/day). Linear and logistic regression were used to assess the associations between genetic variants and platelet reactivity and poor-responder status.

Results

In both treatment groups, *CYP2C19*2*-carriage was associated with higher platelet reactivity ($p<0.002$) and poor-responder status; 75mg-group: OR_{adj} 3.8 95% CI, 2.0-7.2; 300mg-group: OR_{adj} 4.1 95% CI, 1.6-10.4. In the 300mg-group, *CYP2C9*3*-carriage was associated with higher platelet reactivity ($p<0.05$) and poor-responder status (OR_{adj} 11.1 95% CI, 1.6-78.8, $p=0.016$).

Conclusions

Besides *CYP2C19*2*, the variant allele *CYP2C9*3* plays an important role in the response to clopidogrel in patients on dual antiplatelet therapy undergoing coronary stenting.

INTRODUCTION

Clopidogrel plays an important role in the prevention of thrombotic events in patients undergoing coronary stenting.[1] However, a substantial number of atherothrombotic events still occur, which can be partially explained by a heightened residual platelet reactivity despite aspirin and clopidogrel treatment. This phenomenon may be related to genetic variations, alternative pathways of platelet activation, patients non-compliance or drug-drug interactions.
Clopidogrel is a thienopyridine that inhibits platelet activation through an irreversible blockade of the platelet adenosine diphosphate (ADP) P2Y12 receptor. Clopidogrel is an inactive prodrug that requires several biotransformation steps to become active.[2,3] After intestinal absorption, which is dependent on the transporter protein P-glycoprotein, the biotransformation to the active metabolite is mainly mediated by the hepatic cytochrome (CYP) P450 system. The genetic variation in the CYP iso-enzyme CYP2C19 (*CYP2C19*2*) has been repeatedly shown to be associated with a diminished pharmacodynamic and pharmacokinetic response to clopidogrel and with an increased risk of adverse cardiovascular events in clopidogrel-treated patients.[4-15] However, contradictory results regarding genetic variations in proteins that are involved in the metabolism of clopidogrel (e.g. CYP2C9, CYP3A4, CYP3A5), clopidogrel absorption (P-glycoprotein) and platelet receptors such as P2Y1 are found.[4-9,16-19] The aim of the present study was to evaluate the influence of genetic variations involved in clopidogrel's absorption, metabolism and pharmacodynamics on top of the influence of *CYP2C19*2* on platelet reactivity in patients on dual antiplatelet therapy undergoing elective coronary stent implantation.

METHODS

Design and setting

In a prospective observational study we measured platelet response to clopidogrel in a large cohort of consecutive patients undergoing elective coronary stent implantation. Patients either were on chronic clopidogrel maintenance therapy, defined as 75 mg/day for more than 5 days prior to the coronary stent implantation, or received a loading dose of 300 mg clopidogrel that was administered 24 hours to 5 days before the coronary stent implantation followed by 75 mg/day. All patients received aspirin (80 - 100 mg) for more than 5 days prior to the coronary stent implantation. Information on the use of medication was obtained by questionnaires and verified by medication history records from community pharmacies.. Exclusion criteria were: acute myocardial infarction with ST-segment elevation within 48 hours from symptom onset, allergies or contra-indications to either aspirin, clopidogrel or heparin, increased risk of bleeding, malignancies, pregnancy or hematological disorders including thrombocytopenia. The study protocol was approved by the hospital's Medical Ethics Committee, and informed consent was obtained from each patient.

Blood sampling

Prior to the coronary stent implantation procedure and before heparinization, blood was drawn from the femoral arterial sheath in 3.2% citrate tubes for platelet function testing. The first 10 ml of free-flowing blood was discarded. Blood for DNA analysis was sampled using K3-EDTA tubes.

Genotyping

Genomic DNA was isolated from K3-EDTA blood (MagNA Pure LC DNA Isolation kit 1, MagNA Pure; Roche Diagnostics; Basel, Switzerland). *CYP2C9, CYP2C19, CYP3A4* and the *ABCB1 G2677T/A* and *C3435T* alleles were identified by real time PCR. The other alleles were identified by using restriction fragment length polymorphism (RFLP). Information on the primers, restriction enzymes and probes are available upon request. Method validation was carried out by DNA sequence analyses.

Platelet function assays

The magnitude of on-clopidogrel platelet reactivity was assessed by light transmission aggregometry (LTA) using the APACT 4004 four-channel light transmission aggregometer (LABiTec, Ahrensburg, Germany). Samples were centrifuged for 10 min at 150g to obtain native platelet rich plasma (PRP). Maximal platelet aggregation (defined as the maximum extent of platelet aggregation achieved in any time during the run of 10 minutes) was quantified in non-adjusted PRP after stimulation with 5 and 20 µmol/L ADP. The magnitude of on-clopidogrel platelet reactivity in whole blood, expressed as P2Y12 Reaction Units (PRU), was measured with the VerifyNow P2Y12 test cartridge system, as described previously.[20,21] LTA is considered to be the gold standard for determining the effects of antiplatelet therapy on platelet function, but the logistical demands make it impossible to use in daily practice. The VerifyNow P2Y12 assay® is a point-of-care platelet function assay which has the specific purpose to rapidly inform the clinician about the magnitude of platelet inhibition that is achieved with the individual antiplatelet regimen.[22] A strong correlation between the two methods of measurement has been found.[20] All measurements were completed within 2 hours of blood collection.

Statistical analysis

Continuous variables were expressed as mean ± SD or median [range]. Categorical variables were expressed as frequencies and percentages. Categorical data were analyzed by chi-square test and continuous data by Student t test when appropriate. Chi-square tables were used to compare the observed number of each genotype with those expected for a population in Hardy-Weinberg equilibrium ($p>0.05$).

Haplotypes were formed if more than one genetic variation within the same gene was found statistically significant associated with the response to clopidogrel as measured with LTA or VerifyNow. The PHASE II program was used to infer haplotypes.[23,24] The influence of *CYP2C19*2* was investigated in the total cohort. The influence of the other genetic variations was firstly investigated in noncarriers of the *CYP2C19*2* allele. Then, the multiplicative interactions between associated genetic variants and *CYP2C19*2* were tested in the total cohort and expressed as synergy index (SI).[25] Linear regression was used to calculate the mean difference in on-clopidogrel platelet reactivity (indicated as ΔPA for LTA values and ΔPRU for VerifyNow P2Y12 assay® results) between carriers and noncarriers of a variant allele, and to adjust for confounding factors. The following confounding factors were included in the analyses: gender, age, body mass index, diabetes mellitus, prior MI, duration of clopidogrel administration before the coronary stent implantation (in days), CYP3A4-metabolized statins, proton pump inhibitors, calcium channel blockers, SSRI's and NSAIDs. For determining the influence of genotype/haplotype on the clopidogrel responder status, crude and adjusted odds ratios (ORs) with their 95% confidence interval (CI) were calculated using logistic regression analysis. A poor-responder was defined as a clopidogrel pretreated subject with more than 70% aggregation using 20

µmol/L ADP (LTA) or with a PRU value > 235 (VerifyNow P2Y12 assay). Both parameters have been associated with adverse cardiovascular events, including stent thrombosis, in previous studies.[26-28] If a genetic association was found, we corrected for multiple testing by performing the false discovery rate test (q value threshold 0.20).[29] A p value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL).

RESULTS

Characteristics of the study population and genotyping

A total of 428 consecutive clopidogrel and aspirin pretreated patients were included in the study. More than two-third (n=297) of the patients were on chronic clopidogrel maintenance therapy before the intervention. The remaining 131 patients received a clopidogrel loading dose of 300 mg. Table 1 shows the demographic and clinical characteristics of the study cohort.

Table 1: Baseline demographics and clinical characteristics of the study cohort

Characteristic	Chronic clopidogrel maintenance therapy (75mg/day), n=297	Clopidogrel loading dose (300 mg), n=131
Age, years (mean ± SD)	63.4 ± 9.6	61.8 ± 10.12
Men, n (%)	230 (77.4)	105 (80.2)
Risk factors, n (%)		
Current smoker	41 (13.8)	12 (9.2)
Hypertension	232 (78.1)	108 (82.4)
Diabetes Mellitus	59 (19.9)	25 (19.1)
Dyslipidemia	249 (83.8)	102 (77.9)
Family history of CAD	185 (62.3)	94 (71.8)
Previous MI	154 (51.9)	48 (36.6)
Body mass index (mean ± SD)	27.4 ± 3.8	27.6 ± 3.6
Medication, n (%)		
Statins	257 (86.5)	107 (81.7)
CYP3A4-metabolized	203	75
Not -CYP3A4-metabolized	54	32
Betablockers	231 (77.8)	97 (74.0)
NSAIDs	14 (4.7)	5 (3.8)
ACE-inhibitors	123 (41.4)	39 (29.8)
SSRIs	12 (4.0)	4 (3.1)
PPIs	70 (23.6)	28 (21.4)
Aspirin	297 (100)	131 (100)

CAD: coronary artery disease, PPI: proton pump inhibitor; NSAIDs: non steroidal anti-inflammatory drugs, ACE: angiotensin converting enzyme, SSRIs: selective serotonin reuptake inhibitor, student t test for continuous variables and chi-square test for categorical variables, p>0.05 for all

No significant deviations from Hardy Weinberg equilibrium were observed for any of the genetic variants (table 2). Genotype and allele frequencies were not different from previously reported frequencies in healthy Caucasian populations.[18, 30] Since we found only 2 carriers of the *CYP2C19*3*-allele and no subjects with the *CYP3A5*1/*1* genotype, we did not include these subjects in our analysis. Genetic linkage was observed for the variations in the *ABCB1*-gene, with the *C1236T* and *G2677T* variants showing the strongest linkage (R^2=0.87; p<0.0001), followed by *C3435T* and *G2677T* (R^2=0.49; p<0.01) and *C1236T* and *C3435T* (R^2=0.45; p<0.01). Three common haplotypes constituted by 3 genetic variations in the ABCB1-gene represented 42% (*C1236, G2677, C3435*), 39% (*1236T, 2677T, 3435T*) and 13% (*1236T, G2677, C3435*) of the studied population. The other six haplotypes were very rare (<5%) and were excluded from further analysis.

Table 2: Genotype distributions and allele frequencies of the investigated genetic variations

Allele	Homozygous noncarriers of variant allele	Heterozygous carriers of variant allele	Homozygous carriers of variant allele	Allele frequency (%)
CYP2C19 G681A (*1>*2) rs4244285	296 (69.2)	120 (28.0)	12 (2.8)	17
CYP2C19 G636A (*1>*3) rs4986893	426 (99.5)	2 (0.5)	0	0.3
CYP2C9 C430T (*1>*2) rs1799853	334 (78.0)	86 (20.1)	8 (1.9)	12
CYP2C9 A1075C (*1>*3) rs1057910	384 (89.8)	43 (10.0)	1 (0.2)	5
CYP3A4 A290G (*1>*1B) rs2740574	402 (93.9)	26 (6.1)	0 (0)	3
CYP3A5 A6986G (*1>*3) rs776746	1 (0.2)	64 (15.0)	363 (84.8)	92
ABCB1 C1236T rs1128503	137 (32.0)	224 (52.3)	67 (15.7)	42
ABCB1 G2677T/A rs2032582	129 (30.1)	218 (50.9)/9 (2.1)	69 (16.1) / 3 (0.7)	42 / 2
ABCB1 C3435T rs1045642	78 (18.2)	232 (54.2)	118 (27.6)	55
P2Y1 A1622G rs701265	304 (71.0)	115 (26.9)	9 (2.1)	16

Genotype distributions expressed as number (percentage), allele frequency expressed as percentage

Platelet reactivity in patients on chronic clopidogrel maintenance therapy

The median duration of clopidogrel treatment in these patients was 15.0 days [range 6-1200]. An attenuated response to clopidogrel was observed in carriers compared with noncarriers of the *CYP2C19*2* allele (all p<0.0001; table 3). This difference remained significant after adjustment for confounding factors (ΔPA_{adj} 6.9% 95% CI, 3.7-10.0, p<0.001 for 5 µmol/L ADP; ΔPA_{adj} 7.0% 95% CI, 4.0-10.0, p<0.001 for 20 µmol/L ADP and ΔPRU_{adj} 34.8 95% CI, 16.8-52.8, p<0.001 for VerifyNow P2Y12 assay).

Table 3: Influence of *CYP2C19*2* on on-clopidogrel platelet reactivity

	Chronic clopidogrel maintenance therapy (75mg/day)		Clopidogrel loading dose (300 mg)	
	mean differences	p-value	mean differences	p-value
ADP 5 µmol/L (%)	6.7 [3.6-9.8]	<0.001	7.8 [3.9-12.6]	0.002
ADP 5 µmol/L (%)#	6.9 [3.7-10.0]	<0.001	5.7 [0.6-10.8]	0.028
ADP 20 µmol/L (%)	6.3 [3.4-9.3]	<0.001	7.4 [3.3-11.6]	0.001
ADP 20 µmol/L (%)#	7.0 {4.0-10.0]	<0.001	7.0 [2.6-11.5]	0.002
VerifyNow (PRU)	35.1 [17.2-53.0]	<0.001	37.5 [16.5-58.5]	0.001
VerifyNow (PRU) #	34.8 [16.8-52.8]	<0.001	28.5 [7.1-49.6]	0.009

The magnitude of on-clopidogrel platelet reactivity in subjects receiving chronic clopidogrel maintenance therapy as measured with light transmittance aggregometry (maximal platelet aggregation to 5 and 20 µmol/L ADP) and with the VerifyNow P2Y12 assay. Results expressed as mean differences [95% confidence interval], comparisons between carriers and noncarriers of the variant allele, p < 0.05 considered statistically significant, ADP adenosine diphosphate, PRU P2Y12 reaction units

Multivariate analysis: adjusted for gender, age, body mass index, diabetes mellitus, previous MI, duration of clopidogrel administration before the intervention in days, CYP3A4-metabolized statins, calcium channel blockers, proton pump inhibitors, SSRIs and NSAIDs

In patients with the *CYP2C19*1/*1* genotype (*n*=216), carriers of the *ABCB1 1236T* and *2677T/A* allele showed decreased efficacy of clopidogrel when measured with VerifyNow P2Y12 assay (table 4), which remained significant after adjustment for confounders ($\Delta PRU_{adj\ C1236T}$: 20.5 95% CI, 0.6-42.4, p=0.044 and $\Delta PRU_{adj\ G2677T/A}$ 24.7 95% CI, 2.4-47.1, p=0.030). Carriage of 1236T was also associated with higher PRU values in the entire cohort (196.2 ± 6.7 vs. 171.6 ± 68.3, p=0.006 for carriers and noncarriers of *1236T*, respectively), which remained significant after adjustment for confounders ($\Delta PRU_{adj\ C1236T:}$ 20.5 95% CI, 3.3-39.4, p=0.021). Also the carriage of a *2677G/A* variant allele was associated with impaired response to clopidogrel when measured with the VerifyNow P2Y12 assay: 196.1 ± 68.5 vs. 170.7 ± 68.6, p=0.005, respectively. This difference remained significant in multivariate analysis ($\Delta PRU_{adj\ G2677T/A}$ 21.5 95% CI, 3.1-40.0, p=0.022).

No differences between platelet reactivity and other genetic variants were found (table 4).

Table 4: Influence of other genetic variants on platelet reactivity in patients on chronic clopidogrel maintenance therapy

Genetic variant		LTA 5 µmol/L ADP (%)	LTA 20 µmol/L ADP (%)	VerifyNow (PRU)
CYP2C9*2	*1/*1 *1/*2 and *2/*2 p-value	42.2 ± 11.4 39.5 ± 12.5 NS	58.4 ± 11.3 57.1 ± 11.9 NS	182.1 ± 67.6 170.0 ± 75.4 NS
CYP2C9*3	*1/*1 *1/*3 and *3/*3 p-value	41.5 ± 11.9 40.7 ± 10.7 NS	58.2 ± 11.6 56.8 ± 10.8 NS	181.1 ± 67.2 164.6 ± 84.5 NS
CYP3A4*1B	*1/*1 *1/*1B and *1B/*1B p-value	41.3 ± 11.8 42.4 ± 10.8 NS	58.0 ± 11.6 57.8 ± 11.5 NS	177.4 ± 70.9 193.6 ± 53.0 NS
ABCB1 C1236T	C/C C/T and T/T p-value	39.8 ± 10.7 42.2 ± 12.2 NS	56.3 ± 10.8 58.9 ± 11.8 NS	161.6 ± 70.8 187.0 ± 68.0 0.018
ABCB1 G2677T/A	G/G G/A + G/T + A/A + T/T p-value	39.8 ± 10.9 42.1 ± 12.1 NS	56.4 ± 11.1 58.8 ± 11.6 NS	159.0 ± 71.3 187.4 ± 67.5 0.009
ABCB1 C3435T	C/C C/T and T/T p-value	42.1 ± 8.5 41.2 ± 12.4 NS	58.3 ± 9.9 57.9 ± 11.8 NS	182.3 ± 70.0 177.6 ± 69.9 NS
P2Y1 A1622G	A/A A/G and G/G p-value	41.0 ± 11.7 42.2 ± 12.0 NS	57.5 ± 11.5 59.0 ± 11.4 NS	175.1 ± 68.1 185.6 ± 73.2 NS

The magnitude of on-clopidogrel platelet reactivity in subjects receiving chronic clopidogrel maintenance therapy as measured with light transmittance aggregometry (maximal platelet aggregation to 5 and 20 µmol/L ADP) and with the VerifyNow P2Y12 assay. Results expressed as mean ± standard deviation, comparisons between carriers and noncarriers of the variant allele in patients with the *CYP2C19*1/*1* genotype, ADP adenosine diphosphate, PRU P2Y12 reaction units, NS: not significant, $p < 0.05$ considered statistically significant

In patients on chronic clopidogrel maintenance therapy, 20% of the patients were categorized as poor-responders (according to LTA). Figure 1A shows that carriers of the *CYP2C19*2* variant allele were more likely to be classified as clopidogrel poor-responders than noncarriers: OR 3.7 95% CI, 2.0-6.9 p<0.001, OR_{adj} 3.8 95% CI, 2.0-7.2, p<0.001 for LTA and for the VerifyNow P2Y12 assay: OR 2.8 95% CI, 1.6-5.0, p<0.001, OR_{adj} 3.4 95% CI, 1.8-6.4, p<0.001. No associations were found between a poor-responder status and the other genetic variants in patients on chronic clopidogrel maintenance therapy. The analysis performed on the identified haplotypes showed no significant associations with on-clopidogrel platelet reactivity and poor-responder status (data not shown). For all significant associations in this treatment group we found the multiple testing parameter q to be < 0.20.

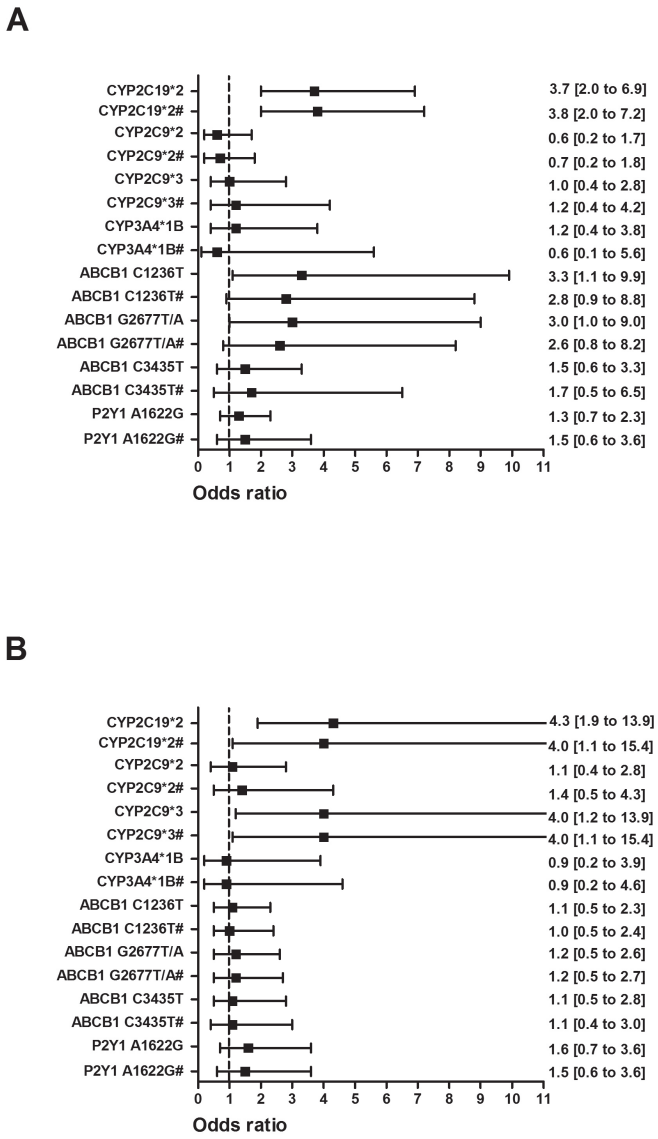

Figure 1: Odds ratios for poor-responder status according to genotype
Odds ratios (ORs) with 95% confidence intervals for poor-responder status according carriage of variant alleles. Poor-responder: clopidogrel pretreated subject with more than 70% aggregation to 20 µmol/L ADP. Panel A: patients receiving clopidogrel maintenance therapy; panel B: patients receiving clopidogrel 300 mg loading dose. Other than *CYP2C19*2* genetic variants assessed in subjects with the *CYP2C19*1/*1* genotype
Multivariate analysis: adjusted for gender, age, body mass index, diabetes mellitus, previous MI, duration of clopidogrel administration before the intervention in days CYP3A4-metabolized statins, calcium channel blockers, proton pump inhibitors, SSRIs and NSAIDs

Platelet reactivity in patients receiving a 300 mg clopidogrel loading dose

Patients in this group received a 300 mg clopidogrel loading dose at a median time of 2.0 days [range 1-5] prior to the coronary stent implantation.

The magnitude of on-clopidogrel platelet reactivity was significantly higher in carriers of the *CYP2C19*2* variant allele than in noncarriers (p<0.03 for all; table 3). These differences remained significant after adjustment for confounding factors (ΔPA_{adj} 5.7% 95% CI, 0.6-10.8, p=0.028 for 5 µmol/L ADP; ΔPA_{adj} 7.0% 95% CI, 2.6-11.5, p=0.002 for 20 µmol/L ADP and ΔPRU_{adj}: 28.5 95% CI, 7.1-49.6, p=0.009 for the VerifyNow P2Y12 assay, table 3).

In subjects with the *CYP2C19*1/*1* genotype (*n*=80), carriers of the *CYP2C9*3* variant allele showed overall higher on-clopidogrel platelet reactivity (table 5), which remained significant after the adjustment for confounders (ΔPA_{adj} 8.6% 95% CI, 0.2-18.5, p=0.043 for 5 µmol/L ADP; ΔPA_{adj} 10.4% 95% CI, 1.1-19.7, p=0.029 for 20 µmol/L ADP and ΔPRU_{adj}: 73.1 95% CI, 26.4-119.9, p=0.003 for the VerifyNow P2Y12 assay). In the entire cohort, the influence of *CYP2C9*3* was less profound (51.4% ± 4.1 vs. 46.4 ± 11.3, p=NS for 5 µmol/L ADP; 71.3% ± 10.5 vs. 64.5% ± 11.0, p=0.043 for 20 µmol/L ADP and 237.6 ± 15.7 vs. 208.6 ± 5.6, p=NS for the VerifyNow P2Y12 assay). No associations were found for the other genetic variants or haplotypes (table 5).

Table 5: Influence of other genetic variants on platelet reactivity in patients receiving a 300 mg clopidogrel loading dose

Genetic variant		LTA 5 µmol/L ADP (%)	LTA 20 µmol/L ADP (%)	VerifyNow (PRU)
CYP2C9*2	*1/*1	42.2 ± 12.2	61.2 ± 11.0	195.2 ± 54.0
	*1/*2 and *2/*2	49.7 ± 15.5	66.5 ± 12.0	207.8 ± 63.5
	p-value	NS	NS	NS
CYP2C9*3	*1/*1	43.2 ± 12.6	61.4 ± 10.9	192.4 ± 53.8
	*1/*3 and *3/*3	51.9 ± 18.2	72.3 ± 11.6	261.5 ± 46.8
	p-value	0.028	0.010	0.003
CYP3A4*1B	*1/*1	44.1 ± 13.6	62.7 ± 11.6	198.4 ± 56.5
	*1/*1B and *1B/*1B	43.7 ± 8.8	58.8 ± 9.2	200.0 ± 65.0
	P-value	NS	NS	NS
ABCB1 C1236T	C/C	42.2 ± 12.4	61.9 ± 10.9	191.9 ± 66.6
	C/T and T/T	44.5 ± 14.0	62.8 ± 11.8	202.1 ± 50.6
	p-value	NS	NS	NS
ABCB1 G2677T/A	G/G	41.7 ± 11.4	61.0 ± 10.2	189.2 ± 68.0
	G/A + G/T + A/A + T/T	45.1 ± 14.2	63.2 ± 12.0	202.6 ± 50.8
	p-value	NS	NS	NS
ABCB1 C3435T	C/C	40.2 ± 16.1	60.1 ± 15.3	203.5 ± 78.3
	C/T and T/T	44.9 ± 12.8	630. ± 10.6	197.6 ± 52.7
	p-value	NS	NS	NS
P2Y1 A1622G	A/A	42.1 ± 12.0	61.7 ± 10.8	196.0 ± 54.8
	A/G and G/G	47.3 ± 16.0	65.2 ± 13.2	206.5 ± 63.0
	p-value	NS	NS	NS

The magnitude of on-clopidogrel platelet reactivity in subjects receiving a 300 mg clopidogrel loading dose as measured with light transmittance aggregometry (maximal platelet aggregation to 5 and 20 µmol/L ADP) and with the VerifyNow P2Y12 assay. Results expressed as mean ± standard deviation, comparisons between carriers and noncarriers of the variant allele in patients with the *CYP2C19*1/*1* genotype, ADP adenosine diphosphate, PRU P2Y12 reaction units, NS: not significant, p < 0.05 considered statistically significant

In patients receiving a loading dose of 300 mg clopidogrel, 40% of the patients were categorized as poor-responders (according to LTA). Figure 1B shows that carriers of the *CYP2C19*2* variant allele were more likely to be classified as clopidogrel poor-responders than noncarriers: OR 3.7 95% CI, 2.0-6.9 p<0.001, OR_{adj} 4.1 95% CI, 1.6-10.4, p=0.003.

In subjects with the *CYP2C19*1/*1* genotype, carriers of the *CYP2C9*3*-allele had also an increased risk of clopidogrel poor-response: OR 11.6 95% CI, 2.1-63.6, p=0.005, which remained significant after adjustment for confounders (OR_{adj} 11.1 95% CI, 1.6-78.8, p=0.016). The association of *CYP2C9*3* and clopidogrel poor-response was also observed when on-clopidogrel platelet reactivity was measured by the VerifyNow P2Y12 assay (OR 6.3 95% CI, 1.3-29.8, p=0.021, OR_{adj} 12.0 95% CI, 1.7-87.0, p=0.014. For both assays, the association of CYP2C9*3 and clopidogrel poor-response was found in the entire cohort (OR 4.0 95% CI, 1.2-13.8, p=0.029 for LTA and OR 4.7 95% CI, 1.4-16.4, p=0.015 for the VerifyNow P2Y12 Assay. Gene-gene interactions between *CYP2C9*3* and *CYP2C19*2* were found, which showed a significant decrease on the multiplicative scale SI_{LTA} 0.1 95% CI, 0.004-0.79, p=0.033 and $SI_{VerifyNow}$ 0.1 95% CI, 0.002-0.7, p=0.035. No association was observed between on-clopidogrel platelet reactivity and the other genetic variants. The analysis performed on the identified haplotypes showed no significant associations (data not shown). For all significant associations in this treatment group we found the multiple testing parameter q to be < 0.20.

DISCUSSION

The present study aimed to determine the effect of genetic variations related to the pharmacokinetics and pharmacodynamics of clopidogrel in patients undergoing elective coronary stent implantation. We found that the presence of the *CYP2C19*2* loss-of-function allele was associated with a higher magnitude of on-clopidogrel platelet reactivity, both when administered as chronic maintenance therapy as well as in patients receiving a 300 mg clopidogrel loading dose. *CYP2C19*2* was associated with an approximately 4-fold increased risk of clopidogrel poor-response in both treatment groups. These findings confirm the results from previous studies in which genetic variations of *CYP2C19* modulated clopidogrel pharmacokinetics[5,7] and pharmacodynamics[6,8,30] both in healthy subjects and in patients. Up to date, in five studies the *CYP2C19*2* genetic variation is directly shown to be associated with adverse cardiovascular events, including stent thrombosis.[10-14] CYP2C19 contributes in both of the two sequential oxidative metabolic steps of clopidogrel activation. An impaired first metabolic step due to a reduced CYP2C19 metabolic capacity would tend to shunt the prodrug preferentially to an esterase-mediated pathway, thereby forming pharmacologically inactive metabolites.[10]

Furthermore, we found *CYP2C9*3* to result in an attenuated response to clopidogrel in patients receiving a 300 mg clopidogrel loading dose, resulting in a more than 10-fold increased risk of clopidogrel poor-response in *CYP2C19*2* noncarriers. In contrast, no association between *CYP2C9*3* and attenuated response to clopidogrel was found in patients on chronic clopidogrel maintenance therapy.

The prodrug clopidogrel is converted in vivo to its active metabolite by the highly polymorphic hepatic cytochrome P450 (CYP) system (including CYP3A, CYP2C9 and CYP2C19) in a 2–step process. First, clopidogrel is metabolized into 2-oxo-clopidogrel. This intermediate metabolite is

then hydrolyzed and generates a highly unstable active metabolite, which irreversibly reacts as a thiol reagent with the G-protein coupled $P2Y_{12}$-receptor on platelets.[2,3] Brandt *et al*. showed that healthy subjects carrying the *CYP2C9*3* allele, receiving a clopidogrel 300 mg loading dose, had an attenuated response to clopidogrel.[7] In the present study, *CYP2C9*3* also modulated the response to clopidogrel in patients receiving a 300 mg loading dose. However, *CYP2C9*3* lacked this effect in patients on chronic clopidogrel maintenance therapy. To our knowledge, this is the first study that reports the association of *CYP2C9*3* and response to clopidogrel in patients with cardiovascular disease. We hypothesize that the reduced enzyme capacity of *CYP2C9*3* only becomes critical in the presence of higher clopidogrel plasma concentrations achieved by the 300 mg loading dose of clopidogrel. Unlike CYP2C19, CYP2C9 is thought to only play a role in the second metabolisation step in the activation of clopidogrel.[10] In the present study, the influence *CYP2C9*3* on clopidogrel poor-response was smaller (but also statistically significant) in the entire cohort than in subjects with the *CYP2C19*1/*1* genotype, which resulted in a gene-gene interaction with a significant decrease on the multiplicative scale. We hypothesize that when formation to the active metabolite is reduced in the first metabolisation step by the presence of *CYP2C19*2*, the nett influence of *CYP2C9*3* in the second metabolisation step might be smaller.

The drug-efflux transporter P-glycoprotein (encoded by *ABCB1* gene), is a physiologic intestinal barrier against the absorption of several drugs, including clopidogrel. Taubert *et al*. found that the noncoding *C3435T* SNP in the *ABCB1*-gene significantly reduced the absorption of clopidogrel.[17] Simon *et al* reported patients with the *3435TT* and *3435CT* genotypes to have worse clinical outcomes than those with a CC genotype.[11] In the present study, we found no evidence of *C3435T* modulating response to clopidogrel. However, two coding SNPs in the *ABCB1*-gene *G2677T/A* and *C1236T*, which are both in strong linkage disequilibrium with C3435T, were associated with an impaired response to clopidogrel when measured with the VerifyNow P2Y12-assay®.

Recently, the *P2Y1* gene *A1622G* dimorphism was shown to influence ADP-induced platelet activation in healthy subjects.[18] Although P2Y1 is not a clopidogrel target receptor, it is hypothesized that mutations conferring increased function of the P2Y1 gene may allow an escape from P2Y12 blockade by clopidogrel and therefore be associated with the response to the drug.[31] Subsequently, in two studies the association of this genetic variant and the pharmacodynamic response to a single 300 and 600 mg clopidogrel loading dose in patients undergoing PCI was determined.[32,33] No association between *A1622G* genotypes and the response to clopidogrel in cardiac patients was found. Our results, demonstrating that the *P2Y1 A1622G* variant did not modulate platelet response, confirm these previous observations.

There are some limitations of this study. First, in this observational study, we cannot completely exclude possible bias by various risk factors and patient characteristics although the multivariable adjustment models confirmed the primary analyses. Unfortunately, the time from the last administration of clopidogrel was not standardized. However, as this parameter is not dependent on genotype, we expect this variation is equally distributed among the different genotypes and treatment groups. Furthermore, we found no association of the *CYP3A4*1B* genetic variation and response to clopidogrel. Since *CYP3A4*1B* occurs with low allele frequency, a larger patient population may need to be screened to definitely determine the impact of this genetic variation on the response to clopidogrel. Finally, we did not investigate the influence of

the genetic variations on clinical outcome in clopidogrel-treated subjects.

In conclusion, in our study with patients undergoing elective coronary stent implantation who were treated with clopidogrel and aspirin from 10 genes encoding for proteins involved in the absorption, metabolism and pharmacodynamics of clopidogrel, we found that, besides *CYP2C19*2*, the genetic variant *CYP2C9*3* plays an important role in the response to clopidogrel.

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2.2

THE USE OF AMLODIPINE, BUT NOT OF P-GLYCOPROTEIN INHIBITING CALCIUM CHANNEL BLOCKERS IS ASSOCIATED WITH CLOPIDOGREL POOR-RESPONSE

Ankie M. Harmsze
Karen Robijns
Jochem W. van Werkum
Nicoline J. Breet
Christian M. Hackeng
Jurriën ten Berg
Hendrik J.T. Ruven
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

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ABSTRACT

Introduction

Clopidogrel is a prodrug that has to be converted in vivo to its active metabolite by several cytochrome (CYP) P450 iso-enzymes. As calcium channel blockers (CCBs) are inhibitors of CYP3A4, concomitant use of these drugs might play a role in the wide interindividual variability in the response to clopidogrel. However, some CCBs also have strong inhibitory effects on the drug transporter P-glycoprotein (Pgp), which mediates the intestinal absorption of clopidogrel.

Aim

Aim of this study was to evaluate the effect of co-administration of Pgp-inhibiting and non-Pgp-inhibiting CCBs on platelet reactivity in patients on dual antiplatelet therapy undergoing elective percutaneous coronary intervention (PCI).

Methods

In a total of 623 consecutive patients undergoing elective PCI treated with clopidogrel and aspirin, platelet aggregation to 5 and 20 µmol/L adenosine diphospate (ADP) and clopidogrel poor-response (defined as > 70% platelet aggregation to 20 µmol/L ADP) were evaluated by light transmittance aggregometry.

Results

A total of 222 patients (35.6%) were on CCB treatment, of which 98 used Pgp-inhibiting CCBs (verapamil, nifedipine, diltiazem, barnidipine) and 124 patients used the non-Pgp-inhibiting CCB amlodipine.

Adjusted mean ADP-induced on-clopidogrel platelet aggregation was significantly higher in both users of Pgp-inhibiting CCBs and amlodipine as compared to CCB nonusers (all $p<0.05$). However, only the use of amlodipine was significantly associated with a 2.3-fold increased risk of clopidogrel poor-response**.**

Conclusions

This study demonstrates that concomitant use of Pgp-inhibiting CCBs and amlodipine increase on-clopidogrel platelet reactivity. Only amlodipine was associated with clopidogrel poor-response. The drug-drug interaction between clopidogrel and amlodipine might be more clinically relevant as compared to Pgp-inhibiting CCBs.

INTRODUCTION

Dual antiplatelet therapy with clopidogrel and aspirin has become standard treatment after percutaneous coronary interventions (PCI).[1] The intestinal efflux of clopidogrel is dependent on P-glycoprotein (Pgp). Pgp-mediated efflux reduces the intracellular accumulation of clopidogrel, thereby diminishing its efficacy.[2] Clopidogrel is a prodrug that needs to be converted in vivo to generate its active metabolite. Conversion into the active compound occurs in a two-step process, in which the hepatic cytochrome (CYP) P450 iso-enzymes CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4,5 are involved.[3] The active thiol metabolite irreversibly inhibits the adenosine diphosphate (ADP) P2Y12 receptor on the platelets surface.[4,5] Platelet response to clopidogrel is highly variable between individuals.[6] The activity of the CYP-enzymes and overexpression of Pgp are thought to influence the antiplatelet effect of clopidogrel. Genetic variants of genes encoding CYP iso-enzymes and Pgp are associated with diminished platelet inhibition during clopidogrel treatment and some with an increased risk of atherothrombotic events.[3,7-15] Drugs that are substrates or inhibitors of the same CYP iso-enzymes or Pgp might also influence the antiplatelet effect of clopidogrel. Calcium channel blockers (CCBs) have been used for many years to treat angina pectoris, hypertension and other cardiovascular diseases.[16] Two recent studies suggested that CCBs reduce the pharmacodynamic response to clopidogrel and increase the risk of adverse atherothrombotic events by the inhibition of CYP3A4.[17,18] However, within the class of CCBs, there are substantial pharmacokinetic differences. All CCBs are substrates and inhibitors of CYP3A4.[19] Importantly, some CCBs (nifedipine, nicardipine, barnidipine, felodipine, lercidipine, verapamil and diltiazem) also have strong inhibitory effects on Pgp activity ("Pgp-inhibiting CCBs").[19] Other CCBs like nimodipine, nisoldipine, isradipine and amlodipine exhibit no inhibitory effects on Pgp activity ("non-Pgp-inhibiting CCBs").[19] Due to these differences within the class of CCBs, different clinical relevance of drug interactions with clopidogrel are expected. Siller-Matula *et al*. and Gremmel *et al.* did not perform comparative analyses within the class of CCBs due to small sample size.
The aim of this study was to investigate the impact of co-administration of different CCBs on on-clopidogrel platelet reactivity in a large cohort of patients on dual antiplatelet therapy undergoing elective PCI.

METHODS

Study design

In a prospective observational study we measured on-clopidogrel platelet reactivity in a large cohort of consecutive patients undergoing elective coronary stent implantation. All patients were on dual antiplatelet therapy with aspirin and clopidogrel at the time of inclusion. All patients received clopidogrel (75 mg) and aspirin (80 mg) daily for more than 5 days prior to the intervention. Exclusion criteria were: acute myocardial infarction with ST-segment elevation within 48 hours from symptom onset, allergies or contra-indications to heparin, increased risk of bleeding, malignancies, pregnancy or hematological disorders including thrombocytopenia and treatment with GP IIb/IIIa inhibitors during the 14 days before platelet function testing. Information on the use of CCBs and other co-medication was obtained from community pharmacies. A CCB-user was defined as a subject who was on CCB treatment for ≥ 7 days prior to the coronary stent implantation. The study protocol was approved by the hospital's Medical Ethics Committee, and informed consent was obtained from each patient.

Blood sampling

Prior to PCI and before heparinization, blood was drawn from the femoral arterial sheath in 3.2% citrate tubes for platelet function testing. The first 10 ml of free-flowing blood was discarded.

Platelet function assays

The magnitude of on-clopidogrel platelet reactivity was assessed by light transmission aggregometry (LTA) using the APACT 4004 four-channel light transmission aggregometer (LABiTec, Ahrensburg, Germany). Samples were centrifuged for 10 min at 150g to obtain native platelet rich plasma (PRP). Maximal platelet aggregation (defined as the maximum extent of platelet aggregation achieved in any time during the run of 10 minutes) was quantified in non-adjusted PRP after stimulation with 5 and 20 µmol/L ADP. The magnitude of on-clopidogrel platelet reactivity in whole blood, expressed as P2Y12 Reaction Units (PRU), was measured with the VerifyNow P2Y12 Point-of-Care test cartridge system, as described previously.[20,21] LTA is considered to be the gold standard for determining the effects of antiplatelet therapy on platelet function, but logistical demands make it difficult to use in daily practice. The VerifyNow P2Y12 assay® is a point-of-care platelet function assay which has the specific purpose to rapidly inform the clinician about the magnitude of platelet inhibition that is achieved with the individual antiplatelet regimen.[22] All measurements were completed within 2 hours of blood collection.

Statistical analysis

Continuous variables were expressed as mean ± SD or median with interquartile range [IQR]. Categorical variables were expressed as frequencies and percentages. For baseline characteristics, continuous data were analyzed by analysis of variance (ANOVA) and categorical data by chi-square test when appropriate. Kolmogorov-Smirnov test was used to test for normal distribution of continuous data.

ANOVA and, in case of a significant result followed, LSD post-hoc tests, were used to analyze mean differences in normally distributed on-clopidogrel platelet reactivity between treatment groups. Multivariate linear regression was used to adjust for confounding factors (gender, age, body mass index, diabetes mellitus, prior myocardial infarction, hypertension, current smoking, left ventricular ejection fraction (LVEF) < 45%, duration of clopidogrel administration before the coronary stent implantation (in days) and the use of proton pump inhibitors). For determining the influence of concomitant use of the CCBs on the clopidogrel responder status, crude and adjusted odds ratios (ORs) with their 95% confidence interval (CI) were calculated using logistic regression analysis. A poor-responder was defined as a clopidogrel-treated subject with more than 70% aggregation using 20 µmol/L ADP (LTA) or with VerifyNow P2Y12 PRU value of more than 235.

Sample size calculation for the present study was based on results of the study of Siller-Matula *et al*. in which an approximately 25% relative increase of ADP-induced platelet aggregation was observed in the group of patients on concomitant CCB treatment.[17] Under the assumption that approximately 35% of the patients is on concomitant CCB treatment, CCB treatment is associated with a 25% relative increase (from 52% ± 25 to 65% ± 25) of ADP-induced platelet aggregation and a power of 90% with a two-sided α-value of 0.05, a sample size of at least 60 patients in each CCB-treatment group and 180 nonusers of CCBs (overall sample size of 300 patients) was required. A p value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL).

RESULTS

Patient characteristics

A total of 623 consecutive patients who were on maintenance therapy with aspirin and clopidogrel were enrolled in this study. From the study population, 222 (35.6%) patients were on CCB treatment at the time of platelet function testing. Among them, 98 patients used Pgp-inhibiting CCBs (verapamil 320 mg (n=1), diltiazem (n=57, mean dose 213.4 ± 52.5 mg), barnidipine 10 mg (n=2) and nifedipine (n=38, 43.0 ± 15.2 mg)). The remaining 124 patients were treated with amlodipine (mean dose 5.5 ± 1.5 mg), which does not inhibit Pgp. The median duration time [IQR] of CCB treatment before platelet function testing was 48 days [294 days]. The baseline characteristics of the study population according to CCB treatment are shown in table 1. In univariate analysis, significant differences between the groups regarding the variables age (p=0.011),hypertension (p<0.0001) and the use of beta-blockers (p<0.0001) were observed.

Table 1: Baseline demographics and clinical characteristics of the study cohort

Variable	No CCB (n=410)	Pgp-inhibiting CCBs (diltiazem, nifedipine, verapamil, barnidipine), n=98	Amlodipine, n=124	p-value
Age (years)	62.5 ± 10.6	65.7 ± 10.0	64.7 ± 10.8	0.011
Men, n (%)	312 (76.1)	77 (78.6)	84 (67.7)	0.11
Body mass index (kg/m2)	27.1 ± 3.9	27.0 ± 3.7	27.3 ± 3.6	0.81
Diabetes mellitus, n (%)	72 (17.6)	17 (17.3)	23 (18.5)	0.96
Current smokers, n (%)	52 (12.7)	6 (6.1)	9 (7.3)	0.07
Hypertension, n (%)	303 (73.9)	86 (87.8)	108 (87.1)	<0.0001
Hypercholesterolemia, n (%)	346 (84.4)	83 (84.7)	94 (76.4)	0.11
Family history of CAD, n (%)	251 (62.1)	59 (61.5)	75 (62.0)	0.99
Previous MI, n (%)	191 (47.3)	54 (56.8)	50 (41.3)	0.08
LVEF < 45%	66 (16.1)	13 (13.3)	18 (14.5)	0.33
Proton pump inhibitors, n (%)	94 (22.9)	29 (29.6)	34 (27.4)	0.30
CYP3A4-metabolized statins, n (%)	280 (68.3)	61 (62.2)	81 (65.3)	0.48
Beta-blockers, n (%)	349 (85.1)	62 (63.3)	114 (91.9)	<0.0001

Data are expressed as mean value ± SD or number of patients n (%); p-value: ANOVA for continuous variables and chi-square test for categorical variables between the three groups, CAD: coronary artery disease, MI: myocardial infarction; CCB: calcium channel blocker, Pgp: P-glycoprotein

On-clopidogrel platelet reactivity and CCB treatment

Figure 1 shows that users of amlodipine and Pgp-inhibiting CCBs exhibited higher on-clopidogrel platelet reactivity as compared to CCB nonusers. On-clopidogrel platelet reactivity differed statistically significant between the three treatment groups ($p<0.0001$ for all platelet function assays). Pairwise comparisons showed that the mean ADP-induced on-clopidogrel maximal platelet aggregation was significantly higher in users of amlodipine as compared to CCB nonusers (41.9% ± 13.1 vs. 36.7% ± 13.5, for 5 µmol/L, $p<0.0001$ and 60.3% ± 13.2 vs. 54.5% ± 14.2, for 20 µmol/L ADP, $p<0.0001$, fig 1). After adjustment for the confounding variables, the use of amlodipine remained significantly associated with an increased on-clopidogrel platelet reactivity (mean difference: 4.4% (95% CI 1.6-7.2, $p=0.002$), for 5 µmol/L ADP, and 5.0% (95% CI 2.1-7.9, $p=0.001$), for 20 µmol/L. Likewise, the mean on-clopidogrel platelet reactivity when measured with VerifyNow P2Y12 assay was also significantly higher in users of amlodipine as compared to patients without CCB treatment (224.1 ± 73.4 vs. 191.1 ± 74.5, $p<0.0001$, fig 1). The adjusted mean difference in VerifyNow P2Y12 PRU results between patients on amlodipine and CCB nonusers was 26.9, 95% CI 11.9-36.2, $p<0.0001$.

Pairwise comparisons showed that the mean ADP-induced on-clopidogrel platelet reactivity for users of Pgp-inhibiting CCBs was also significantly higher as compared to patients without CCB treatment (figure 1). After adjustment for confounders, the use of Pgp-inhibiting CCBs remained significantly associated with an increased on-clopidogrel platelet reactivity (mean difference: 5.7% (95% CI 1.9-9.6, $p=0.003$), for 5 µmol/L ADP, and 3.7% (95% CI 0.3-7.7, $p=0.035$) for 20 µmol/L. However, platelet reactivity according to the VerifyNow P2Y12 assay did not differ between users of Pgp-inhibiting CCBs and CCB nonusers (204.5 ± 73.2 vs. 191.1 ± 74.5, $p=0.11$, fig 1). No significant differences in platelet reactivity between users of Pgp-inhibiting CCBs and amlodipine were found in pairwise comparisons (figure 1).

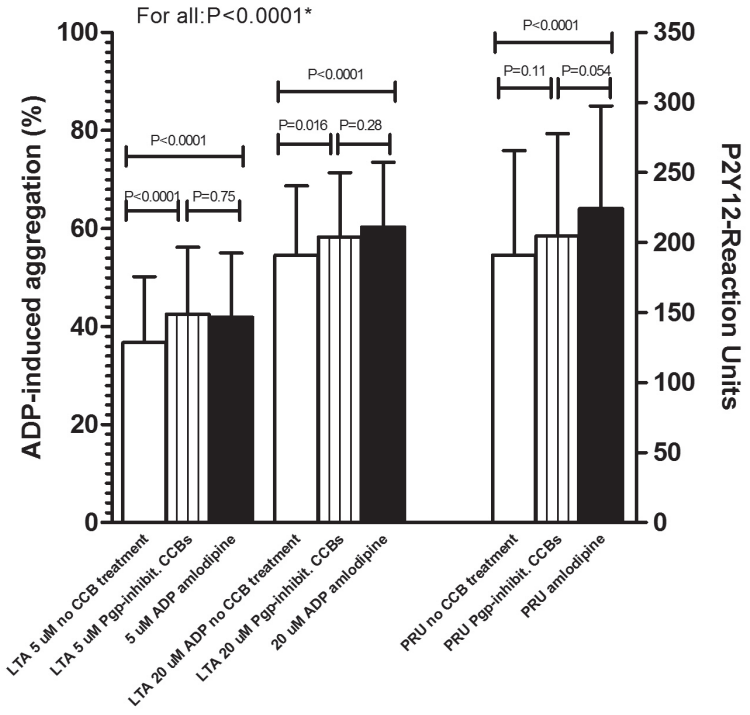

Figure 1: On-clopidogrel platelet reactivity according to the use of calcium channel blockers
Platelet aggregation (as measured with LTA after 5 and 20 µmol/L ADP and VerifyNow P2Y12 assay) in patients with no CCB treatment (open bars), treatment with Pgp-inhibiting CCBs (striped bars) and with amlodipine (non-Pgp-inhibiting CCB) (solid bars). CCB: calcium channel blocker. ADP: adenosine diphosphate, p-values ANOVA with LSD.

Clopidogrel poor-response and CCB treatment

Based on LTA measurements, 97 patients (15.6% of the total cohort) were classified as clopidogrel poor-responders. The proportion of clopidogrel poor-responders was significantly higher in users of amlodipine compared to patients without CCB treatment (25.6% vs. 12.9%), resulting in an odds ratio of 2.3 (95% CI 1.4-3.9, p=0.001, table 2). This association remained significant after the adjustment for confounders: OR_{adj} 2.3 95% CI, 1.4-3.9, p=0.001. According to the VerifyNow P2Y12 assay, the use of amlodipine was associated with clopidogrel poor response (OR 2.4 95% CI, 1.6-3.7, p<0.0001 and OR_{adj} 2.3 95% CI, 1.5-3.6, p<0.0001). In the group of patients on Pgp-inhibiting CCBs, 14.6% was classified as clopidogrel poor-responder. No association between the risk of clopidogrel poor-response and the use of Pgp-inhibiting CCBs was found (OR 1.2 95% CI, 0.6-2.2, p=0.66 and OR_{adj} 0.9 95% CI, 0.4-2.2, p=0.92, table 2). In concordance with results from the LTA, no association between concomitant use of Pgp-inhibiting CCBs and clopidogrel poor-response as measured with the VerifyNow P2Y12 assay was found (OR 1.3 95% CI, 0.8-2.1, p=0.33 and OR_{adj} 1.5 95% CI, 0.8-2.8, p=0.19, table 2). Subanalysis within

the group of Pgp-inhibiting CCBs showed that the dihydropyridins nifedipine and barnidipine (n=40), did not have any influence on clopidogrel poor-responder status (LTA: OR 1.4 95% CI, 0.6-3.4, p=0.41 and OR_{adj} 1.4 95% CI, 0.6-3.4, p=0.49 and VerifyNow: OR 1.0 95% CI, 0.5-2.1, p=0.97 and OR_{adj} 0.9 95% CI, 0.4-2.1, p=0.96). The use of diltiazem and verapamil (n=58) also did not have any influence on clopidogrel poor-response (LTA: OR 1.0 95% CI, 0.4-2.3, p=0.94 and OR_{adj} 1.0 95% CI, 0.4-2.3, p=0.91 and VerifyNow: OR 1.5 95% CI, 0.8-2.7, p=0.17 and OR_{adj} 1.5 95% CI, 0.8-2.7, p=0.24).

Table 2: Odds ratios for clopidogrel poor-responder status according to CCB treatment

CCB	Poor responder	Crude OR [95% CI]	p value	Adjusted OR [95% CI]#	p value
Amlodipine	LTA – ADP	2.3 [1.4-3.9]	0.001	2.3 [1.4-3.9]	0.001
	VerifyNow - PRU	2.4 [1.6-3.7]	<0.0001	2.3 [1.5-3.6]	<0.0001
Pgp-inhibiting CCBs	LTA – ADP	1.2 [0.6-2.2]	0.66	0.9 [0.4-2.2]	0.92
	VerifyNow - PRU	1.3 [0.8-2.1]	0.33	1.5 [0.8-2.8]	0.19

Odds ratios (ORs) with 95% confidence intervals for poor-responder status according to CCB treatment. Poor-responder: clopidogrel pretreated subject with more than 70% aggregation to 20 μM ADP (LTA) or more than 235 P2Y12 Reaction Units (PRU, VerifyNow). CCBs: calcium channel blockers, OR: odds ratio

Multivariate analysis: adjusted for gender, age, body mass index, diabetes mellitus, previous myocardial infarction, LVEF < 45%, hypertension, current smoking, duration of clopidogrel administration before the coronary stent implantation (in days) and the use of proton pump inhibitors

DISCUSSION

In the present study we assessed the influence of the concomitant use of different CCBs on on-clopidogrel platelet reactivity in a large cohort of patients on dual antiplatelet therapy undergoing elective PCI. Co-administration of amlodipine, which does not inhibit Pgp, was associated with increased on-clopidogrel platelet reactivity. Furthermore, the use of amlodipine was associated with an 2.4-fold increased risk of clopidogrel poor-response using the predefined criteria for poor-response as more than 70% platelet aggregation to 20 μmol/L ADP (LTA) or a VerifyNow P2Y12 PRU-value of more than 235. In other studies, this parameter is associated with adverse cardiovascular events, including stent thrombosis.[23,24] The use of the Pgp-inhibiting CCBs diltiazem, verapamil, barnidipine and nifedipine was found to increase on-clopidogrel platelet reactivity as measured with LTA. However, no influence was observed when platelet reactivity was measured with the VerifyNow P2Y12 assay and importantly, this subclass of CCBs was not associated with an increased risk of clopidogrel poor-response.

The inhibitory effect of CCBs on the platelet response to clopidogrel is thought to be caused at the level of CYP3A4.[17] Clopidogrel is a prodrug, which requires hepatic biotransformation by CYP3A4 to generate the active metabolite.[3] As all CCBs are substrates and inhibitors of CYP3A4[19], concomitant use could inhibit clopidogrel's metabolism. The intestinal absorption of clopidogrel is limited by P-glycoprotein by increasing the intestinal efflux.[2] The CCBs verapamil, diltiazem, nifedipine en barnidipine are potent inhibitors of Pgp and have been shown to increase the responsiveness to several drugs e.g. digoxin and anticancer agents by this

mechanism.[16,25-28] Inhibition of Pgp by the concomitant use of Pgp-inhibiting CCBs may lead to a decreased intestinal efflux of clopidogrel, thereby increasing clopidogrel plasma concentrations and counteracting the effect of CCB induced CYP3A4 inhibition. Therefore, concomitant use of Pgp-inhibiting CCBs might have less clinical relevance than co-administration of amlodipine. However, the clinical use of diltiazem and verapamil is not completely comparable with the use of amlodipine. Amlodipine is solely used in the treatment of hypertension and coronary artery disease while diltiazem and verapamil are also used for rate control in atrial fibrillation. However, subanalysis within the group of Pgp-inhibiting CCBs showed that nifedipine and barnidipine, drugs that have the same clinical use as amlodipine, also have no influence on clopidogrel poor-response. In the study of Siller-Matula *et al*, co-administration of CCBs was found to be associated with a diminished pharmacodynamic response to clopidogrel and with an increased risk of adverse cardiovascular events.[17] The authors made no distinction between the different CCBs but the majority of their study population used amlodipine. These results are consistent with our observation that the use of amlodipine is associated with clopidogrel poor-response. In our study, amlodipine was the only representative of the CCB subclass with no inhibiting effect on Pgp. Other non-Pgp-inhibiting CCBs like nimodipine, nisoldipine and isradipine, were not studied. There are some limitations of this study. First, in this observational study, we cannot completely exclude possible bias by various risk factors and patient characteristics although the multivariable adjustment models confirmed the primary analyses. Furthermore, we did not investigate the influence of CCBs on plasma concentrations of clopidogrel's active metabolite nor on clinical outcome. An additional limitation is that we did not adjust for the carriage of genetic variants of e.g. CYP2C9, CYP2C19 and Pgp (ABCB1), which are found to play a role in the antiplatelet properties of clopidogrel.

In conclusion, concomitant use of Pgp-inhibiting CCBs and amlodipine increases on-clopidogrel platelet reactivity. However, only amlodipine was associated with a higher risk of clopidogrel poor-response. These findings may have important implications with regards to which type of CCB is preferred in clopidogrel-treated patients.

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2.3

THE INFLUENCE OF OMEPRAZOLE ON ON-TREATMENT PLATELET REACTIVITY MIGHT BE DEPENDENT ON CLOPIDOGREL'S DOSING REGIMEN

Ankie M. Harmsze
Jochem W. van Werkum
Christian M. Hackeng
Jurriën M. ten Berg
Hendrik J.T. Ruven
Nicoline J. Breet
Heleen J. Bouman
Miranda G.M. Kok
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

Submitted

ABSTRACT

Introduction

Concomitant use of omeprazole has been shown to decrease the antiplatelet properties of clopidogrel. Aim of this study was to determine whether the influence of omeprazole on the antiplatelet effect of clopidogrel is dependent on clopidogrel's dosing regimen.

Materials and Methods

On-treatment platelet reactivity was measured with ADP-induced light transmittance aggregometry and the VerifyNow P2Y12-assay in 431 patients undergoing elective PCI. High on-treatment platelet reactivity was defined as >64.5% platelet reactivity to 20 µmol/L ADP. In total, 135 patients received a 300 mg clopidogrel loading dose 2-5 days prior to PCI, followed by 75 mg daily. The remaining 296 patients were on chronic clopidogrel maintenance therapy for at least 10 days.

Results

Fiftysix patients (13%) were on omeprazole treatment. After a recent 300 mg clopidogrel loading dose, platelet reactivity was significantly higher in omeprazole-users as compared to proton pump inhibitor nonusers: 54.1% ± 11.7 vs. 42.6% ± 14.4, p=0.001 (5 µmol/L ADP), 70.1% ± 8.0 vs. 61.8% ± 13.2, p=0.008 (20 µmol/L ADP) and 265 ± 58 vs. 213 ± 70, p=0.003 (VerifyNow P2Y12-assay). The use of omeprazole was associated with a 6.3-fold increased risk of high on-treatment platelet reactivity (p=0.003). In contrast, omeprazole was not associated with increased platelet reactivity in patients on chronic clopidogrel maintenance therapy.

Conclusions

In conclusion, the drug-drug interaction between clopidogrel and omeprazole might be more prominent when clopidogrel is administered as a 300 mg loading dose compared to clopidogrel maintenance therapy.

INTRODUCTION

Clopidogrel is a prodrug that needs to be metabolized by the iso-enzymes of the hepatic cytochrome (CYP) P450 system to become active.[1] The response to clopidogrel is therefore dependent on the activity of CYP isoenzymes. This is illustrated with the loss-of-function allele *CYP2C19*2*, which has been associated with an impaired response to clopidogrel and with worse clinical outcome in clopidogrel-treated patients.[1-5] Given the increased risk of gastro-intestinal bleeding associated with antiplatelet therapy, proton pump inhibitors (PPI's) are often co-prescribed in clopidogrel-treated patients.[6] Omeprazole is known to be a potent CYP2C19 inhibitor.[7] In 2006, an interaction between clopidogrel and omeprazole was reported for the first time.[8] Recently, omeprazole was found to decrease the exposure of clopidogrel's active metabolite by 47%.[9] Contradictory results have been published on whether co-administration of clopidogrel and omeprazole affects cardiovascular outcomes.[10-14] In the prematurely terminated COGENT trial, at this moment the only trial in which patients are randomized to PPI-treatment, co-administration of omeprazole was not associated with the risk of adverse cardiovascular events in clopidogrel-treated patients.[15]
In the context of these controversial results and the importance of the potential drug-drug interaction, we investigated in a prospective, observational study, whether the influence of concomitant omeprazole treatment on on-treatment platelet reactivity is dependent on clopidogrel's dosing regimen.

MATERIALS AND METHODS

Study population
The magnitude of on-treatment platelet reactivity prior to PCI was measured in a large cohort of 1069 consecutive patients undergoing elective PCI with stenting.[16] For this pre-specified analysis, patients were selected when they either received a loading dose of 300 mg clopidogrel that was administered 2 to 5 days before PCI, followed by 75 mg/day or were on chronic clopidogrel maintenance therapy, defined as 75 mg/day for more than 10 days prior to PCI. From these, patients were selected when they used omeprazole (for at least 14 days prior to platelet reactivity measurements took place) or were nonusers of PPI's (defined as no PPI-use for at least 14 days prior to measurements). Information on the use of omeprazole and other co-medication was assessed by questionnaires at the time of inclusion and was verified by medication histories from community pharmacies. All patients received aspirin (80-100 mg) for more than 7 days prior to PCI. Exclusion criteria were: acute myocardial infarction (MI) with ST-segment elevation within 48 hours from symptom onset, allergies or contra-indications to either aspirin, clopidogrel or heparin, increased risk of bleeding, malignancies, pregnancy or hematological disorders including thrombocytopenia and treatment with glycoprotein IIb/IIIa inhibitors during the 14 days prior to platelet function testing. The study protocol was approved by the hospital's Medical Ethics Committee, and informed consent was obtained from each patient.

Blood sampling
Before heparinization, blood was drawn from the femoral arterial sheath in 3.2% citrate tubes for platelet function testing. The first 10 ml of free-flowing blood was discarded. Blood for DNA analysis was sampled using EDTA tubes.

Platelet function testing
Platelet reactivity was assessed by light transmission aggregometry (LTA) using the APACT 4004 four-channel light transmission aggregometer (LABiTec, Ahrensburg, Germany). The magnitude of on-treatment peak platelet reactivity (defined as the maximum extent of platelet aggregation achieved in any time during the run of 10 minutes) was quantified in non-adjusted platelet rich plasma after stimulation with 5 and 20 µmol/L ADP. The magnitude of on-treatment platelet reactivity in whole blood, expressed as P2Y12 Reaction Units (PRU), was measured with the VerifyNow P2Y12 point-of-care test cartridge system, as described previously.[17,18] All measurements were completed within 2 hours of blood collection.

Genotyping
Genomic DNA was isolated from EDTA blood (MagNA Pure LC DNA Isolation kit 1, MagNA Pure; Roche Diagnostics; Basel, Switzerland). *CYP2C19*2* alleles (G681A; rs4244285) were detected by using the LightCycler CYP2C19 Mutation Detection Kit on the LightCycler instrument (Roche Diagnostics). Method validation of the kit for *CYP2C19* analyses was carried out by DNA sequence analysis.

Statistical analysis
Continuous variables were expressed as mean ± SD or median with interquartile range [IQR] when appropriate. Kolmogorov-Smirnov test was used to test for normal distribution of continuous data. Categorical variables were expressed as frequencies and percentages. For baseline characteristics, continuous data were analyzed by Student's t-test and categorical data by chi-square test. Student's t-tests were also used to analyze differences in on-treatment platelet reactivity between omeprazole treatment groups. Multiple linear regression was used to adjust for potential confounders (gender, age, body mass index, diabetes mellitus, previous MI, indication for PCI (acute coronary syndrome (ACS) or stable angina pectoris), carriage of *CYP2C19*2* and the concomitant use of calcium channel blockers). For determining the influence of omeprazole on the risk of high on-treatment platelet reactivity (HPR) in both clopidogrel treatment groups, crude and adjusted odds ratios (ORs) with their 95% confidence intervals (CI) were calculated using logistic regression analysis. HPR was defined as an aggregation of more than 64.5% to 20 µmol/L ADP (LTA), as -based on receiver operating characteristic curve (ROC) analyses- this cut-off point was shown to be associated with a one-year composite endpoint of ischemic events.[16] Sample size calculation was based on the results of the placebo-controlled trial by Gilard *et al*., in which approximately 25% relative increase of P2Y12 platelet reactivity was observed in the group of patients with concomitant omeprazole treatment.[19] Under the assumption that approximately 15% of all patients is on concomitant treatment with omeprazole, a relative increase of 20 µmol/L ADP-induced platelet aggregation of 25% caused by omeprazole, and choosing a power of 90% with a two-sided alpha-value of 0.05, a sample size of at least 12 patients on concomitant omeprazole treatment and 84 PPI nonusers in each clopidogrel treatment group was required. Statistical analysis was performed using SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL).

RESULTS

Patient characteristics

From the entire cohort, 431 PPI nonusers and omeprazole users were selected. In total, 135 patients received a 300 mg clopidogrel loading dose 2 to 5 days prior to PCI (median time 3.0 days [IQR: 2.0-4.0 days]), followed by 75 mg/day. The remaining 296 patients were on chronic clopidogrel maintenance therapy for at least 10 days prior to PCI (median time 31.0 days [IQR: 14-105 days]). Table 1 shows the baseline demographic and clinical characteristics of the study population. In patients receiving a 300 mg clopidogrel loading dose, no significant differences between PPI nonusers and users of omeprazole were found. In patients on chronic clopidogrel maintenance therapy, omeprazole users had a higher BMI, had more often diabetes mellitus and were less frequently carriers of *CYP2C19*2* compared to PPI nonusers. These parameters were included as potential confounders in multiple regression analysis. Patients on chronic clopidogrel maintenance therapy had more often experienced a prior MI compared to patients receiving a clopidogrel loading dose (p=0.005) and were more often current smokers (p=0.021). No other significant differences in baseline characteristics were found between the two clopidogrel treatment groups.

In the total study population, 56 patients (13.0%) were on concomitant omeprazole treatment. Mean omeprazole doses did not differ significantly between the two clopidogrel treatment groups (patients receiving clopidogrel loading dose were on a mean dose omeprazole of 28.1 mg ± 9.8 and patients on clopidogrel maintenance therapy: 28.6 mg ± 13.8).

The influence of omeprazole treatment on the effect of clopidogrel

Clopidogrel 300 mg loading dose group

In patients receiving a 300 mg clopidogrel loading dose, the use of omeprazole was associated with significantly higher magnitude of on-treatment platelet reactivity as compared to PPI nonusers: 54.1% ± 11.7 vs. 42.6% ± 14.4, p=0.001 for 5 µmol/L ADP and 70.1% ± 8.0 vs. 61.8% ± 13.2, p=0.008 for 20 µmol/L ADP (figure 1). Also platelet reactivity as measured with the VerifyNow P2Y12 assay was significantly higher in users of omeprazole as compared to PPI nonusers: 265 ± 58 vs. 213 ± 70, p=0.003 (figure 1). After the adjustment for potential confounders i.e. gender, age, body mass index, diabetes mellitus, previous MI, indication for PCI (acute coronary syndrome (ACS) or stable angina pectoris), carriage of *CYP2C19*2* and the concomitant use of calcium channel blockers, the use of omeprazole remained significantly associated with increased on-treatment platelet reactivity: for 5 µmol/L ADP adjusted mean difference [95% CI]: 13.0% [6.2-19.8], p<0.0001; for 20 µmol/L ADP: 10.4% [4.6-16.2], p=0.001 and for VerifyNow PRU: 54 [21-88], p=0.002.

Table 1: Baseline demographics and clinical characteristics

	Clopidogrel loading dose (300 mg) (n=135)			Chronic clopidogrel maintenance therapy (75mg/day) (n=296)		
	PPI-nonusers (n=114)	Omeprazole users (n=21)	p-value	PPI-nonusers (n=261)	Omeprazole users (n=35)	p-value
Age, years (mean ± SD)	63.1 ± 9.4	63.9 ± 11.3	0.50	63.5 ± 10.5	65.9 ± 10.6	0.26
Men, n (%)	97 (85.1)	16 (76.2)	0.25	205 (78.5)	26 (74.3)	0.52
Risk factors, n (%)						
Current smoker	7 (6.1)	3 (14.3)	0.30	35 (13.4)	4 (11.4)	0.99
Hypertension	94 (82.5)	16 (76.2)	0.36	196 (75.1)	29 (82.9)	0.25
Diabetes Mellitus	19 (16.7)	3 (14.3)	0.57	45 (17.2)	12 (34.3)	0.019
Dyslipidemia	85 (74.6)	16 (76.2)	0.59	215 (82.4)	29 (82.9)	0.99
Family history of CAD	69 (60.5)	12 (57.1)	0.63	147 (56.3)	17 (48.6)	0.93
Previous myocardial infarction	37 (32.5)	9 (42.9)	0.42	132 (50.6)	22 (62.9)	0.72
ACS as indication of PCI	22 (19.3)	3 (14.3)	0.30	68 (26.1)	12 (34.3)	0.37
Body mass index, kg/m^2 (mean ± SD)	27.4 ± 3.1	27.3 ± 3.4	0.84	27.3 ± 4.4	29.4 ± 4.2	0.009
Genotype, n (%)						
CYP2C19*2 carriers	37 (32.5)	5 (23.8)	0.35	87 (33.3)	5 (14.3)	0.024
Medication, n (%)						
Aspirin	114 (100)	21 (100)	1.00	261 (100)	35 (100)	1.00
Statins	94 (82.5)	17 (78.6)	0.26	222 (85.0)	32 (91.4)	0.21
Betablockers	80 (70.2)	16 (76.2)	0.43	204 (78.2)	26 (74.3)	0.35
ACE-inhibitors	33 (28.9)	8 (38.1)	0.22	106 (40.6)	9 (25.7)	0.06
Calcium channel blockers	41 (36.0)	5 (23.8)	0.12	101 (38.7)	17 (48.6)	0.14

Data are expressed as mean value ± SD or number of patients n (%); p-value: Student's test for continuous variables and chi-square test for categorical variables between users of omeprazole and PPI-nonusers, CAD: coronary artery disease, PPI: proton pump inhibitor; ACE: angiotensin converting enzyme, ACS: acute coronary syndrome

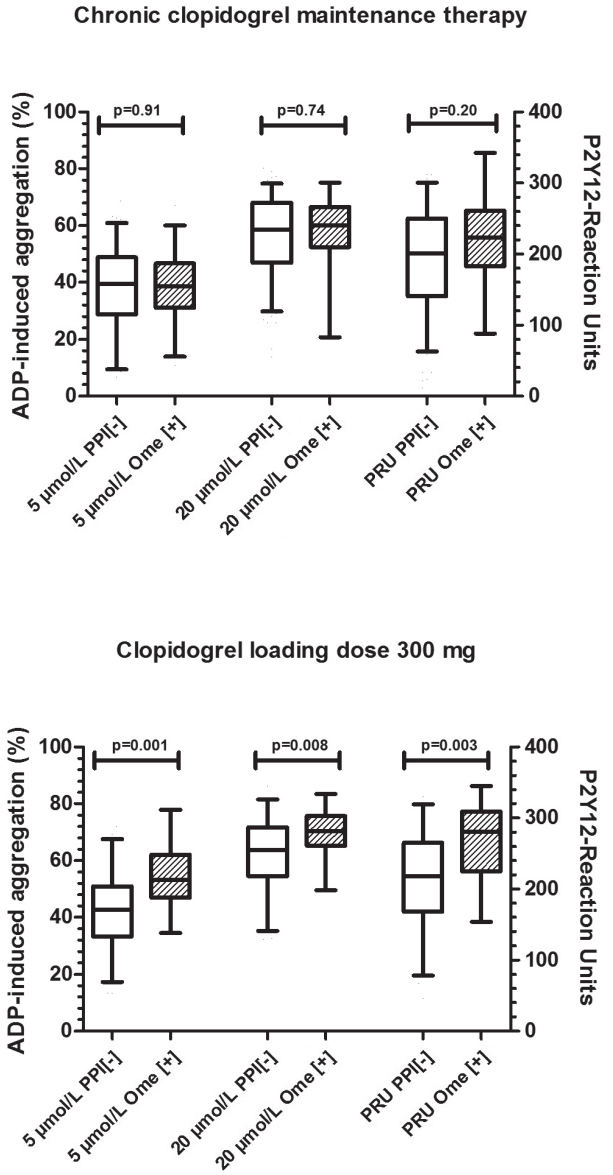

Figure 1: Influence of concomitant use of omeprazole on on-treatment platelet reactivity
On-treatment platelet reactivity (as measured with LTA after 5 and 20 µmol/L ADP and VerifyNow P2Y12 Assay) in patients treated with omeprazole (striped bars and indicated as: Ome[+]) and in patients not using any proton pump inhibitors (open grey bars and indicated as PPI [-]) in two clopidogrel dosing regimens. ADP: adenosine diphosphate, P-values obtained with Student's t-test

Chronic clopidogrel maintenance therapy group
In patients on chronic clopidogrel maintenance therapy, the use of omeprazole was not associated with increased on-treatment platelet reactivity as compared to PPI nonusers: 38.3% ± 11.8 vs. 38.6% ± 14.7, p=0.91 for 5 µmol/L ADP, 57.3% ± 14.5 vs. 56.4% ± 14.4, p=0.74 for 20 µmol/L ADP and 219 PRU ± 67 vs. 194 PRU ± 74, p=0.20 (figure 1).
In subgroup analyses, we investigated the influence of omeprazole treatment in patients on clopidogrel maintenance therapy 10-30 days prior to the intervention (n=151) and in patients who were on clopidogrel maintenance therapy for more than 30 days prior to PCI (n=145). No association of omeprazole and increased on-treatment platelet reactivity was found in both chronic clopidogrel maintenance treatment subgroups: in patients on clopidogrel for more than 30 days prior to PCI (omeprazole vs. PPI nonuse): 37.9% ± 10.5 vs. 40.1% ± 14.7, p=0.56 for 5 µmol/L ADP; 56.1% ± 15.8 vs. 58.2% ± 14.1, p=0.60 for 20 µmol/L ADP and 217.3 PRU ± 63.2 vs. 203.9 PRU ± 73.2, p=0.46. In patients on clopidogrel for 10-30 days prior to PCI: 36.9% ± 13.7 vs. 37.4% ± 14.7, p=0.77 for 5 µmol/L ADP; 58.8% ± 13.1 vs. 55.0% ± 14.6, p=0.38 for 20 µmol/L ADP and 221 PRU ± 74 vs. 196 PRU ± 75, p=0.22.

High on-treatment platelet reactivity (HPR) and omeprazole treatment
In patients receiving a 300 mg clopidogrel loading dose, concomitant omeprazole treatment was associated with an increased risk of HPR, OR 4.9, 95% CI 1.6-15.4, p=0.006. After the adjustment for all potential confounders, the use of omeprazole remained statistically significant associated with HPR: OR_{adj} 6.3 95% CI, 1.9-21.4, p=0.003.
In contrast, in patients on chronic clopidogrel maintenance therapy, the use of omeprazole was not associated with an increased risk of HPR as compared to patients without PPI-treatment (OR 1.3, 95% CI 0.6-2.9). In both treatment groups, the covariates body mass index, diabetes mellitus and carriage of *CYP2C19*2* were associated with HPR in univariate analysis.

DISCUSSION

In this study we demonstrated that the impact of co-administration of omeprazole on the magnitude of on-treatment platelet reactivity might be dependent on the dosing regimen of clopidogrel. In patients with a recent 300 mg clopidogrel loading dose (median time 3 days prior to PCI, followed by 75 mg/day), concomitant use of omeprazole was associated with a relative increase in platelet reactivity of more than 20%, as measured with two different platelet function tests. This augmentation in platelet reactivity resulted in a more than 6-fold increased risk of high on-treatment platelet reactivity (HPR). HPR has shown to be associated with adverse cardiovascular events, including stent thrombosis.[16,20,21] However, in patients on chronic clopidogrel maintenance therapy (median time 31 days prior to PCI), omeprazole was not associated with increased platelet reactivity.
Similar to clopidogrel, the metabolisation of PPI's is CYP2C19-dependent.[7,22] On top of that, omeprazole is a potent inhibitor of the CYP2C19-enzyme.[7] CYP2C19 has a very important role in the metabolisation of clopidogrel. In several study populations it is demonstrated that carriage of the loss-of-function allele *CYP2C19*2* leads to an approximately 1.3-fold increased risk on the occurrence of major adverse cardiovascular events as compared to noncarriers.[23] Carriers of *CYP2C19*2* who underwent stent implantation are shown to have a more than 3-fold increased risk of stent thrombosis compared with noncarriers.[23] In this study we observed a signifi-

cant reduction of clopidogrel's platelet inhibition when administered to omeprazole users, as a loading dose but not as maintenance therapy. We hypothesize that the inhibition of CYP2C19 might only become critical when a high clopidogrel loading dose has to be converted into the active metabolite at once. In the majority of the published platelet function studies in which concomitant use of omeprazole was found to reduce clopidogrel's efficacy, platelet reactivity was measured within several days after the administration of a 300 or 600 mg clopidogrel loading dose or was observed in patients treated with a double maintenance dose of 150 mg clopidogrel daily.[12,19,24-27] In the prematurely terminated double-blind, placebo-controlled COGENT trial, 3761 patients with either ACS or PCI were randomized to a fixed-dose combination of clopidogrel and omeprazole (75/20mg) or clopidogrel alone.[15] No effect of omeprazole on the risk of major adverse cardiovascular events during a median follow-up of 106 days was observed. Interestingly, almost 70% of the patients in the COGENT trial already used clopidogrel (with a maximum of 21 days) prior to inclusion. The lack of an effect of omeprazole might be caused by the fact that the majority of the patients did not receive a clopidogrel loading dose shortly before or after randomization but was already on long-term clopidogrel maintenance therapy. According to the results of our study, omeprazole does not diminish the antiplatelet properties of clopidogrel maintenance therapy but only after a recent loading dose.

There are some limitations of our study. First, in this (pre-specified) post-hoc analysis of a large observational study, we cannot completely exclude possible bias by various risk factors and patient characteristics between omeprazole users and nonusers of PPI's. Nonetheless, the multivariable adjustment models confirmed the primary analyses. We decided to analyze solely the interaction between omeprazole and clopidogrel in this study because omeprazole is the only PPI for which a large influence on the formation of clopidogrel's active thiol metabolite has been objectified.[9] Unfortunately, we did not assess plasma levels of the active metabolite of clopidogrel in the present study, which would have provided more mechanistic insights into the observed differences in platelet reactivity.

In conclusion, the drug-drug interaction between clopidogrel and omeprazole might be more prominent when clopidogrel is administered as a 300 mg loading dose compared to clopidogrel maintenance therapy.

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2.4

SULFONYLUREAS AND ON-TREATMENT PLATELET REACTIVITY IN TYPE 2 DIABETES MELLITUS PATIENTS

Ankie M. Harmsze
Jochem W. van Werkum
Fulya Moral
Jurriën M. ten Berg
Christian M. Hackeng
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

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ABSTRACT

Objective

Clopidogrel is a prodrug that needs to be converted in vivo by several cytochrome (CYP) P450 iso-enzymes to become active. Both clopidogrel and the oral hypoglycemic drug class sulfonylureas are metabolized by the iso-enzyme CYP2C9. The objective of this study was to evaluate the relation of sulfonylureas and on-treatment platelet reactivity in type 2 diabetes mellitus patients undergoing elective coronary stent implantation.

Methods

In this prospective, observational study, on-treatment platelet reactivity was quantified using adenosine diphosphate (ADP)-induced light transmittance aggregometry in 139 type 2 diabetes mellitus patients undergoing elective coronary stent implantation treated with clopidogrel and aspirin. High on-treatment platelet reactivity was defined as >70.7% platelet reactivity to 20 µmol/L ADP.

Results

A total of 53 patients (38.1%) were on concomitant treatment with sulfonylureas. The remaining 86 patients were on other hypoglycemic drugs. On-treatment platelet reactivity was significantly higher in patients with concomitant sulfonylurea treatment as compared to patients without concomitant sulfonylurea treatment (for 5 µmol/L ADP: 46.0% ± 11.8 vs. 40.6% ± 16.0; p=0.035, adjusted p=0.032 and for 20 µmol/L ADP: 64.6% ± 10.8 vs. 58.7% ± 15.5; p=0.019, adjusted p=0.017). The concomitant use of sulfonylureas was associated with a 2.2-fold increased risk of high on-treatment platelet reactivity (OR 2.2, 95% CI 1.1-4.7, p=0.039 and after adjustment for confounders: OR_{adj} 2.0 95% CI, 1.0-5.7, p=0.048).

Conclusions

Concomitant treatment with sulfonylureas might be associated with decreased platelet inhibition by clopidogrel in type 2 diabetes mellitus patients on dual antiplatelet therapy undergoing elective coronary stent implantation.

INTRODUCTION

Clopidogrel on top of aspirin is standard treatment after coronary stent implantation.[1] Several studies report a high interindividual variability in the response to antiplatelet therapy.[2-4] Increased on-treatment platelet reactivity has been associated with adverse cardiovascular events.[5-7] Several clinical determinants contributing to a suboptimal response to antiplatelet therapy have been identified, including age, gender, body mass index (BMI) and diabetes mellitus.[8,9] Clopidogrel is a prodrug that needs to be converted in vivo by several cytochrome (CYP) P450 iso-enzymes to become active. This activation takes place in a 2-step process. The first step, the conversion to the inactive metabolite 2-oxo-clopidogrel, is mainly mediated by CYP2C19, CYP1A2 and CYP2B6. The iso-enzymes CYP2C19, CYP2C9, CYP3A and CYP2B6 are involved in the second step, in which the active metabolite is formed.[10] Concomitant use of certain CYP-metabolized drugs is found to attenuate the effect of clopidogrel. Significant drug-drug interactions between clopidogrel and omeprazole (CYP2C19), calcium channel blockers (CYP3A4) and phenprocoumon (CYP2C9 and CYP3A4) have been reported in literature.[11-13] Various other commonly prescribed drugs are metabolized by the hepatic CYP-enzymes and do therefore have the potential to interfere with clopidogrel metabolism.
Sulfonylureas are also metabolized by the hepatic CYP system and specifically by the CYP2C9 iso-enzyme. Whether concomitant use of sulfonylureas interferes with the antiplatelet effects of clopidogrel has never been investigated before. The aim of the present study is to evaluate the relation of sulfonylureas and on-treatment platelet reactivity in type 2 diabetes mellitus patients undergoing elective coronary stent implantation.

METHODS

Study design

From a large cohort of consecutive patients undergoing elective coronary stent implantation with stenting, patients with type 2 diabetes mellitus were selected and enrolled in the present study.[5] All patients were pretreated with clopidogrel (defined as maintenance of 75 mg/d therapy for > 5 days or a loading dose of 300 mg ≥ 24 hours before PCI or 600 mg ≥ 4 hours before PCI) and aspirin (80-100 mg ≥ 10 days). Information on the use of aspirin, clopidogrel, sulfonylureas and other co-medication was assessed by questionnaires at the time of inclusion and verified by medication history records obtained from community pharmacies. A sulfonylurea-user was defined as a subject who was on sulfonylurea treatment for at least one week prior to the coronary stent implantation. Exclusion criteria were: acute myocardial infarction with ST-segment elevation within 48 hours from symptom onset, allergies or contra-indications to heparin, increased risk of bleeding, malignancies, pregnancy or hematological disorders including thrombocytopenia and treatment with GP IIb/IIIa inhibitors during the 14 days before platelet function testing and patients with a known platelet function disorder or a whole blood count of less than $150x10^3$/ µL. The study protocol was approved by the hospital's Medical Ethics Committee, and informed consent was obtained from each patient.

Blood sampling
Prior to PCI and before heparinization, blood was drawn from the femoral arterial sheath in 3.2% citrate tubes for platelet function testing. The first 10 ml of free-flowing blood was discarded.

Platelet function testing
The magnitude of on-treatment platelet reactivity was assessed by light transmittance aggregometry (LTA) using the APACT 4004 four-channel light transmission aggregometer (LABiTec, Ahrensburg, Germany). Samples were centrifuged for 10 min at 150g to obtain native platelet rich plasma (PRP). Maximal platelet aggregation (defined as the maximum extent of platelet aggregation achieved in any time during the run of 10 minutes) was quantified in non-adjusted PRP after stimulation with 5 and 20 µmol/L adenosine diphosphate (ADP). All measurements were completed within 2 hours of blood collection.

Statistical analysis
Continuous variables were expressed as mean ± SD and categorical variables were expressed as frequencies and percentages. The Kolmogorov-Smirnov test was used to test for normal distribution. All continuous data were normally distributed. For baseline characteristics, continuous data were analyzed by Students t-test and categorical data by chi-square test when appropriate.
Student's t-tests were used to analyze differences in on-treatment platelet reactivity between patients on concomitant treatment with sulfonylureas and patients without sulfonylureas. Multiple linear regression was used to adjust for potential confounders (age, gender, BMI, impaired renal function, i.e. estimated glomerular filtration rate eGFR<60mL/min, concomitant use of proton pump inhibitors and the use of either more than one oral hypoglycemic drug or insulin as an indication of the severity of the diabetes disease).
Univariate and multivariate logistic regression analysis was used to assess the effect of the use of sulfonylureas on the risk of high on-treatment platelet reactivity and to adjust for potential confounders. Patients with high on-treatment platelet reactivity were defined as the upper quintile of patients according to their on-treatment platelet reactivity values, as assessed in the total study cohort (n=1069; upper quintile LTA: 70.7% - 96.6% to 20 µmol/L ADP).[5] All analyses were performed with SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL). A p value <0.05 was considered statistically significant.

RESULTS

Patient characteristics
A total of 139 consecutive type 2 diabetes mellitus patients were enrolled in this study. The mean age of patients was 65.8 ± 9.6 years and the mean on-treatment platelet reactivity was 42.1% ± 14.8 for 5 µmol/L ADP and 60.7% ± 14.2 for 20 µmol/L ADP. In total, 53 (38.1%) patients were on treatment with sulfonylureas (tolbutamide n=13, glimepiride n=24, gliclazide n=13 and glibenclamide n=3) at the time of platelet function testing, whereas 86 patients were treated with other antidiabetic drugs. The baseline characteristics of the study cohort according to the use of sulfonylureas are shown in table 1. Patients with sulfonylureas were older and were less frequently treated with insulin and metformin. The other variables were well balanced between both groups.

Table 1: Baseline characteristics of the study cohort

Variable	With sulfonylureas (n=53)	Without sulfonylureas (n=86)	p-value
Age, years	68.4 ± 7.1	64.2 ± 9.6	0.006
Gender, male (%)	40 (75.5)	56 (65.1)	0.26
BMI (kg/m2)	28.6 ± 4.2	29.1 ± 4.7	0.50
Hypercholesterolemia	44 (83.0)	80 (93.0)	0.09
Hypertension	47 (88.7)	70 (81.4)	0.34
Current smoking	7 (9.3)	8 (10.5)	0.58
Familial history CAD	35 (68.6)	58 (67.4)	0.99
Prior myocardial infarction	24 (47.1)	44 (51.8)	0.60
Impaired renal function (eGFR<60mL/min)	10 (18.9)	11 (12.8)	0.33
Co-medication at inclusion:			
Aspirin	53 (100)	86 (100)	>0.99
Proton pump inhibitors	13 (24.5)	31 (36.0)	0.19
Statins	43 (81.1)	74 (86.0)	0.48
Calcium channel blockers	19 (35.8)	30 (34.9)	0.99
Metformin	30 (56.6)	74 (86.0)	<0.0001
Insulin	6 (11.3)	29 (33.7)	0.004
Thiazolidinediones	1 (1.9)	5 (5.8)	0.41
ACE-inhibitors	30 (56.6)	39 (45.3)	0.28
Beta-blockers	44 (83.0)	69 (80.2)	0.82
Coumarins	0 (0)	0 (0)	>0.99

Data presented are means ± SDs or number of patients (percentages). BMI, body mass index. CAD, coronary artery disease. eGFR<60mL/min, estimated glomerular filtration rate smaller than 60mL/min. P-value: Student t-test for continuous variables and chi-square test for categorical variables between the groups

On-treatment platelet reactivity and treatment with sulfonylureas

As shown in figure 1, the magnitude of on-treatment platelet reactivity was significantly higher in patients on concomitant treatment with sulfonylureas as compared to the remaining patients (46.0% ± 11.8 vs. 40.6% ± 16.0, p=0.035 for 5 µmol/L ADP and 64.6% ± 10.8 vs. 58.7% ± 15.5, p=0.019 for 20 µmol/L ADP). Multivariate analyses demonstrated that concomitant treatment with sulfonylureas was independently associated with an attenuated platelet response to clopidogrel (mean differences (sulfonylureas vs. no sulfonylureas): 6.3% for 5 µmol/L ADP (95% CI, 0.8-11.8, p=0.032) and 6.5% for 20 µmol/L ADP (95% CI, 1.4-11.8, p=0.017)).

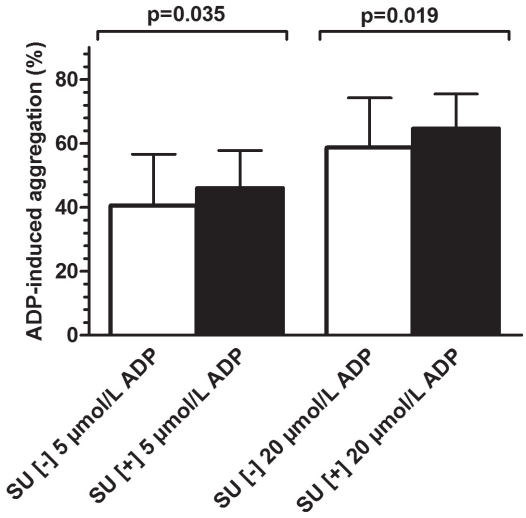

Figure 1: On-treatment platelet reactivity according to the use of sulfonylureas
On-treatment platelet reactivity (as measured with LTA after 5 and 20 µmol/L ADP) expressed as mean ± standard deviation in sulfonylurea nonusers ("SU [-]"; open bars) and sulfonylurea users ("SU [+]"; solid bars). ADP: adenosine diphosphate. Student t-test, $p<0.05$ considered statistically significant

We assessed on-treatment platelet reactivity among a subgroup of patients on sulfonylurea monotherapy (patients who use only sulfonylureas as antidiabetic therapy; n=20) and metformin monotherapy (patients who only use metformin as antidiabetic therapy; n=53). Patients on sulfonylurea monotherapy exhibited higher on-treatment platelet reactivity than patients treated with metformin as the only antidiabetic drug (45.2% ± 12.3 vs. 37.8% ± 16.5 for 5 µmol/L ADP and 64.1% ± 10.3 vs. 56.8% ± 16.2 for 20 µmol/L ADP). Due to small sample sizes, these differences failed to reach statistical significance (p=0.070 and p=0.065 respectively).

Based on the cutoff value of 70.7% in response to 20 µmol/L ADP, 37 (26.6%) patients were found to have high on-treatement platelet reactivity. The proportion of patients with high on-treatment platelet reactivity was significantly higher in patients with concomitant sulfonylurea treatment as compared with patients without sulfonylureas, 19 (35.8%) vs. 18 (20.9%); OR 2.2, 95% CI 1.1-4.7, p=0.039, which remained statistically significant after adjustment for potential confounders (OR_{adj} 2.0 95% CI, 1.0-5.7, p=0.048).

DISCUSSION

To the best of our knowledge, this is the first study investigating the impact of concomitant treatment with sulfonylureas on the antiplatelet effects of clopidogrel. The major finding of our study is that the use of sulfonylureas might be associated with decreased platelet inhibition by clopidogrel in type 2 diabetic patients on dual antiplatelet therapy undergoing elective coronary stent implantation. Patients under concomitant treatment with sulfonylureas exhibited a mean relative increase ADP-induced platelet reactivity of approximately 12% as compared with the remaining patients. The influence of sulfonylureas on on-treatment platelet reactivity is comparable to that of *CYP2C19*2*, the genetic variant which has shown to be of great clinical importance in clopidogrel-treated patients.[10,14] Furthermore, the concomitant use of sulfonylureas was associated with a more than twofold increased risk on high on-treatment platelet reactivity. This parameter is shown to be a strong predictor of ischemic events in patients who underwent elective coronary stent implantation.[5] Sulfonylureas are mainly metabolized by the CYP2C9 enzyme.[15,16] This iso-enzyme also plays an important role in the activation of clopidogrel.[14,17] Results of our study suggest that the use of sulfonylureas significantly alters the biotransformation of clopidogrel into its active metabolite by competition for CYP2C9, resulting in higher on-treatment platelet reactivity. In literature, examples of drug-drug interactions due to competition for CYP2C9 between sulfonylureas and other drug compounds are found. In one study, the use of sulfonylureas was associated with faster onset of anticoagulation in patients during the first 30 days of CYP2C9-metabolized warfarin therapy.[18] Furthermore, in an in vitro study, glibenclamide strongly inhibited CYP2C9-metabolized phenytoin and warfarin in a competitive manner.[19] Also, the same common loss-of-function genetic variants in the *CYP2C9* gene have been shown to influence the metabolisation of both sulfonylureas and clopidogrel.[17,20] The genetic variant *CYP2C9*3* has also been associated with a 10% mean relative increase in on-treatment platelet reactivity in a cohort of patients undergoing elective PCI.[14]

Various studies have demonstrated that diabetic patients exhibit a suboptimal response to clopidogrel as compared to non-diabetics.[9,21,22] Besides an impaired response to clopidogrel, diabetes is characterized by a more prothrombotic state, reflected by a shortened platelet lifespan, a heightened thrombin generation and an increased glycoprotein IIb/IIIa expression.[21,22] Type 2 diabetes mellitus is a progressive disease in which the impairment of insulin secretion worsens. Consequently, dosages of hypoglycemic drugs need to be increased over time, often followed by the addition of a second oral hypoglycemic drug. If all oral hypoglycemic drugs fail to regulate blood glucose levels adequately, adding or switching to insulin therapy is necessary.[15,16] With increasing severity of diabetes mellitus, the response to clopidogrel might even become worse. Angiolillo *et al*. demonstrated that patients on dual antiplatelet therapy with insulin-treated type 2 diabetes mellitus, representing a subpopulation with more advanced stages of insulin resistance and biological disorders, exhibited higher platelet aggregation than non-insulin-treated diabetics.[23] In our study we used two strategies to investigate whether the differences in on-treatment platelet reactivity between patients on concomitant treatment with sulfonylureas and the remaining patients were driven by the drug interaction or by differences in the severity of the diabetic disease. First, we adjusted for the severity of the diabetic disease by including the use of either a second oral hypoglycemic drug or insulin as a confounder in the multivariate analysis models. The inclusion of this potential confounder did not change findings, which indicates that sulfonylureas cause higher platelet reactivity which is

independent of the state of the diabetic disease. Second, we performed a subanalysis comparing platelet reactivity between patients on monotherapy with sulfonylureas and patients on monotherapy with metformin. These two groups represent patients who are in an initial state of diabetes type 2. Again, the results pointed out that sulfonylureas are associated with decreased antiplatelet effects of clopidogrel.

A limitation of our study is that the number of patients on sulfonylureas is relatively small. Therefore, the study was underpowered to investigate the influence of the individual sulfonylureas on platelet reactivity. Although all sulfonylureas are CYP2C9-substrates, differences in affinity for the enzyme do exist. This is illustrated by the fact that carriage of loss-of-function alleles of *CYP2C9* (*2 and *3) diminishes the clearance of the individual sulfonylureas to different extents.[20] This might lead to varying potentials of the individual sulfonylureas to interact with the antiplatelet properties of clopidogrel. In addition, we did not investigate the influence of sulfonylureas on plasma concentrations of clopidogrel's active metabolite nor on clinical outcome. Finally, we did not adjust for carriage of genetic variants in *CYP2C9* in the multivariate analyses.

CONCLUSION

In conclusion, the concomitant use of sulfonylureas might be associated with increased on-treatment platelet reactivity. Our study shows that a drug-drug interaction study measuring both clopidogrel's active metabolite and clopidogrel's antiplatelet effects is indicated.

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2.5

ESOMEPRAZOLE BUT NOT PANTOPRAZOLE IS ASSOCIATED WITH LOWER PLASMA CONCENTRATIONS OF CLOPIDOGREL'S ACTIVE METABOLITE

Ankie M. Harmsze
Jochem W. van Werkum
Dirk Taubert
Christian M. Hackeng
Hendrik J.T. Ruven
Heleen J. Bouman
Nicoline J. Breet
Jurriën M. ten Berg
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

Submitted

ABSTRACT

Background

Contradictory results regarding the effect of co-administration of CYP2C19-metabolized proton-pump-inhibitors (PPIs) on platelet reactivity and clinical outcome in clopidogrel-treated patients are reported in literature. Recently, omeprazole was found to reduce the formation of the active thiol metabolite of clopidogrel (AMC) with 45%.

Objectives

The aim of this study was to assess the impact of esomeprazole and pantoprazole on the plasma concentrations of AMC and ex-vivo platelet reactivity.

Methods

Forty-nine patients with a history of stent thrombosis were enrolled in this single center study. All patients received a 600 mg clopidogrel loading dose. Plasma concentrations of clopidogrel, the AMC and the inactive carboxylic acid metabolite were determined. On-treatment platelet reactivity was measured by adenosine diphospate (ADP)-induced light transmittance aggregometry.

Results

Of all patients, 20 were on pantoprazole and 6 were on esomeprazole treatment. Users of esomeprazole had a reduction of 45% in maximal plasma concentrations of the AMC as compared with PPI nonusers (geometric mean [range] C_{max}(AMC) 4.3 ng/mL [1.9-9.3] vs. 7.8 ng/mL [3.5-19.5], p=0.005). Esomeprazole-users exhibited higher on-treatment platelet reactivity as compared to PPI nonusers (61.1 ± 16.5% vs. 41.8 ± 18.1%, p=0.026 for 20 µmol/L ADP). All associations remained significant after adjustment for confounders. Pantoprazole had no influence on the formation of the AMC nor on on-treatment platelet reactivity. No influence of PPI use on maximal plasma concentrations of clopidogrel and its inactive carboxylic acid metabolite were found.

Conclusions

We demonstrated that co-administration of esomeprazole was associated with a decreased formation of the active metabolite of clopidogrel and attenuated clopidogrel-induced platelet inhibition. On the contrary, pantoprazole had no influence on C_{max} of the AMC and was not associated with a significant increase in on-treatment platelet reactivity.

INTRODUCTION

Clopidogrel is a prodrug that needs to be metabolized by the hepatic cytochrome (CYP) P450 enzymesystem to become active. [1] CYP2C19 plays an important role in the formation of the active thiol metabolite of clopidogrel (AMC). This is illustrated by several studies showing that carriage of the loss-of-function allele *CYP2C19*2* is associated with less inhibition of platelet aggregation by clopidogrel and with an increased risk on adverse cardiovascular events in clopidogrel-treated patients. [1-5] CYP2C19 is also a major enzyme in the metabolism of proton pump inhibitors (PPIs), which are often prescribed in patients on dual antiplatelet therapy for the prevention of gastrointestinal ulcera.[6,7] In various platelet function studies, concomitant use of PPIs was associated with a decreased inhibition of platelet aggregation by clopidogrel. [8,9,11] Contradictory findings have been reported on whether co-administration of clopidogrel and PPIs is associated with an increased risk of adverse cardiovascular events.[10,12,13,14,15] Recently, Angiolillo and co-workers conducted a series of placebo-controlled, crossover studies in which was demonstrated that omeprazole decreased the plasma concentration of the AMC by about 45% and increased on-treatment platelet reactivity to a similar extent.[16] In contrast, the use of pantoprazole was associated with a much smaller effect on clopidogrel's pharmacokinetic and –dynamic parameters.[16] Based on these findings, the FDA recommends to avoid the use of omeprazole in clopidogrel-treated patients.[17] Furthermore, the FDA stated that the use of esomeprazole should also be avoided, however this is not supported by any pharmacokinetic data. The aim of the present study was to determine the influence of the concomitant use of esomeprazole and pantoprazole on plasma concentrations of the AMC and on-treatment platelet reactivity.

METHODS

Study population

Eligible for this study were all patients with coronary artery disease and a history of angiographically confirmed stent thrombosis between January 2004 and December 2006. Definite stent thrombosis was defined according to the Academic Research Consortium (ARC).[18] The ARC protocol defines stent thrombosis as definite when confirmed by angiography or when pathologic confirmation of acute thrombosis in patients with acute coronary syndromes is made. All patients were on 80-100 mg aspirin therapy daily. A part of the eligible patients was on chronic clopidogrel maintenance therapy (75 mg daily) as they had experienced the stent thrombosis less than one year prior to inclusion in this study. These patients were included if they discontinued their clopidogrel maintenance therapy 24 hours before the measurements took place. Information on the use of medication was obtained by questionnaires and verified by medication history records from community pharmacies. A PPI-user was defined as a patient who had been on PPI-treatment for at least one month prior to measurements.

Exclusion criteria were an acute coronary syndrome in the last 6 months, recent bleeding diathesis, bleeding disorder, known platelet dysfunction or an abnormal platelet count ($<150x10^9$/L) and the use of a glycoprotein IIb/IIIa inhibitor within the last 14 days prior to measurements. The study protocol was approved by the hospital's Medical Ethics Committee, and informed consent was obtained from each patient before enrollment.

Study procedure and blood sampling

Patients who met the inclusion criteria visited the outpatient clinic for platelet function measurements, physical examination and a standardized interview. Blood was drawn for baseline measurements and for DNA analysis. All patients subsequently received a witnessed 600 mg clopidogrel loading dose and a 100 mg dose of aspirin. The maximal time interval between the administration of PPIs and the 600 mg clopidogrel loading dose was 4 hours.

Blood samples for plasma concentration measurements were collected from the antecubital vein in tubes containing K_3-EDTA prior to the clopidogrel loading dose (t=0) and 20, 40, 60, 90, 120, 180, 240 and 360 minutes after the clopidogrel loading dose. Samples were immediately centrifuged at 1500 g for 10 minutes and plasma was pipetted into tubes containing a stabilizing agent (Pat. No. DE 10 2004 046 159.7) in order to prevent degradation of the AMC. After vortexing for 60 seconds, samples were stored at -80°C until analysis took place.

Blood samples for platelet function evaluation were drawn from the antecubital vein with a loose tourniquet and collected in a citrated (3.2%) non-vacuum tubes (Starstedt, Nümbrecht, Germany) before and after 6 hours after the clopidogrel loading dose. All blood samples were processed within 2 h after collection.

Determination of the plasma concentrations of clopidogrel, its carboxylic acid metabolite and its AMC

Concentrations of clopidogrel, the inactive carboxylic acid metabolite and the AMC were determined in EDTA-plasma on a triple-quadruple tandem mass spectrometer (TSQ Quantum, Thermo Electron, Dreieich, Germany) as previously described.[19] Plasma concentration versus time data of each patient were fitted by a one-compartment first order lag-time model using WinNonlin TM Software (Pharsight, Palo Alto, CA, USA). The maximal plasma concentration (C_{max}, ng/mL) was calculated from the individual regression fits ($r > 0.95$). In this study, C_{max} is reported since this appeared to be the pharmacokinetic parameter correlating best with inhibition of platelet aggregation in previous studies.[19,20]

Platelet function measurements

The magnitude of on-treatment platelet reactivity was assessed by light transmission aggregometry (LTA) on an APACT 4004 four-channel light transmission aggregometer (LABiTec, Ahrensburg, Germany). Platelet-poor-plasma was set as 100% aggregation and after stimulation of platelet-rich-plasma with ADP in final concentrations of 5 and 20 µmol/L ADP, maximal (peak) aggregation (%) was measured.

Genotyping

Genomic DNA was isolated from K_3-EDTA blood by using the MagNA Pure LC DNA Isolation kit 1 (MagNA Pure; Roche Diagnostics; Basel, Switzerland). *CYP2C19*2* alleles (G681A; rs4244285) were detected by using the LightCycler CYP2C19 Mutation Detection Kit on the LightCycler instrument (Roche Diagnostics). Method validation was carried out by DNA sequence analyses.

Statistical analysis

Continuous variables are expressed as mean ± SD or geometric mean [range]. Categorical variables are expressed as frequencies and percentages. For baseline characteristics, continuous data were analyzed by analysis of variance (ANOVA) and categorical data by the Fisher's Exact test when appropriate. A chi-square table was used to compare the observed number of the *CYP2C19* genotype with that expected for a population in Hardy-Weinberg equilibrium. Kolmogorov-Smirnov test was used to test for normal distribution of continuous data. To meet the distributional assumptions of the statistical models, C_{max} values were log-transformed for the statistical models and antilog-transformed for descriptive purposes, yielding geometric means, ranges and 95% confidence intervals. ANOVA and LSD post-hoc tests were used to study differences in log-transformed C_{max} and on-treatment platelet reactivity between treatment groups. Mean differences and 95% confidence intervals were calculated. As not every patient was clopidogrel-naive at the time the measurements took place, we only report on-treatment platelet reactivity as measured 6 hours after administration of the 600 mg clopidogrel loading dose. Linear regression analysis was used to identify independent correlates of $logC_{max}$(AMC): age, body mass index (BMI), gender, diabetes mellitus, hypertension, hypercholesterolemia, history of MI, active smoking, carriage of *CYP2C19*2*, recent clopidogrel maintenance therapy prior to inclusion and the use of beta-blockers, calcium channel blockers and statins. All variables with a p-value <0.10 were included in the multivariate analysis. ANCOVA was used to adjust for confounders. The mean differences and 95% confidence intervals calculated by LSD post-hoc tests were also adjusted for confounders. Power calculation was based on findings reported by the FDA on the interaction of clopidogrel and omeprazole in which was observed that co-administration of omeprazole was associated with a 45% reduction of the C_{max} (AMC). Power analysis indicated that there was sufficient power (0.80) with an alpha value of 0.05 to detect a 45% reduction of C_{max} (AMC) in esomeprazole users as compared with PPI nonusers in the present study.

A p value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL).

RESULTS

Characteristics of the study population

A total of 49 patients with a history of stent thrombosis were enrolled. Of these, 27 patients were on PPI treatment at the time the measurements took place. Among them, 20 patients used pantoprazole 40 mg/day, 6 patients used esomeprazole (4 subjects were on 40 mg/day and 2 subjects were on 20 mg/day), and one patient used omeprazole 20 mg/day. This last patient was excluded from all analyses. Baseline characteristics of the study population according to PPI treatment are shown in table 1. The groups were well balanced except for hypercholesterolemia (p=0.002). In total, 21 patients were still on chronic clopidogrel maintenance therapy. For *CYP2C19*, no significant deviation from Hardy Weinberg equilibrium was observed. (p=0.80). The co-variables calcium channel blockers (p=0.10), *CYP2C19*2* (p=0.013), BMI (p=0.012), recent clopidogrel intake (p=0.09) and age (p=0.029) were found to be associated with $logC_{max}$(AMC) (p<0.10) and were therefore included as potential confounders in the multivariable models.

Table 1: Baseline demographics and clinical characteristics

Variable	No PPI (n=22)	Pantoprazole (n=20)	Esomeprazole (n=6)	p-value
Age (years)	63.0 ± 12.1	59.8 ± 9.1	68.3 ± 8.2	0.22
Men	20 (90.9)	16 (80.0)	5 (83.3)	0.53
Body mass index (kg/m2)	26.2 ± 4.5	27.5 ± 3.7	26.5 ± 1.6	0.55
Diabetes mellitus	3 (13.6)	5 (25.0)	0 (0)	0.43
Active smokers	12 (54.5)	10 (50.0)	6 (100)	0.09
Hypertension	13 (59.1)	8 (40.0)	3 (50.0)	0.56
Hypercholesterolemia	9 (40.9)	18 (90.0)	2 (33.3)	0.0004
Carriage of CYP2C19*2	5 (22.7)	9 (45.0)	2 (33.3)	0.25
Previous MI	6 (27.3)	8 (40.0)	2 (33.3)	0.54
Recent clopidogrel maintenance therapy prior to inclusion	11 (50.0)	9 (45.0)	1 (16.7)	0.34
Beta-blockers	18 (81.8)	15 (75.0)	4 (66.7)	0.71
Calcium channel blockers	4 (18.2)	2 (10.0)	3 (50.0)	0.11
Statins	21 (95.5)	18 (90.0)	5 (83.3)	0.47

Data are expressed as mean ± standard deviation or number of patients (%); p-value: ANOVA for continuous variables and Fisher's Exact test for categorical variables between the three groups, MI: myocardial infarction, PPI: proton pump inhibitor

The influence of PPI use on the plasma concentrations of clopidogrel and its metabolites

The maximal plasma concentrations of clopidogrel, its inactive carboxyl metabolite and the AMC were compared among users of pantoprazole, esomeprazole and PPI nonusers (figure 1). Maximal plasma concentrations of the AMC differed statistically significant between the three treatment groups (p=0.005). Pairwise comparisons showed that users of esomeprazole had a reduction of 45% in maximal plasma concentrations of the AMC as compared to PPI nonusers (geometric mean [range] C_{max} (AMC) 4.3 ng/mL [1.9-9.3] vs. 7.8 ng/mL [3.5-19.5], p=0.005, figure 1). After adjustment for the confounding variables, the use of esomeprazole remained significantly associated with a decreased maximal plasma concentration of the AMC (mean difference C_{max} (AMC): -1.6 ng/mL (95% CI, -2.3 to -1.1), p=0.020).

Maximal plasma concentrations of clopidogrel and the inactive carboxylic acid metabolite were not significantly different in esomeprazole users and PPI nonusers (figure 1).

In pairwise comparisons we found that the use of pantoprazole was not associated with a decreased maximal plasma concentration of the AMC compared to PPI nonusers (geometric mean [range] C_{max}(AMC): 8.7 ng/mL [5.2-22.0] vs. 7.8 ng/mL [3.5-19.5], p=0.41), nor with reduced concentrations of clopidogrel and the carboxylic acid metabolite (figure 1).

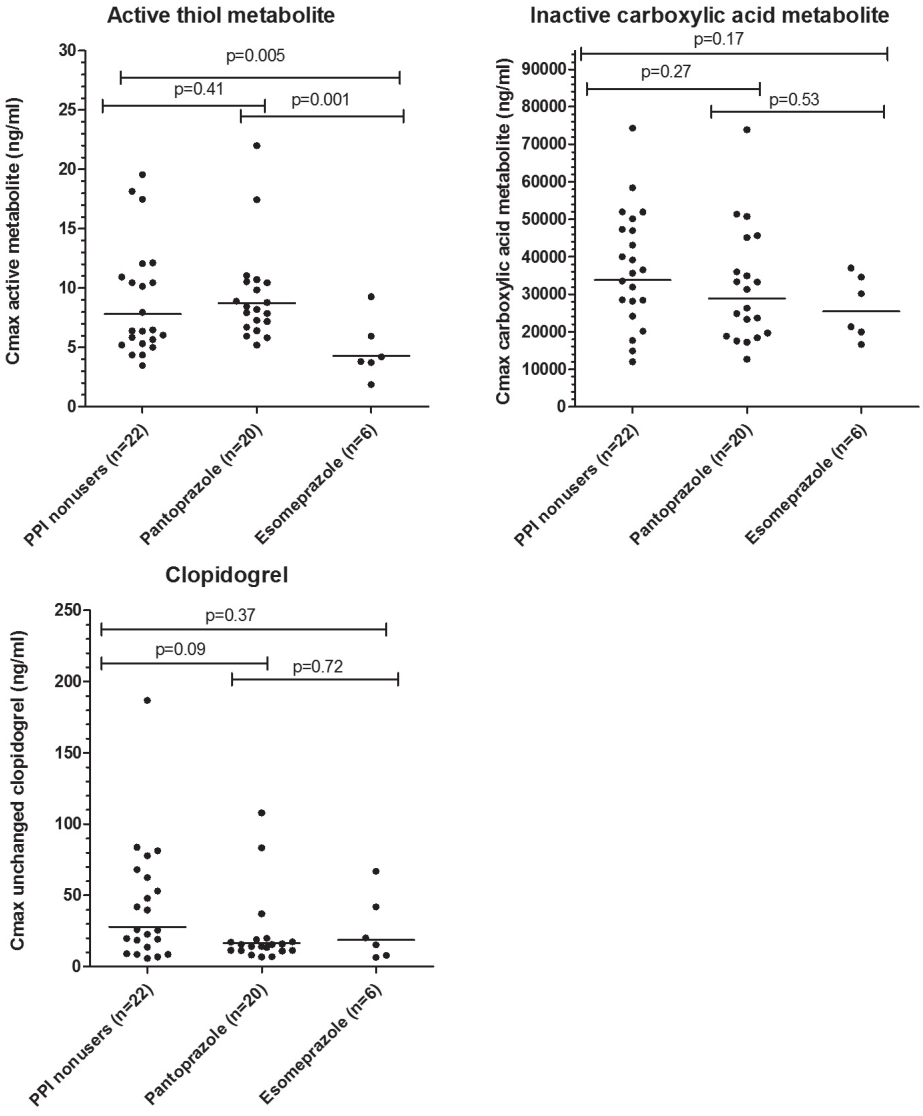

Figure 1: Influence of PPI-use on maximal plasma concentrations after the administration of a 600 mg clopidogrel loading dose

Scatter plots with geometric means of the maximal plasma concentrations of clopidogrel's active thiol and inactive carboxylic acid metabolites and unchanged clopidogrel after receiving a 600 mg clopidogrel loading dose, according to PPI use. ANOVA with LSD post-hoc tests. Overall p-values ANOVA: C_{max}(AMC): p=0.005; C_{max}(inactive metabolite): p=0.30; C_{max}(clopidogrel): p=0.14. Overall p-values ANCOVA: C_{max}(AMC): p=0.001; C_{max}(inactive metabolite): p=0.12; C_{max}(clopidogrel): p=0.24

The effect of pantoprazole versus esomeprazole on the maximal plasma concentration of the AMC was significantly different (p=0.001), which remained significant after the correction for all confounders (mean difference C_{max}(AMC): -1.9 ng/ml (95% CI, -2.9 to -1.3), p=0.002). Comparison of the effect of the higher dosages of both PPIs (pantoprazole 40 mg (n=20) and esomeprazole 40 mg (n=4)) showed even larger differences in maximal plasma levels of the AMC (geometric mean [range] C_{max}(AMC): 8.7 [5.2-22.0] ng/mL vs. 3.2 [1.7-4.2] ng/mL, p=0.001), which remained significant after the correction for confounders (mean difference C_{max}(AMC): 2.0 ng/mL (95% CI, 1.2 to 3.5), p=0.014).

The influence of PPI use on the magnitude of on-treatment platelet reactivity

On-treatment platelet reactivity differed statistically significant between the three treatment groups (p=0.032 for 5 µmol/L ADP and p=0.033 for 20 µmol/L ADP, respectively). Pairwise comparisons showed that the magnitude of on-treatment platelet reactivity was significantly higher in patients using esomeprazole compared to PPI nonusers (44.2 ± 16.1% vs. 27.6 ± 11.9%, p=0.009, for 5 µmol/L ADP and 61.1 ± 16.5% vs. 41.8 ± 18.1%, p=0.026 for 20 µmol/L ADP, figure 2). Mean differences remained significant after the adjustment for confounders (mean difference: 13.2% (95% CI, 1.2 to 27.6), p=0.037 for 5 µmol/L ADP, and 16.9% (95% CI, 1.5 to 34.6), p=0.041 for 20 µmol/L ADP).

Pairwise comparisons revealed that the use of pantoprazole was not associated with significantly higher on-treatment platelet reactivity as compared to PPI nonusers (35.3% ± 15.8 vs. 27.6% ± 11.9, p=0.10 for 5 µmol/L ADP and 52.3% ± 16.4 vs. 41.8% ± 18.1, p=0.07 for 20 µmol/L ADP, figure 2). No significant differences in on-treatment platelet reactivity between esomeprazole and pantoprazole users were found (p-values 0.25 and 0.27 for 5 and 20 µmol/L ADP respectively).

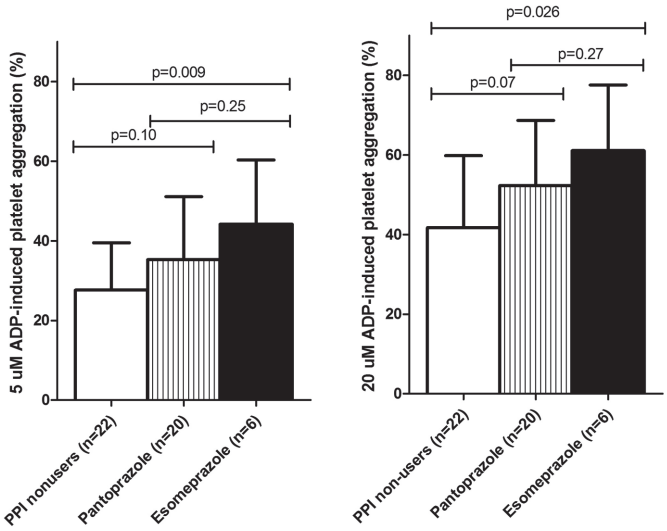

Figure 2 : Influence of PPI-use on on-treatment platelet reactivity

On-treatment platelet reactivity after receiving a 600 mg clopidogrel loading dose, according to PPI use, expressed as mean ± sd, ANOVA with LSD post-hoc tests. Overall p-values ANOVA: p=0.032 for 5 µmol/L ADP and p=0.033 for 20 µmol/L ADP. Overall p-values ANCOVA: p=0.040 for 5 µmol/L ADP and p=0.048 for 20 µmol/L ADP

DISCUSSION

The principal finding of the present study is that concomitant use of esomeprazole leads to a 45% reduction in plasma concentrations of the AMC in patients receiving a 600 mg clopidogrel loading dose as compared to PPI nonusers. In contrast, the use of pantoprazole was not associated with decreased formation of the AMC. Furthermore, esomeprazole users had a relative increase of 46% of on-treatment platelet reactivity in response to 20 μmol/L ADP, while the influence of pantoprazole did not reach statistical significance.

Similar to clopidogrel, the metabolisation of all PPIs is CYP-dependent.[6] In vitro experiments in human liver microsomes indicate that CYP2C19 is responsible for approximately 70% of the metabolism of esomeprazole, with the majority of the remaining 30% being metabolized by CYP3A4.[21] Pantoprazole is unique for its metabolisation since it is not only metabolised by cytochrome P450 iso-enzymes, but also by a cytosolic sulfotransferase, which is non-saturable.[22] Pantoprazole appears to have the lowest potential for interactions with other drugs.[6,23]

In literature, the strongest evidence for an interaction between clopidogrel and individual PPIs is found for omeprazole.[8,9,11] This is strengthened by the pharmacokinetic data published recently, showing a 45% decrease in plasma levels of the AMC and a 47% reduction in clopidogrel-induced platelet inhibition in patients on concomitant omeprazole use.[16] Furthermore, the authors reported that there was no clinically relevant interaction between clopidogrel and pantoprazole, which is confirmed by the results of our study. At this moment, the effect of pantoprazole on on-treatment platelet reactivity has been evaluated in four observational studies.[11,24,25,26] One of the studies showed that pantoprazole was associated with a higher magnitude of on-treatment platelet reactivity. In the three remaining studies no influence of pantoprazole on platelet reactivity was found, while in one of these studies the use of omeprazole was associated with increased platelet reactivity.[11]

Based on the pharmacokinetic study, the FDA recommends to avoid the use of CYP2C19-inhibitor omeprazole in clopidogrel-treated patients. Furthermore, the FDA stated that the use of esomeprazole should also be avoided, although this was not supported by pharmacokinetic data.[16] The use of esomeprazole was associated with increased platelet reactivity in a large observational study in patients undergoing PCI[24], while in three smaller studies, no effect on on-treatment platelet reactivity was observed.[11,25,26] Inconsistent data have been published about the influence of individual PPIs on clinical outcome in clopidogrel-treated patients.[10,12,13,14,15] Most studies are hampered by the fact that users of PPIs were older and had more co-morbidities than nonusers. This might have led to channeling bias and makes interpretation of clinical outcome data difficult. Our data strengthen the statement of the FDA that esomeprazole also has CYP2C19-inhibiting properties and is therefore capable of diminishing the concentration of the AMC.

The fact that co-administration of esomeprazole did not cause a decrease in plasma concentrations of unchanged clopidogrel and the inactive metabolite of clopidogrel indicates that the interaction between clopidogrel and esomeprazole is not caused by decreased clopidogrel absorption by the PPI-induced elevation of gastric pH, but rather by a metabolic drug-drug interaction. If the interaction was caused by diminished absorption due to higher pH levels, it would be expected that esomeprazole (and pantoprazole) would induce significant decreasements to plasma concentrations of unchanged clopidogrel and the carboxyl metabolite.

Various studies both in healthy volunteers and in patients showed no significant metabolic

interactions when pantoprazole was used in combination with a wide range of CYP2C19-metabolized drug compounds, while esomeprazole is shown to have the potential to interact with CYP2C19-metabolized drugs such as phenytoin and warfarin.[6] In literature, we found one study directly comparing the effects of co-administration of esomeprazole and pantoprazole on the pharmacokinetics of a single-dose diazepam, a drug which is also metabolized by CYP2C19. Differences in maximum concentrations revealed an increase of 34% in diazepam concentrations when receiving esomeprazole versus pantoprazole.[27] This supports the findings of our study as a significant impact on C_{max} of the AMC and on on-treatment platelet reactivity was observed with the use of esomeprazole, but not with pantoprazole.

To our knowledge, our study is the first that provides pharmacokinetic data to support the assumption that concomitant use of esomeprazole decreases concentrations of the AMC.
The present study has limitations that merit mention. Because of the nonrandomized study design, it is possible that residual confounding could have affected results. Furthermore, one might argue that the relatively low number of patients on recent clopidogrel maintenance therapy in the esomeprazole group accounts for the high magnitude of on-treatment platelet reactivity, as it might be hypothesized that due to maintenance therapy a bigger part of circulating platelets already are affected by clopidogrel. Clopidogrel-naïve patients totally depend on their platelet inhibition by the loading dose, which might not be sufficient due to the competitive character of esomeprazole. However, in multivariate analyses, the adjustment for recent clopidogrel maintenance therapy did not change the influence of esomeprazole on on-treatment platelet reactivity. Furthermore, recent clopidogrel maintenance therapy could not have caused the differences in AMC, since this metabolite has a halflife of less than one hour.[19]
In addition, for the analysis of on-treatment platelet reactivity, the sample size for esomeprazole might have been too small to detect significant differences in platelet reactivity between pantoprazole and esomeprazole . Furthermore, PPIs and the 600 mg clopidogrel loading dose were not administered at the same time. However, the maximal time interval was 4 hours and a recent study reported that similar levels of interaction were observed whether clopidogrel and omeprazole were administered simultaneously or 12 hours apart.[16] Finally, the impact of the concomitant use of clopidogrel and esomeprazole or pantoprazole on clinical outcome cannot be ascertained from the results of this study.
In conclusion, the concomitant use of esomeprazole decreased the plasma levels of the AMC and clopidogrel-induced platelet inhibition. On the contrary, pantoprazole had no influence on C_{max} of the AMC and was not associated with a significant increase in on-treatment platelet reactivity.

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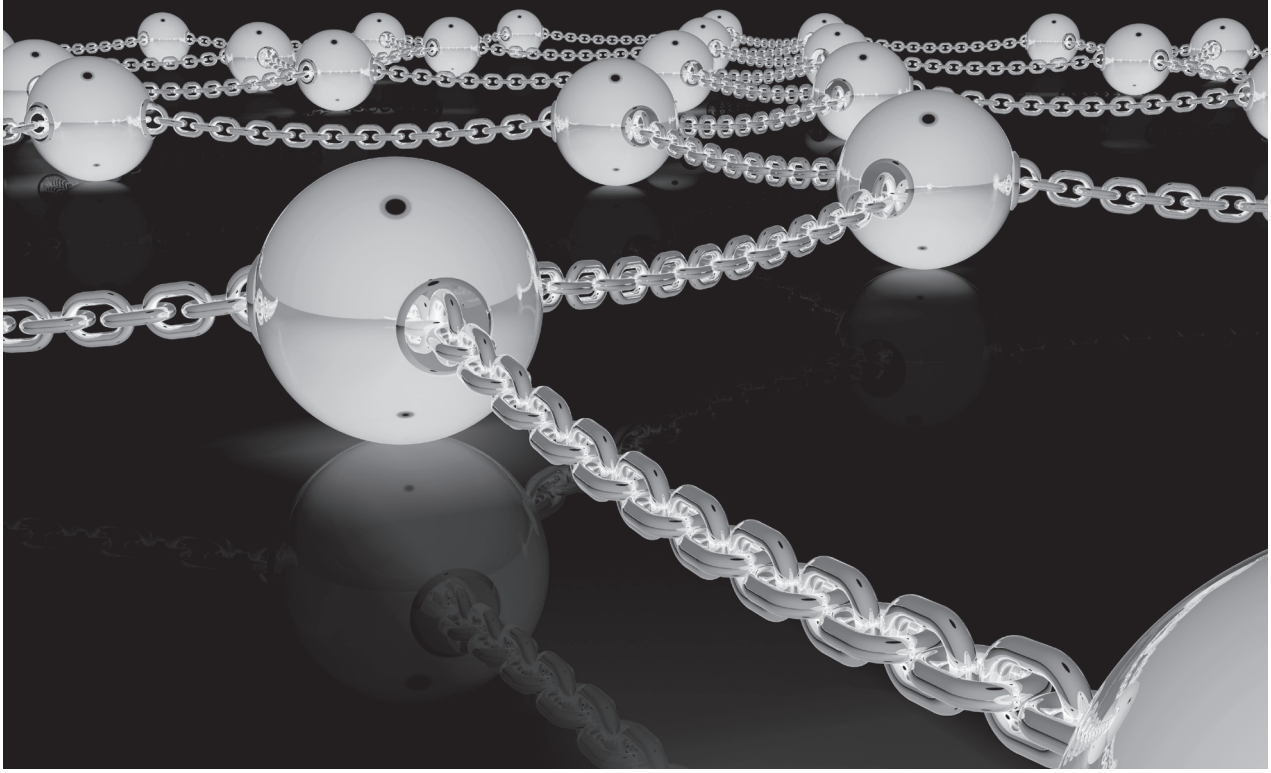
3 FACTORS INFLUENCING CLINICAL OUTCOME IN PATIENTS RECEIVING ANTIPLATELET THERAPY



3.1

*CYP2C19*2* AND *CYP2C9*3* ALLELES ARE ASSOCIATED WITH STENT THROMBOSIS - A CASE-CONTROL STUDY

Ankie M. Harmsze
Jochem W. van Werkum
Jurriën M. ten Berg
Bastiaan Zwart
Heleen J.Bouman
Nicoline J. Breet
Arnoud W.J. van 't Hof
Hendrik J.T. Ruven
Christian M. Hackeng
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

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ABSTRACT

Aims

Despite treatment with clopidogrel on top of aspirin, stent thrombosis (ST) still occurs being the most serious complication after percutaneous coronary interventions (PCI). In this study we aimed to determine the effect of variations in genes involved in the absorption (*ABCB1 C1236T, G2677T/A, C3435T*), metabolism (*CYP2C19*2* and **3*, *CYP2C9*2* and **3*, *CYP3A4*1B* and *CYP3A5*3*) and pharmacodynamics (*P2Y1 A1622G*) of clopidogrel on the occurrence of ST.

Methods and Results

The selected genetic variants were assessed in 176 subjects who developed ST while on dual antiplatelet therapy with aspirin and clopidogrel and in 420 control subjects who did not develop adverse cardiovascular events, including ST, within one year after stenting. The timing of the definite ST was acute in 66, subacute in 87 and late in 23 cases. The presence of the *CYP2C19*2* and *CYP2C9*3* variant alleles was significantly associated with ST (OR_{adj} 1.7 95% CI, 1.0-2.6, p=0.018 and OR_{adj} 2.4 95% CI, 1.0-5.5, p=0.043, respectively). The influence of *CYP2C19*2* (OR_{adj} 2.5 95% CI, 1.1-5.5, p=0.026) and *CYP2C9*3* (OR_{adj} 3.3 95% CI, 1.1-9.9, p=0.031), was most strongly associated with subacute ST. No significant associations of the other genetic variations and the occurrence of ST were found.

Conclusion

Carriage of the loss-of-function alleles *CYP2C19*2* and *CYP2C9*3* increases the risk on ST after PCI.

INTRODUCTION

Clopidogrel plays an important role in the prevention of atherothrombotic events in patients undergoing percutaneous coronary interventions (PCI) with stent implantation.[1] Despite this treatment, a substantial number of thrombotic events still occur. The most serious thrombotic complication is stent thrombosis (ST). This acute re-occlusion of the artery causes acute myocardial infarction and is associated with substantial morbidity and mortality. The reported incidence of ST varies from 0.2% to 4.6%.[2,3] The pathophysiology of ST involves complex and multifactorial mechanisms and many issues are still unresolved.[4-7] Heightened platelet reactivity despite clopidogrel treatment has been associated with the occurrence of ST.[8,9] The magnitude of on-treatment platelet reactivity is highly variable between subjects. Clinical, cellular and genetic factors are thought to play an important role in this phenomenon.[10,11]
Clopidogrel is a thienopyridine that inhibits platelet activation through an irreversible blockage of the platelet adenosine diphosphate (ADP) P2Y12 receptor.[12,13] Clopidogrel is an inactive prodrug that requires several biotransformation steps to become active.[13] After intestinal absorption, which is mediated by P-glycoprotein (Pgp), clopidogrel's conversion into the active metabolite is mediated mainly by the hepatic cytochrome P450 system.[12,13] Variations in genes involved in the absorption, metabolism and pharmacodynamics of clopidogrel are thought to influence the response to the drug.[14-20]
In addition, recent studies have demonstrated a relationship between carriage of *CYP2C19* loss-of-function alleles and adverse cardiovascular events, including ST, in patients on clopidogrel treatment.[19,21-26] However, all of these studies had a limited amount of cases with ST, with the largest number of subjects being 24.[21] In the present study, 176 subjects with ST were included who were all on clopidogrel treatment at the time the event occurred. The aim of the present study was to investigate whether variations in genes involved in clopidogrel absorption (*ABCB1 C1236T, G2677T/A, C3435T*), metabolism (*CYP2C19*2* and **3*, *CYP2C9*2* and **3*, *CYP3A4*1B* and *CYP3A5*3*) and the P2Y1 receptor (*P2Y1 A1622G*), are associated with the occurrence of ST in patients undergoing coronary stent placement who were treated with clopidogrel and aspirin.

METHODS

Study population
All consecutive patients with an angiographically confirmed ST presenting from January 2004 to February 2007 in three high-volume centres in the Netherlands were enrolled.[27] ST was defined according to the Academic Research Consortium (ARC) "definite" definition.[28] ST was categorized according to the time of the event as acute (occurrence within the first 24 h after the index-procedure), subacute (from 24 h to 30 days) and late (from 30 days to 1 year). Patients were only selected as cases when they were still on aspirin and clopidogrel at the time of ST. Control subjects were consecutive patients who underwent PCI with stent implantation between December 2005 to December 2006 in one of the participating centres, with no adverse cardiovascular events, including ST, during a 1-year follow-up post-PCI. All control subjects were on clopidogrel maintenance therapy and aspirin (80-100 mg) during the entire follow-up period. Of all subjects, medication records of community pharmacies were used to verify the use of clopidogrel, aspirin, proton pump inhibitors (PPIs) and calcium channel blockers (CCBs) from

the time of index-PCI until one year post-PCI. The ethnicity of the population in and around the cities of the participating centres is primarily Caucasian (>85%).[29-31] The study complies with the Declaration of Helsinki, the study protocol was approved by the hospital's Medical Ethics Committee, and informed consent was obtained from each patient.

Genotyping

Genomic DNA of all control subjects and of 38 cases was isolated from EDTA blood (MagNA Pure LC DNA Isolation kit 1, MagNA Pure; Roche Diagnostics; Basel, Switzerland). Genomic DNA of the remaining 138 cases was manually extracted from saliva samples (Oragene kit, DNA Genotek, Inc., Ottawa, Ontario, Canada, Laboratory Protocol for Manual Purification of DNA from 4.0 mL of Oragene® DNA/saliva on www.dnagenotek.com).

*CYP2C19*2* and **3*, *CYP2C9*2* and **3*, *CYP3A4*1B* and the *ABCB1 G2677T/A* and *C3435T* alleles were identified by Real time PCR. *CYP3A5*3*, *ABCB1 C1236T* and the *P2Y1 A1622G* alleles were identified by using restriction fragment length polymorphism (RFLP). Method validation was carried out by DNA sequence analyses.

Data analysis

The Kolmogorov-Smirnov test was used to check for normal distribution of continuous data. Continuous data, except for the time to ST, were normally distributed. Normally distributed continuous data were expressed as mean ± standard deviation (SD). Continuous data not meeting the criteria for normal distribution were expressed as median [interquartile range (IQR)]. Comparisons between groups were made with the chi-square test for categorical variables. For continuous variables, comparisons were made with the two-sided Students t-test. Chi-square tables were used to compare the observed number of each genotype with those expected for a population in Hardy-Weinberg equilibrium ($p>0.05$). The linkage disequilibrium (LD) correlation coefficient (r^2) between each pair of variant alleles that was associated with ST was calculated with the Cubic exact solutions for the estimation of pairwise haplotype frequencies.[32] We assumed a dominant model for our genetic analyses. Logistic regression was used to analyse the association between the presence of variant alleles and ST, and to adjust forpotential confounders. Variates that have been associated with an altered response to clopidogrel or with an increased risk of adverse cardiovascular events post-PCI in previous publications were selected as potential confounders. The included confounders were: age, gender, body mass index (BMI), smoking, diabetes mellitus, prior myocardial infarction (MI), the use of proton pump inhibitors (PPIs), the use of calcium channel blockers (CCBs), acute coronary syndrome (ACS) as the indication for PCI and peri-procedural variables being stent length, stent diameter and stent type (bare metal or drug eluting) and the use of glycoprotein IIb/IIIa antagonists during the procedure. A p value < 0.05 was considered statistically significant. All associations which were statistically significant were corrected for multiple testing by performing the false discovery rate test (q-value threshold 0.20).[33] Statistical analysis was performed using SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL).

RESULTS

Characteristics of the study population and genotype

Of a total of 21,009 patients undergoing stent implantations in the participating hospitals, 437 patients presented with an angiographic confirmed ST during the inclusion period. In total, 210 patients were still on dual antiplatelet therapy at the time of ST. From these, DNA was obtained from 176 patients. In total 176 cases and 420 control subjects were included in the study. The timing of the "definite" ST was acute in 66 (37.5%), subacute in 87 (49.4%) and late in 23 (13.1%) subjects. The median time [IQR] for the occurrence of ST in relation to the index procedure was 3.0 [0-9] days. Table 1 summarizes the characteristics of the cases and control subjects. There were no significant differences with regard to gender, age, diabetes mellitus, BMI, hypertension and hypercholesterolemia between the two groups. Cases were more frequently current smokers (p<0.001) than control subjects. The control group consisted of significantly more patients who had suffered from a previous MI. No significant deviations from Hardy Weinberg equilibrium were observed for any of the genetic variants (table 2). Genotype and allele frequencies of control subjects were not different from previously reported frequencies in healthy Caucasian populations.[16,34] As we found only one subject carrying a *CYP2C19*3*-allele, we did not include this allele in our analysis.

Table 1: Baseline characteristics

Variable	Control subjects (n= 420)	Cases (n= 176)	p-value
Age, years	62.1 ± 9.4	64.1 ± 10.5	0.14
Gender, male	334 (79.5)	137 (77.8)	0.66
BMI (kg/m2)	27.4 ± 3.8	27.1 ± 2.2	0.76
Diabetes mellitus	69 (16.4)	31 (17.6)	0.55
Dyslipidemia	212 (50.5)	92 (52.3)	0.72
Hypertension	208 (49.5)	82 (46.6)	0.53
Prior MI	178 (42.4)	42 (23.9)	< 0.0001
Current smoking	51 (12.1)	39 (22.2)	< 0.0001
GP IIb/IIIa receptor antagonist use	39 (9.3)	61 (34.7)	< 0.0001
ACS as indication for PCI	103 (24.6)	136 (77.3)	< 0.0001
Drug eluting stent (DES)	199 (47.4)	55 (31.3)	< 0.0001
Stent length (mm)	29.6 ± 17.6	18.9 ± 5.7	< 0.0001
Stent diameter (mm)	3.1 ± 0.6	3.1 ± 0.4	0.36
Proton pump inhibitors	95 (22.7)	51 (29.0)	0.12
CYP3A4-metabolized statins	297 (70.7)	128 (72.7)	0.44
Calcium channel blockers	120 (28.6)	53 (30.1)	0.77

Data presented are mean ± SD or number of patients (percentage). P-value: student t test for continuous variables and chi-square test for categorical variables. ST, stent thrombosis; PCI, percutaneous coronary intervention; MI, myocardial infarction; BMI, body mass index; GP, glycoprotein; ACS, acute coronary syndrome

Table 2: Genotype frequencies

SNP (Allele) dbSNP Accession No.	Genotype	Frequency control subjects (%)	HWE control subjects	Frequency cases (%)
CYP2C19 G681A (*1>*2) rs4244285	*1/*1 *1/*2 *2/*2 AF	70.5 25.7 3.8 17.0	0.12	60.0 34.9 5.1 22.6
CYP2C19 G636A (*1>*3) rs4986893	*1/*1 *1/*3 *3/*3 AF	99.8 0.2 0 0	0.98	100 0 0 0
CYP2C9 C430T (*1>*2) rs1799853	*1/*1 *1/*2 *2/*2 AF	77.2 21.5 1.2 12.0	0.66	77.7 20.0 2.3 12.3
CYP2C9 A1075C (*1>*3) rs1057910	*1/*1 *1/*3 *3/*3 AF	90.0 9.8 0.2 5.2	0.99	83.5 15.3 1.1 8.6
CYP3A4 A290G (*1>*1B) rs2740574	*1/*1 *1/*1B *1B/*1B AF	91.7 6.7 1.6 5.0	0.28	92.6 7.4 0 3.7
CYP3A5 A6986G (*1>*3) rs776746	*1/*1 *1/*3 *3/*3 AF	0 12.7 87.3 94.0	0.17	0.6 11.0 88.4 93.9
ABCB1 C1236T rs1128503	CC CT TT AF	29.5 54.0 16.5 43.5	0.07	32.0 53.7 14.3 41.2
ABCB1 G2677T/A rs2032582	GG GT+GA TT+TA+AA AF	29.7 53.8 16.5 43.0	0.06	28.6 56.0 15.4 43.4
ABCB1 C3435T rs1045642	CC CT TT AF	16.8 56.6 26.6 54.9	0.12	21.6 54.0 24.4 51.4
P2Y1 A1622G rs701265	AA AG GG AF	72.0 26.0 1.9 15.0	0.63	70.1 29.3 0.6 15.3

SNP, single nucleotide polymorphism; HWE, Hardy Weinberg Equilibrium; AF, allele frequency. All frequencies are expressed as percentages.

Association between genotype and the occurrence of ST

As shown in table 2, 40.0% of the cases had at least one *CYP2C19*2* allele, compared with 29.5% of the control subjects (p=0.013). The *CYP2C19*2* allele was associated with ST in univariate analysis, with an OR of 1.6 (95% CI 1.1-2.3, p=0.013, table 3). This association remained significant after the adjustment for confounders (OR_{adj} 1.7 95% CI, 1.0-2.6, p=0.018). When cases were divided according to the time of ST after PCI, carriers of *CYP2C19*2* were at an approximately twofold higher risk of developing a subacute ST (OR 2.0 95% CI 1.3-3.3 p=0.003), which remained significant after the adjustment for confounders (OR_{adj} 2.5 95% CI, 1.1-5.5, p=0.026, table 4). Subanalyses in cases with acute or late ST did not reveal any significant associations of genotypes with the occurrence of these types of ST (table 4).

Table 3: Associations of genetic variants and risk on stent thrombosis

Carriers ≥ 1 variant allele	Crude OR [95% CI]	P-value	Adjusted OR [95% CI]*	p-value
CYP2C19 G681A (*1>*2)	1.6 [1.1-2.3]	0.013	1.7 [1.0-2.6]	0.018
CYP2C19 G636A (*1>*3)	ND	ND	ND	ND
CYP2C9 A1075C (*1>*3)	1.8 [1.1-3.0]	0.027	2.4 [1.0-5.5]	0.043
CYP2C9 C430T (*1>*2)	1.0 [0.6-1.5]	0.90	0.6 [0.2-1.7]	0.12
CYP3A4 A290G (*1>*1B)	0.8 [0.5-1.8]	0.76	0.6 [0.3-2.0]	0.45
CYP3A5 A6986G (*1>*3)	0.2 [0.1-1.2]	0.99	0.2 [0.1-1.3]	0.99
ABCB1 C1236T	0.9 [0.6-1.4]	0.74	0.7 [0.4-1.2]	0.48
ABCB1 G2677T/A	1.0 [0.7-1.6]	0.79	0.9 [0.5-1.6]	0.89
ABCB1 C3435T	0.8 [0.5-1.2]	0.30	0.6 [0.3-1.2]	0.18
P2Y1 A1622G	1.1 [0.7-1.6]	0.64	1.2 [0.6-2.2]	0.28

OR, odds ratio; CI, confidence interval.

*Adjusted for age, gender, body mass index, smoking, diabetes mellitus, prior MI, use of PPIs, use of CCBs, use of glycoprotein IIb/IIIa receptor antagonists, stent length, type and diameter and ACS as indication for PCI; ND, not determined;

Table 4: Associations of genetic variants and risk on stent thrombosis, stratified by the timing of stent thrombosis

	Acute ST (n = 66)		Subacute ST (n = 87)		Late ST (n = 23)	
Genetic variants	OR [95% CI], OR_{adj} [95% CI]*	P-value	OR [95% CI], OR_{adj} [95% CI]*	P-value	OR [95% CI], OR_{adj} [95% CI]*	p-value
CYP2C19 G681A (*1>*2)	1.3 [0.8-2.3] 1.7 [0.8-3.5]	0.34 0.11	2.0 [1.3-3.3] 2.5 [1.1-5.5]	0.003 0.026	[0.4-2.6] 1.4 [0.6-9.5]	0.92 0.54
CYP2C9 A1075C (*1>*3)	1.5 [0.7-3.1] 2.2 [0.9-6.8]	0.15 0.10	2.2 [1.1-4.4] 3.3 [1.1-9.9]	0.024 0.031	0.4 [0.06-3.1] 1.1 [0.1-12.5]	0.39 0.73

OR, odds ratio; CI, confidence interval; ST, stent thrombosis.

*, Adjusted for age, gender, body mass index, smoking, diabetes mellitus, prior MI, use of PPIs, use of CCBs, use of glycoprotein IIb/IIIa receptor antagonists, type, length and diameter of the stent and ACS as indication for PCI.

For *CYP2C9*, carriage of the **3* allele was associated with an increased risk of ST when compared to *CYP2C9*3* noncarriers: OR 1.8 95% CI, 1.1-3.0, p=0.027; OR_{adj} 2.4 95% CI, 1.0-5.5, p = 0.043. The influence of *CYP2C9*3* was most prominent on the occurrence of subacute ST (OR 2.2 95% CI, 1.1-4.4, p=0.024; OR_{adj} 3.3 95% CI, 1.1-9.9, p=0.031), while the associations of this variant allele and the occurrence of acute and late ST were not statistically significant (table 4).
In multivariate analysis, in which besides the non-genetic covariates, both *CYP2C19*2* and *CYP2C9*3* were included as covariates, the two genetic variants were found to be independent predictors of ST (for *CYP2C19*2*: OR_{adj} 1.7 95% CI, 1.0-3.1, p=0.040 and for *CYP2C9*3:* OR_{adj} 2.5 95% CI, 1.1-5.8, p=0.035).
We found no evidence of linkage disequilibrium for the pair *CYP2C19*2-CYP2C9*3* (r^2=0.01). The distribution of *CYP2C19* and *CYP2C9* genotypes among cases and control subjects is shown in table 5. In *CYP2C19*2* noncarriers, *CYP2C9*3* was associated with an almost twofold increased risk of ST: OR 1.9 95% CI, 1.0-3.4, p=0.042, which remained statistically significant after the adjustment for confounders: OR_{adj} 3.0 95% CI, 1.1-8.6, p=0.037. Cases were more often carriers of both *CYP2C19*2* and *CYP2C9*3*-alleles as compared to control subjects: 4.5% vs. 1.7% (OR 1.9 95% CI, 1.2-10.0, p=0.029, OR_{adj} 2.1 95% CI, 1.3-3.5, p=0.003; table 5).

Table 5: Distribution of *CYP2C19* and *CYP2C9* variant alleles in control subjects and cases

Genotype groups	Control subjects, n (%)	Cases, n (%)	OR [95% CI], OR_{adj} [95% CI]*	p-value
Subjects carrying neither CYP2C19*2 nor CYP2C9*3	262 (62.4)	85 (48.3)	0.6 [0.4-0.8] 0.4 [0.2-0.7]	0.002 0.003
Subjects carrying CYP2C19*2 but not CYP2C9*3	116 (27.6)	62 (35.2)	1.6 [1.1-2.4] 2.1 [1.1-3.9]	0.013 0.018
Subjects carrying CYP2C9*3 but not CYP2C19*2	35 (8.3)	21 (11.9)	1.9 [1.0-3.4] 3.0 [1.1-8.6]	0.042 0.037
Subjects carrying both CYP2C19*2 and CYP2C9*3	7 (1.7)	8 (4.5)	1.9 [1.1-3.2] 2.4 [1.3-4.3]	0.018 0.004

Cases and control subjects are divided into four subgroups: (1) subjects carrying neither *CYP2C19*2* nor *CYP2C9*3*, (2) subjects carrying *CYP2C19*2* but not *CYP2C9*3*, (3) subjects carrying *CYP2C9*3* but not *CYP2C19*2*, and (4) subjects carrying both *CYP2C19*2* and *CYP2C9*3*. Data expressed as number (%). OR, odds ratio; CI, confidence interval. *, Adjusted for age, gender, body mass index, smoking, diabetes mellitus, prior MI, use of PPIs, use of CCBs, use of glycoprotein IIb/IIIa receptor antagonists, type, length and diameter of the stent and ACS as indication for PCI.

No interaction between the indication for PCI (ACS versus stable angina pectoris (SAP)) and the carriage of *CYP2C19*2* or *CYP2C9*3* was found (p-values 0.97 and 0.18 respectively). In addition, stratified analysis according to the indication of PCI were performed. In subjects with ACS as the indication for PCI (136 cases and 103 control subjects), *CYP2C19*2* and *CYP2C9*3* both increased the risk on the occurrence of ST (OR_{adj} 2.0 95% CI, 1.1-4.5, p=0.032 and OR_{adj} 2.9 95% CI, 1.0-9.3, p=0.039, respectively).
In the subgroup of subjects with SAP (40 cases and 317 control subjects), a trend towards an association for *CYP2C19*2* was found (OR 1.7 95% CI, 0.9-4.1, p=0.076, while for *CYP2C9*3* no association with ST (OR 1.2 95% CI, 0.4-6.5, p=0.56) was observed.
No significant associations of the other genetic variations and the occurrence of ST were found (table 3). For all significant associations, the multiple testing parameter q was found to be < 0.20.

DISCUSSION

This case-control study aimed to determine the influence of genetic variations related to the pharmacokinetics and pharmacodynamics of clopidogrel on the occurrence of ST in patients who were on clopidogrel and aspirin treatment at the time of the event. We found that carriers of the *CYP2C19*2* and *CYP2C9*3* loss-of-function alleles were at an 1.7 and 2.4-fold increased risk of developing ST, respectively. The influence of these genetic variants was most profound on the risk of subacute ST. We found no significant associations between the other investigated genetic variants and the occurrence of ST.
Of all genotypes included in this study, *CYP2C19* has been by far the most extensively investigated. After absorption, 85% of clopidogrel is metabolized into an inactive compound. The remaining 15% of clopidogrel is metabolized into 2-oxo-clopidogrel. This intermediate metabolite is then hydrolyzed and generates a highly unstable active thiol (R-130964) metabolite.[12,13] CYP2C19 contributes in both of the two sequential metabolic steps of clopidogrel activation. Data from several studies report that carriage of the *CYP2C19*2* allele is associated with an impaired pharmacodynamic response to different dosing regimens of clopidogrel, as measured with various platelet function assays.[14,15,20] In two studies in healthy subjects, carriers of *CYP2C19*2* exhibited significantly lower area under the plasma concentration time curves (AUCs) and lower maximal plasma concentrations of clopidogrel's metabolites than subjects homozygous for the *CYP2C19* wildtype.[25,35] The results of our study regarding *CYP2C19*2* are consistent with recent studies investigating the effect of *CYP2C19*2* on clinical endpoints, including ST.[21-23,26]

To our knowledge, this is the first study showing that carriage of *CYP2C9*3* is associated with an increased risk of ST. The association of *CYP2C9* genetic variants and ST is only explored in the study reported by Mega *et al*. in which no associations of *CYP2C9*3* and ST were found.[25] However, the number of subjects with ST was rather small (*n*=18). Together with the low allele frequency of *CYP2C9*3* (7.0-9.0% in Caucasians[34], 4% in Asians and not present in African populations[36]), this study was underpowered to detect the association. Our observation regarding *CYP2C9*3* is supported by the results of two studies. In patients undergoing elective PCI, *CYP2C9*3* carriers had a mean relative increase of 10% in on-treatment platelet reactivity as measured with ADP-induced light transmittance aggregometry and the VerifyNow P2Y12 assay, compared to *CYP2C9*3* noncarriers.[20] Carriage of *CYP2C9*3* was associated with a fourfold increased risk on high on-treatment platelet reactivity (HPR). In the same study, carriage of *CYP2C19*2* was also associated with a more than 10% mean relative increase of on-treatment platelet reactivity. *CYP2C19*2*-carriers had an approximately 3.5-fold increased risk of HPR.[20] Brandt *et al*. found healthy subjects carrying the *CYP2C9*3* loss-of-function allele to have a significantly lower AUC and lower maximal plasma concentrations of clopidogrel's active metabolite as compared to noncarriers. Furthermore, they also found *CYP2C9*3* to be associated with an impaired pharmacodynamic response to a 300 mg clopidogrel loading dose.[35] CYP2C9 is thought to play a role in only clopidogrel's secondary metabolic step of activation.[25]

The other investigated genetic variant in the *CYP2C9* gene, *CYP2C9*2*, was not associated with the risk of ST. This is in concordance with other pharmacogenetic studies of CYP2C9-metabolized drugs, for example coumarins. The presence of the *CYP2C9*2* allele also has less impact on the anticoagulation effect of acenocoumarol than *CYP2C9*3*.[37]
The influence of genetic variations is most prominent on subacute ST. However, it should be

noted that subanalyses in the different groups of ST had less power due to lower number of cases in each of the ST subgroups.
No significant associations were found in patients presenting with acute ST. This observation is in line with previous findings that indicate that mechanical and procedural factors are the predominant cause of acute ST.[27,38] We found no associations of genetic variations on the occurrence of late ST. This phenomenon might partly be caused by the fact that only 23 patients with late ST were included in our study. Furthermore, when the time-interval after the index-PCI increases, it is likely that other mechanisms (e.g. late stent malapposition) might play a more prominent role. Our findings confirm recently published data from Geisler *et al.* showing no predictive value of residual platelet aggregation for the incidence of late ST. The authors concluded that other mechanisms might be involved in the development of late ST.[39]
Drug eluting stents (DES) are considered to be associated with the occurrence of particularly late ST. The lower percentage of cases who received DES might be caused by the fact that we observed mainly acute and subacute ST (in total 87% of the cases). These types of ST are more common with the use of bare metal stents (BMS).[40]

There are some limitations of this study. First, in this observational case-control study, we cannot completely exclude possible bias by various risk factors and patients characteristics. Nonetheless, the multivariable adjustment models confirmed the primary analyses. Second, our cases had more often ACS as the indication for PCI. ACS is a known risk factor for the development of ST.[6] However, adjustment for this confounder and including interaction terms did not change findings. In addition, stratified analyses showed that the genetic variants *CYP2C19*2* and *CYP2C9*3* were associated with ST in the subgroup of patients with ACS. In the subgroup consisting of patients with SAP as the indication for PCI, a trend towards a significant association for *CYP2C19*2* but no association for *CYP2C9*3* was found. As only 40 cases had SAP as the indication for PCI and the fact that *CYP2C9*3* has a low allele frequency, this subgroup was too small to detect significant associations. Finally, the cases more often received glycoprotein IIb/IIIa antagonists than the control subjects. Both in patients with ACS and SAP as the indication for PCI, the use of glycoprotein IIb/IIIa antagonists was limited to the provisional (bail-out) use at the discretion of the operator, after PCI. Nevertheless, we observed that ACS patients more often received glycoprotein IIb/IIIa antagonists than patients with SAP (29% of the patients with ACS and 10% of the patients with SAP). However, adjustment for the use of glycoprotein IIb/IIIa antagonists did not change the associations between the two genetic variants and ST. Also, in stratified analysis according to the indication of PCI, the adjustment for glycoprotein IIb/IIIa antagonists did not change findings.
Given the devastating consequences of ST, great efforts should be made to identify those patients at highest risk, who would benefit most from an alternative strategy. Specifically, the frequent presence of the *CYP2C19*2* allele, seen in approximately 30% of the Caucausian and 60% of the Asian population, may require an alternative strategy in the prevention of atherothrombotic complications after stent implantation.[41] A randomized trial in 60 patients undergoing elective PCI, reported that *CYP2C19*2*-carriers had a greater platelet inhibition after a split 1200 mg clopidogrel loading dose or 150 mg clopidogrel maintenance doses than after a 600 mg loading dose and 75 mg maintenance dose, respectively. Interestingly, in patients with the *CYP2C19*1/*1* genotype, no dose-dependent response was observed. This might indicate that subjects with a poor-response genotype may specifically benefit from a higher dose of clopidogrel.[42] However, large clinical trials are needed to confirm these observations.

In conclusion, we have shown that carriage of the loss-of-function alleles *CYP2C19*2* and *CYP2C9*3* increases the risk on ST. Personalized therapy targeting patients who carry these genetic variants might help to improve clinical outcome after stent implantation.

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3.2

COMBINED INFLUENCE OF PROTON PUMP INHIBITORS, CALCIUM CHANNEL BLOCKERS AND *CYP2C19*2* ON ON-TREATMENT PLATELET REACTIVITY AND ON THE OCCURRENCE OF ATHEROTHROMBOTIC EVENTS IN PATIENTS UNDERGOING CORONARY STENT IMPLANTATION

Ankie M. Harmsze
Jochem W. van Werkum
Patrick C. Souverein
Nicoline J. Breet
Heleen J. Bouman
Christian M. Hackeng
Hendrik J.T. Ruven
Jurriën M. ten Berg
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

Submitted

ABSTRACT

Background

Carriage of *CYP2C19*2* and the use of proton pump inhibitors (PPIs) and calcium channel blockers (CCBs) has been associated with diminished efficacy of clopidogrel. However, previous studies only assessed the isolated impact of these risk factors for clopidogrel poor-response.

Aim

To investigate the impact of combined presence of three pharmacokinetic risk factors for clopidogrel poor-response, i.e. the use of CCBs, PPIs and carriage of *CYP2C19*2*, on on-treatment platelet reactivity and the occurrence of atherothrombotic events in 725 patients on dual antiplatelet therapy undergoing elective coronary stenting.

Methods

In a prospective follow up study, on-treatment platelet reactivity was quantified using ADP-induced light transmittance aggregometry and the VerifyNow P2Y12 assay. The clinical study endpoint was the composite of all-cause mortality, myocardial infarction, stent thrombosis and stroke at one year after stenting.

Results

Patients with either one or more than one risk factor exhibited increased platelet reactivity (mean relative increase one risk factor: 11% and >1 risk factor: 28%, respectively). Sixty-four events occurred during follow-up (8.8% of the study population). Patients with one risk factor for clopidogrel poor-response did not have an increased risk of the composite endpoint. However, patients using both CCBs and PPIs and carriers of *CYP2C19*2* who used CCBs had a statistically significant increased risk of the composite endpoint (HR_{adj} 2.1 95% CI, 1.0-4.4, p=0.037 and HR_{adj} 3.3 95% CI, 1.1-9.5, p=0.029, respectively).

Conclusions

The presence of more than one of the three investigated pharmacokinetic risk factors for clopidogrel poor-response is associated with an increased risk of adverse cardiovascular events within one year after elective coronary stenting.

INTRODUCTION

Clopidogrel plays an important role in the secondary prevention of atherothrombotic events in patients with acute coronary syndromes (ACS) and following percutaneous coronary interventions (PCI).[1-3] However, a wide interindividual variability in the response to clopidogrel exists.[4] A significant proportion of clopidogrel-treated patients do not respond optimally to the drug and are therefore classified as 'clopidogrel poor-responders'. Clopidogrel is a prodrug that has to be metabolized by the cytochrome (CYP) P450 enzymesystem in 2 sequential oxidative steps to become active. The first metabolic step, which leads to 2-oxo-clopidogrel, is mediated by CYP1A2, CYP2B6 and CYP2C19, whereas the second step, in which the active thiol metabolite is formed, is mediated by CYP2B6, CYP2C9, CYP2C19 and CYP3A4.[5] The active metabolite binds irreversibly to the P2Y12-receptor on the platelets surface, thereby inhibiting ADP-induced platelet aggregation.[6, 7]

Recently, several pharmacokinetic risk factors for clopidogrel poor-response have been identified. First, it has been shown that carriage of the loss-of-function *CYP2C19*2* allele leads to a diminished formation of clopidogrel's active metabolite, a reduced pharmacodynamic response to clopidogrel and a higher rate of recurrent cardiovascular events in clopidogrel-treated patients.[8-10] Second, concomitant use of drugs that inhibit CYP iso-enzymes which are involved in clopidogrel metabolism (e.g. calcium channel blockers (CCBs) and proton pump inhibitors (PPIs)) is shown to be associated with a higher magnitude of on-treatment platelet reactivity and with an increased risk of adverse cardiovascular events in clopidogrel-treated patients who underwent PCI.[11-14] Both drug classes are often co-prescribed in clopidogrel-treated patients with coronary artery disease.[15] In previous studies, only the isolated impact of these pharmacokinetic risk factors for clopidogrel poor-response has been assessed.

The primary aim of this study was to get more insight in not only the isolated effect but also the impact of the combined presence of pharmacokinetic risk factors for clopidogrel poor-response, i.e. CCBs, PPIs and carriage of the *CYP2C19*2* allele, on on-treatment platelet reactivity and on the occurrence of atherothrombotic events in a cohort of patients on dual antiplatelet therapy undergoing elective PCI. Second, we investigated whether the gain-of-function *CYP2C19*17* allele has a protective effect on the occurrence of atherothrombotic events.

METHODS

Study design and patients

In total, 1069 patients with established coronary artery disease scheduled for elective PCI with stent implantation were consecutively recruited in the setting of the prospective POPular study with platelet function testing for the prediction of clinical outcome.[16] Blood for DNA analysis was available from 820 patients who were still on therapy with clopidogrel and aspirin at the end of the one-year follow-up period or at the time the clinical endpoint occurred. Of these patients, detailed pharmacy records were available for 725 patients (88.4%). This constitutes the study population for the present study. All patients used clopidogrel during the entire follow-up period (according to patient information and verified by medication histories obtained from community pharmacies (drug dispenses for at least 328 days post-PCI, i.e. >90% adherence)) or at least until the clinical endpoint took place. Clopidogrel and aspirin maintenance doses were 75 mg and 80 to 100 mg daily, respectively.

Prior to PCI, all patients were pretreated with clopidogrel (defined as 75 mg/day therapy for >5 days or a loading dose of 300 mg >24 hours before PCI or 600 mg >4 hours before PCI) and aspirin (80-100 mg/day >10 days). A small subset of the patients (7.0%) received intravenous glycoprotein IIb/IIIa inhibitors during the intervention, but these drugs were always administered after blood collection for platelet function testing.
Patients were excluded if they used concomitant medication known to affect platelet function other than aspirin (i.e. nonsteroidal anti-inflammatory agents, dipyramidole, upstream glycoprotein IIb/IIIa inhibitors) or had a known platelet function disorder or a whole blood platelet count of less than 150 x 10^3/μL.
Written informed consent was obtained before PCI. The study was conducted according to the principles of the Declaration of Helsinki and the local institutional review board approved the study.

Blood sampling and genotyping
Before heparinization and prior to PCI, whole blood samples were drawn from the femoral or radial artery sheath into 3.2% citrate tubes for light transmittance aggregometry. Testing with VerifyNow P2Y12 (Accumetrics, San Diego, California) was performed using Greiner tubes. Genomic DNA was isolated from K3-EDTA blood (MagNA Pure LC DNA Isolation kit 1, MagNA Pure; Roche Diagnostics; Basel, Switzerland). The *CYP2C19* alleles *CYP2C19*2* (rs4244285) and *CYP2C19*17* (rs12248560) were identified by real time PCR. DNA sequence analysis was used to validate the genotyping procedure.

Platelet function testing
The magnitude of on-treatment platelet reactivity was quantified using light transmittance aggregometry (LTA) with adenosine diphosphate (ADP) in a final concentration of 20 μmol/L as the agonist (maximum extent of platelet aggregation achieved in any time during the run of 10 minutes) and the VerifyNow P2Y12 assay. Details of these methods have been reported previously.[16-18] All measurements were completed within 2 hours of blood collection.

Definition of the clinical endpoint
The clinical endpoint of this study was defined as the composite of all-cause death, nonfatal myocardial infarction (MI), stent thrombosis, and ischemic stroke at 1-year after PCI. Information on the occurrence of these events was obtained by telephone contact to all patients at 30 days and 12 months and verified using source documents from medical records from the referring hospitals. An independent committee which was blinded for platelet function data adjudicated the clinical endpoints through review of medical record source documents.

Exposure assessment
Detailed pharmacy records were obtained from community pharmacies. The records included the name of the drug, the day of dispensing, the dosage form, the number of units dispensed, the prescribed daily dose and the Anatomical Therapeutic Chemical code of the drug. For each patient we identified all dispenses for CCBs and PPIs. The theoretical duration of each prescription was assessed by dividing the number of dispensed tablets or capsules by the prescribed daily dose. For both types of drugs, treatment episodes were calculated, defined as a series of subsequent refills, independent of changes in dose regimen or drug switches within the same class. A new episode was assumed if an interval of 30 days or more occurred between the the-

oretical end date of one prescription and new prescription. The period of follow-up was divided into periods of exposure and non-exposure. Patients could move between exposure categories over the duration of follow-up. When the clinical endpoint occurred, the date was defined as the event date.

Data analysis

Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables were expressed as frequencies and percentages. Baseline continuous data were analyzed by ANOVA and categorical data by chi-square test when appropriate. Chi-square tables were used to compare the observed number of the *CYP2C19* genotypes with that expected for a population in Hardy-Weinberg equilibrium ($p>0.05$). The Kolmogorov-Smirnov test was used to check for normal distribution of continuous data. All data were normally distributed. Continuous platelet function data were compared between eight groups according to the presence of risk factors for clopidogrel poor-response with ANOVA followed by the least significant difference test (LSD). The eight groups in which the subjects were divided were: 1) non-CCB- and non-PPI-exposed *CYP2C19*2*-noncarriers, 2) CCB-exposed *CYP2C19*2*-noncarriers, 3) PPI-exposed *CYP2C19*2*-noncarriers, 4) CCB- and PPI-exposed *CYP2C19*2* noncarriers, 5) non-CCB- and non-PPI-exposed *CYP2C19*2*-carriers, 6) CCB-exposed *CYP2C19*2*-carriers, 7) PPI-exposed *CYP2C19*2*-carriers and 8) CCB- and PPI-exposed *CYP2C19*2*-carriers. CCB and PPI users were defined as patients who used CCBs or PPIs for more than seven days prior to PCI and platelet function testing. ANCOVA with LSD was used to adjust for the potential confounding factors age, BMI, gender, diabetes mellitus, smoking, left ventricular ejection fraction (LVEF) <45%, hypertension, clopidogrel loading dose and renal function (estimated glomerular filtration rate cut-off 60 mL/min, MDRD4). All included potential confounders have been associated with on-treatment platelet reactivity in previous publications.[19] The influence of PPIs, CCBs and *CYP2C19*2* on high on-treatment platelet variability (HPR) was assessed with univariate and multivariate logistic regression analysis. As cut-off value for the LTA we used 64.5% and for VerifyNow P2Y12 236 PRU. These thresholds were derived from ROC curve analysis and were shown have predictive value on the composite endpoint in the total POPular cohort.[16]

Cox proportional hazard models with time-varying exposure for CCBs and PPIs were used to evaluate the risk of exposure to these drugs on the composite endpoint. Cox proportional hazard models were used to investigate the influence of *CYP2C19*2* and *CYP2C19*17* on the composite endpoint.

Furthermore, the influence of exposure to CCBs and PPIs was determined in stratified groups according to *CYP2C19*2* carrier status. To assess the effect of confounders on the composite endpoint, we analyzed the following covariates (assessed at the time of PCI): age, body mass index (BMI), gender, diabetes mellitus, smoking, hypertension, hypercholesterolemia, history of MI, history of CABG, family history of coronary artery disease (CAD), renal failure, left ventricular ejection fraction (LVEF) < 45%, total stent length, number of lesions treated, number of stents, implanted bifurcation lesions, clopidogrel loading dose, left anterior descending coronary artery, or graft-stenting. Covariates that induced a difference of 5% or more in the original hazard ratio were included in the final multivariate model.

Statistical analyses were performed using STATA (version 10.1) and SPSS software (version 17.0.1 for Windows; SPSS Chicago, IL). A p-value < 0.05 was considered statistically significant.

RESULTS

Study population

The total study population consisted of 725 patients. The baseline characteristics of the study population are shown in Table 1. In total, 525 (72.4%) subjects were noncarriers of the *CYP2C19*2* variant allele and 200 (37.6%) were carriers of *CYP2C19*2* (of whom 24 homozygous for the *2 variant allele). Furthermore, 462 patients were *CYP2C19*17* noncarriers, 234 patients had the *CYP2C19*1/*17* genotype and 29 the *17/*17 genotype. The distributions were in Hardy Weinberg equilibrium (p>0.05).

Between the eight groups there were significant differences in age, gender, renal function, hypertension and the use of beta-blockers. Other variables were well-balanced between the different groups (Table 1). The CCB used most often was amlodipine (used by 55.3% of the CCB-users). Omeprazole and esomeprazole were the most frequently used PPIs (59.2%).

During the follow-up period, 298 patients were exposed to CCBs (of which 55.0% amlodipine) and 305 to PPIs (of which 58.7% omeprazole or esomeprazole) at any time. In total, 126 patients were exposed to the combination of CCBs and PPIs at any time during follow-up.

Exposure to drugs, genotype and on-treatment platelet reactivity

For the total study population, the mean values ± SD of on-treatment platelet reactivity were 57.2% ± 14.5 (LTA) and 208 ± 77 PRU (VerifyNow P2Y12 assay).

Light transmittance aggregometry

For the LTA, on-treatment platelet reactivity across the 8 groups was as follows: 53.0% ± 14.4 for non-drug-exposed *CYP2C19*2*-noncarriers, 57.2% ± 13.9 for CCB-exposed *CYP2C19*2*-noncarriers, 58.0% ± 14.6 for PPI-exposed *CYP2C19*2*-noncarriers, 62.8% ± 11.3 for CCB- and PPI-exposed *CYP2C19*2*-noncarriers, 61.1% ± 13.8 for nonexposed *CYP2C19*2*-carriers, 64.9% ± 11.4 for CCB-exposed *CYP2C19*2*-carriers, 65.3% ± 11.8 for PPI-exposed *CYP2C19*2*-carriers and 65.8 ± 11.9 for CCB- and PPI-exposed *CYP2C19*2*-carriers. Platelet reactivity was significantly different between the 8 groups (p<0.0001, Figure 1A).

In pairwise comparisons we found that both in *CYP2C19*2*-noncarriers and -carriers, the exposure to PPIs, exposure to CCBs as well as the combined use of CCBs and PPIs was associated with significantly increased on-treatment platelet reactivity as compared to the reference group of non-drug-exposed *CYP2C19*2*-noncarriers (Figure 1A). All associations remained significant after the adjustment for confounders (Table 2).

According to the LTA, 36.1% (*n*=262) of the study cohort exhibited HPR. All patients with more than one risk factor for clopidogrel poor-response were at an increased risk of HPR as compared to non-drug-exposed *CYP2C19*2*-noncarriers (Figure 2). Associations remained significant after the adjustment for confounders (data not shown).

Table 1: Baseline characteristics of the study population (part 1 of 2)

	All (n=725)	CCB[-] PPI[-] *2[-] (n=307)	CCB[+] PPI[-] *2[-] (n=108)	CCB[-] PPI[+] *2[-] (n=75)	CCB[+] PPI[+] *2[-] (n=35)
Age, years	63.2±10.2	62.1±10.3	64.6±9.7	64.2±11.0	64.7±9.4
Gender, male	551 (76)	247 (80.5)	85 (78.7)	51 (68.0)	21 (60.0)
BMI, kg/m2	27.1 ± 3.9	27.0±4.1	27.2±3.5	27.4±3.8	28.7±4.7
Hypertension	546 (75.3)	214 (69.7)	92 (85.2)	54 (72.0)	30(85.7)
Hypercholestero-lemia	584 (80.6)	243(79.1)	88(81.5)	64(85.3)	26(74.3)
Diabetes mellitus	123 (17.0)	46 (15.0)	21 (19.4)	14 (18.7)	9 (25.7)
Renal dysfunction	92 (12.7)	35 (11.4)	12 (11.1)	10 (13.3)	7 (20.0)
CABG	52 (7.2)	20 (6.5)	9 (8.3)	6 (8.0)	5 (14.3)
Family history of CAD	456 (62.9)	205 (66.8)	66 (6.1)	47 (62.7)	23 (65.7)
History of MI	311 (42.9)	128 (41.7)	44 (40.7)	39 (52.0)	16 (45.7)
Current smoker	69 (9.5)	34 (11.1)	6 (5.6)	7 (9.3)	0 (0)
Type stent	421/47	180/20	71/7	41/5	15/2
Length stent, mm	28.2±6.8	27.4±15.4	29.6±17.8	26.3±15.0	33.8±23.6
Diameter stent, mm	3.1±0.93	3.1±1.2	3.2±0.98	3.1±0.42	3.0±0.45
LAD stenting	339 (46.8)	147 (47.9)	43 (39.8)	40 (53.3)	14 (40.0)
RCX stenting	219 (30.2)	86 (28.0)	35 (32.4)	23 (30.7)	11 (31.4)
RCA stenting	263 (36.3)	110 (35.8)	45 (41.7)	22 (29.3)	17 (48.6)
Graft stenting	23 (3.2)	9 (2.9)	7 (6.5)	1 (1.3)	0 (0)
Gp IIb/IIIa inhibitors	51 (7.0)	20 (6.5)	9 (8.3)	6 (8.0)	3 (8.6)
Clopidogrel loading dose	371 (51.2)	148 (48.2)	53 (49.1)	43 (57.3)	20 (57.1)
Beta blockers	574 (79.2)	247 (80.5)	76 (70.4)	67 (89.3)	24 (68.6)
ACE-inhibitors	279 (38.5)	127 (41.4)	36 (33.3)	34 (45.3)	12 (34.2)
CYP3A4-metab. statins	419 (57.8)	165 (53.7)	66 (61.1)	40 (53.3)	22 (62.9)
LVEF < 45%	120 (16.6)	51 (16.6)	16 (14.8)	14 (18.7)	1 (2.9)

Continuation Table 1: Baseline characteristics of the study population (part 2 of 2)

	CCB[-] PPI[-] *2[+] (n=119)	CCB[-] PPI[+] *2[+] (n=23)	CCB[+] PPI[-] *2[+] (n=47)	CCB[+] PPI[+] *2[+] (n=11)	p-value
Age, years	61.6±9.8	65.8±7.1	66.3±10.1	64±13.6	0.025
Gender, male	92 (77.3)	12 (52.2)	37 (78.7)	6 (54.5)	0.003
BMI, kg/m2	26.4±3.6	26.6±3.0	27.6±4.6	27.8±3.2	0.12
Hypertension	91(76.5)	20(87.0)	36(76.6)	9(81.8)	0.036
Hypercholestero-lemia	99(83.2)	20(87.0)	35(74.5)	9(81.8)	0.71
Diabetes mellitus	20 (16.8)	5 (21.7)	7(14.9)	1(9.1)	0.74
Renal dysfunction	8 (6.7)	4 (17.4)	13(27.7)	3(27.3)	0.011
CABG	7 (5.9)	1 (4.3)	3(6.4)	1(9.1)	0.80
Family history of CAD	66 (55.5)	14 (60.9)	31 (66.0)	4(36.4)	0.58
History of MI	50 (42.0)	7 (30.4)	24 (51.1)	3(27.3)	0.44
Current smoker	14 (11.8)	0 (0)	6 (12.8)	2 (18.2)	0.13
Type stent	72/6	11/1	27/5	4/1	0.53
Length stent, mm	28.7±19.4	24.7±11.2	30.3±14.4	27.7±15.8	0.32
Diameter stent, mm	3.0±0.34	3.0±0.45	3.2±0.65	3.2±0.65	0.74
LAD stenting	61 (51.2)	8 (34.8)	20 (42.6)	6 (54.5)	0.42
RCX stenting	39 (32.8)	7 (30.4)	17 (36.2)	1 (9.1)	0.73
RCA stenting	38 (31.9)	12 (52.2)	15 (31.9)	4 (36.4)	0.25
Graft stenting	2 (1.7)	0 (0)	3 (6.4)	1 (9.1)	0.17
Gp IIb/IIIa inhibitors	10 (8.4)	1 (4.3)	2 (4.3)	0 (0)	0.92
Clopidogrel loading dose	65 (54.6)	13 (56.5)	21 (44.7)	8 (72.8)	0.49
Beta blockers	99 (83.2)	20 (87.0)	32 (68.1)	9 (81.8)	0.011
ACE-inhibitors	38 (31.9)	10 (43.5)	18 (38.3)	4 (36.4)	0.48
CYP3A4-metab. statins	74 (62.2)	15 (65.2)	30 (63.8)	7 (63.6)	0.58
LVEF < 45%	26 (21.8)	5 (21.7)	6 (12.8)	1 (9.1)	0.25

Data are expressed as mean ± SD or number of patients (%); Definition of eight groups: (1) CCB[-] PPI[-]*2[-]: non-drug-exposed *CYP2C19*2*-noncarriers, (2) CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-noncarriers, (3) CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-noncarriers, (4) CCB[+] PPI[+]*2[-]: CCB and PPI-exposed *CYP2C19*2*-noncarriers, (5) CCB[-] PPI[-]*2[+]: non-drug-exposed *CYP2C19*2*-carriers, (6) CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-carriers, (7) CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-carriers and (8) CCB[+] PPI[+]*2[+]: CCB and PPI-exposed *CYP2C19*2*-carriers

p-value: ANOVA for continuous variables and chi-square test for categorical variables between the eight groups, renal dysfunction: glomerular filtration rate less than 60mL/min, MI: myocardial infarction, CABG: coronary artery bypass grafting, CAD: coronary artery disease, PPI: proton pump inhibitor, LVEF: left ventricular ejection fraction, ACS: acute coronary syndrome, PCI: percutaneous coronary intervention, LAD: left anterior descending artery, RCX: right circumflex artery, RCA: right coronary artery, type of stent: bare metal/drug eluting stent

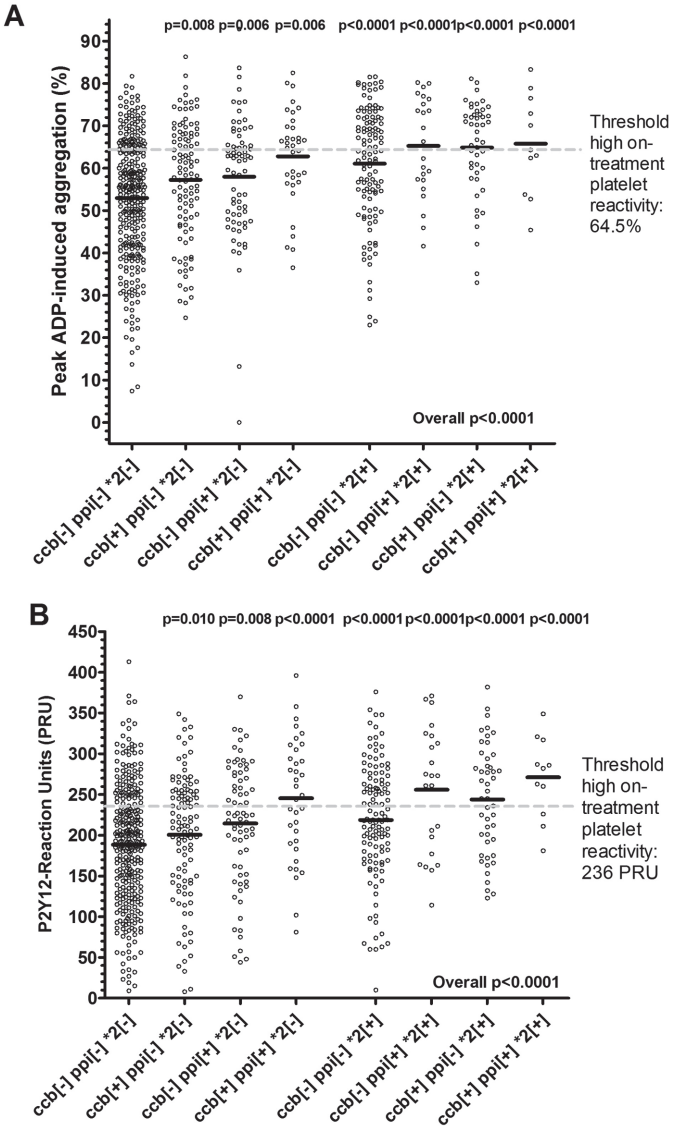

Figure 1: On-treatment platelet reactivity stratified by exposure to CCBs and PPIs and CYP2C19 genotype
Individual values (n=725) of on-treatment platelet reactivity stratified for 8 groups as measured with 20 µmol/L ADP-induced LTA (panel A), and the VerifyNow P2Y12 assay (panel B). Mean values are represented by solid lines. ADP: 5'-adenosine diphosphate, LTA: light transmittance aggregometry, PRU: P2Y12 reaction units, p-values: ANOVA with LSD Definition of eight groups: (1) CCB[-] PPI[-]*2[-]: non-drug-exposed *CYP2C19*2*-noncarriers, (2) CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-noncarriers, (3) CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-noncarriers, (4) CCB[+] PPI[+]*2[-]: CCB and PPI-exposed *CYP2C19*2*-noncarriers, (5) CCB[-] PPI[-]*2[+]: non-drug-exposed *CYP2C19*2*-carriers, (6) CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-carriers, (7) CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-carriers and (8)CCB[+] PPI[+]*2[+]: CCB and PPI-exposed *CYP2C19*2*-carriers.

Table 2: Mean differences in on-treatment platelet reactivity

Group	Light transmittance aggregometry		VerifyNow P2Y12 assay	
	Mean difference (%)	p-value	Mean difference (PRU)	p-value
CCB[+] PPI[-] *2[-]	-3.6 (-6.7 to -0.56)	0.020	-8.0(-16 to –0.06)	0.013
CCB[-] PPI[+] *2[-]	-3.8 (-7.4 to -0.31)	0.033	-18 (-34 to -2.2)	0.026
CCB[+] PPI[+] *2[-]	-7.7 (-12.5 to -2.9)	0.002	-42 (-67 to -17)	0.001
CCB[-] PPI[-] *2[+]	-8.0 (-10.9 to -5.1)	<0.0001	-30 (-45 to -15)	<0.0001
CCB[+] PPI[-] *2[+]	-11.0 (-16.9 to – 5.2)	<0.0001	-50 (-71 to -28)	<0.0001
CCB[-] PPI[+] *2[+]	-11.3 (-15.6 to -7.1)	<0.0001	-53 (-83 to -23)	0.001
CCB[+] PPI[+] *2[+]	-11.6 (-19.5 to -2.9)	0.008	-70 (-113 to -27)	0.001

Data are expressed as adjusted mean differences with (95% confidence intervals).
Mean differences are calculated between the seven groups versus the reference group of non-drug-exposed *CYP2C19*2*-noncarriers. Adjusted for gender, age, body mass index (in kg/m2, per unit), renal function, clopidogrel loading dose, diabetes mellitus, left ventricular ejection fraction of less than 45% and hypertension. P-values: ANCOVA with LSD. Definition of groups: CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-noncarriers, CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-noncarriers, CCB[+] PPI[+]*2[-]: CCB and PPI-exposed *CYP2C19*2*-noncarriers, CCB[-] PPI[-]*2[+]: non-drug-exposed *CYP2C19*2*-carriers, CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-carriers, CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-carriers and CCB[+] PPI[+]*2[+]: CCB and PPI-exposed *CYP2C19*2*-carriers

VerifyNow P2Y12 assay

For the VerifyNow P2Y12 assay, on-treatment platelet reactivity across the 8 groups was as follows: 189 ± 74 PRU for non-drug-exposed *CYP2C19*2*-noncarriers, 201 ± 77 PRU for CCB-exposed *CYP2C19*2*-noncarriers, 214 ± 76 PRU for PPI-exposed *CYP2C19*2*-noncarriers, 245 ± 74 PRU for CCB- and PPI-exposed *CYP2C19*2*-noncarriers, 219 ± 72 PRU for non-exposed *CYP2C19*2*-carriers, 244 ± 67 PRU for CCB-exposed *CYP2C19*2*-carriers, 256 ± 70 PRU for PPI-exposed *CYP2C19*2*-carriers and 271 ± 50 PRU for CCB- and PPI-exposed *CYP2C19*2*-carriers. Platelet reactivity was significantly different between the groups (p<0.0001, Figure 1B).

In pairwise comparisons we found that both in *CYP2C19*2*-noncarriers and -carriers, the exposure to PPIs, exposure to CCBs as well as the combined use of CCBs and PPIs was associated with significantly increased on-treatment platelet reactivity as compared to the reference group of non-exposed *CYP2C19*2*-noncarriers (Figure 1B). All associations remained statistically significant after the adjustment for confounders (Table 2).

According to the VerifyNow P2Y12 assay, 37.6% (*n*=272) of the study cohort exhibited HPR. All patients with more than 1 risk factor for clopidogrel poor-response were at a 1.6 to 7.4 times increased risk of HPR as compared to non-drug-exposed *CYP2C19*2*-noncarriers (Figure 2). Associations remained significant after the adjustment for confounders (data not shown).

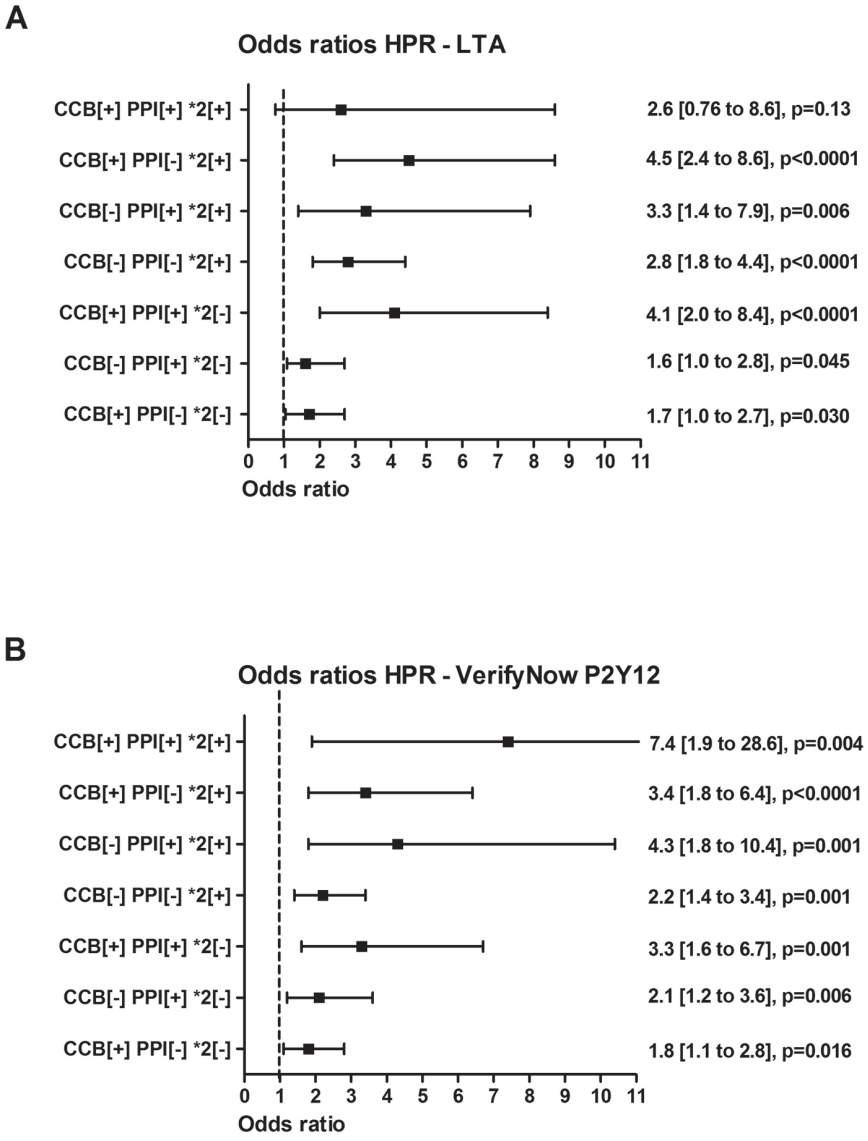

Figure 2: High on-treatment reactivity
The risk on high on-treatment platelet reactivity (HPR) of patients with one or more risk factors for clopidogrel poor-response (LTA >64.5%, VerifyNow P2Y12 > 236 PRU) as compared to non-drug-exposed *CYP2C19*2*-noncarriers. Definition of groups: CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-noncarriers, CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-noncarriers, CCB[+] PPI[+]*2[-]: CCB and PPI-exposed *CYP2C19*2*-noncarriers, CCB[-] PPI[-]*2[+]: non-drug-exposed *CYP2C19*2*-carriers, CCB[-] PPI[+]*2[-]: PPI-exposed *CYP2C19*2*-carriers, CCB[+] PPI[-]*2[-]: CCB-exposed *CYP2C19*2*-carriers and CCB[+] PPI[+]*2[+]: CCB and PPI-exposed *CYP2C19*2*-carriers.

Exposure to drugs, genotype and clinical outcome

During the 1-year follow-up period, a total of 64 events occurred (cumulative incidence 8.8%), of which 7 deaths, 43 nonfatal MIs, 5 cases with stent thrombosis, and 9 nonfatal ischemic strokes.

The variables diabetes mellitus, hypercholesterolemia, LVEF < 45%, left anterior descending artery stenting, graft stenting, and the diameter of the stent were included as confounders in the multivariate Cox proportional hazard models.

In the total study population, patients who were exposed to CCBs were not at an increased risk of the composite endpoint as compared to non-drug-exposed patients (Table 3). Similarly, exposure to PPIs was not associated with an increased risk of the composite endpoint (Table 3). Patients who were exposed to the combination of CCBs and PPIs were at a more than two-fold increased risk of developing the composite endpoint as compared to non-drug-exposed patients: HR 2.2 95% CI, 1.1-4.4, p=0.034. This association remained statistically significant after the adjustment for confounders: HR_{adj} 2.1 95% CI, 1.0-4.4, p=0.037.

Table 3: Hazard ratios for the composite endpoint

	Risk factor for clopidogrel poor response	HR (95% CI)	p-value
Total study population	CCB	1.8 (0.93-3.2)	0.084
	PPI	1.1 (0.51-2.2)	0.86
	CYP2C19*2-carriage	1.4 (0.82-2.3)	0.23
	CCB + PPI	2.2 (1.1-4.4)	0.034
	CYP2C19*17-carriage	0.74 (0.43-1.3)	0.26
CYP2C19*2-carriers	CCB	3.1 (1.1-9.0)	0.036
	PPI	1.9 (0.52-6.7)	0.34
	CCB + PPI	2.6 (0.70-9.3)	0.15
CYP2C19*2-noncarriers	CCB	1.2 (0.52-2.7)	0.68
	PPI	0.83 (0.34-2.0)	0.68
	CCB + PPI	1.9 (0.88-4.6)	0.078

Risk of the composite endpoint in the total study population and in two subgroups based on *CYP2C19*2*-status. CCB: calcium channel blocker, PPI: proton pump inhibitor, HR: hazard ratio, CI: confidence interval. P values: Cox proportional hazard models

In the total study population, carriage of *CYP2C19*2* was not associated with an increased risk of clinical endpoints: HR 1.4 95% CI, 0.82-2.3, p=0.23 (Table 3). Analyses of the three genotype groups showed that patients homozygous for the *CYP2C19*2* variant allele had no significantly increased risk of the endpoint (HR 2.3 95% CI, 0.81-6.3, p=0.12) nor had patients with one *2 allele (HR 1.3 95% CI, 0.74-2.2) as compared to *CYP2C19*2* noncarriers. The *CYP2C19*17* genotype was not associated with a decreased risk on the composite endpoint (for *1/*17: HR 0.79 95% CI, 0.45-1.4, p=0.39 and for *17/*17: HR 0.30 95% CI, 0.05-2.5, p=0.30), as compared to *CYP2C19*17* noncarriers).

When patients were divided into subgroups based on *CYP2C19*2* carrier status, CCB-exposed *CYP2C19*2*-carriers had a more than threefold increased risk on the endpoint than non-drug-exposed *CYP2C19*2*-carriers: HR 3.1 95% CI, 1.1-9.0, p=0.036, which remained significant after the adjustment for confounders: HR_{adj} 3.3 95% CI, 1.1-9.5, p=0.029. The exposure to PPIs was not associated with an increased risk on the outcome in *CYP2C19*2*-carriers, nor was the exposure to the combination of CCBs and PPIs in *CYP2C19*2*-carriers (Table 3).

In the subgroup of *CYP2C19*2*-noncarriers, no significant influence of CCB- or PPI-exposure was observed (table 3). *CYP2C19*2*-noncarriers that were exposed to the combination of CCBs

and PPIs were at an 1.9-fold increased risk of the composite endpoint, however, this did not reach statistical significance (Table 3).
Stratification according to different types of CCBs or PPIs did not lead to significant associations with clinical outcome.
For alternative definitions for clopidogrel adherence (95% and 100%), effect sizes were qualitatively similar (data not shown).

DISCUSSION

To the best of our knowledge this is the first study investigating the impact of combinations of pharmacokinetic risk factors for clopidogrel poor-response on platelet function and on the occurrence of adverse cardiovascular events in patients who underwent elective coronary stent implantation. We observed that patients with one pharmacokinetic risk factor for clopidogrel poor-response, i.e. exposure to CCBs, PPIs or carriage of *CYP2C19*2*, exhibited a mean relative increase of 11% in on-treatment platelet reactivity as compared to patients without one of these risk factors. The presence of more than one risk factor resulted in a mean relative increase of 28% in on-treatment platelet reactivity.
Our study demonstrated that patients with only one risk factor for clopidogrel poor-response were not at an increased risk of the composite endpoint of all-cause death, nonfatal myocardial infarction, stent thrombosis and ischemic stroke. However, patients who were exposed to the combination of CCBs and PPIs were at a more than 2-fold increased risk of the composite endpoint as compared to patients with no risk factors for clopidogrel poor-response. Also, exposure to CCBs in *CYP2C19*2*-carriers was associated with a more than 3-fold increased risk of the composite clinical endpoint. Carriage of *CYP2C19*17* was not associated with the composite endpoint. According to both platelet function and clinical outcome data, particularly patients who had two risk factors that interfere with clopidogrel metabolism at two different target sites were at increased risk of clopidogrel poor-response.

CYP2C19 plays an important role in both metabolic steps of the activation of clopidogrel.[5] It has been shown that CYP2C19 is responsible for up to 45% of the oxidation of the thiophene ring of clopidogrel to 2-oxo-clopidogrel and for almost 21% of the second step in which the thiopene ring is opened and both a carboxyl and the active thiol group is formed.[5] Several publications have demonstrated that carriers of *CYP2C19*2* exhibit a reduced formation of clopidogrel's active metabolite and a reduced pharmacodynamic platelet response to clopidogrel.[8-10, 20] Clinical data on the relevance of this genetic variation are conflicting. Studies of Collet *et al.*, Shuldiner *et al.* and Mega *et al.* have shown a significant association between carriage of *CYP2C19*2* and major adverse cardiovascular events (MACE).[8, 10, 21] In contrast, in a recent study of a high-risk population of patients with acute myocardial infarction undergoing PCI with stenting, *CYP2C19*2* was not associated with the incidence of MACE.[22] Furthermore, in the post-hoc genetic analysis of the CURE trial, no effect of *CYP2C19* loss-of-function alleles on cardiovascular risk was found.[23] However, it should be noted that the CURE population is different from the above-mentioned studies with regard to the rates of PCI with stenting, which was only 14.5% in the CURE trial.[23] The results regarding the association between *CYP2C19*2* and stent thrombosis as primary study endpoint are more consistent.[9, 24, 25] Based on the results of six trials, a recent meta-analysis concludes that carriers of *CYP2C19*2* are at an overall 2.8-fold increased risk of stent thrombosis as compared to noncarriers.[26] In the present study, only 0.7% (*n*=5) of the study population suffered from stent thrombosis. No significant association between *CYP2C19*2* and the occurrence of the composite endpoint was observed. Also,

*CYP2C19*17* did not have a protective effect, in contrast to findings of other studies.[22, 23]
Studies with surrogate laboratory endpoints have reported that CYP2C19-metabolized PPIs are associated with the response to PPIs but contradictory findings have been found for PPIs and MACE.[11, 12, 27] Particularly retrospective cohort studies have found a significant influence of concomitant PPI treatment on the occurrence MACE.[28, 29] In contrast, in the prematurely terminated COGENT trial, in which clopidogrel-treated patients were randomized according to omeprazole treatment, an effect of omeprazole on the risk of adverse cardiovascular events was absent during a median follow-up of 106 days.[30] In the present study, the increase in on-treatment platelet reactivity in PPI-users, was not translated into a higher risk of the composite endpoint in PPI-users. However, the use of PPIs combined with CCBs did lead to an increased risk of the composite endpoint.
The inhibitory effect of CCBs on the platelet response to clopidogrel is considered to be caused at the level of CYP3A4. CYP3A4 is considered to contribute the most of all iso-enzymes to the second step in clopidogrel's metabolism in which the active thiol metabolite is formed.[5] As all CCBs are substrates and inhibitors of CYP3A4, concomitant use is likely to inhibit the metabolism of clopidogrel.[31] Several prior publications have reported that CCBs are associated with increased on-treatment platelet reactivity.[13, 14, 32, 33] In our previous work we have demonstrated that the subgroup of CCBs that possesses no inhibiting properties on the intestinal efflux transporter P-glycoprotein (Pgp), i.e. amlodipine, has largest potential to interfere with clopidogrel's metabolism. Inhibition of Pgp by the concomitant use of Pgp-inhibiting CCBs (diltiazem, verapamil, nifedipine, barnidipine, felodipine and lercanidipine) may lead to a decreased intestinal efflux of clopidogrel, thereby increasing clopidogrel plasma concentrations and partly counteracting the effect of CCB-induced CYP3A4 inhibition.[13]

An important strength of our study is the availability of community pharmacy records to define drug exposure during the entire follow-up period post-PCI. Exposure to clopidogrel, CCBs and PPIs was defined based on pharmacy records, which validity to measure drug exposure has shown to be good.[34] A limitation of this study might be related to the fact that we only investigated the influence of two genetic variants on platelet reactivity and clinical outcome. For example, genetic variations in the *CYP2C9* and *ABCB1* genes have also been associated with clinical outcome in clopidogrel-treated patients.[9,35, 36] Furthermore, the number of events in our study was limited. Unfortunately, the present study was underpowered to investigate differences between subgroups of CCBs and PPIs on the influence on the occurrence of adverse cardiovascular events.

In conclusion, the main finding of our study is that the presence of more than one of the investigated pharmacokinetic risk factors for clopidogrel poor-response is associated with an increased risk of adverse cardiovascular events within one year after elective coronary stenting. On the contrary, patients with only one pharmacokinetic risk factor did not have an increased risk of adverse cardiovascular events. In the prediction of adverse cardiovascular events in patients undergoing coronary stenting, it is important to examine combinations of pharmacokinetic risk factors instead of a single one.

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3.3

INTERACTIONS BETWEEN *CES2, CYP2C9, UGT1A6* AND *COX1* GENETIC VARIANTS AND ACETYLSALICYLIC ACID MODIFY THE RISK OF MYOCARDIAL INFARCTION

Ankie M. Harmsze
Vera H.M. Deneer
Anke-Hilse Maitland-van der Zee
Bas J.M. Peters
Patrick C. Souverein
Jochem W. van Werkum
Jurriën M. ten Berg
Mathieu M. Tjoeng
Anthonius de Boer
Olaf H. Klungel

Submitted

ABSTRACT

Aim

To investigate whether genetic variants in the enzymes CES2, CYP2C9, UGT1A6, COX1 and COX2 modify the effectiveness of acetylsalicylic (ASA) therapy in the prevention of myocardial infarction (MI).

Methods

In a population-based registry of pharmacy records linked to hospital discharge records (PHARMO RLS), a nested case-control study was performed. Cases had a first MI between 1991 and 2005, controls were matched to MI cases by age, gender and region. Patients were genotyped for tagging SNPs in genes coding for *CES2, CYP2C9, UGT1A6, COX1* and *COX2*. Logistic regression was used to assess the interaction between ASA and genetic variants on the risk of MI and to adjust for confounding.

Results

The influence of 22 tagging SNPs was assessed in 853 cases and 887 control subjects. ASA-use was associated with a reduced risk of MI (adjusted odds ratio (OR_{adj}) 0.74 (95% CI 0.56-0.97), p=0.032). The *CES2* rs11568311 and *CYP2C9* rs1057910 variants were found to interact with ASA treatment (adjusted synergy index (SI_{adj}) 0.43 (0.21-0.90),p=0.025 and SI_{adj} 0.44 (0.22-0.91), p=0.026, respectively). Two variants in the *UGT1A6* gene showed a significant interaction with ASA (rs11563251, p=0.044 and rs3771342, p=0.023). In addition, two *COX1* variants were associated with modified effectiveness of ASA (rs10306135: SI_{adj} 1.5 (1.0-2.7), p=0.042 and rs5788: SI_{adj} 1.5 (1.0-2.6), p=0.023). No significant interactions between other genetic variants and the effect of ASA were observed.

Conclusion

Common genetic variants in the *CES2*, *CYP2C9*, *UGT1A6* and *COX1* genes might be associated with modified effectiveness of ASA in the prevention of MI.

INTRODUCTION

Acetylsalicylic acid (ASA) is the most commonly used antiplatelet drug in both primary and secondary prevention of cardiovascular disease.[1] ASA has been found to reduce the risk of myocardial infarction (MI), stroke, or death by approximately 25% in patients who are at increased risk of cardiovascular events.[2, 3] Nonetheless, a considerable number of patients still suffers from cardiovascular events while on ASA-treatment. The variability in response to ASA is likely to be multifactorial in origin. Besides inadequate dosing and non-compliance, genetic variations in ASA's pathway of action are considered to play a role.[4]

The enzymes human carboxylesterase 2 (CES2), UGT1A6 and CYP2C9 are involved in ASA metabolism.[5, 6] In literature, several *CES2* genetic variations were found to decrease the hydrolysis of ASA.[6] Furthermore, it has been shown that genetic variations in *UGT1A6* modulate the protective effect of ASA on colon adenoma risk.[7] The dependence on CYP2C9 enzyme activity is illustrated by studies showing that carriers of *CYP2C9* variant alleles were more prone to develop acute gastrointestinal bleeding when they received ASA as compared to noncarriers.[8, 9] Other relevant targets in the pharmacogenetics of ASA are those related to ASA pharmacodynamics. The antiplatelet effect of ASA is a result of direct and irreversible inhibition of the activity of cyclooxygenase-1 (COX1), thereby decreasing the formation of precursors of prostaglandins and thromboxanes from arachidonic acid.[1, 4] Thromboxane A2 (TXA_2) induces platelet aggregation and is a powerful vasoconstrictor.[10] Genetic variants in *COX1* were shown to modulate arachidonic acid-induced platelet aggregation and serum thromboxane B2 (TXB_2) levels in patients treated with ASA.[11, 12] The COX2 enzyme is known to produce TXA_2 from arachidonic acid in monocytes, macrophages and vascular endothelial cells.[1] ASA is more potent in inhibiting the COX1 enzyme compared with the COX2 isoform.[1] However, carriage of a functional genetic variant in *COX2* was found to result in greater benefits from ASA treatment in the primary prevention of MI.[13, 14]

Although it has been demonstrated that variability within the genes encoding for the enzymes CES2, CYP2C9, UGT1A6, COX1 and COX2 affects the response to ASA, the impact of these gene-treatment interactions on ischemic cardiovascular events remains unknown. Therefore, the aim of this case-control study was to investigate whether variations in genes coding for the enzymes CES2, CYP2C9, UGT1A6, COX1 and COX2 modify the effectiveness of ASA therapy in the prevention of a first MI.

DESIGN AND METHODS

Design and setting

This study was performed as part of the Utrecht Cardiovascular Pharmacogenetics (UCP) study.[15, 16] Participants from the UCP studies were enrolled from the population-based PHARMO-Morbidity Record Linkage System (PHARMO RLS, www.pharmo.nl).[17] The PHARMO RLS links drug dispensing histories from a representative sample of Dutch community pharmacies to the national registration of hospital discharges (Dutch National Medical Registry). Approval for this study was obtained from the Medical Ethics Committee of the University Medical Center Utrecht, The Netherlands.

Case and control definition

Patients who received a prescription for an antihypertensive drug or had hypercholesterolemia (prescription for a cholesterol-lowering drug or total cholesterol > 5.0 mmol/L), were selected from the PHARMO RLS for pharmacogenetic studies on antihypertensive drugs and statins respectively. Details have been described elsewhere.[16] From this cohort, a nested case-control study was designed in which patients hospitalized for nonfatal MI (ICD-9 code 410) were included as cases. The index date was defined as the date of hospitalization for the first MI. Cases were eligible if they were registered in the database for at least one year and were older than 18 years at the index date. Controls met the same eligibility criteria as the cases, but had not developed MI. They were matched to the patients on age (±1 year), gender and region. They were assigned the same index date as the patient to whom they had been matched. Participants were contacted through community pharmacies.

Ascertainment of drug exposure and questionnaires

Coded pharmacy records were used to ascertain exposure to ASA and other drugs. In PHARMO, complete pharmacy records are available as of 1991, including the day of delivery, daily dose, and duration of therapy. For each patient we identified all prescriptions for concomitant drug use and coded each patient as current, past, or no usage of a certain drug. Questionnaires were used to assess cardiovascular risk factors such as smoking, hypertension, hypercholesterolemia, diabetes mellitus, use of alcohol, diet, history of cardiovascular disease, family history of cardiovascular disease, weight and height.

DNA collection, selection of genetic variants, genotyping.

Patients were sent three cotton swabs and tubes containing buffer to collect buccal cell samples or an Oragene collection kit (DNA Genotek, Ottawa, Canada). To assess all common genetic variants for *CES2, CYP2C9, UGT1A6, COX1* and *COX2*, tagging SNPs (single nucleotide polymorphisms) within 200 bp (up- and downstream) with a minor-allele frequency (MAF) higher than 0.05 and r2 >0.8 were selected with QuickSNP version 1.1.[18] With this strategy, both synonymous and non-synonymous SNPs were obtained. If the selected tagging SNPs were not present on the Illumina chip, they were substituted with a SNP in linkage disequilibrium ($r^2 > 0.8$). When no SNP's in linkage disequilibrium were found, the selected SNP was removed from the SNP list. A final set of 22 genetic variants was obtained. Genotyping of the genetic variants was performed using the Illumina (Illumina Inc. San Diego, CA, USA) IBC Candidate Gene array, version 3.

Statistical analysis

The Kolmogorov–Smirnov test was used to check for normal distribution of continuous data. Continuous data were normally distributed and were expressed as mean ± standard deviation (SD). Comparisons between groups were made with the chi-square test for categorical variables. For continuous variables, comparisons were made with the two-sided Student's t-test. Chi-square tables were used to compare the observed number of each genotype with those expected for a population in Hardy–Weinberg equilibrium ($p > 0.05$).

Logistic regression analysis was used to study the association between ASA and the risk of MI and to adjust for potential confounders. The matching variables age, gender, region and index date were included in each statistical model. The covariates ischemic heart disease, hypertension, diabetes mellitus, hypercholesterolemia, the use of ACE inhibitors, diuretics, coumarins and beta blockers showed at least a 10% change in the regression coefficient (beta) for ASA use and

were included in the logistic regression models. We estimated the multiplicative synergy index (SI), which is the ratio of the odds ratio (OR) in those with the genetic variant to the OR in those without the genetic variant. A p value <0.05 was considered statistically significant. In addition, q values (the positive false discovery rate (pFDR) analogue of the p value) were calculated for each gene-treatment interaction that was tested to account for multiple testing.
Statistical analysis was performed using SPSS software (version 17.0.1 for Windows; SPSS Chicago, IL).

RESULTS

Characteristics of the study population and genotype

In total, 853 cases and 887 control subjects were included in the analysis. Table 1 summarizes the characteristics of the cases and control subjects. There were no significant differences with regard to gender, age, diabetes mellitus, and body mass index (BMI) between both groups. The control group consisted of significantly more patients with hypertension and ischemic heart disease and were more often treated with diuretics, beta blockers, ACE inhibitors and coumarins than the cases. Cases were more frequently current smokers and had hypercholesterolemia more often. Approximately 25% of the study population was treated with low dose ASA. The use of ASA was associated with a reduced risk of MI (OR_{adj} 0.74 95% CI, 0.56-0.97, p=0.032).

Drug-gene interactions and risk of MI

In total, 22 genetic variants were selected. All genetic variants were in Hardy-Weinberg equilibrium (p>0.05, tables 2, 3, 4). The tables show the SI's for the gene-drug interactions between ASA and the genetic variants in the *CES2*, *CYP2C9*, *UGT1A6*, *COX1* and *COX2* gene, respectively. In addition, the OR's for the efficacy of ASA in reducing the risk of MI for carriers and noncarriers of the minor (variant) allele are shown.
Table 2 shows that an intronic SNP in the *CES2* gene (rs11568311) exhibited a significant interaction with ASA treatment: SI_{adj} 0.43 (95% CI 0.21-0.90), p=0.025. After stratification on the *CYP2C9* 1075A>C genotype (rs1057910; *CYP2C9*3*), the adjusted OR for ASA effectiveness in carriers of the 1075C variant allele was 0.43 (95% CI 0.19-0.94) compared to 0.78 (95% CI 0.58-1.1) for noncarriers of the 1075C allele, resulting in a SI_{adj} of 0.44 (95% CI 0.22-0.91, p=0.026) (table 2).
Two variants in the *UGT1A6* gene showed a significant interaction with ASA treatment (rs11563251 and rs3771342, table 3). In carriers of the *UGT1A6* variant alleles, the effect of ASA was more profound than among noncarriers of the variant alleles, which resulted in significant drug-gene interactions (for rs11563251: SI_{adj} 0.66 95% CI, 0.36-0.98 and for rs3771342: SI_{adj} 0.50 95% CI 0.28-0.91, p=0.023).
In the *COX1* gene, two variants showed a significant interaction with ASA treatment, indicating that the protective effect of ASA on the prevention of MI is larger in noncarriers of these variant alleles than in carriers (for rs10306135: SI_{adj} 1.5 95% CI, 1.0-2.7), p=0.042 and for rs5788: SI_{adj} 1.5 95% CI, 1.0-2.6, p=0.036, table 4). The two variants in the *COX2* gene did not modulate the effectiveness of ASA therapy in the prevention of MI (table 4). Q values for the five significant gene-treatment interactions were 0.16 (tables 2, 3, 4). Subsequently, stratified analysis in patients with and without ischemic heart disease were performed. Similar findings were observed in both groups (data not shown).

Table 1: Baseline characteristics by case-control status

	Cases n=853	Control subjects n=887
Age, years	63.8 ± 10.2	63.5 ± 10.1
Men	614(72.0)	637 (71.8)
BMI>30kg/m2	161 (18.9)	126 (14.2)
Hypertension	639 (74.9)	821 (92.6)*
Smoking status		
Current	184 (21.6)	117 (13.2)*
Past	418 (49.0)	482 (54.4)
Diabetes mellitus		
No medication	82 (9.6)	55 (6.2)
Medication	107 (12.6)	76 (8.6)
Hypercholesterolemia		
No medication	299 (35.1)	178 (20.1)*
Medication	341 (39.9)	237 (26.7)
Ischemic heart disease	306 (35.9)	198 (22.3)*
Use of alcohol		
<2 units/day	303 (35.5)	291 (37.3)*
>2 units/day	64 (7.5)	109 (14.0)
Family history of CVD		
Yes < 60 years	67 (7.9)	2 (0.2)*
Yes > 60 years	175(21.6)	82 (9.2)
Physical activity leisure >4 hrs a week	628 (73.6)	676 (76.2)
Diuretics		
Current use	124 (14.5)	214 (24.1)*
Past use	121 (14.2)	132 (14.9)
Calcium channel blockers		
Current use	193 (22.6)	176 (19.8)
Past use	93 (10.9)	114 (12.9)
Beta blockers		
Current use	346 (40.6)	413 (46.6)*
Past use	154 (18.1)	170 (19.2)
ACE inhibitors		
Current use	166 (19.5)	290 (32.7)*
Past use	91 (10.7)	108 (12.2)
Angiotensin 2 antagonists		
Current use	66 (7.7)	98 (11.0)*
Past use	102 (12.0)	116 (13.1)
Coumarins		
Current use	41 (4.8)	63 (7.1)
Past use	59 (6.9)	73 (8.2)
Statins		
Current use	226 (26.5)	173 (19.5)*
Past use	60 (7.0)	36 (3.1)
Acetylsalicylic acid	251 (29.4)	220 (24.8)*

ACE: Angiotensin Converting Enzyme, BMI: body mass index, CVD: cardiovascular disease. Continuous data expressed as mean ± standard deviation. Categorical data expressed as number (%).*p<0.05, Students t-test for continuous variables, chi-square test for categorical variables

Table 2: Association between the use of acetylsalicylic acid and the incidence of myocardial infarction stratified by CYP2C9 and CES2 genotype

Gene	SNP	MAF	Minor allele	n	OR (95% CI)b	p	SI (95% CI)a	p	SI (95% CI)b	p	q
CYP2C9	rs1057910	0.06	NC	1532	0.78 (0.58-1.1)	0.10					
			C	208	0.43 (0.19-0.94)	0.036	0.56 (0.30-1.1)	0.073	0.44 (0.22-0.91)	0.026	0.16
CYP2C9	rs1856908	0.39	NC	651	0.75 (0.48-1.2)	0.21					
			C	1089	0.77 (0.54-1.1)	0.13	0.91 (0.58-1.4)	0.66	0.88 (0.54-1.5)	0.62	0.63
CYP2C9	rs4086116	0.19	NC	1128	0.67 (0.47-0.97)	0.032					
			C	612	0.81 (0.52-1.2)	0.32	0.85 (0.55-1.3)	0.45	0.90 (0.55-1.5)	0.68	0.63
CYP2C9	rs9332197	0.05	NC	1557	0.76 (0.57-1.0)	0.070					
			C	183	0.67 (0.28-1.6)	0.38	0.83 (0.42-1.6)	0.58	0.81 (0.37-1.8)	0.59	0.63
CYP2C9	rs2153628	0.22	NC	1074	0.81 (0.58-1.1)	0.23					
			C	666	0.64 (0.40-1.0)	0.060	0.96 (0.62-1.5)	0.85	1.1 (0.65-1.8)	0.80	0.63
CYP2C9	rs10509679	0.17	NC	1199	0.81 (0.59-1.1)	0.22					
			C	541	0.62 (0.38-1.0)	0.068	0.88 (0.55-1.4)	0.59	0.87 (0.51-1.5)	0.61	0.63
CYP2C9	rs9332238	0.19	NC	1129	0.67 (0.47-0.96)	0.027					
			C	611	0.82 (0.53-1.3)	0.36	0.85 (0.55-1.3)	0.47	0.91 (0.56-1.5)	0.71	0.63
CES2	rs11568311	0.07	NC	1510	1.0 (0.77-1.3)	0.90					
			C	230	0.47 (0.23-0.98)	0.045	0.45 (0.23-0.91)	0.025	0.43 (0.2-0.90)	0.025	0.16
CES2	rs2241409	0.18	NC	1163	0.97 (0.71-1.3)	0.84					
			C	577	0.72 (0.16-1.1)	0.15	0.79 (0.49-1.3)	0.31	0.78 (0.47-1.3)	0.33	0.60

CES2: human carboxylesterase 2, SNP : single nucleotide polymorphism, MAF: minor allele frequency, OR: odds ratio, SI: synergy index, NC: noncarriers, C: carriers
a= adjusted for age, gender, index date and region
b= adjusted for age, gender, index date, region, hypercholesterolemia, hypertension, diabetes mellitus, ischemic heart disease, use of coumarins, beta-blockers, ACE inhibitors and diuretics

Table 3: Association between the use of acetylsalicylic acid and the incidence of myocardial infarction stratified by UGT1A6 genotype

SNP	MAF	Minor allele	n	OR (95% CI)b	p	SI (95% CI)a	p	SI (95% CI)b	p	q
rs11563251	0.10	Noncarriers	1389	0.83 (0.61-1.1)	0.23					
		Carriers	351	0.44 (0.23-0.85)	0.015	0.64 (0.37-0.98)	0.042	0.66 (0.36-0.98)	0.044	0.16
rs3771342	0.12	Noncarriers	1351	0.87 (0.64-1.2)	0.37					
		carriers	389	0.41 (0.21-0.78)	0.006	0.67 (0.42-1.1)	0.086	0.50 (0.28-0.91)	0.023	0.16
rs6759892	0.41	Noncarriers	587	0.79 (0.48-1.3)	0.37					
		carriers	1153	0.70 (0.50-0.97)	0.031	0.84 (0.52-1.3)	0.44	1.0 (0.60-1.7)	0.99	0.68
rs2070959	0.33	Noncarriers	800	0.78 (0.51-1.2)	0.24					
		Carriers	940	0.71 (0.50-1.0)	0.069	0.80 (0.52-1.2)	0.31	1.0 (0.62-1.6)	0.96	0.68
rs28946889	0.26	Noncarriers	954	0.83 (0.57-1.2)	0.32					
		Carriers	786	0.67 (0.44-1.0)	0.058	1.1 (0.69-1.6)	0.80	0.83 (0.51-1.4)	0.45	0.63
rs4143828	0.38	Noncarriers	665	0.89 (0.57-1.4)	0.59					
		Carriers	1075	0.67 (0.47-0.95)	0.025	1.0 (0.66-1.6)	0.90	0.78 (0.47-1.3)	0.34	0.60
rs10929303	0.21	Noncarriers	1103	0.66 (0.47-0.93)	0.017					
		Carriers	637	0.90 (0.57-1.4)	0.66	1.6 (1.0-2.5)	0.035	1.4 (0.87-2.4)	0.15	0.40

SNP : single nucleotide polymorphism, MAF: minor allele frequency, , OR: odds ratio, SI: synergy index.
a= adjusted for age, gender, index date and region
b= adjusted for age, gender, index date, region, hypercholesterolemia, hypertension, diabetes mellitus, ischemic heart disease, use of coumarins, beta-blockers, ACE inhibitors and diuretics

Table 4: Association between the use of acetylsalicylic acid and the incidence of myocardial infarction stratified by COX1 and COX2 genotype

Gene	SNP	MAF	Minor allele	n	OR (95% CI)b	p	SI (95% CI)a	p	SI (95% CI)b	p	q
COX1	**rs1213266**	0.07	Noncarriers	1494	0.73 (0.54-0.98)	0.035					
			Carriers	246	0.82 (0.39-1.7)	0.60	1.1 (0.61-2.1)	0.71	1.1 (0.54-2.1)	0.84	0.63
COX1	**rs10306135**	0.14	Noncarriers	1299	0.67 (0.49-0.92)	0.012					
			Carriers	441	1.0 (0.58-1.8)	0.98	1.4 (1.0-2.3)	0.038	1.5 (1.0-2.7)	0.042	0.16
COX1	**rs5788**	0.13	Noncarriers	1312	0.65 (0.48-0.88)	0.006					
			Carriers	428	1.2 (0.67-2.3)	0.50	1.5 (1.0-2.5)	0.037	1.5 (0.83-2.6)	0.036	0.16
COX1	**rs12238505**	0.06	Noncarriers	1522	0.80 (0.64-1.0)	0.055					
			Carriers	218	0.60 (0.25-1.4)	0.24	1.0 (0.52-2.0)	0.98	1.1 (0.51-2.3)	0.85	0.63
COX2	**rs2066826**	0.11	Noncarriers	1375	0.71 (0.53-0.97)	0.032					
			Carriers	365	0.78 (0.44-1.4)	0.42	1.1 (0.65-1.9)	0.68	1.2 (0.69-2.2)	0.48	0.63
COX2	**rs2745557**	0.17	Noncarriers	1138	0.71 (0.52-0.98)	0.038					
			Carriers	532	0.78 (0.47-1.3)	0.33	0.87 (0.53-1.4)	0.58	0.85 (0.50-1.5)	0.56	0.63

SNP : single nucleotide polymorphism, MAF: minor allele frequency, OR: odds ratio, SI: synergy index, a= adjusted for age, gender, index date and region, b= adjusted for age, gender, index date, region, hypercholesterolemia, hypertension, diabetes mellitus, ischemic heart disease, use of coumarins, beta-blockers, ACE inhibitors and diuretics

DISCUSSION

This case-control study aimed to determine whether genetic variants in enzymes involved in ASA pharmacokinetics and -dynamics were associated with the effectiveness of ASA in the prevention of MI. Out of 22 SNPs that were tested, we found six significant drug-gene interactions that include the *CES2* 11568311, *CYP2C9* rs1057910, the *UGT1A6* rs11563251 and rs3771342, and the *COX1* rs5788 and rs10306135 genetic variations.
After absorption, ASA is rapidly deacetylated to salicylic acid, primarily by human carboxylesterase-2 (CES2).[6] In our study, we found that carriers of the rs11568311 variant in the *CES2* gene exhibited more benefit from ASA in the prevention of MI as compared to noncarriers of the genetic variant, which resulted in a significant drug-gene interaction (SI_{adj} 0.43, p=0.025).
CYP2C9 and UGT1A6 play an important role in the metabolism of salicylic acid.[5] It is thought that the effects of ASA are due to actions by both the acetyl portion of the intact molecule as well as by the active salicylic acid metabolite.[5, 20] ASA inhibits platelets by acetylating COX1, thereby blocking the production of TXA_2.[21] Salicylic acid is also reported to reduce the COX-mediated prostaglandin E_2 synthesis, thereby decreasing the formation of TXA_2.[20, 22]

The *CYP2C9* 1075G variant allele (rs1057910) has been associated with decreased metabolism of several drugs including clopidogrel and acenocoumarol.[23, 24] In our study, we observed a significant interaction between the use of ASA and *CYP2C9* 1075A>G (SI 0.44 95% CI 0.22-0.91, p=0.026). Carriers of the 1075G variant allele exhibited increased efficacy of ASA in the prevention of MI as compared to noncarrriers. In literature, several studies have found a high frequency of the *CYP2C9* variant allele among patients who developed gastrointestinal bleeding with NSAIDs including ASA.[8, 9] These observations are in line with our findings, indicating an enhanced efficacy of ASA by reduced metabolism caused by the loss-of-function allele 1075G.
For UGT1A6, significant drug-gene interactions between two *UGT1A6* variants and ASA were observed (for rs11563251 SI_{adj}: 0.66 and for rs3771342 SI_{adj}: 0.50, respectively). Carriage of these variants resulted in an increased effect of ASA on the prevention of MI compared to noncarriers. Lower UGT1A6 activity in subjects carrying these genetic variants may inhibit the metabolism of ASA resulting in higher exposure of salicylic acid. Regarding the metabolism of ASA, the most investigated variant allele of UGT1A6 is *UGT1A6*2* (rs2070959). As compared with the *UGT1A6* wildtype, the genetic variant *UGT1A6*2* is associated with 30-50% lower enzyme activity, leading to reduced glucuronidation rates of salicylic acid.[5] This is illustrated by several studies indicating that *UGT1A6* genetic variants have a modulating effect on the effectiveness of ASA in the chemoprevention of colon adenomas.[7, 25] Carriers of at least one *UGT1A6*2* allele were found to benefit more from ASA as compared to noncarriers of this genetic variant. However, it has recently been shown that healthy volunteers carrying the *UGT1A6*2* allele had lower salicylic acid plasma concentrations, as compared to noncarriers of the variant allele.[26] This indicates that *UGT1A6*2*-carriers exhibit higher glucuronidation rates of salicylic acid than *UGT1A6* wildtype homozygotes. In our study, we did not observe a significant drug-gene interaction between the *UGT1A6*2* and ASA therapy.

We found two variations in the *COX1* gene to be associated with reduced efficacy of ASA. We hypothesize that the suppression of *COX1* is diminished as a consequence of variation in the *COX1* gene. Other studies also reported genetic variations as relevant factors for platelet response to ASA.[11, 12] Particularly, the minor allele of the promotor variant 842A>G (rs1236913) in

COX1 was found to be associated with increased platelet reactivity.[12] This variant is in complete linkage disequilibrium with C50T (rs3842787) in the signal peptide. Unfortunately, neither of these variants was present on the Illumina IBC Candidate Gene array. The best proxy to tag these variants was rs1213266 (r^2=0.787). This variant however, was not associated with the efficacy of ASA in our study.

To the best of our knowledge, this is the first study to show that common genetic variability within the *CES2*, *CYP2C9*, *UGT1A6* and *COX1* genes is associated with the modification of the effectiveness of ASA in the prevention of MI. An important strength of our study is the availability of community pharmacy records to define drug exposure and clinical outcome data (hospital records). In the Netherlands low-dose ASA is only provided on prescription and ASA exposure was defined based on pharmacy records, which validity to measure drug exposure has shown to be good.[27] Furthermore, we comprehensively covered the common genetic variability in five important genes encoding for enzymes involved in the pharmacokinetics and –dynamics of ASA by using tagging SNPs. However, a limited number of initially selected tagging SNPs, or genetic variants in linkage disequilibrium with the tagging SNPs, were not present on the Illumina IBC Candidate Gene array. It is therefore possible that we missed potentially significant genetic variations (for example the promoter variant 842A>G in the *COX1* gene). In addition, q values were calculated to address the multiple testing issue. For the significant interactions the q value was approximately 0.2, suggesting possibly one to be a false positive.[28] Furthermore, we acknowledge that in the setting of the prevention of a first MI, ASA therapy was possibly more often initiated in patients at highest risk of cardiovascular events, which could explain the higher exposure to ASA in cases as compared to control subjects. For example, cases had more often ischemic heart disease than control subjects. However, after adjustment for confounders, ASA treatment was associated with a 26% reduction in the risk of a first MI. This effect size is comparable to that was found in a meta analysis based on 5 large randomized clinical trials, in which ASA was found to be associated with a 32% reduction in the risk of a first MI.[29] Furthermore, drug-gene interactions are most likely not influenced by this potential bias, as the physician is unaware of a patient's genotype.

In conclusion, this study suggests that common genetic variants in the *CES2*, *CYP2C9*, *UGT1A6* and *COX1* gene are important modifiers of the effectiveness of ASA in the prevention of MI. Validation in well-powered, independent populations will be ultimately necessary to confidently conclude that these genetic variations are significantly associated with the effectiveness of ASA in the prevention of cardiovascular events.

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3.4

THE INFLUENCE OF *CYP2C19*2* AND **17* ON ON-TREATMENT PLATELET REACTIVITY AND BLEEDING EVENTS IN PATIENTS UNDERGOING ELECTIVE CORONARY STENTING

Ankie M. Harmsze
Jochem W. van Werkum
Christian M. Hackeng
Hendrik J.T. Ruven
Johannes C. Kelder
Heleen J. Bouman
Nicoline J. Breet
Jurriën M. ten Berg
Olaf H. Klungel
Anthonius de Boer
Vera H.M. Deneer

Submitted

ABSTRACT

Objectives

To investigate the impact of genotypes based on the loss-of-function variant *CYP2C19*2* and the gain-of-function variant *CYP2C19*17* on on-treatment platelet reactivity and on the occurrence of TIMI major bleedings in 820 clopidogrel-treated patients who underwent elective coronary stenting.

Methods

On-treatment platelet reactivity was quantified using ADP-induced light transmittance aggregometry (LTA) and the VerifyNow P2Y12 assay. Postdischarge TIMI major bleedings within one year after enrollment were recorded.

Results

In total, 25 major bleedings (3.0% of the study population) were observed. Patients with the *CYP2C19*1/*17* and **17/*17* genotypes exhibited a lower magnitude of platelet reactivity as compared to patients with the *CYP2C19*1/*1* genotype (for the LTA adjusted mean difference: -5.8% 95% CI -9.6 to -2.1, p=0.002) . Patients with the **1/*17* and **17/*17* genotype had a 2.7-fold increased risk on the occurrence of major bleedings (HRadj: 2.7 95% CI 1.1 to 7.0, p=0.039). The genotypes **2/*17*, **1/*2* and **2/*2* exhibited higher on-treatment platelet reactivity as compared to wildtype (p<0.0001). However, this was not translated into an altered risk on major bleedings as compared to wildtype (HR: 1.3 (0.45 to 4.0), p=0.60).

Conclusion

Patients with the *CYP2C19*1/*17* and **17/*17* genotype have a lower magnitude of on-treatment platelet reactivity and are at a 2.7-fold increased risk on postdischarge TIMI major bleedings events after coronary stenting than patients with the *CYP2C19*1/*1* genotype. The genotypes **2/*17*, **1/*2* and **2/*2* are associated with increased on-treatment platelet reactivity, however, this is not translated into a lower risk on bleeding events.

INTRODUCTION

In the secondary prevention of thrombotic events following percutaneous coronary interventions (PCI), patients are treated with aspirin and clopidogrel. This combination has shown to be effective in reducing the risk of recurrent thrombotic events.[1] However, the use of dual antiplatelet therapy is also associated with an increased risk of bleeding complications.[2,3] Recent findings indicate that both thrombotic events such as myocardial infarction, and bleeding events post-PCI have a comparable prognostic impact on mortality-rates one year after the intervention.[3,4]

Clopidogrel is a prodrug that needs to be metabolized by the hepatic cytochrome P450 enzyme system to become active.[5] CYP2C19 plays a key role in this process.[5,6] This is illustrated by the fact that the loss-of-function variant allele of *CYP2C19*, *CYP2C19*2*, is associated with a reduced formation of the clopidogrel's active thiol metabolite, a higher on-treatment platelet reactivity, and an increased risk of atherothrombotic events after PCI.[6-9]

Another common *CYP2C19* variant allele, *CYP2C19*17*, is associated with an increased enzymatic activity which has been linked to a lower magnitude of on-treatment platelet reactivity.[10,11-13] Furthermore, carriage of *CYP2C19*17* was associated with an increased risk of combined TIMI major and minor bleeding events in patients within 30 days after PCI.[11] However, the influence of combined *CYP2C19*17* and *CYP2C19*2* variant alleles on bleeding events remains unknown. For clinical practice it is important to gain more information on how different *CYP2C19*-genotypes translate into phenotypic response.

The aim of this present study was to investigate the impact of *CYP2C19* genotypes based on *CYP2C19*2* and *CYP2C19*17* on on-treatment platelet reactivity in patients undergoing elective coronary stenting and on the occurrence of postdischarge major bleeding events.

METHODS

Patients

In total, 1069 patients with established coronary artery disease scheduled for elective PCI with stent implantation were consecutively recruited in the setting of the prospective POPular study.[14] Blood for DNA analysis was available of 820 patients who were still on therapy with clopidogrel and aspirin at the end of the one-year follow-up period or at the time the bleeding event occurred. This constitutes the study population for the present study. All patients used clopidogrel during the entire follow-up period (according to patient information and verified by medication histories obtained from community pharmacies (drug dispenses for at least 351 days post-PCI)) or at least until the bleeding event took place. Clopidogrel and aspirin maintenance doses were 75 mg and 80 to 100 mg daily, respectively.

Prior to PCI, all patients were pretreated with clopidogrel (defined as 75 mg/day therapy for >5 days or a loading dose of 300 mg >24 hours before PCI or 600 mg >4 hours before PCI) and aspirin (80-100 mg/day >10 days). A small subset of the patients (6.5%) received intravenous glycoprotein IIb/IIIa inhibitors during the intervention, but these drugs were always administered after blood collection for platelet function testing.

Patients using concomitant medication known to affect platelet function other than aspirin (ie, nonsteroidal anti-inflammatory agents, dipyramidole, upstream glycoprotein IIb/IIIa inhibitors) and patients with a known platelet function disorder or a whole blood platelet count of less

than 150 x 10^3/μL were excluded.
Written informed consent was obtained before PCI. The study was conducted according to the principles of the Declaration of Helsinki and the local institutional review board approved the study.

Blood sampling and genotyping
Before heparinization, whole blood samples were drawn from the femoral or radial artery sheath into 3.2% citrate tubes for light transmittance aggregometry. Testing with the VerifyNow P2Y12 assay (Accumetrics, San Diego, California) was performed using Greiner tubes. Genomic DNA was isolated from K3-EDTA blood (MagNA Pure LC DNA Isolation kit 1, MagNA Pure; Roche Diagnostics; Basel, Switzerland). The *CYP2C19* alleles *CYP2C19*2* (rs4244285) and *CYP2C19*17* (rs12248560) were identified by real time PCR. DNA sequence analysis was used to validate the genotyping procedure.

Platelet function testing
The magnitude of on-treatment platelet reactivity was quantified using light transmittance aggregometry (LTA) with adenosine diphosphate (ADP) in a final concentration of 20 μmol/L as the agonist (platelet aggregation at 360 sec) and the VerifyNow P2Y12 assay. Details of these methods have been reported previously.[14,15] All measurements were completed within 2 hours of blood collection.

Definition of bleeding events
To evaluate the impact of combined *CYP2C19*2* and *CYP2C19*17* allelic variants on the occurrence of postdischarge (> 48 hours) major bleedings we used the Thrombolysis in Myocardial Infarction (TIMI) criteria ("*TIMI major bleeding*").[16] Information on the occurrence of bleeding events was obtained by telephone contact to all patients at 30 days and 12 months and verified using source documents from medical records from the referring hospitals. An independent committee that was blinded for platelet function data adjudicated the bleeding events through review of medical record source documents.

Statistical methods
Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables were expressed as frequencies and percentages. Baseline continuous data were analyzed by ANOVA and categorical data by chi-square test when appropriate. Chi-square tables were used to compare the observed number of the *CYP2C19* genotypes with that expected for a population in Hardy-Weinberg equilibrium ($p>0.05$). The Kolmogorov-Smirnov test was used to check for normal distribution of continuous data. All data were normally distributed. Continuous platelet function data were compared between groups with ANOVA followed by the least significant difference test (LSD). ANCOVA was used to adjust for the potential confounding factors gender, age, body mass index (BMI, in kg/m2), current smoking, renal dysfunction (estimated glomerular filtration rate <60 mL/min), clopidogrel loading dose and the use of coumarins at study entry. The mean differences and 95% confidence intervals calculated by LSD post hoc tests were also adjusted for these confounders.
Univariate and multivariate Cox proportional hazards models were used to investigate the effect of the *CYP2C19* genotypes on bleeding events. In models with bleeding as the dependent variable, the use of glycoprotein IIb/IIIa antagonists was also added as confounder. This poten-

tial confounder was not included in models with platelet function as the dependent variable because all blood samples for platelet function testing were collected prior to the administration of these drugs. All selected potential confounders were shown to be associated with the occurrence of bleeding events in patients with ACS or patients with coronary artery disease undergoing PCI or with an aggravated response to clopidogrel, according to recent publications.[2,3,17-20]

Statistical analysis was performed using SPSS software (version 15.0.1 for Windows; SPSS Chicago, IL). A p value <0.05 was considered statistically significant.

RESULTS

Study population and CYP2C19 genotyping

In total, *CYP2C19*2* and *CYP2C19*17* genotypes were available for 820 patients who were on clopidogrel treatment during the entire 1-year follow up after PCI or at the time the bleeding event occurred. Baseline characteristics of the study population are shown in table 1. Of the 820 patients, 351 (42.8%) had the *CYP2C19*1/*1* genotype, 207 (25.2%) had the *CYP2C19*1/*17* genotype, 33 (4.2%) had the *CYP2C19*17/*17* genotype, 47 (5.7%) had the *CYP2C19*2/*17* genotype, 157 (19.1%) patients had the *CYP2C19*1/*2* genotype and 25 (3.0%) patients had the *CYP2C19*2/*2* genotype. The **2* and **17* alleles were in complete linkage equilibrium (D'=1, r^2=0.04). No significant deviations from Hardy-Weinberg equilibrium were observed (p=0.17 for *CYP2C19*2* and p=0.63 for *CYP2C19*17* genotypes). Except for the use of proton pump inhibitors (p=0.039), clinical variables and co-medication were well balanced between the six genotype groups.

Table 1: Baseline characteristics of the study population according to CYP2C19 genotype

Variable	*17*/17 n=33 (4.2%)	*1/*17 n=207 (25.2%)	*1/*1 n=351 (42.8%)	*2/17 n=47 (5.7%)	*1/*2 n=157 (19.1%)	*2/*2 n=25 (3.0%)	p-value
Age (years)	61.9±9.5	63.3±10.5	63.1±10.9	64.1±8.9	63.8±9.9	63.3±12.0	0.94
Gender (male)	27 (79.4)	155 (74.9)	266 (75.8)	34 (72.3)	110 (70.1)	18 (72.0)	0.77
BMI (kg/m2)	27.2±4.3	27.4±4.3	27.1±3.8	27.2±3.8	26.5±3.9	28.7±4.3	0.13
GFR < 60 mL/min	5 (12.1)	28 (13.5)	55 (15.7)	7 (14.7)	23 (14.6)	4 (16.0)	0.99
Diabetes mellitus	7 (20.6)	30 (14.6)	71 (20.3)	10 (21.3)	26 (16.6)	5 (20.0)	0.59
Smoking	3 (8.8)	25 (12.1)	30 (8.5)	6 (12.8)	15 (9.6)	3 (12.0)	0.80
Hypertension	22 (64.7)	151 (72.9)	273 (77.8)	39 (83.0)	116(73.9)	22 (88.0)	0.17
Hypercholestero-lemia	27 (79.4)	161(77.8)	285 (81.2)	38 (80.9)	130 (82.8)	18 (72.0)	0.75
Previous MI	19 (55.9)	87 (42.0)	148 (42.2)	15 (31.9)	66 (42.0)	10 (40.0)	0.45
Previous CABG	3 (8.8)	14 (6.8)	25 (7.1)	1 (2.1)	11 (7.0)	3 (12.0)	0.65
Family history of CAD	27 (79.4)	131 (63.3)	220 (62.7)	27 (57.4)	92 (58.6)	15 (60.0)	0.32
LVEF <45%	3 (8.8)	30 (14.5)	55 (15.7)	10 (21.3)	30 (19.1)	3 (12.0)	0.52
Clopidogrel loading dose	18 (52.9)	100 (48.3)	185 (52.7)	24 (51.1)	72 (45.9)	18 (72.0)	0.20
Co-medication							
Aspirin	33 (100)	207 (100)	351 (100)	47 (100)	157 (100)	25 (100)	1.00
Beta-blockers	26 (76.5)	160 (77.3)	274 (78.1)	38 (80.9)	124(79.0)	15 (60.0)	0.39
Statins	28 (84.8)	165 (79.7)	286 (81.5)	42 (89.4)	127 (80.9)	20 (80.0)	0.58
ACE-inhibitors	15 (44.1)	72 (34.8)	132 (37.6)	18 (38.3)	48 (30.6)	11 (44.0)	0.51
Proton pump inhibitors	5 (14.7)	49 (23.7)	105 (29.9)	8 (17.0)	32 (20.4)	9 (36.0)	0.039
Calcium channel blockers	13 (39.4)	68 (32.9)	110 (31.3)	18 (38.3)	57 (36.3)	10 (40.0)	0.70
Coumarins	1 (2.9)	19 (9.2)	22 (6.3)	3 (6.4)	20 (12.7)	2 (8.0)	0.17
Gp IIb/IIIa antagonists	3 (8.8)	12 (5.8)	23 (6.6)	2 (4.3)	8 (5.1)	4 (16.0)	0.53

Data are expressed as mean ± SD or number of patients (%); p-value: ANOVA for continuous variables and chi-square test for categorical variables between the six groups, BMI: body mass index, GFR: glomerular filtration rate, MI: myocardial infarction, CABG: coronary artery bypass grafting, PPI: proton pump inhibitor, LVEF: left ventricular ejection fraction, ACS: acute coronary syndrome, PCI: percutaneous coronary intervention

***CYP2C19* genotypes and platelet function**

For the total study population, the mean values ± SD of on-treatment platelet reactivity as assessed with the LTA was 40.6% ± 24.3 and for the VerifyNow P2Y12 assay 211 ± 76 PRU.

Light transmittance aggregometry

For the LTA, on-treatment platelet reactivity across the six *CYP2C19* genotypes was as follows: 32.2% ± 23.1 for **17/*17* patients, 33.9% ± 23.9 for **1/*17* patients, 39.2% ± 23.6 for **1/*1* patients, 49.5% ± 24.0 for **2/*17* patients, 48.8% ± 22.8 for **1/*2* patients and 64.3% ± 12.5 for **2/*2* patients. Platelet reactivity was significantly different between the 6 genotype groups (p<0.0001, figure 1A).

In pairwise comparisons we found that **1/*17* and **17/*17* had decreased platelet reactivity as compared to **1/*1* patients (figure 1A). The three genotype groups **2/*17*, **1/*2* and **2/*2* showed increased platelet reactivity (figure 1A). All differences remained significant after the adjustment for confounders (table 2).

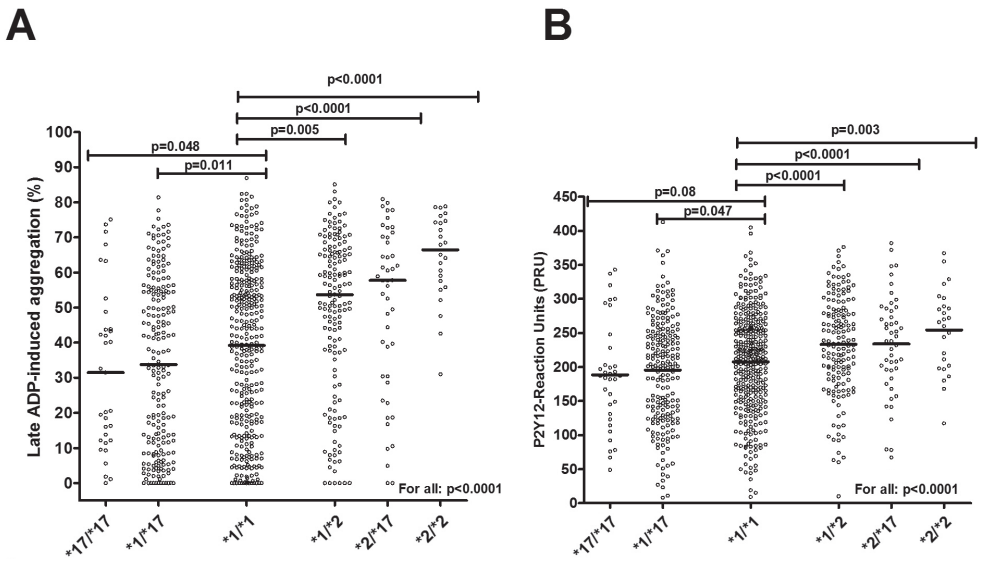

Figure 1: On-treatment platelet reactivity according to *CYP2C19* genotype

Individual values (n=820) of on-treatment platelet reactivity stratified by *CYP2C19* genotype as measured with 20 µmol/L ADP-induced LTA (panel A), and the VerifyNow P2Y12 assay (panel B). Mean values are presented by solid lines. ADP: 5'-adenosine diphosphate, LTA: light transmittance aggregometry, PRU: P2Y12 reaction units., p-values: ANOVA with LSD

Table 2: Differences in on-treatment platelet reactivity according to *CYP2C19* genotype

	Light transmittance aggregometry		VerifyNow P2Y12 assay	
Genotype	**Mean difference (%)**	**p-value**	**Mean difference (PRU)**	**p-value**
CYP2C19*17/*17	-6.3 (-8.7 to -0.3)	0.043	-17 (-42 to 7.7)	0.17
CYP2C19*1/*17	-5.7 (-9.6 to -1.8)	0.004	-14 (-26 to -1.3)	0.031
CYP2C19*2/*17	9.7 (2.8 to 16.6)	0.006	24 (2.4 to 46)	0.030
CYP2C19*1/*2	9.8 (5.5 to 14.0)	<0.0001	24 (11 to 38)	<0.0001
CYP2C19*2/*2	21.9 (12.6 to 31.2)	<0.0001	38 (8.8 to 66)	0.011

Data are expressed as adjusted mean differences (95% confidence intervals) after adjustment for gender, age (per unit), body mass index (in kg/m2, per unit), current smoking, renal dysfunction, clopidogrel loading dose and the use of coumarins at study entry. Mean differences are calculated between CYP2C19*2 and *CYP2C19*17* genotypes versus the *CYP2C19*1/*1* genotype. P-values: ANCOVA with LSD

VerifyNow P2Y12 assay

For the VerifyNow P2Y12 assay, on-treatment platelet reactivity across 6 *CYP2C19* genotypes was as follows: 189 ± 79 PRU for **17/*17* patients, 196 ± 79 PRU for **1/*17* patients, 208 ± 74 PRU for **1/*1* patients, 234 ± 74 PRU for **2/*17* patients, 233 ± 72 PRU for **1/*2* patients and 254 ± 62 PRU for **2/*2* patients. Platelet reactivity was significantly different between the 6 genotype groups (p<0.0001, figure 1B).

Pairwise comparisons showed that the differences between **1/*17, *2/*17, *1/*2* and **2/*2* versus **1/*1* were statistically significant (figure 1B). For patients with the **17/*17* genotype a trend towards a significant association with platelet reactivity was observed (p=0.08). The differences in platelet reactivity between **1/*17, *2/*17, *1/*2* and **2/*2* versus the **1/*1* genotype remained statistically significant after the adjustment for confounders (table 2).

Conversion of six genotypes into three phenotypes for clopidogrel metabolism

Based on platelet reactivity values and the expected activities of the enzymes, the six genotypes were merged into 3 categories, which define 3 main phenotypes: ultrarapid metabolizers (*CYP2C19*1/*17* and **17/*17* patients), extensive metabolizers (*CYP2C19*1/*1* patients) and intermediate/poor metabolizers (*CYP2C19*1/*2*, **2/*17* and **2/*2* patients). Differences in on-treatment platelet reactivity between the three genotype groups were statistically significant (figure 2A-B).

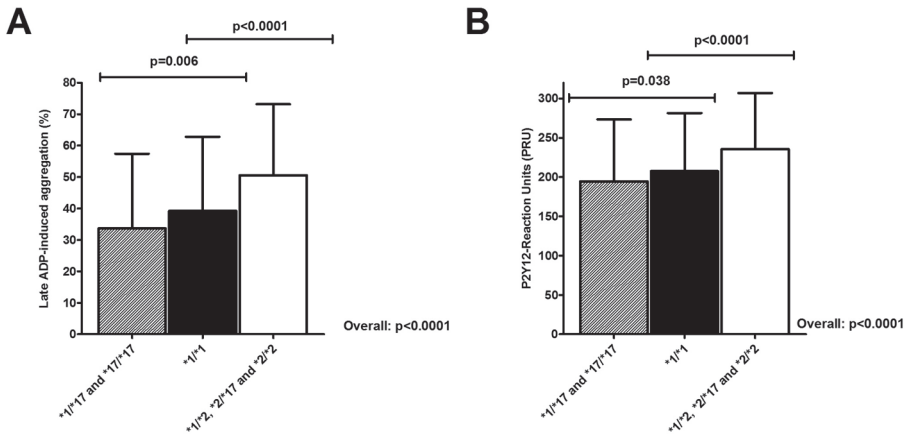

Figure 2: On-treatment platelet reactivity stratified according to CYP2C19 phenotype being ultrarapid, extensive and intermediate/poor metabolizers

On-treatment platelet reactivity stratified by three (A, ultrarapid metabolizers: *CYP2C19*1/*17* and **17/*17*; B, extensive metabolizers: *CYP2C19*1/*1* and C, intermediate/poor metabolizers *CYP2C19*1/*2, *2/*17* and *2/*2) phenotype groups as measured with 20 µmol/L ADP-induced LTA (panel A), and the VerifyNow P2Y12 assay (panel B). Data are presented as mean ± standard deviation, p-values: ANOVA with LSD

For the LTA, mean differences (95% CI) in platelet reactivity were as follows: for ultrarapid metabolizers vs. extensive metabolizers: -5.5% (-9.3 to -0.50), p=0.006 (adjusted mean difference -5.8% (-9.6 to -2.1), p=0.002) and for intermediate/poor metabolizers vs. extensive metabolizers 11.4% (7.4 to 15.3), p<0.0001 (adjusted mean difference 11.1% (7.3 to 14.9), p<0.0001). When measured with the VerifyNow P2Y12 assay, mean differences (95% CI) in platelet reactivity were as follows: for ultrarapid metabolizers vs. extensive metabolizers -13 PRU (-25 to -0.7), p=0.038 (adjusted mean difference -14 PRU (-26 to –2.4), p=0.018) and for intermediate/poor metabolizers vs. extensive metabolizers mean difference 28 PRU (16 to 41), p<0.0001 (adjusted mean difference 26 PRU (14 to 38), p<0.0001).

CYP2C19 genotypes, platelet function and bleeding events

In total, 25 postdischarge (>48 hours) TIMI major bleedings were observed (3.0% of the total study population). Patients who developed a TIMI major bleeding event during follow-up exhibited lower on-treatment platelet reactivity as compared to patients without a TIMI major bleeding event; after the adjustment for confounders (major bleeding vs. no bleeding): 30.5% (21.7 to 39.4) vs. 40.9% (39.3 to 42.5), p=0.040 for LTA and 183 PRU (156 to 211) vs. 212 PRU (207-217), p=0.044 for the VerifyNow P2Y12 assay.

The risk for TIMI major bleedings differed statistically significant between the three genotypes (p=0.048). Patients with the ultrarapid metabolizer phenotype exhibited a more than twofold increased risk on major bleedings as compared to extensive metabolizers (HR 2.6, 95% CI 1.0 to 6.4, p=0.046), which remained significant after the adjustment for potential confounders (HR_{adj} 2.7 95% CI, 1.1-7.0, p=0.039, figure 3).

As compared to extensive metabolizers, intermediate/poor metabolizers had no altered risk of major bleedings (HR 1.3 95% CI, 0.45-4.0, p=0.60).

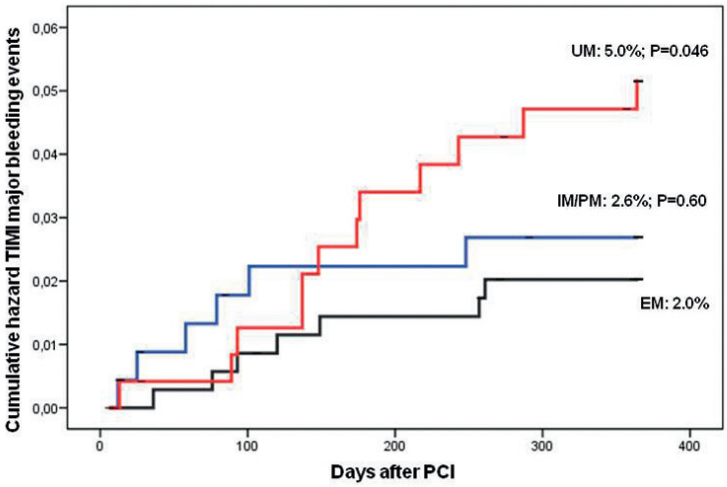

Figure 3: Rate of TIMI major bleedings in subgroups of CYP2C19 phenotypes

The rate of TIMI major bleedings was 5.0% among ultrarapid metabolizers (UM: *CYP2C19*1/*17* and **17/*17*), 2.6% among intermediate/poor metabolizers (IM/PM; *CYP2C19*1/*2, *2/*17* and **2/*2*) and 2.0% in extensive metabolizers (EM; *CYP2C19*1/*1*). PCI: percutaneous coronary intervention

DISCUSSION

In this study we found that on-treatment platelet reactivity as measured with two well-established platelet function tests, significantly differed across 6 *CYP2C19* genotype groups. It was demonstrated that the genotypes *CYP2C19*1/*17* and **17/*17* were associated with decreased on-treatment platelet reactivity as compared to patients with the *CYP2C19*1/*1* genotype. In contrast, patients with the *CYP2C19*2/*17*, **1/*2* and **2/*2* genotype had a significantly higher magnitude of on-treatment platelet reactivity than *CYP2C19*1/*1* subjects.
According to the results of the platelet function tests we converted six genotypes into three phenotypes for clopidogrel metabolism; (1) ultrarapid, which consisted of patients with the **17/*17* and **1/*17* genotypes, (2) extensive (*CYP2C19*1/*1*) and (3) intermediate/poor metabolizers, consisting of patients with the *CYP2C19*2/*17, *1/*2* and **2/*2* genotype. Ultrarapid metabolizers had a more than twofold increased risk of major bleedings whereas intermediate/poor metabolizers had no altered risk as compared to extensive metabolizers. We observed lower platelet reactivity values in patients developing major bleedings as compared to the remaining patients. This strengthens the hypothesis that the **1/*17* and **17/*17* genotypes influence bleeding risk by increasing the formation of clopidogrel's active thiol metabolite.
For the implementation of pharmacogenetics of clopidogrel in daily clinical practice it is relevant to translate *CYP2C19* genotypes into predicted phenotypes of clopidogrel metabolism. Based on the results of this study we conclude that patients with the **17/*17* and **1/*17* genotypes have a predicted phenotype of ultrarapid metabolizer and the *CYP2C19*2/*17* genotype corresponds with a predicted phenotype of intermediate metabolizer.

CYP2C19 plays an important role in het metabolism of clopidogrel. Recent studies have clearly demonstrated that carriage of the loss-of-function variant allele *CYP2C19*2* was found to be associated with reduced formation of the active thiol metabolite of clopidogrel, increased on-treatment platelet reactivity, as well as worse clinical outcome in clopidogrel-treated patients.[6-9]
On the other hand, *CYP2C19*17* is associated with increased enzyme activity.[10] The **17* variation in the 5'-flanking region of the gene can specifically bind nuclear proteins, which leads to increased gene transcription and expression.[10] The influence of *CYP2C19*17* on the magnitude of on-treatment platelet reactivity and clinical outcome has been evaluated in previous studies.[11-13] In three studies, carriage of *CYP2C19*17* was found to be associated with decreased values of on-treatment platelet reactivity.[11-13] One study, however, did not find the association with platelet aggregation. This might be caused by the limited power of this small study (*n*=268).[21] Recently, the isolated and interactive impact of *CYP2C19*2* and **17* variant alleles on platelet reactivity in patients on chronic clopidogrel therapy was assessed.[12] The authors found that both genetic variations were independent predictors for the antiplatelet properties of clopidogrel. Furthermore, a recent study reported that carriers of *CYP2C19*17* had an almost twofold increased risk on the 30-day incidence of combined TIMI major and minor bleedings after PCI, however the influence of carriage of *CYP2C19*2* in addition to **17* was not explored.[11]
Recently, Gurbel and co-workers reported that carriage of one *CYP2C19*2* and one **17* variant allele would result in normal enzymatic activity phenotype.[22] However, our results indicate that the **2/*17* genotype is significantly associated with higher platelet reactivity values as compared to patients with the **1/*1* genotype. Our findings are confirmed by the recently published platelet function data by Sibbing and co-workers.[12] These authors also found that the heighte-

ning effect of *CYP2C19*2* was only partly diminished by concomitant presence of *CYP2C19*17*. This might be explained by the fact that the presence of **2* leads to a complete loss of enzyme function whereas *CYP2C19*17* only enhances existing enzyme capacity.[10,12] Therefore the overall impact of *CYP2C19*2* on platelet function is thought to be far more prominent as compared to the impact of *CYP2C19*17*.[12] In both our and Sibbing's cohort, this is illustrated by the fact that intermediate/poor metabolizers have a mean relative increase in platelet reactivity compared to extensive metabolizers that is approximately twice as high as the mean relative decrease of platelet reactivity in ultrarapid metabolizers compared to extensive metabolizers.

The importance of bleeding complications after coronary stent implantations has been demonstrated by several studies indicating that bleeding is strongly associated with adverse cardiovascular events, including death.[3,23] The prognostic implications of bleeding on one-year mortality are similar or even higher than that of having a recurrent myocardial infarction.[24] The results from clinical studies showing the poor prognosis of patients with bleeding events underline the need to define a specific subgroup of patients in whom the risk of developing bleeding events is substantially increased. Knowledge of the *CYP2C19*17* and **2* genotype, in combination with other clinical data, might be clinically useful in determinating the risk of bleeding complications in patients undergoing coronary stent implantation.

Our study has limitations that merit mention. First, we investigated the influence of only two genetic variants on platelet reactivity and the occurrence of bleeding complications. We did not explore interactions with other genetic variants on both endpoints. In addition, although the number of included patients in this study is large, we observed a relatively small number of patients with bleeding complications.

In conclusion, patients with the *CYP2C19*1/*17* and **17/*17* genotype have a lower magnitude of on-treatment platelet reactivity and are at a 2.7-fold increased risk on postdischarge TIMI major bleedings events after coronary stenting than patients with the **1/*1* genotype. The genotypes **2/*17, *1/*2* and **2/*2* are associated with increased on-treatment platelet reactivity, however, this is not translated into a lower risk on bleeding events.

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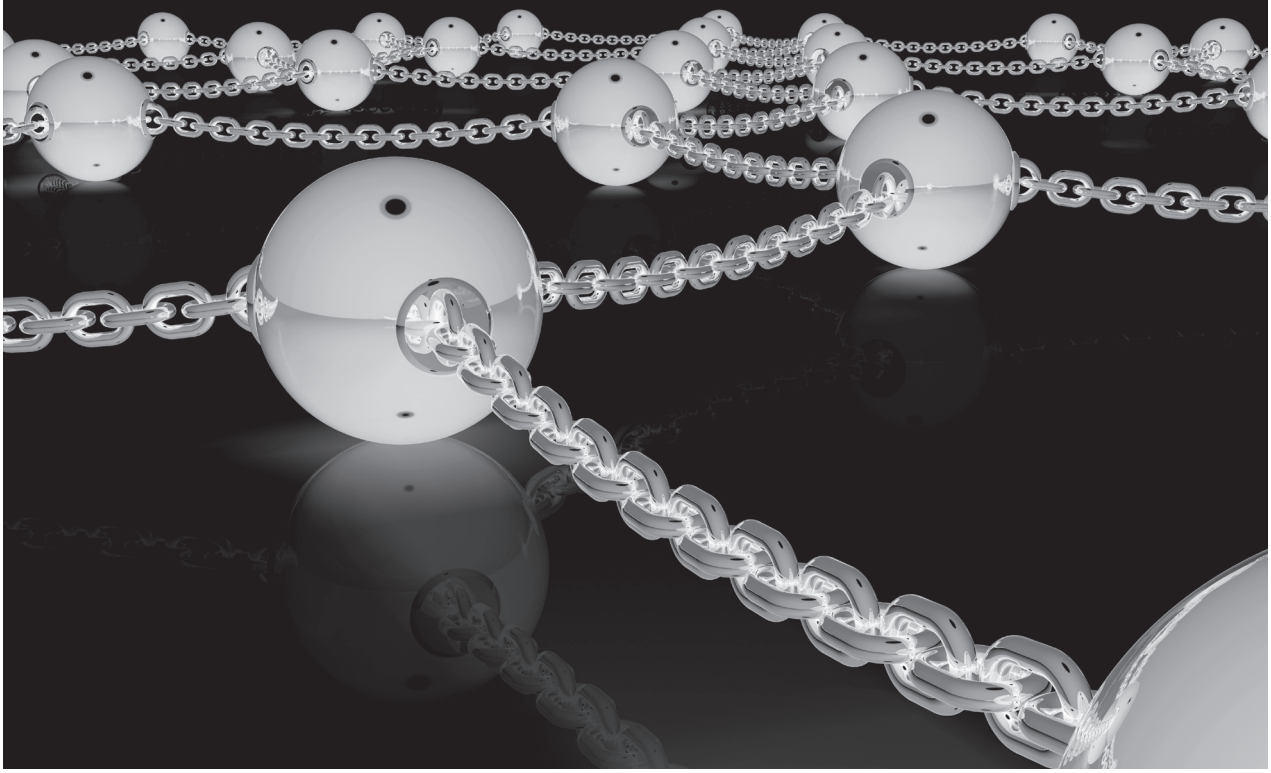
4

GENERAL DISCUSSION



INTRODUCTION

Antiplatelet therapy plays an important role in the treatment of cardiovascular disease. The combination of acetylsalicylic acid (ASA) and clopidogrel ("dual antiplatelet therapy") is routine care in patients with acute coronary syndromes (ACS) or undergoing percutaneous coronary interventions (PCI). Although the efficacy of ASA and clopidogrel is well established, several studies have shown interindividual variability in the response to these antiplatelet drugs.[1, 2] In this thesis, studies are presented in which the impact of genetic variants and co-medication on the response to these antiplatelet agents was investigated. In this chapter, the main findings of this thesis will be discussed and put into a broader context of clinical implications and further research.

MAIN FINDINGS

Pharmacogenetics and clopidogrel

One of the aims in this thesis was to investigate the impact of genetic variations on the antiplatelet properties of clopidogrel and ASA. ABCB1 is the main drug transporter associated with clopidogrel absorption.[3] Once absorbed, only approximately 15% of clopidogrel is transformed in the liver in a 2-step process that is mediated by several cytochrome (CYP) P450 enzymes (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4).[4] Of these, CYP2C19 is responsible for approximately 45% of the first step (the formation of 2-oxo-clopidogrel) and approximately 20% of the final step – the generation of the pharmacologically active thiol metabolite.[4]

CYP2C19

The *CYP2C19*2* variant is a G681A nucleotide substitution at the junction of intron 4 and exon 5 on chromosome 10q24.1, which introduces a splicing defect resulting in a truncated, non-functional protein. There are ethnic differences in its distribution; approximately 50% of the East-Asian, 34% of the African American and 25% of the Caucasian population carries at least one copy of the loss-of-function *CYP2C19*2* allele. Other genetic variations associated with impaired CYP2C19 activity (*CYP2C19*3, *4, *5, *8*) are much less common in Caucasians and African Americans.[5]

In a cohort of 428 patients on dual antiplatelet therapy with clopidogrel and ASA undergoing elective PCI, we demonstrated that carriage of *CYP2C19*2* was associated with increased on-treatment platelet reactivity as measured with light transmittance aggregometry (LTA) and the point-of-care VerifyNow P2Y12 assay (Chapter 2.1). In line with these results we observed in a case-control study that carriage of *CYP2C19*2* was associated with an 1.7-fold increased risk of stent thrombosis (Chapter 3.1). Stent thrombosis is considered to be the most serious thrombotic complication after PCI. This acute re-occlusion of the artery causes acute myocardial infarction and is associated with substantial morbidity and mortality. The reported incidence of stent thrombosis varies from 0.2% to 4.6%.[6, 7] However, in Chapter 3.2 we did not observe a significant effect of *CYP2C19*2* on the 1-year combined endpoint of major adverse cardiovascular events (MACE; all-cause death, myocardial infarction, stent thrombosis and ischemic stroke) in a cohort of 725 patients who underwent elective coronary stenting (hazard ratio (HR) 1.4, 95% confidence interval (CI) 0.82 to 2.3).

The influence of *CYP2C19*2* has been evaluated in several studies. Carriage of at least one variant allele has been associated with lower active clopidogrel metabolite levels and increased on-treatment platelet reactivity.[8, 9] Clopidogrel pharmacogenetic studies have reported divergent results for the influence of the carriage of *CYP2C19*2* variant alleles on the combined endpoint of cardiovascular death, myocardial infarction or ischemic stroke. Half of the presently published studies found that carriage of a least one CYP2C19-variant allele was associated with an increased risk of adverse cardiovascular events (Table 1).
The impact of *CYP2C19*2* is more pronounced for the outcome of stent thrombosis than for the broader outcome of (cardiovascular) death, myocardial infarction and stroke. The majority of the studies, including ours[10], indicate that carriage of *CYP2C19*2* is associated with an increased risk of stent thrombosis (table 1). This observation logically follows from the larger risk reduction that has been documented with clopidogrel on the outcome stent thrombosis than on the other outcomes.[21, 22] In the PLATO, CURE and FAST-MI studies only 66, 16 and 70% of the patients underwent PCI with stenting, respectively (table 1).[14, 15, 18] The absence of an association between *CYP2C19*2* and the 1-year incidence of MACE in these studies might be partly explained by the fact that there was a lower percentage of cases with stent thrombosis. However, in the PLATO study, there was a significant difference in the rate of MACE at 30 days after randomization between carriers and noncarriers of *CYP2C19* loss-of-function-enzymes (5.7% in carriers of *CYP2C19* loss-of-function-alleles vs. 3.8% in noncarriers (p=0.028).[15] Another difference between the studies is the proportion of patients with ACS. In the study described in Chapter 3.2, about one quarter of the patients had ACS as the indication for PCI. The remaining patients were diagnosed with coronary artery disease. The ISAR and FAST-MI studies, in which the association between *CYP2C19*2* and clinical outcome was also absent, included less than 50% of patients with ACS.[17, 18] It is possible that the detrimental effect of *CYP2C19*2* may predominantly exist in high-risk patients.

In March 2010, the US Food and Drug Administration (FDA) announced a boxed warning on clopidogrel, stating that the drug has a diminished effect in individuals based on their *CYP2C19* genotype, specifically in those who carry 2 reduced-function CYP2C19 alleles (www.fda.gov). The FDA referenced a crossover study of 40 healthy subjects who were treated with 300 mg of clopidogrel followed by 75 mg per day and with 600 mg clopidogrel followed by 150 mg per day. The study found that individuals with 2 reduced-function *CYP2C19* alleles, as compared with carriers of one or none, exhibited substantially decreased active drug metabolite levels and inhibition of platelet aggregation. However, it should be noted that a large number of pharmacokinetic and pharmacodynamic studies have also found that individuals (including healthy volunteers but also patients with coronary artery disease) who carry only one reduced function CYP2C19 allele already have a blunted pharmacologic response to treatment to clopidogrel.[20, 23-29]

Table 1: *CYP2C19*2* carrier status and the risk of major adverse cardiovascular events and stent thrombosis in patients treated with clopidogrel

Author, year of publication	Name study	Study design	PCI (%)	ACS (%)	N total	HR MACE	N stent thrombosis	HR stent thrombosis
Collet, 2009[11]	AFIJI	Prospective, observational	73	100	259	5.4 (2.3-12.5)	12	6.0 (1.8-20.8)
Giusti, 2009[12]	RECLOSE	Prospective, observational	100	70	772	2.3 (1.1-4.8)	24	2.6 (1.1-5.7)
Harmsze, 2010[10]		Case-control	100	40	596	-	176	1.6 (1.1-2.3)
Harmsze, this thesis	POPULAR	Prospective, observational	100	27	725	1.4 (0.82-2.3)	5	ND
Mega, 2009[13]	TRITON-TIMI	Randomized controlled	100	100	1459	1.5 (1.1-2.2)	17	3.1 (1.2-8.0)
Paré, 2010[14]	CURE	Randomized controlled	16	100	5059	0.86 (0.63-1.2)	ND	ND
Wallentin, 2010[15]	PLATO	Randomized controlled	66	100	5148	1.2, NS	56	ND
Shuldiner, 2009[16]	CLEAR-PLATELETS	Prospective, observational	100	0	228	4.0 (1.1-14.0)	3	4.8 (0.43-52.7)
Sibbing, 2009[17]	ISAR	Prospective, observational	100	34	2485	1.2 (0.89-1.7)	23	2.5 (1.1-5.6)
Simon, 2009[18]	FAST-MI	Prospective, observational	70	53	2208	0.79 (0.59-4.8)	ND	ND
Tiroch, 2010[19]		Prospective, observational	100	100	928	0.89 (0.64-1.3)*	10	1.2 (0.30-4.6)
Trenk, 2008[20]	EXCELSIOR	Prospective, observational	100	0	797	1.6 (0.52-5.1)	5	0.57 (0.06-5.1)

PCI: percutaneous coronary intervention, ACS: acute coronary syndrome, HR: hazard ratio, MACE: major adverse cardiovascular events, n: number, ND: not determined, NS: not significant

* Calculated from data taken from the original text

Of note, it should be acknowledged that only a limited part of the variability in response to clopidogrel can be explained by the *CYP2C19* loss-of-function alleles. In one study, *CYP2C19* genetic variations accounted for only 12% of the variability in the effect of clopidogrel, as measured using ADP-induced platelet aggregation, while the clinical factors BMI, age and levels of high-density lipoprotein cholesterol and triglycerides accounted for less than 10% of the variability.[16]

In 2006, a novel gain-of-function allele, *CYP2C19*17*, was identified. The **17* variation in the 5'-flanking region of the gene can specifically bind nuclear proteins, which leads to increased gene transcription and expression.[30] About 27% of the Caucasian population has the *CYP2C19*1/*17* or **17/*17* genotype compared to 1% of the Chinese and 3% of the Japanese population.[31] Although the carriage of *CYP2C19*17* has been associated with a reduced risk on the occurrence of ischemic events in two studies, we did not observe such an association in our study (Chapter 3.2).[14, 19] However, in Chapter 3.5 we showed that patients with the *CYP2C19*1/*17 or *17/*17* had a more than twofold risk of 1-year post-discharge TIMI major bleedings than patients with the *CYP2C19*1/*1* genotype in a cohort of patients undergoing elective PCI. These results were accompanied by reduced platelet reactivity values in patients with the *CYP2C19*1/*17* and **17/*17* genotype, which was also observed by others.[32-34] In the same study, we assessed the influence of combined CYP2C19*2 and*17 alleles on the response to clopidogrel. The *CYP2C19*2* and *CYP2C19*17* alleles were found to be in complete linkage disequilibrium (r^2=0.04). Carriers of the *CYP2C19*2/*17* genotype exhibited increased on-treatment platelet reactivity, as compared to patients with the *CYP2C19*1/*1* genotype. The heightening effect of *CYP2C19*2* was only partly diminished by concomitant presence of *CYP2C19*17*. This might be explained by the fact the presence of *2 leads to a complete loss of enzyme function, while *CYP2C19*17* only enhances existing enzyme activity. Carriage of *CYP2C19*2* did not result in a decreased risk of major bleedings as compared to *CYP2C19* wild-type patients. From these findings, which were confirmed by others,[34] it can be concluded that when patients are genotyped for *CYP2C19*17* to estimate the risk of bleeding after PCI, the presence of *CYP2C19*2* should also be assessed. On the other hand, when patients are genotyped for CYP2C19*2 to predict the risk of ischemic events after PCI, genotyping of *CYP2C19*17* is not indicated.

CYP2C9

CYP2C9 is involved in the second step of clopidogrel activation. The *CYP2C9* gene is located on chromosome 10q24.2. *CYP2C9* is highly polymorphic; more than 30 non-synonymous variations have been described. Their prevalence shows considerable interethnic differences. The two most important allelic variants are *CYP2C9*2* (Arg144Cys) and *CYP2C9*3* (Ile359Leu), encoding enzymes with a decreased activity compared to wild-type alleles. In Caucasian populations allele frequencies for *CYP2C9*2* range from 10 to 18% and for *CYP2C9*3* from 7 to 9%. In 2007, it was reported that the carriage of *CYP2C9* loss-of-function variants in healthy individuals was associated with lower exposure to clopidogrel's active metabolite and diminished clopidogrel-induced platelet inhibition.[23] In Chapter 2.1, we have shown for the first time that carriage of the *CYP2C9*3* variant allele is associated with a 10% increase in on-treatment platelet reactivity in patients undergoing elective PCI, as compared to noncarriers. Furthermore, besides *CYP2C19*2*, we found the *CYP2C9*3* allele to be associated with the occurrence of stent thrombosis (Chapter 3.1). The association of *CYP2C9* genetic variants and stent thrombosis was only explored in one other study in which no associations of *CYP2C9*3* and stent thrombosis

were found. However, the number of subjects with stent thrombosis was very small (*n*=17); together with the low allele frequency of *CYP2C9*3*, that study was underpowered to detect the association.[13]

ABCB1

A key protein involved in clopidogrel absorption is the efflux pump P-glycoprotein (Pgp), which is encoded by *ABCB1*, which is located on chromosome 7q21.12. Pgp is an ATP-dependent efflux pump that transports various molecules across extracellular and intracellular membranes. It is expressed, among other places, on intestinal epithelial cells, where increased expression or function can affect bioavailability of drugs that are substrates. Some studies have suggested that when treated with clopidogrel, patients with genetic variants in *ABCB1* (specifically those who are *TT* homozygotes for the *3435C>T* variant) have reduced concentrations of the active drug metabolite and increased rates of adverse clinical events.[3, 18] The silent *ABCB1 3435C>T* variant affects the timing of co-translational folding and has been shown to alter substrate specificity.[35] In the Chapters 2.1 and 3.1 we investigated the influence of the *ABCB1* genetic variants *3435C>T*, *1236C>T* and *G2677T>A* (the two latter nonsynonymous SNPs are in linkage disequilibrium with the first[3]) on on-treatment platelet reactivity and on the occurrence of stent thrombosis in clopidogrel-treated patients. Although we observed significantly higher on-treatment platelet reactivity in carriers of *1236T* and *2677T/A* as measured with the VerifyNow P2Y12 assay, this was not confirmed by the LTA. None of the *ABCB1* genetic variants was found to be associated with stent thrombosis. The lack of influence on ischemic events is confirmed by other studies.[16, 19] On the other hand, in the TRITON-TIMI 38 trial it was shown that homozygous *3435 TT* was associated with an increased risk of adverse cardiovascular events compared with *CT/CC* individuals. Furthermore, the effects were found to be independent of and complementary to those of *CYP2C19*. However, the rates of stent thrombosis did not differ significantly between *3435 TT* and *CT/CC* individuals.[36]

Pharmacogenetics and ASA

After absorption, ASA is rapidly deacetylated to salicylic acid, primarily by human carboxylesterase-2 (CES2).[37] CYP2C9 and UGT1A6 play an important role in the metabolism of salicylic acid.[38] It is thought that the effects of ASA are due to actions by both the acetyl portion of the intact molecule as well as by the active salicylic acid metabolite. ASA inhibits platelets by acetylating cyclo-oxygenase-1 (COX1), thereby blocking the production of thromboxane A_2(TXA_2). Salicylic acid is also reported to reduce the COX-mediated prostaglandin E_2 synthesis, thereby decreasing the formation of TXA_2.[38, 39] In previous studies it was demonstrated that genetic variability within the enzymes CES2, CYP2C9, UGT1A6, COX1 and COX2 affected the response to ASA. Several *CES2* genetic variations were found to decrease the hydrolysis of ASA.[37] Genetic variations in *UGT1A6* modulated the protective effect of ASA on colon adenoma risk.[40] Furthermore, carriers of *CYP2C9* variant alleles were more prone to develop acute gastrointestinal bleeding when they received ASA as compared to noncarriers.[41, 42] Genetic variants in *COX1* were shown to modulate arachidonic acid-induced platelet aggregation and serum thromboxane B_2 (TXB_2) levels in patients treated with ASA.[43, 44] In Chapter 3.3, we were the first to show that genetic variability within the *CES2*, *CYP2C9*, *UGT1A6* and *COX1* genes was associated with the modification of the efficacy of ASA in the prevention of MI.

Drug interactions and clopidogrel

Another aim was to investigate the impact of co-medication on the antiplatelet properties of clopidogrel. In this thesis we reported on drug-interactions between clopidogrel and proton pump inhibitors, calcium channel blockers and sulfonylureas.

Proton pump inhibitors

Proton pump inhibitors (PPIs) are often co-prescribed in patients on dual antiplatelet therapy for the prevention of gastrointestinal ulcera.[45] The interaction between clopidogrel and PPIs has been investigated in several studies.[46] Drug-drug interactions between PPIs and clopidogrel are considered to be caused at the level of CYP2C19. The CYP2C19 enzyme is not only important for the pharmacokinetics of clopidogrel but is also a major enzyme in the metabolism of proton pump inhibitors. Furthermore, omeprazole and esomeprazole are considered to be CYP2C19 inhibitors.[47] This is illustrated by drug-interaction studies, in which was observed that omeprazole and esomeprazole changed the pharmacokinetics of CYP2C19-substrates such as diazepam, phenytoin and warfarin.[47, 48]

In 2008, the first attention to the drug-drug interaction between PPIs and clopidogrel was drawn by showing in a randomized and double-blind study that omeprazole significantly attenuated the antiplatelet effects of clopidogrel.[49] In subsequent studies of platelet function, it was shown that this interaction is probably not a class effect inherent to all PPIs.[50-53] Our results for the effect of omeprazole on on-treatment platelet reactivity (Chapter 2.3) showed that omeprazole only diminished the antiplatelet properties of clopidogrel when clopidogrel was administered as a 300 mg loading dose, but not in patients receiving clopidogrel maintenance therapy (more than 10 days 75 mg per day). We hypothesized that the inhibition of CYP2C19 might only become critical when a high clopidogrel loading dose has to be converted into the active metabolite at once.

Data about the effect of various PPIs on clinical outcomes in clopidogrel-treated patients are conflicting. Particularly retrospective observational studies have shown that the use of PPIs was associated with an increased risk of major adverse cardiovascular events.[54-58] However, the results of these studies might be confounded by the fact that patients on concomitant PPIs are generally older and have more co-morbidities than those not on a PPI. The need for a PPI itself might be an indicator of worse clinical outcome. This hypothesis is strengthened by the results of a post-hoc analysis from the CREDO trial, which showed that the use of PPIs was associated with worse clinical outcome, independently of the use of clopidogrel.[59] Only one randomized controlled trial has examined the interaction between clopidogrel and PPIs with cardiovascular events as the outcome. In the prematurely terminated double-blind, placebo-controlled COGENT trial, 3761 patients with either ACS or PCI were randomized to a fixed-dose combination of clopidogrel and omeprazole (75/20mg) or clopidogrel alone. No effect of omeprazole on the risk of MACE during a median follow-up of 106 days was observed.[60] However, the number of cardiovascular events was low, therefore the study was underpowered to detect an increase in risk smaller than 44%.[61] In a cohort of patients undergoing elective coronary stenting (Chapter 3.2) we did not observe an effect of PPIs on clinical outcome either (HR 1.1, 95% CI 0.51 to 2.2).

In a series of placebo-controlled, crossover pharmacokinetic and –dynamic studies it was demonstrated that omeprazole decreased the plasma concentration of the active metabolite of clopidogrel by about 45% and increased on-treatment platelet reactivity to a similar extent,

irrespectively whether clopidogrel and omeprazole were administered simultaneously or 12 hours apart.[62] The concomitant use of pantoprazole did not result in a clinically relevant interaction with clopidogrel.[62] The absence of a significant interaction between clopidogrel and pantoprazole is consistent with the low potential of pantoprazole to inhibit CYP2C19, which is illustrated by the fact that pantoprazole had no influence on the pharmacokinetics of other CYP2C19 substrates.[47, 63] Based on these findings, the FDA posted a safety warning in November 2009 about the concomitant use of clopidogrel and omeprazole or esomeprazole, although the latter was not supported by any pharmacokinetic data (www.fda.gov). In Chapter 2.5 we present a study which provides pharmacokinetic data to support the assumption that concomitant use of esomeprazole decreases concentrations of clopidogrel's active metabolite. In patients with a history of stent thrombosis, the concomitant use of esomeprazole resulted in a reduction of 45% in maximal plasma concentrations of clopidogrel's active metabolite, as compared to PPI nonusers. On the contrary, we found that the use of pantoprazole was not associated with clopidogrel metabolism. These findings confirm the importance of the FDA-warning. However, it remains controversial whether the impact of concomitant use of clopidogrel and omeprazole/esomeprazole can be fully extrapolated to clinical outcome in clopidogrel-treated patients.
In November 2010, an update of the ACCF/ACG/AHA Expert Consensus Document on the concomitant use of PPIs and thienopyridines was published to provide provisional guidance for clinical management. [61]The authors suggest that the risk/benefit of the addition of a PPI to antiplatelet therapy should be determined for individual patients. In patients with a history of gastro-intestinal bleeding and patients with risk factors for gastro-intestinal bleeding (advanced age, concomitant use of coumarins, steroids or NSAIDs or Helicobacter pylori infection), a PPI should be added to antiplatelet therapy. Other patients receive little absolute risk reduction from a PPI and the risk/benefit balance would seem to favor use of antiplatelet therapy without concomitant PPI treatment.[61]

Calcium channel blockers

The inhibitory effect of calcium channel blockers (CCBs) on the platelet response to clopidogrel is considered to be caused at the level of CYP3A4.[64] CYP3A4 is considered to contribute the most of all CYP enzymes to the second step in clopidogrel's metabolim in which the active metabolite is formed.[4] A part of the CCBs has inhibiting properties on the intestinal efflux transporter P-glycoprotein (nifedipine, barnidipine, felodipine, lercanidipine, verapamil and diltiazem). In Chapter 2.2 we demonstrated that the subgroup of CCBs that possesses no inhibiting properties on P-glycoprotein (in this study only amlodipine) has the largest potential to interfere with clopidogrel's metabolism. Inhibition of Pgp by the concomitant use of Pgp-inhibiting CCBs may lead to a decreased intestinal efflux of clopidogrel, thereby increasing clopidogrel plasma concentrations and partly counteracting the effect of CCB-induced CYP3A4 inhibition. Other publications have also reported that CCBs are associated with increased on-treatment platelet reactivity but did not differentiate between subgroups.[65-67] In the future, this drug-drug interaction should be further investigated, for example to examine whether dihydropyridins like nifedipine and barnidipine should be preferred over amlodipine in the treatment of hypertension in patients with clopidogrel.

Sulfonylureas
The sulfonylureas tolbutamide, glibenclamide, glimepiride and gliclazide are mainly metabolized by the CYP2C9 enzyme, which was shown to play an important role in the metabolism of clopidogrel.[68, 69] In Chapter 2.4 it is demonstrated that the concomitant use of sulfonylureas is associated with increased on-treatment platelet reactivity in a cohort of patients with type 2 diabetes mellitus undergoing elective PCI. As patients with diabetes already exhibit a suboptimal response to clopidogrel and a more prothrombotic state, an additional drug-interaction diminishing the antiplatelet effects of clopidogrel should be avoided.[70, 71] Besides a study in which was demonstrated that the concomitant use of CYP2C9- metabolized coumarins diminished clopidogrel's antiplatelet properties, we are the first to report an interaction with clopidogrel caused at the CYP2C9-level.[72] The sulfonylureas should be considered as important candidates for further drug-drug interaction studies measuring both clopidogrel's active metabolite and its antiplatelet effects.

Combinations of factors and response to clopidogrel
In Chapter 3.2 it was demonstrated that combinations of the pharmacokinetic risk factors for clopidogrel poor response (the use of CCBs, PPIs and carriage of *CYP2C19*2*) were associated with the occurrence of MACE in a cohort of patients undergoing elective PCI. According to both platelet function and clinical outcome data, particularly patients who had two pharmacokinetic risk factors that interfere with clopidogrel metabolism at two different target sites are at increased risk of clopidogrel poor response. Patients who carried at least one *CYP2C19*2* allele and were on treatment with CCBs and patients who were treated with PPIs and CCBs, had a statistically significant 2.1 and 3.3-fold increased risk on the occurrence of MACE, respectively. In this study, the presence of only one pharmacokinetic risk factor for clopidogrel poor-response was not found to be associated with worse clinical outcome. This underlines the importance of incorporating multiple risk factors in the estimation of risk on adverse cardiovascular events in patients undergoing PCI.

STRENGTHS AND LIMITATIONS

In this thesis, the pharmacogenetics of clopidogrel and ASA was studied in relation to a broad range of outcomes varying from the surrogate markers on-treatment platelet reactivity and plasma concentrations of clopidogrel's active metabolite to the clinical endpoints major bleeding, stent thrombosis, first myocardial infarction and a composite of all-cause death, nonfatal MI, stent thrombosis and stroke. Both platelet function tests that were used in the studies of this thesis, have been associated with the occurrence of clinical events.[73, 74] An important strength of the two studies described in the Chapters 3.2 and 3.4, is the fact that associations between genetic variants, co-administered drugs and clinical outcome were accompanied by platelet function data. These data suggested that the influence on clinical outcome was explained by diminished/enhanced platelet inhibition by clopidogrel. Another strength of our studies was the availability of complete pharmacy records, which included dosage data and information about adherence.
All studies were observational and may therefore be hampered by information bias, selection bias and confounding. In all studies, patients filled in questionnaires with questions regarding cardiovascular risk factors and the use of co-medication. This gave us the opportunity to adjust

for potential confounders in our analyses. Information bias for drug exposure, the occurrence of clinical events and hospitalization data was not expected because these data were verified by community pharmacy records and hospital records.

Regarding the influence of genetic variants on the response to antiplatelet therapy, selection bias might have occurred if the genetic variation under study was associated with the drug exposure itself. However, this was not a problem for our studies in which the effect of genetic variants on the response to clopidogrel was investigated, as all patients were on clopidogrel treatment. For the study described in Chapter 3.3 (genetic variants and ASA), factors that determined whether patients received ASA or not could have resulted in differences between groups in prognostic factors related to the outcome (confounding by indication). Confounding by indication, sometimes also referred to as channeling bias, is caused by the tendency of clinicians to prescribe treatment based on a patient's prognosis.[75] As a result of this bias, treated patients might be more (or less) likely to be at higher risk of a particular outcome than non-treated patients, which might lead to a biased interpretation of treatment outcomes. However, the prescriber of ASA was unaware of a patient's genotype, therefore we considered it unlikely that the drug-gene interaction was influenced by this type of confounding.
Regarding the influence of co-medication on the response to clopidogrel, confounding by indication should also be addressed. In clopidogrel-treated patients, it is possible that PPI therapy was initiated in patients who were expected to be at a higher than average risk for adverse gastrointestinal outcomes based on their higher age, worse cardiovascular disease, and poorer prognosis than those who were not given a PPI. These factors—rather than the PPI *per se*—might explain the worse cardiovascular outcomes among PPI users as reported in several observational studies.[46, 55-57] We aimed to address this item by incorporating platelet function data and by adjusting for confounders in the multivariate analysis.
Moreover, genetic association studies are liable to false positive results. To deal with multiple testing, we calculated the positive false discovery rate (pFDR) to assess the proportion of false positives among those declared significant in the Chapters 2.1, 3.1 and 3.3.[76] Another approach to prevent false positive results is replicating findings on similar populations.[77]

In this thesis, genetic associations were investigated with the candidate gene approach. In literature, several genetic variations were proposed to play a role in the response to the antiplatelet drugs clopidogrel and ASA. This strategy has the advantage of focusing on a manageable number of genes and variants that are likely to be important and keeping sufficient statistical power to detect associations.[78] A limitation of candidate gene analyses is that associations will not be found in genes which were not selected based on prior knowledge.
In the studies investigating the pharmacogenetics of clopidogrel, candidate genetic variants were chosen based on the information that the variants had been associated with the response to clopidogrel in previous studies. In Chapter 3.3, the study in which was investigated if genetic variants were able to modulate the effectiveness of ASA in the prevention of MI, we used tagging SNPs in the candidate gene approach. We selected a number of genes which were previously shown to be associated with the response to ASA. In each gene, tagging SNPs were selected which represent variation in SNPs in the rest of the gene. This representation arises because genetic variation is transmitted in haplotype blocks. Within these haplotypes, variant alleles are associated with each other (linkage disequilibrium). The tagging SNPs were selected with the information on HapMap.[79] The major advantage of this method is that with a small

number of genetic tests, a large degree of genetic variation can be analyzed. We did not only focus on gene regions coding for amino acids (exons) but also on intronic SNPs situated in the gene regions not coding for amino acids. Intronic SNPs affect transcription rates and may also have an impact on drug response. With the use of tagging SNPs, new clinically relevant SNPs may be identified in genes of interest.
A more advanced method to study genetic associations is the genome wide association study (GWAS). In GWA studies, the SNPs are not limited to previously selected genes but cover the whole genome. One of the advantages of GWA studies is that genes might be discovered which were not previously associated with drug response. The large number of tested SNPs, however, increases the risk of false positive results. To avoid this, only associations with very low p-values ($<10^{-6}$ - 10^{-8}) are regarded as significant. These low p-values can be attained either by very large study populations or by studying very strong associations. In 2009, a GWAS for the effect of clopidogrel on ADP-induced platelet aggregation identified only one associated locus extending across the *CYP2C19–CYP2C9* gene cluster. The most significantly associated variant rs12777823 ($P \approx 10^{-13}$) was found to be in strong linkage disequilibrium with *CYP2C19*2* (r^2=0.87).[16]

CLINICAL IMPLICATIONS

Pharmacogenetics and antiplatelet therapy
In this thesis we found the genetic variants *CYP2C19*2* and *CYP2C9*3* to be associated with atherothrombotic events in patients on dual antiplatelet therapy who had undergone PCI. We were the first to report the adverse effect of *CYP2C9*3* on the prevention of stent thrombosis in clopidogrel-treated patients. To date, this finding has not been replicated yet. It should be investigated in large, well-designed studies whether *CYP2C9*3* modifies the effectiveness of clopidogrel in the prevention of major adverse cardiovascular events. Likewise, our findings regarding the genetic variants influencing the effectiveness of ASA in the prevention of MI should be replicated in other studies. Furthermore, the impact of the drug-gene interaction of *CYP2C19*17* and clopidogrel on the occurrence of bleeding should be further explored.

The question on whether *CYP2C19* genotypes should assist clinical decision making is heavily debated despite the large amount of evidence for their clinical relevance. A recent meta-analysis indicated that patients with one or more *CYP2C19* loss-of-function variants had a statistically significant overall 57% increase in the risk on the occurrence of major cardiovascular events and an even 2.8-fold increased risk of stent thrombosis.[21] Carriage of *CYP2C19* loss-of-function alleles accounts for approximately 12% of variability in clopidogrel platelet response[16] and the positive predictive value for clinical events is estimated to be between 12% and 20% in patients with ACS undergoing PCI.[11, 13] The positive predictive values observed with a point-of-care P2Y12 assay in a similar population were only 12%.[80, 81] Neither genotyping nor platelet function testing is a perfect discriminator of subsequent clinical outcomes, underscoring the complex, multifactorial nature of cardiovascular risk. Therefore, other risk factors (such as the use of CCBs or PPIs, ACS and diabetes mellitus) should also be assessed to make a proper decision about the optimal antiplatelet treatment strategy in patients undergoing PCI.

An alternative treatment option for patients undergoing PCI is the more potent antiplatelet drug prasugrel. Prasugrel is a prodrug that needs a single-step hepatic conversion to an active metabolite before binding to the platelet P2Y12-receptor. The drug appears to have very few poor responders in patients with stable coronary artery disease and in patients with ACS.[82, 83] The response seems independent of genetic variations in CYP enzymes.[84] Standard dosing of prasugrel (60 mg loading, 10 mg daily) is associated with more potent platelet inhibition than clopidogrel, even at high doses (600 mg loading, 150 mg daily). This enhanced platelet inhibition with prasugrel was documented in a small substudy of the TRITON-TIMI 38 trial.[83] Importantly, significantly reduced rates of ischemic events (20% reduction) compared with those seen with clopidogrel, including stent thrombosis (>50% reduction), were reported in TRITON-TIMI-38.[82] However, this reduction in ischemic events was accompanied by an increased rate of major bleeding, including life-threatening bleeding. The three groups at highest risk for bleeding in TRITON-TIMI 38 included patients older than 75 years, with body weight less than 60 kg and with a history of stroke or transient ischemic attack (TIA).[82] The first two groups should receive decreased prasugrel maintenance doses (5 mg daily). The use of prasugrel is contraindicated in patients with a history of stroke or TIA. Currently, prasugrel is only approved for the use in patients with ACS undergoing PCI.
A second potent P2Y12-receptor antagonist is ticagrelor, which is not yet approved for clinical use. Ticagrelor is an oral, reversible P2Y12 receptor antagonist that blocks ADP-induced platelet aggregation and does not require metabolic activation. Ticagrelor has been found to be effective in improving platelet inhibition in patients who respond not optimally to clopidogrel.[85] In addition, compared with clopidogrel in the large PLATO trial of patients with ACS, ticagrelor significantly reduced the rate of the primary composite endpoint of death from vascular causes, MI, or stroke.[86] While there was no increase in the rate of overall major bleeding, there was an increase in the rate of nonprocedure-related bleeding.[86]

Another treatment strategy is an alternative dosing regimen for clopidogrel. Several studies have evaluated the effect of different combinations of clopidogrel loading and maintenance doses on the formation of clopidogrel's active metabolite and on-treatment platelet reactivity. Some studies were performed specifically in patients with a documented suboptimal response to 'normal' dosing protocols for clopidogrel.[87-89] A 600 mg clopidogrel loading dose was found to improve the degree of acute platelet inhibition as compared to 300 mg.[90] Likewise, a double 600 mg clopidogrel loading dose performed better than a single 600 mg dose.[91] A clopidogrel maintenance dose of 150 mg daily resulted in a greater degree of platelet inhibition in many studies in patients with a reduced response to the usual 75 mg maintenance dose.[88, 89, 91] Furthermore, it was demonstrated that increased clopidogrel loading doses could overcome a suboptimal response to clopidogrel in carriers of *CYP2C19*2*.[73] However, even at the higher dose, some patients do not reach an optimal level of platelet inhibition. [87]
At this moment, two large trials investigated the effect of double-doses clopidogrel on clinical outcome. In the first, the CURRENT-OASIS-7 (Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events-Optimal Antiplatelet Strategy for Interventions-7) trial, patients were randomly assigned to double-dose (600 mg on day 1, 150 mg on days 2-7, then 75 mg daily) versus standard-dose clopidogrel (300 mg on day 1 then 75 mg daily). There was no increase in efficacy of double-dose versus standard-dose in the overall study cohort. However, patients undergoing PCI (nearly 75% of the overall cohort) who received double-dose clopidogrel had a significantly decreased risk of MACE but an increased risk of bleeding.[92] In November 2010,

the results of the GRAVITAS trial were presented.[93] In this trial, 5429 patients on standard-dose clopidogrel underwent platelet function tests with the VerifyNow assay P2Y12 assay 12 to 24 hours after PCI. Of these, 2214 (41%) had *high on-treatment platelet reactivity* and were randomized to continue on the standard-dose clopidogrel or to receive another 600-mg loading dose and a clopidogrel maintenance dose of 150 mg daily. At six months of follow-up, the composite endpoint of cardiovascular death, MI and stent thrombosis was identical in both groups (HR 1.0, p=0.98). No clinical trials investigating the effect of double-dose clopidogrel versus the standard-dose in carriers of *CYP2C19* loss-of-function alleles are reported yet.

Ideally, the choice of the optimal antiplatelet treatment strategy in patients undergoing PCI would be directed by validated predictive algorithms based on information of genetic variants, platelet function testing, co-medication and other risk factors such as diabetes, BMI and ACS. However, such validated algorithms are not available yet. Furthermore, there are still no results from randomized prospective trials with clinical endpoints in which treatment strategies are based on *CYP2C19* carrier status. However, with stent thrombosis rates in carriers of a *CYP2C19* loss-of-function allele as high as 13%[11, 21] and mortality associated with stent thrombosis close to 50%, one can question whether it is ethical to wait for the results of additional trials. In the interim, with the evidence that is already available, all potential interventions should be implemented to prevent the serious outcomes of stent thrombosis and other cardiovascular events in patients at risk. The FDA boxed warning advocates implementing alternative strategies in these high-risk individuals. One logical strategy might be to switch carriers of a *CYP2C19* loss-of-function allele to the more potent antiplatelet drug prasugrel (except patients with a history of stroke or TIA) and to give noncarriers standard treatment with clopidogrel. Routine genotyping requires information on cost effectiveness and cost-consequences, which is not available yet.[94] Interestingly, clopidogrel is off patent and is much cheaper than prasugrel, which is recently approved by regulatory authorities. Furthermore, physicians and clinical pharmacists need to become informed about the usefulness and also the limitations of genotyping in patient care.[94] Finally, it will be a challenge to have genotype information available on time to assist the choice of antiplatelet treatment, particularly in patients undergoing urgent PCI.

Drug-interactions and clopidogrel

In March 2010, the Royal Dutch Association for the Advancement of Pharmacy (KNMP) incorporated the drug-interaction between (es)omeprazole and clopidogrel into a database that is linked to the national medication surveillance programs. Although the clinical relevance of the interaction between (es)omeprazole and clopidogrel is still debated[61], both community and hospital pharmacists are warned when patients are prescribed the combination of (es)omeprazole and clopidogrel. The KNMP states that the concomitant use of (es)omeprazole and clopidogrel should be avoided and that when the use of a PPI is needed, another PPI should be prescribed. In practice, pharmacists and clinicians often decide to substitute (es)omeprazole to pantoprazole. At this moment, there is for pantoprazole the most evidence that it exhibits no clinically relevant effect on the antiplatelet effects of clopidogrel.

The drug-drug interaction between clopidogrel and CCBs and sulfonylureas should be further investigated. Properly designed drug-drug interaction studies measuring both the plasma concentrations of clopidogrel's active metabolite and on-treatment platelet reactivity are indicated. Furthermore, sufficiently powered studies with clinical endpoints are needed to replicate our findings and to analyze whether the subgroup of Pgp inhibiting CCBs has less potential to interfere with clopidogrel's antiplatelet properties compared to non-Pgp-inhibiting CCBs.

FUTURE RESEARCH

The variability in response to clopidogrel and ASA is considered to be caused by several factors. In the future, well designed, large sample sized studies should further investigate potential (both genetic and non-genetic) factors associated with the variability in response to clopidogrel and ASA. Future prospective studies that include cardiovascular outcome are necessary to design and validate predictive algorithms based on genetic variants, platelet function testing, comedication and other demographic, clinical and procedural risk factors such as diabetes, BMI and ACS as the indication for PCI. The prediction of future cardiovascular events in patients receiving antiplatelet therapy will likely benefit from such a global risk assessment score based on combinations of risk factors. Randomized controlled trials should be designed to investigate the effect of tailored genotype-based antiplatelet therapy compared to standard treatment strategies on the occurrence of adverse cardiovascular events. Furthermore, the cost-effectiveness of genotype-based antiplatelet therapy should be investigated.

The selection of SNPs and the amount of SNPs which are considered for genotyping remains an important issue in the future. The response to antiplatelet agents is likely to be influenced by additional genes as well. To explore a larger amount of genes, new strategies need to be explored, such as haplotypes and genome wide association studies. In genome wide association studies however, it is important to adapt to the computational burden, as up to more than one million variables are available in the dataset. Furthermore, even in large sample sizes, the study power is limited to the detection of common variants with large effects only. To increase statistical power, it is important that research groups collaborate to replicate findings, or to conduct a joint meta-analysis.[95] Conducting meta-analyses is considered to be the most powerful strategy.[96] Prospective randomized trials directly comparing the influence of different PPIs on clinical outcome in patients treated with clopidogrel are necessary. The ongoing randomized SPICE-trial may provide additional evidence regarding the clinical relevance of the interactions.[97] This trial will directly compare the effects of commonly prescribed PPIs and the H2-recepter antagonist ranitidine on platelet aggregation among 320 post-PCI patients on dual antiplatelet therapy. Secondary outcomes include assessment of clopidogrel poor-response, the effect of *CYP2C19*2* on PPI and antiplatelet therapy and the occurrence of MACE at 1-year post-PCI. Finally, as mentioned before, the drug-drug interactions between clopidogrel and CCBs and sulfonylureas should be further investigated.

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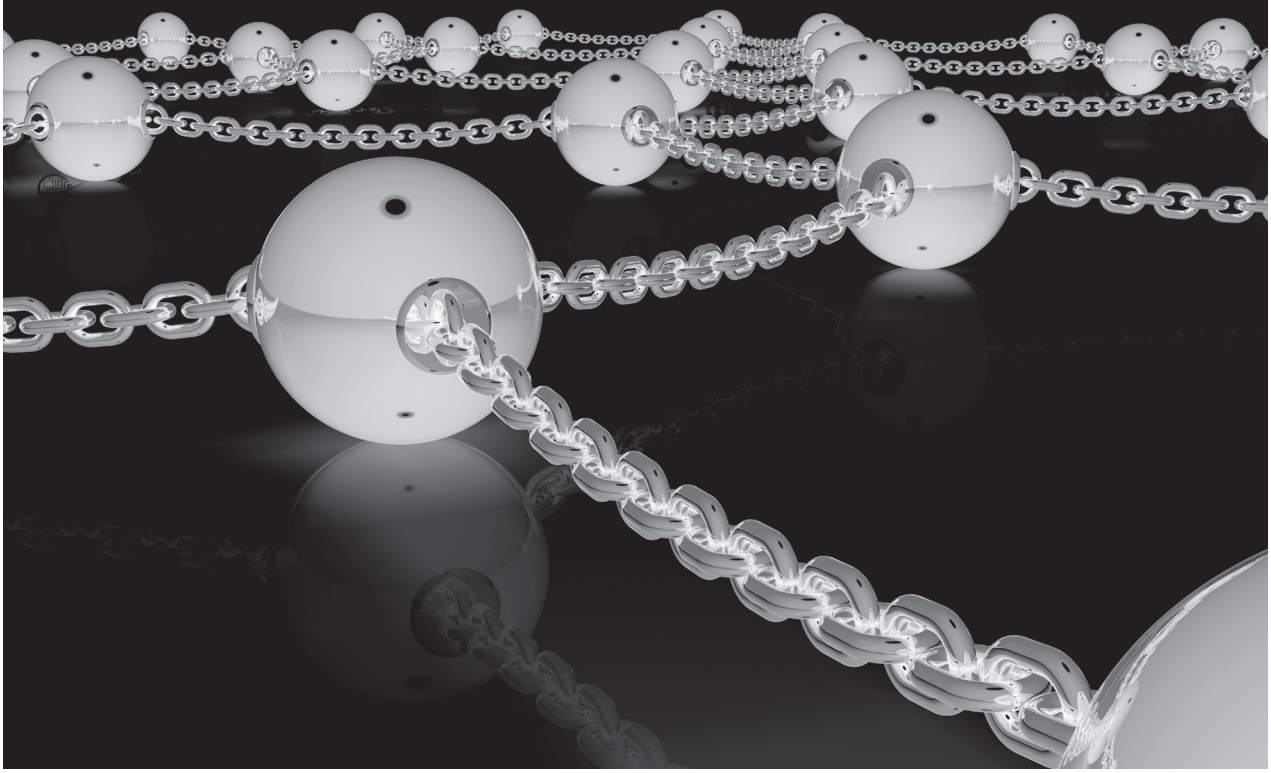
5

SUMMARY



SUMMARY

Antiplatelet therapy plays an important role in the treatment of cardiovascular disease. Although the efficacy of acetylsalicylic acid (ASA) and clopidogrel is well established, several studies have shown interindividual variability in the response to these antiplatelet drugs. This variability in response results in complications at both ends of the therapeutic spectrum (bleeding or thrombosis). In this thesis, the impact of genetic variations and co-prescribed drugs on the response to clopidogrel and ASA was investigated.

In **Chapter 1.1** the principal mechanisms of the point-of-care VerifyNow platelet function assay were reviewed. Its clinical utility for the monitoring of antiplatelet therapy and proposed cut-off levels to identify high on-treatment platelet reactivity for the different types of antiplatelet therapy were discussed.

In **Chapter 2**, studies were described in which we examined the influence of genetic variants and drug-interactions on on-treatment platelet reactivity and plasma concentrations of the active metabolite of clopidogrel.

In **Chapter 2.1** we examined the effect of genetic variants in *ABCB1*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5* and *P2Y1* on on-treatment platelet reactivity in 428 patients undergoing elective percutaneous interventions (PCI) on dual antiplatelet therapy. Patients either received a recent 300 mg clopidogrel loading dose or were on chronic clopidogrel maintenance therapy. In both treatment groups, *CYP2C19*2*-carriage was associated with higher platelet reactivity ($p<0.002$) and with an approximately 4-fold increased risk of high on-treatment platelet reactivity. In the 300mg-group, *CYP2C9*3* was associated with 4-fold increased risk of high on-treatment platelet reactivity ($p=0.016$).

In **Chapter 2.2** the influence of concomitant use of calcium channel blockers (CCBs) on on-treatment platelet reactivity was explored in a cohort of 623 patients undergoing elective PCI on dual antiplatelet therapy. All CCBs are considered to be CYP3A4-inhibitors but some CCBs also have strong inhibitory effects on the drug transporter P-glycoprotein (Pgp), which mediates the intestinal absorption of clopidogrel. All CCBs were associated with increased on-treatment platelet reactivity. However, only the use of the non-Pgp-inhibiting CCB amlodipine was significantly associated a statistically significant increased risk of high on-treatment platelet reactivity (adjusted odds ratio (OR_{adj} 2.3 95% confidence interval (CI), 1.4-3.9, $p=0.001$).

In **Chapter 2.3** we determined whether the influence of the proton pump inhibitor (PPI) omeprazole on the antiplatelet effect of clopidogrel was dependent on clopidogrel's dosing regimen in 431 patients undergoing elective PCI. After a recent 300 mg clopidogrel loading dose, platelet reactivity was significantly higher in omeprazole-users as compared to PPI-nonusers: 70.1% ± 8.0 vs. 61.8% ± 13.2, $p=0.008$ as measured with 20 µmol/L ADP-induced light transmittance aggregometry (LTA). The use of omeprazole was associated with a 6.3-fold increased risk of high on-treatment platelet reactivity ($p=0.003$). In contrast, omeprazole was not associated with increased platelet reactivity in patients on chronic clopidogrel maintenance therapy.

In **Chapter 2.4** we evaluated the association of CYP2C9-metabolized sulfonylureas and on-treatment platelet reactivity in 139 type 2 diabetes mellitus patients undergoing elective coronary stent implantation. On-treatment platelet reactivity was significantly higher in patients treated with sulfonylureas as compared to patients without sulfonylurea treatment (for 20 µmol/L ADP-LTA: 64.6% ± 10.8 vs. 58.7% ± 15.5; p=0.019). Concomitant use of sulfonylureas was associated with a 2.2-fold increased risk of high on-treatment platelet reactivity (OR_{adj} 2.0 95% CI, 1.0-5.7, p=0.048).

In **Chapter 2.5**, we assessed the impact of esomeprazole and pantoprazole on the plasma concentrations of the active metabolite of clopidogrel (AMC) and on on-treatment platelet reactivity. Of the 49 patients, 20 were on pantoprazole and 6 were on esomeprazole treatment. Users of esomeprazole had a reduction of 45% in maximal plasma concentrations of the AMC as compared with PPI nonusers (geometric mean [range] C_{max}(AMC) 4.3 ng/mL [1.9-9.3] vs. 7.8 ng/mL [3.5-19.5], p=0.005). Esomeprazole-users exhibited higher on-treatment platelet reactivity as compared to PPI nonusers (61.1 ± 16.5% vs. 41.8 ± 18.1%, p=0.026 for 20 µmol/L ADP-LTA). In contrast, pantoprazole had no influence on the formation of the AMC nor on on-treatment platelet reactivity.

In **Chapter 3**, the effect of drug-interactions and genetic variants on clinical outcome in patients receiving antiplatelet therapy was investigated.

In **Chapter 3.1**, a case-control study is described in which we determined the effect of variations in the *ABCB1*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5* and *P2Y1* genes on the occurrence of stent thrombosis. The selected genetic variants were assessed in 176 subjects who developed stent thrombosis while on dual antiplatelet therapy with ASA and clopidogrel and in 420 control subjects who did not develop adverse cardiovascular events, including ST, within one year after stenting. The *CYP2C19*2* and *CYP2C9*3* variant alleles were significantly associated with stent thrombosis (OR_{adj} 1.7 95% CI, 1.0-2.6, p=0.018 and OR_{adj} 2.4 95% CI 1.0-5.5, p=0.043, respectively). No significant associations of the other genetic variations and the occurrence of stent thrombosis were found.

In **Chapter 3.2** the impact of combined presence of three pharmacokinetic risk factors for clopidogrel poor-response, i.e. the use of CCBs, PPIs and carriage of *CYP2C19*2*, on on-treatment platelet reactivity and the occurrence of atherothrombotic events in 725 patients on dual antiplatelet therapy undergoing elective coronary stenting was investigated. Patients with either one or more than one risk factor exhibited increased platelet reactivity. Patients with one risk factor for clopidogrel poor-response did not have an increased risk of the composite endpoint. However, patients using both CCBs and PPIs and carriers of *CYP2C19*2* who used CCBs had a statistically significant increased risk of the composite endpoint of all-cause death, nonfatal myocardial infarction, stent thrombosis and stroke (adjusted hazard ratio (HR_{adj}) 2.1 95% CI, 1.0-4.4, p=0.037 and HR_{adj} 3.3 95% CI, 1.1-9.5, p=0.029, respectively).

In **Chapter 3.3**, it was investigated whether genetic variants in the enzymes CES2, CYP2C9, UGT1A6, COX1 and COX2 modified the effectiveness of ASA therapy in the prevention of myocardial infarction (MI). In a population-based registry of pharmacy records linked to hospital discharge records (PHARMO), a nested case-control study in 853 cases and 887 control sub-

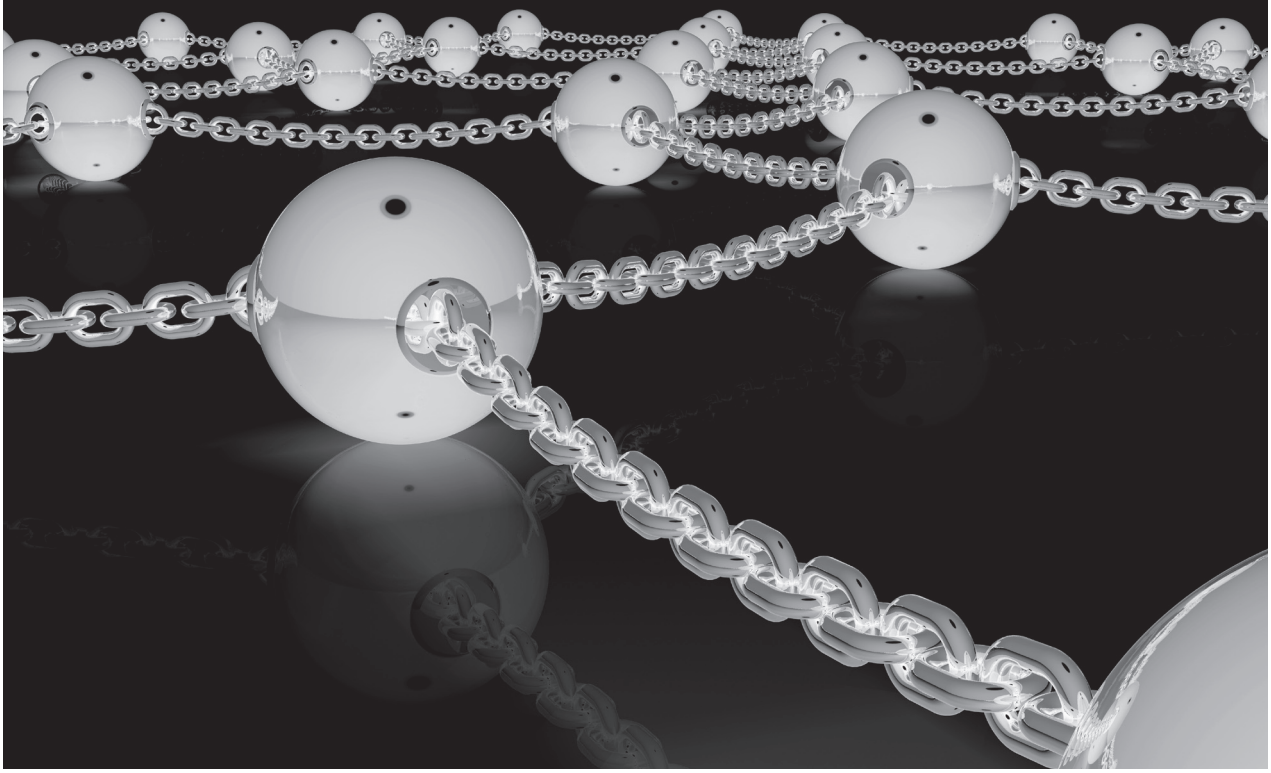
jects was performed. The use of ASA was associated with a reduced risk of MI (OR_{adj} 0.74 95% CI 0.56-0.97, p=0.032). The *CES2* rs11568311 and *CYP2C9* rs1057910 variants were found to interact with ASA treatment (adjusted syngery index (SI_{adj}) 0.43 95% CI, 0.21-0.90, p=0.025 and SI_{adj} 0.44 95% CI, 0.22-0.91, p=0.026, respectively). Two variants in the *UGT1A6* gene showed a significant interaction with ASA (rs11563251, p=0.044 and rs3771342, p=0.023). In addition, two *COX1* variants were associated with the effect of ASA (rs10306135: SI_{adj} 1.5 95% CI, 1.0-2.7, p=0.042 and rs5788: SI_{adj} 1.5 95% CI, 1.0-2.6, p=0.023). No significant interactions between other genetic variants and the effect of ASA were observed.

In **Chapter 3.4** we examined the impact of genotypes based on the loss-of-function variant *CYP2C19*2* and the gain-of-function variant *CYP2C19*17* on on-treatment platelet reactivity and on the occurrence of TIMI major bleedings in 820 patients undergoing elective PCI. Patients with the *CYP2C19*1/*17* and **17/*17* genotypes exhibited a statistically significant lower magnitude of platelet reactivity as compared to patients with the *CYP2C19*1/*1* genotype. Patients with the **1/*17* and **17/*17* genotype had a 2.7-fold increased risk on the occurrence of major bleedings (HR_{adj} 2.7 95% CI, 1.1-7.0, p=0.039). The genotypes **2/*17, *1/*2* and **2/*2* exhibited higher on-treatment platelet reactivity as compared to wildtype (p<0.0001). However, this was not translated into an altered risk on major bleedings as compared to wildtype (HR 1.3 95% CI, 0.45-4.0, p=0.60).

In **Chapter 4** we summarized the results of our studies and placed them into the broader perspective of clinical implications and further research.

6

SAMENVATTING



SAMENVATTING

De bloedplaatjesremmers ("trombocytenaggregatieremmers") clopidogrel en acetylsalicylzuur (ook wel aspirine genoemd) spelen een belangrijke rol in de behandeling van hart- en vaatziekten. Clopidogrel en acetylsalicylzuur worden gecombineerd gebruikt bij het voorkomen van trombotische complicaties (stolselvorming) na een acuut coronair syndroom (hartinfarct of een voorstadium hiervan) en na een percutane coronaire interventie (ook wel dotterbehandeling genoemd, waarbij met een ballonnetje de kransslagader wordt opgerekt en vaak een veertje (een "stent") wordt achtergelaten).

Clopidogrel is een prodrug, dat wil zeggen dat het een geneesmiddel is dat eerst door het lichaam moet worden omgezet voordat het werkzaam is. De opname van clopidogrel in de darmen wordt geregeld door het transporteiwit P-glycoproteine (het stukje DNA wat de samenstelling van dit eiwit bepaalt, wordt ABCB1 genoemd). Na de opname wordt clopidogrel door verschillende enzymen in de lever (ondermeer CYP2C9, CYP2C19 en CYP3A4/5) omgezet in de werkzame stof. De werkzame stof voorkomt de binding van ADP, een stof die bloedplaatjes aanzet tot stolselvorming, aan receptoren op het bloedplaatje, waardoor de vorming van een stolsel wordt geremd.

De bloedplaatjesremmende werking van acetylsalicylzuur berust op het feit dat acetylsalicylzuur het enzym cyclo-oxygenase (COX) in het bloedplaatje onwerkzaam maakt. Hierdoor wordt er minder tromboxaan A_2 gemaakt. Tromboxaan A_2 zet de bloedplaatjes aan tot stolselvorming. Bij de afbraak van acetylsalicylzuur zijn de enzymen CYP2C9, UGT1A6 en CES2 betrokken.
Verschillende onderzoeken hebben laten zien dat clopidogrel en acetylsalicylzuur erg goed zijn in het voorkomen van trombotische complicaties bij patiënten met hart- en vaatziekten. Echter, de werking van clopidogrel en acetylsalicylzuur verschilt tussen mensen onderling (interindividuele variatie). Patiënten waarbij clopidogrel en/of acetylsalicylzuur minder goed werkt, hebben een grotere kans op het krijgen van een trombotische complicatie terwijl patiënten die heel goed reageren op deze middelen een hogere kans op bloedingen hebben. Een groot aantal factoren bepaalt hoe de individuele patiënt op geneesmiddelen reageert. Hoe meer men weet over deze factoren, des te beter de werking voorspeld kan worden. Deze kennis kan in de praktijk gebruikt worden om geneesmiddelen efficiënter en veiliger in te zetten.

Het doel van dit proefschrift was om het inzicht te vergroten in de effecten van twee factoren op de werking van clopidogrel en acetylsalicylzuur, namelijk de invloed van erfelijke factoren en de invloed van andere geneesmiddelen die gelijktijdig worden gebruikt.

Erfelijke factoren kunnen van invloed zijn op de werking van geneesmiddelen. De erfelijke informatie is opgeslagen in het DNA. Het DNA bepaalt de samenstelling van eiwitten, waaronder de eiwitten die betrokken zijn bij de afbraak van geneesmiddelen (enzymen), en de eiwitten die een transportfunctie hebben en geneesmiddelen in en uit cellen transporteren. Kleine veranderingen in het DNA, zogenaamde *genetische variaties*, kunnen leiden tot eiwitten die beter of juist minder goed werken. In het geval van enzymen die geneesmiddelen afbreken, kunnen genetische variaties leiden tot een verhoogde of verlaagde afbraak van geneesmiddelen. Het gevolg van veranderingen in de werking van transporteiwitten is een verhoging of een verlaging van de geneesmiddelconcentratie in de cellen van het betrokken orgaan en in het bloed, en dit kan leiden tot een veranderde werkzaamheid. Onderzoek naar de invloed van genetische variaties op de werking van geneesmiddelen is het terrein van de farmacogenetica.

Geneesmiddelen kunnen de werking van andere geneesmiddelen beïnvloeden, zogenaamde geneesmiddelinteracties. Dit kan bijvoorbeeld gebeuren als het geneesmiddel A de afbraak van geneesmiddel B remt of juist versnelt, waardoor de concentraties in het bloed van geneesmiddel B respectievelijk hoger en lager zullen zijn indien beide geneesmiddelen tegelijkertijd worden gebruikt. Hogere concentraties kunnen leiden tot bijwerkingen, en lagere concentraties tot een verminderde werkzaamheid.

De bloedplaatjesremmende werking van clopidogrel en acetylsalicylzuur kan in het laboratorium met zogenaamde bloedplaatjesfunctietesten worden bepaald. Bij deze testen wordt de reactiviteit van bloedplaatjes in het bloed van de met clopidogrel en/of acetylsalicylzuur behandelde patiënt gemeten. Het doel van het gebruik van clopidogrel en acetylsalicylzuur is om bloedplaatjes zo min mogelijk reactief te maken (hoe reactiever de bloedplaatjes zijn, des te hoger is de neiging tot stolselvorming). In **hoofdstuk 1.1** gaan we in op de werking, toepasbaarheid en de voor- en nadelen van de VerifyNow® Assay, een relatief nieuwe bloedplaatjesfunctietest. Er wordt ingegaan op de afkapwaardes van bloedplaatjesreactiviteit waarboven patiënten onvoldoende op bloedplaatjesremmende geneesmiddelen reageren. Bij patiënten bij wie de bloedplaatjesreactiviteit hoger is dan die afkapwaarde, is sprake van “bloedplaatjesremmers-resistentie” (dus “clopidogrel resistentie” of “acetylsalicylzuur resistentie”). Deze “resistente” patiënten hebben een grotere kans op trombotische complicaties.

In de **hoofdstukken 2** en **3** worden de studies beschreven waarin de invloed van genetische variaties en geneesmiddelinteracties op de werkzaamheid van clopidogrel en acetylsalicylzuur is onderzocht. In **hoofdstuk 2** wordt in dit verband de invloed op bloedplaatjesreactiviteit en de concentratie van de werkzame stof van clopidogrel in het bloed bepaald (dit zijn studies met zogenaamde “surrogaat eindpunten”). In **hoofdstuk 3** wordt de werkzaamheid van clopidogrel en acetylsalicylzuur bepaald op basis van klinische eindpunten (bijvoorbeeld het krijgen van een hartinfarct, beroerte of overlijden).

In **hoofdstuk 2.1** wordt het effect van genetische variaties in de eiwitten ABCB1, CYP2C9, CYP2C19, CYP3A4, CYP3A5 en in een receptor op het bloedplaatje (P2Y1) onderzocht in een groep van 428 met clopidogrel en acetylsalicylzuur behandelde patiënten die een geplande dotterbehandeling ondergingen. Patiënten kregen al meer dan 5 dagen voor de dotterbehandeling dagelijks 75 mg clopidogrel als onderhoudstherapie of ontvingen een 300 mg clopidogrel oplaaddosis 1-5 dagen voor de dotterbehandeling, gevolgd door 75 mg clopidogrel per dag. Bij alle patiënten is de aanwezigheid van de genetische variatie *CYP2C19*2* geassocieerd met een ongeveer 4-maal verhoogde kans op *clopidogrel resistentie* zoals gemeten met twee verschillende bloedplaatjesfunctietesten. In de groep van patiënten die een 300 mg clopidogrel oplaaddosis ontvingen, hadden patiënten met de genetische variatie *CYP2C9*3* een ongeveer 4-maal zo grote kans op *clopidogrel resistentie*.

In de **hoofdstukken 2.2** tot **2.5** wordt de invloed van het gelijktijdige gebruik van andere geneesmiddelen op de werkzaamheid van clopidogrel onderzocht.

Hoofdstuk 2.2 beschrijft een studie waarin de invloed van het gelijktijdig gebruik van calciumantagonisten op de bloedplaatjesreactiviteit bij met clopidogrel en acetylsalicylzuur behandelde patiënten die een geplande dotterbehandeling ondergaan, wordt bekeken. Calciumantagonisten (ondermeer amlodipine, barnidipine, diltiazem, nifedipine, verapamil) worden

voornamelijk gebruikt voor de behandeling van hoge bloeddruk en pijn op de borst door zuurstoftekort van het hart. Alle calciumantagonisten zijn remmers van het leverenzym CYP3A4. Omdat dit leverenzym is betrokken bij de omzetting van clopidogrel in de werkzame stof zou dit proces kunnen worden geremd door gebruik van een calciumantagonist. Slechts een paar calciumantagonisten hebben daarnaast een sterk remmend effect op het transporteiwit P-glycoproteine (Pgp), dat de opname van clopidogrel in de darmen regelt. Door deze Pgp-remming kan de opname van clopidogrel juist worden bevorderd. Het gebruik van zowel calciumantagonisten mèt (barnidipine, diltiazem, nifedipine, verapamil) als zonder (amlodipine) remmend effect op Pgp was geassocieerd met verhoogde bloedplaatjesreactiviteit. Echter, alleen het gebruik van amlodipine leidde tot een verhoogde kans op *clopidogrel resistentie*. Deze studie suggereert dat het nadelige effect van CYP3A4-remming op de effectiviteit van clopidogrel deels wordt tegengegaan door de remming van Pgp. Verder onderzoek met klinische eindpunten is nodig om na te gaan of het beter zou zijn om calciumantagonisten met Pgp-remmende eigenschappen voor te schrijven bij patiënten die clopidogrel gebruiken.

Andere geneesmiddelinteracties die in de klinische praktijk veel voorkomen zijn die tussen clopidogrel en protonpompremmers. Ter bescherming tegen maag-darmklachten wordt vaak een protonpompremmer aan met clopidogrel en acetylsalicylzuur behandelde patiënten voorgeschreven. De protonpompremmer omeprazol is een sterke remmer van het leverenzym CYP2C19, een enzym wat een belangrijke rol speelt bij de omzetting van clopidogrel in de werkzame stof. **In hoofdstuk 2.3** wordt onderzocht of de invloed van de protonpompremmer omeprazol op de bloedplaatjesremmende werking van clopidogrelafhankelijk is van het dosering van clopidogrel. In deze studie zien we dat bij patiënten die een dotterbehandeling ondergaan en die een recente 300 mg clopidogrel oplaaddosis hebben gehad, het gebruik van omeprazol van invloed lijkt te zijn op verminderde effectiviteit van clopidogrel. In deze groep patiënten was het gebruik van omeprazol geassocieerd met een 6,3-maal verhoogde kans op *clopidogrel resistentie*. Echter, bij patiënten die al langere tijd clopidogrel onderhoudstherapie ontvingen (75 mg/dag), werd geen nadelig effect van omeprazol op de effectiviteit van clopidogrel waargenomen.

In **hoofdstuk 2.4** beschrijven we een studie waarin wordt onderzocht of gelijktijdig gebruik van sulfonylureumderivaten van invloed is op een verminderde werkzaamheid van clopidogrel bij 139 patiënten met diabetes mellitus type 2 (ouderdomssuikerziekte) die een geplande dotterbehandeling ondergaan. Sulfonylureumderivaten (bijvoorbeeld de geneesmiddelen glibenclamide, glimepiride, tolbutamide, gliclazide) zijn bloedsuikerverlagende middelen die bij diabetes mellitus type 2 gebruikt kunnen worden. Deze middelen worden afgebroken door het leverenzym CYP2C9, een enzym wat ook bij het werkzaam maken van clopidogrel is betrokken. In deze studie hadden patiënten die sulfonylureumderivaten een 2,2-maal verhoogde kans op *clopidogrel resistentie* vergeleken met patiënten die geen sulfonylureumderivaten gebruikten. Dit is de eerste studie waarin deze geneesmiddelinteractie werd onderzocht. In de toekomst zullen studies aan moeten tonen of het sulfonylureumderivaten ook in verband kunnen worden gebracht met een hogere kans op trombotische complicaties bij patiënten die clopidogrel gebruiken.

In de studie beschreven in **hoofdstuk 2.5** wordt de invloed van de protonpompremmers esomeprazol en pantoprazol op de vorming van de actieve metaboliet van clopidogrel en op bloedplaatjesreactiviteit onderzocht. Esomeprazol is net als omeprazol een remmer van het leverenzym CYP2C19, terwijl pantoprazol deze eigenschap niet heeft. De studie laat zien dat bij gelijktijdig gebruik van esomeprazol, de concentratie van de werkzame stof van clopidogrel in het bloed, na een toediening van 600 mg clopidogrel oplaaddosis, bijna gehalveerd is ten opzichte van de concentratie in het bloed bij patiënten die geen protonpompremmer gebruiken. Ook was het gebruik van esomeprazol geassocieerd met hogere bloedplaatjesreactiviteit vergeleken met patiënten zonder protonpompremmers. Echter, bij patiënten die pantoprazol gebruikten, was de vorming van de werkzame stof niet verlaagd ten opzichte van patiënten die geen protonpompremmers gebruikten. Ook hadden pantoprazolgebruikers geen verhoogde bloedplaatjesreactiviteit ten opzichte van patiënten zonder protonpompremmers. Deze studie suggereert dat gelijktijdig gebruik van esomeprazol de werkzaamheid van clopidogrel kan verminderen terwijl dat effect bij het gebruik van pantoprazol minder is. Deze resultaten zijn interessant voor de discussie of de interactie tussen clopidogrel en protonpompremmers een groepseffect is (dus voor alle protonpompremmers relevant) of dat er protonpompremmers zijn die mogelijk beter gebruikt kunnen worden bij patiënten die clopidogrel gebruiken.

In **hoofdstuk 3** wordt de invloed van genetische variaties en geneesmiddelinteracties op klinische eindpunten onderzocht.
Hoofdstuk 3.1 is een case-control studie waarin genetische variaties in ABCB1, CYP2C9, CYP2C19, CYP3A4, CYP3A5 en P2Y1 zijn onderzocht in 176 patiënten (cases) die binnen een jaar na een dotterbehandeling, een zogenaamde stent trombose tijdens het gebruik van clopidogrel en acetylsalicylzuur hadden ontwikkeld en in 420 controlepatiënten die tot een jaar na dotterbehandeling niet opnieuw hart- en vaatproblemen, inclusief stent trombose, tijdens het gebruik van clopidogrel en acetylsalicylzuur hadden gekregen. Stent trombose is een zeer ernstige complicatie na plaatsing van een stent in de kransslagader, waarbij door een acute afsluiting van de kransslagader op de plek van de stent een hartinfarct volgt. Stent trombose komt niet heel vaak voor (bij ongeveer 2-4% van de gedotterde patiënten) maar gaat gepaard met een hoge mortaliteit; 40-50% van de patiënten overlijdt hieraan. Patiënten die drager waren van *CYP2C19*2* hadden een 1,7-maal verhoogde kans op het ontwikkelen van stent trombose vergeleken met patiënten die geen niet-dragers van *CYP2C19*2*. Voor *CYP2C9*3* werd een nog groter effect gevonden; dragers van *CYP2C9*3* hadden een 2,4-maal hogere kans op stent trombose dan niet-dragers van *CYP2C9*3*. *CYP2C19*2* wordt door ongeveer 30% van de Westerse bevolking gedragen, terwijl *CYP2C9*3* veel minder vaak voorkomt (7-9% van de Westerse bevolking). De andere geteste genetische variaties hadden geen effect op het optreden van stent trombose.

In de bovengenoemde studies is de geïsoleerde invloed van een aantal genetische variaties en geneesmiddelen de werkzaamheid van clopidogrel onderzocht. In **hoofdstuk 3.2** hebben we onderzocht wat het effect is van *combinaties* van drie van deze factoren, namelijk het *CYP2C19*2*-dragerschap, het gebruik van protonpompremmers en het gebruik van calciumantagonisten. Dit onderzoek is uitgevoerd bij 725 patiënten die een geplande dotterbehandeling ondergingen en daarna gedurende 1 jaar met clopidogrel en acetylsalicylzuur behandeld werden. Het klinische eindpunt van de studie was de optelsom van het aantal patiënten die dood gingen, of een hartinfarct, stent trombose of beroerte binnen 1 jaar na een

dotterbehandeling kregen. Uit deze studie bleek dat de aanwezigheid van slechts 1 factor (dus *CYP2C19*2* òf protonpompremmers òf calciumantagonisten) niet geassocieerd was met een hogere kans op het klinische eindpunt. Echter, patiënten die zowel een calciumantagonist als een protonpompremmer gebruikten, hadden een 2,1-maal verhoogde kans op het klinische eindpunt. Patiënten die drager waren van *CYP2C19*2* en calciumantagonisten gebruikten hadden zelfs een 3,3-maal verhoogde kans op het klinische eindpunt. Deze studie suggereert dat met name patiënten die een combinatie van factoren bezitten die de werkzaamheid van CYP2C19 (protonpompremmers, *CYP2C19*2*) en CYP3A4 (calciumantagonisten) laten afnemen, een verhoogde kans op een slechtere uitkomst hebben.

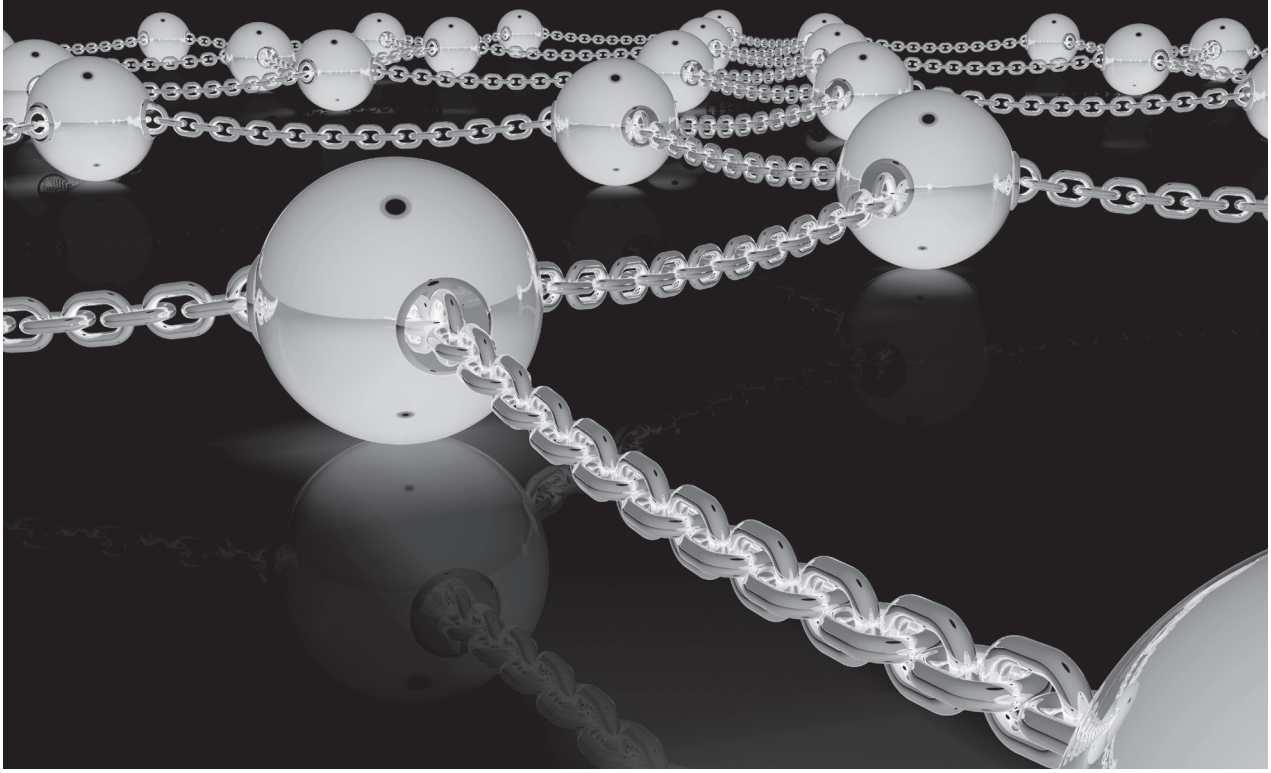
Hoofdstuk 3.3 beschrijft een studie waarin werd onderzocht of genetische variaties in de enzymen CES2, CYP2C9, UGT1A6, COX1 en COX2 de werkzaamheid van acetylsalicylzuur op het voorkomen van een hartinfarct kunnen beïnvloeden. Deze studie werd uitgevoerd in een groep van 853 patiënten die een hartinfarct kregen (cases) en 887 controlepatiënten die geen hartinfarct hadden gekregen. In deze studie, die is uitgevoerd binnen de PHARMO RLS database, vonden we verbanden tussen de werkzaamheid van acetylsalicylzuur en de genetische variaties in de enzymen CES2, CYP2C9, UGT1A6 en COX1.

In **hoofdstuk 3.4** wordt een studie beschreven waarin de invloed van twee genetische variaties - *CYP2C19*2* en *CYP2C19*17* - op bloedplaatjesreactiviteit en op het optreden van ernstige bloedingen is onderzocht. De studie werd uitgevoerd bij 820 patiënten die een geplande dotterbehandeling ondergingen en die met clopidogrel en acetylsalicylzuur werden behandeld. In tegenstelling tot *CYP2C19*2*, leidt *CYP2C19*17* tot een verhoogde werking van het enzym CYP2C19 en dus mogelijk tot een verhoogde werking van clopidogrel. Patiënten met het *CYP2C19*1/*17* en *CYP2C19*17/*17* genotype hadden een lagere bloedplaatjesreactiviteit en een 2,7-maal verhoogde kans op het optreden van ernstige bloedingen binnen 1 jaar na de dotterbehandeling vergeleken met patiënten met het *CYP2C19*1/*1* genotype (*1 heeft normale enzymfunctie). De genotypes *CYP2C19*2/*17*, **1/*2* en **2/*2* hadden een hogere bloedplaatjesreactiviteit dan *CYP2C19*1/*1*. Dit suggereert dat *CYP2C19*17* is geassocieerd met een verhoogde werking van clopidogrel, maar dat dit effect grotendeels teniet wordt gedaan bij patiënten die ook drager zijn van *CYP2C19*2*.

In **hoofdstuk 4** worden de resultaten van onze studies samengevat en in een breder perspectief van klinische relevantie en toekomstig onderzoek geplaatst.

7

DANKWOORD



DANKWOORD

Zonder de hulp en ondersteuning van velen zou het me niet gelukt zijn dit proefschrift af te ronden. Ik kijk terug op een ontzettend leerzame en leuke periode. Iedereen die hierbij betrokken is geweest wil ik daarom heel hartelijk bedanken, in het bijzonder de volgende mensen.

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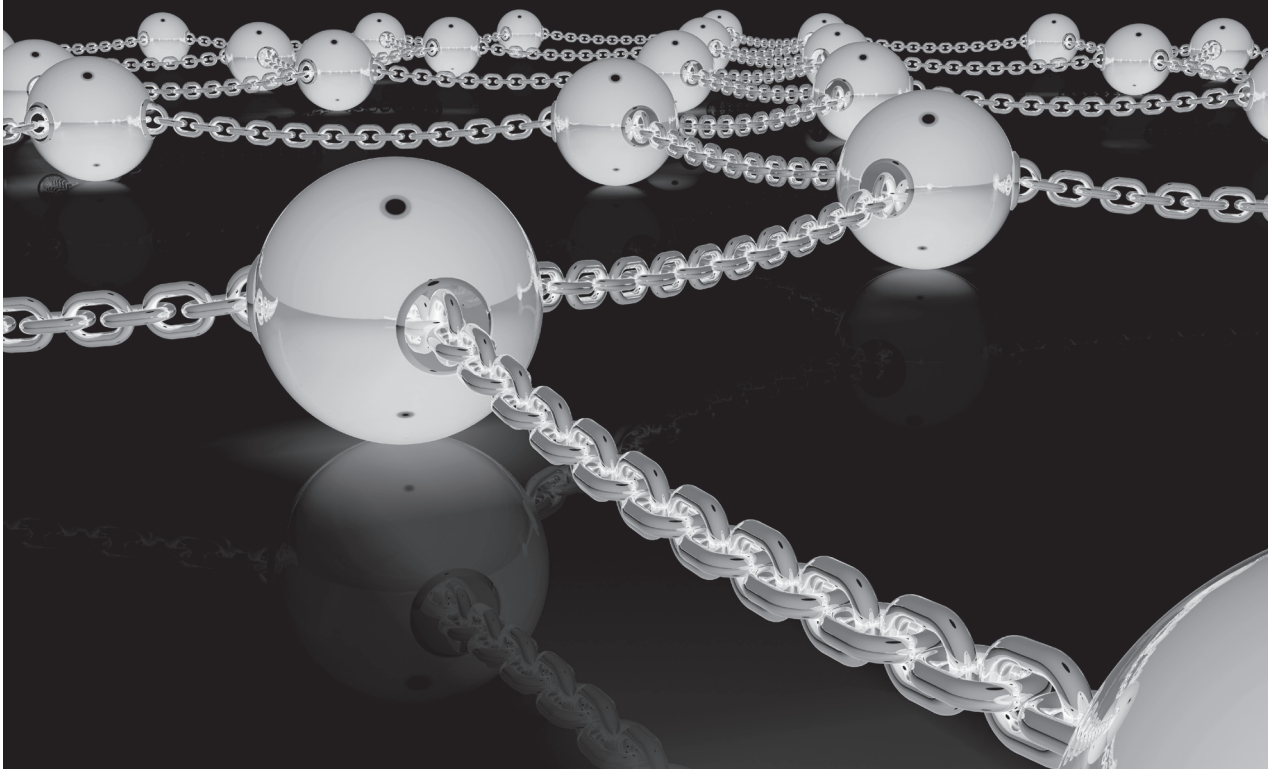
LIST OF CO-AUTHORS



LIST OF CO-AUTHORS

Affiliations during the conduct of the research

Jurriën M. ten Berg
Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Anthonius de Boer
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Heleen J. Bouman
Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Nicoline J. Breet
Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Vera H.M. Deneer
Department of Clinical Pharmacy, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Ellen H.A.M. Elsenberg
Department of Clinical Chemistry, St. Antonius Hospital, Nieuwegein, The Netherlands

Christian M. Hackeng
Department of Clinical Chemistry, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Arnoud W.J. van 't Hof
Department of Cardiology, Isala Clinics, Zwolle, The Netherlands

Johannes C. Kelder
Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Olaf H. Klungel
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Miranda G.M. Kok
Department of Clinical Pharmacy, St. Antonius Hospital, Nieuwegein, The Netherlands

Anke-Hilse Maitland-van der Zee
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Fulya Moral
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Bas J.M. Peters
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Karen Robijns
Department of Clinical Pharmacy, St. Antonius Hospital, Nieuwegein, The Netherlands

Hendrik J.T. Ruven
Department of Clinical Chemistry, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Patrick C. Souverein
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands

Dirk Taubert
Department of Pharmacology, University Hospital, University of Cologne, Germany

Mathieu M. Tjoeng
Department of Clinical Pharmacy, St. Antonius Hospital, Nieuwegein, The Netherlands

Jochem W. van Werkum
Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

Bastiaan Zwart
Department of Cardiology, St. Antonius Hospital, Nieuwegein, The Netherlands
St. Antonius Center for Platelet Function Research, St. Antonius Hospital, Nieuwegein, The Netherlands

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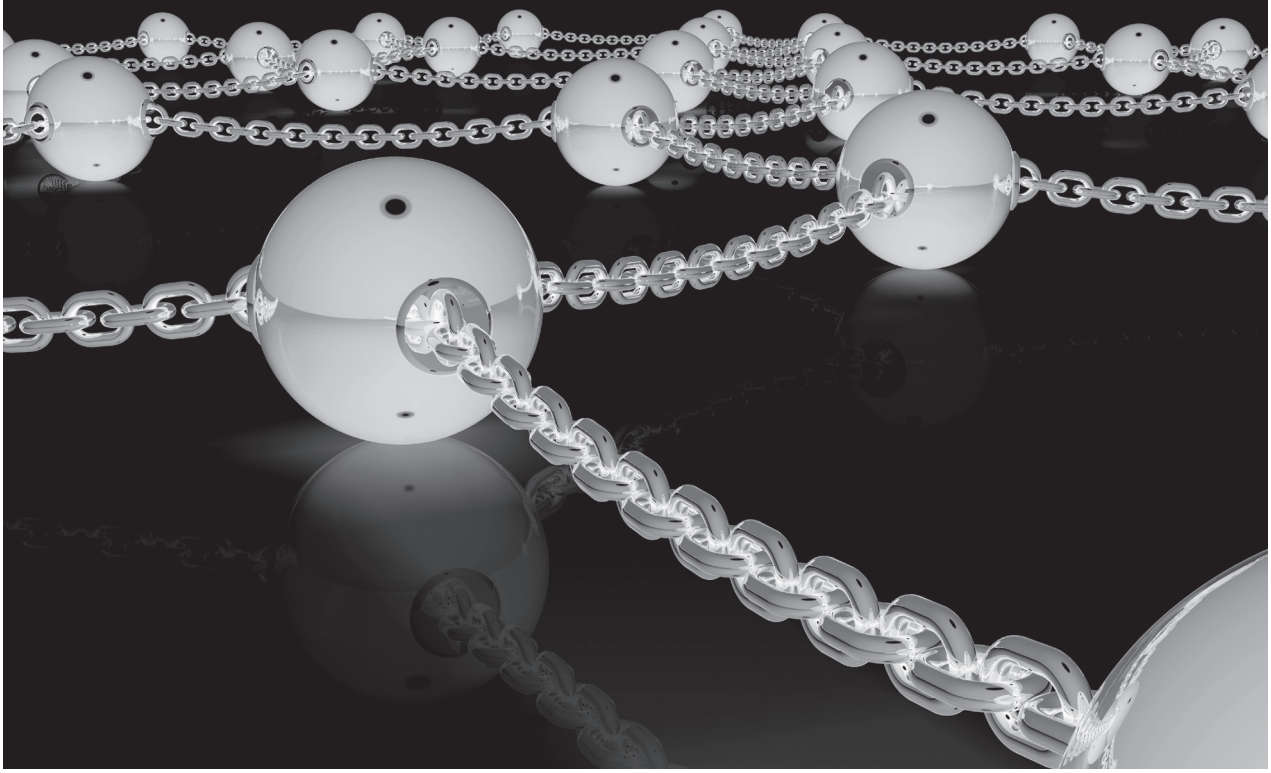
LIST OF PUBLICATIONS



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Peters BJ, Harmsze AM, ten Berg JM, Maitland-van der Zee AH, Deneer VH. The CYP2C19 and ABCB1 genes and individualized treatment with clopidogrel. Accepted Pharmacogenomics 2011

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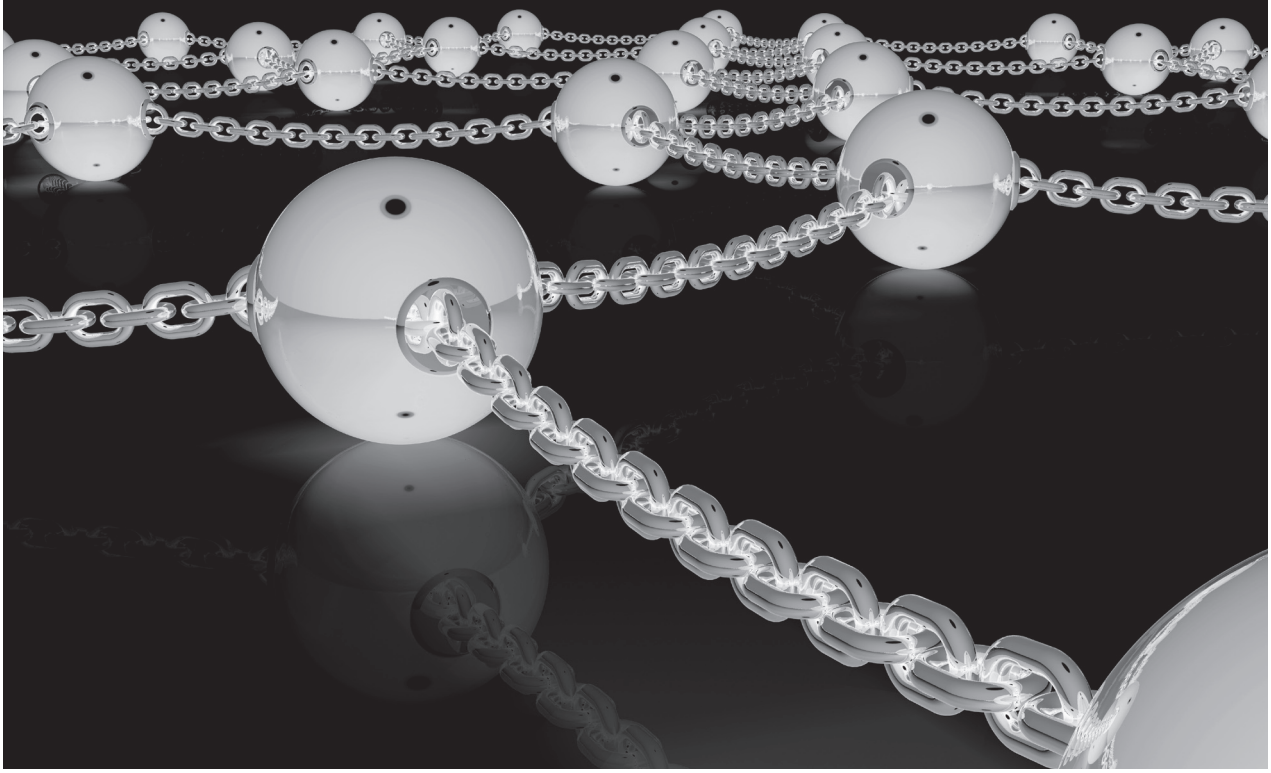
ABOUT THE AUTHOR



ABOUT THE AUTHOR

Ankie Harmsze was born on February 28, 1978 in Gouda, the Netherlands. In 1996, she completed secondary school at the Revius Lyceum in Doorn. She studied pharmacy at the University of Utrecht where she obtained her PharmD in 2004. During her study she did a research project at the Wales Heart Research Institute, Cardiff University, Wales (supervisors Prof. dr. J.R. Cockcroft and Prof. dr. A. de Boer). In 2004, she started working as a pharmacist at the department of Clinical Pharmacy of the St. Antonius Hospital Nieuwegein/Utrecht. In 2006 she initiated her training to become a hospital pharmacist at the Same department. From 2007 she combined this traineeship with the PhD research described in this thesis (ZAPIKO) in affiliation with the Division of Pharmacoepidemiology and Clinical Pharmacology of the Utrecht Institute for Pharmaceutical Sciences of Utrecht University. From January 2011, she holds a position as a registered hospital pharmacist at the Department of Clinical Pharmacy of the Catharina Hospital in Eindhoven.