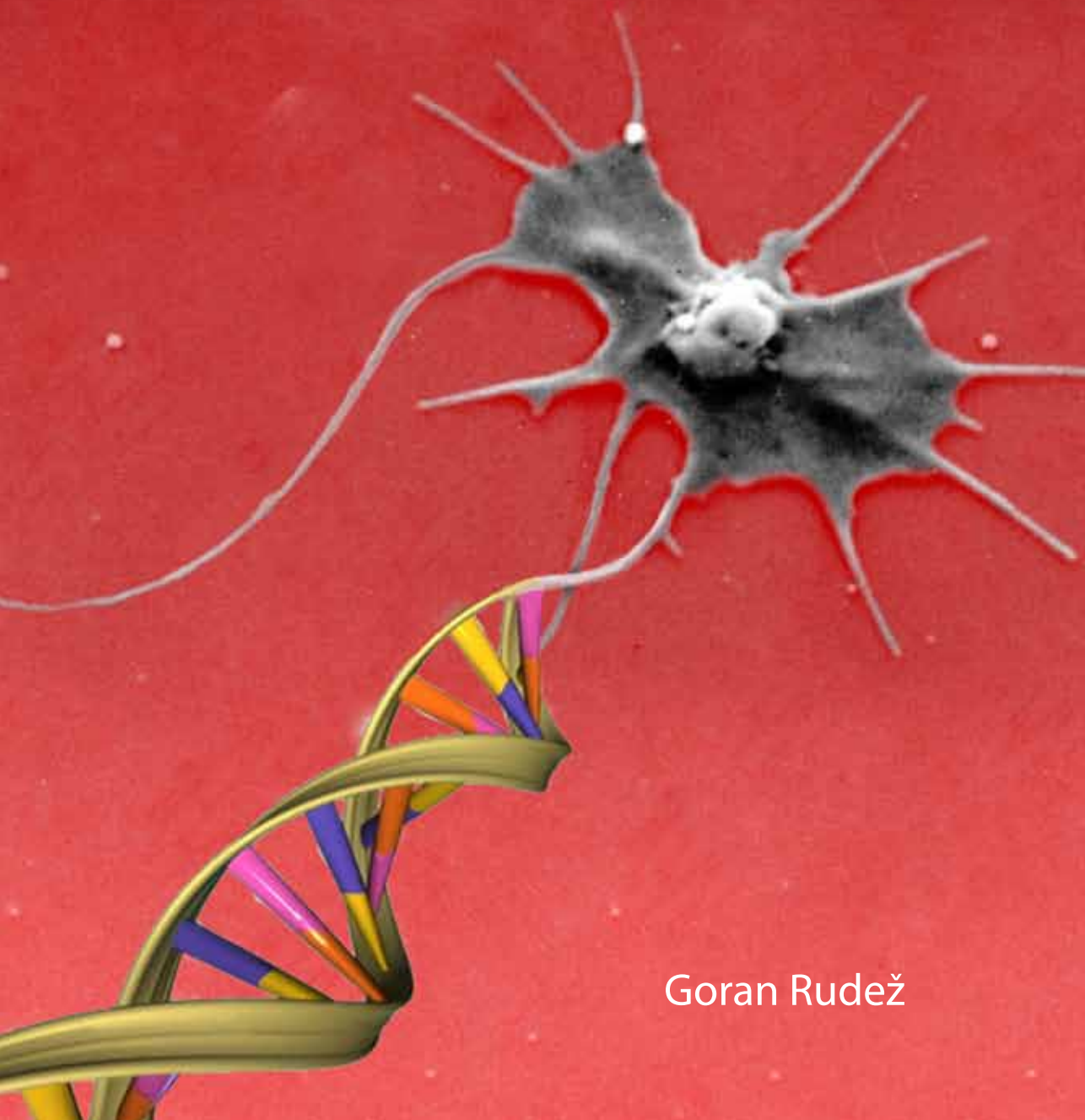
Role of *P2RY12* Gene Variants and Biological Variation in Arterial Thrombosis



Goran Rudež

Role of *P2RY12* Gene Variants and Biological Variation in Arterial Thrombosis

Goran Rudež

Role of *P2RY12* Gene Variants and Biological Variation in Arterial Thrombosis.

ISBN: 978-908559-570-0

Cover design: Goran Rudež
Layout: Egied Simons and Goran Rudež
Printing: Optima Grafische Communicatie, Rotterdam

Role of *P2RY12* Gene Variants and Biological Variation in Arterial Thrombosis

De rol van *P2RY12* genvariaties en biologische variatie in arteriële trombose

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus prof.dr. H.G. Schmidt
en volgens het besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 3 november 2009 om 13:30 uur

door
Goran Rudež
geboren te Zagreb (Kroatië)

PROMOTIECOMMISSIE

Promotor:	Prof.dr. F.W.G. Leebeek
Overige leden:	Prof.dr. H. ten Cate Prof.dr. M.L. Simoons Prof.dr. A.G. Uitterlinden
Copromotor:	Dr. M.P.M. de Maat

The work described in this thesis was performed at the Department of Hematology of Erasmus University Medical Center, Rotterdam, The Netherlands. This work was partly funded by the Netherlands Organization for Scientific Research (NWO) (ZonMW) as a clinical fellow to F.W.G. Leebeek and by the ECAT Foundation.

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Printing of this thesis was financially supported by AstraZeneca, Erasmus University Rotterdam, GlaxoSmithKline, Instrumentation Laboratory, Kordia and Roche Diagnostics.

"Daleko preko granice zapažanja naših čula,
Duh još uvijek može da nas vodi"

"Far beyond the boundary of our senses,
the Spirit can still take us"

Nikola Tesla (1856-1943)

Aan mijn ouders

CONTENTS

1

Chapter 1

General introduction

ARTERIAL THROMBOSIS

Arterial thrombosis is the leading cause of morbidity and mortality in the Western world.[1] Plaque rupture induces arterial thrombus formation, which may result in vascular occlusion.[2] Depending on the localization of the occluded vessel, arterial thrombosis can affect the cardiovascular, cerebrovascular and peripheral arterial systems. Clinical manifestations of arterial thrombosis include acute myocardial infarction (AMI), unstable angina pectoris (UAP), transient ischemic attack (TIA), ischemic stroke (IS) and peripheral arterial disease (PAD).[3] Arterial thrombosis is a multifactorial disease, and both genetic and environmental factors are known to contribute to its pathogenesis.[4, 5] In this thesis we have focused on the role of these genetic factors in arterial thrombosis, with an emphasis on common variation in the platelet receptor *P2RY12* gene. In addition, we studied the role of environmental factors, including the relationship between biological variation and air pollution with several important hemostatic cardiovascular risk factors.

PLATELETS IN PRIMARY HEMOSTASIS

In an intact vascular system blood is maintained in a fluid state, whereas in case of vascular injury a thrombus is formed, resulting in hemostasis (*i.e.* the arrest of bleeding). Hemostasis can be divided into two phases that are called primary and secondary hemostasis. Primary hemostasis is the initial phase of thrombus formation, which leads to the formation of a platelet plug. Secondary hemostasis involves the coagulation system and leads to the formation of a fibrin network that further strengthens the thrombus.[6]

Platelets play a central role in primary hemostasis, and in addition, they facilitate coagulation on the surfaces of their membranes. Platelets are the smallest, anucleated cells in the human circulation, but they bear the highest density of cell membrane receptors, as well as a variety of stimulatory substances within their granulas.[7] These cellular features reflect the main physiological role of platelets in healthy state, i.e. to rapidly adhere to sites of vascular injury and to recruit additional cells and molecules to a growing thrombus. Primary hemostasis is initiated immediately in the event of vessel wall damage, when the endothelial monolayer, which separates the subendothelial layers from flowing blood, is disrupted. Platelets respond to this event in three successive, but closely related steps: platelet adhesion, activation and aggregation (Figure 1).[8, 9]

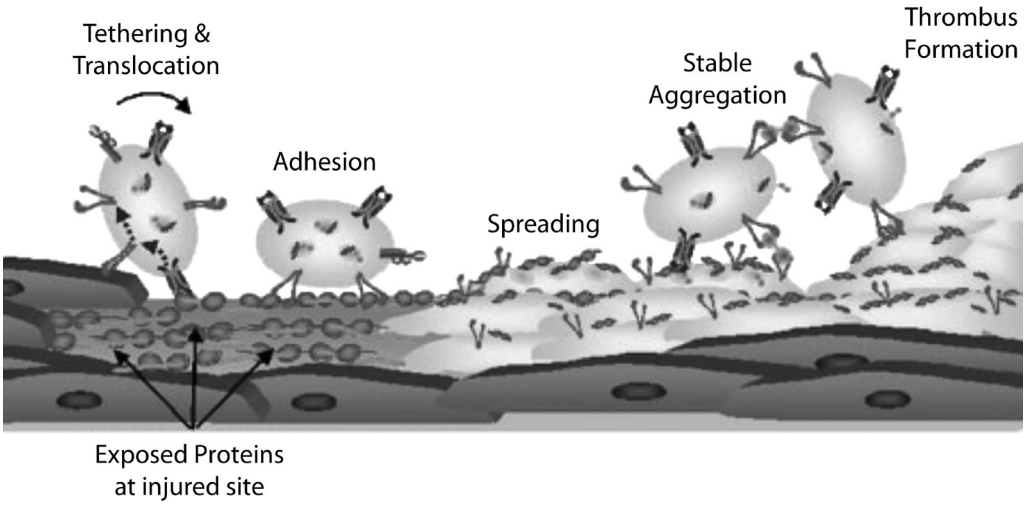


Figure 1. Different stages of platelet response to vascular injury
At sites of vascular injury, subendothelial proteins (primarily collagen and VWF) become exposed to the blood and transiently interact with the rapidly flowing platelet (tethering). In this way the platelet is slowed down (translocation) until it is firmly attached to the vessel wall (adhesion). The initial binding between subendothelial proteins and platelet membrane glycoproteins leads to platelet activation and the formation of a layer of activated platelets that cover the injured site (spreading and aggregation). Simultaneously with platelet aggregation, coagulation is initiated, which together ensures thrombus formation. Adapted from: *http://med.monash.edu.au/assets/images/boxhill/ps-fig1.jpg*

Platelet adhesion

Upon the exposure of the subendothelial layer to the flowing blood, constituents of the subendothelial matrix come into contact with passing platelets. In the arterial vessel, where blood is flowing under high shear stress, von Willebrand factor (VWF) can bind to the exposed subendothelial collagen and is then able to interact with the rapidly passing platelets. First, the passing platelets are slowed down, because the glycoprotein (GP) Ib (GPIb) receptor on the platelet, together with GPV and GPIX, forms a complex on the platelet membrane that can bind to VWF. Once the platelet has been slowed down, it will become firmly attached to the subendothelial layer via binding of its receptors $\alpha_2\beta_1$,GPVI-FcR and $\alpha_{IIb}\beta_3$ to other subendothelial components, such as collagen, fibronectin, laminin, fibrin(ogen) and vitronectin (adhesion). Subsequently, the adhered platelet becomes activated, changes shape and spreads over the exposed subendothelium to increase the surface of coverage.[10, 11]

Platelet activation

During platelet adhesion, the binding of platelet membrane receptors to their subendothelial

ligands initiates several intracellular signaling pathways that ultimately lead to platelet activation.[12] Platelet activation is characterized by the influx of extracellular calcium and the mobilization of intracellular calcium stores. A rise in cytosolic calcium facilitates the rearrangement of platelet cytoskeleton, which is the underlying mechanism of platelet shape change and platelet degranulation. During degranulation, a variety of substances, which are normally contained in platelet granules, are secreted. Among the exocytosed compounds are adenosine diphosphate (ADP), serotonin and thrombin, but also thromboxane A_2 (TxA_2), which is newly synthesized from arachidonic acid (AA) upon activation. Since most of the released compounds are agonists for platelet receptors, their secretion forms a positive feedback loop that is necessary for further autocrine activation of the platelet, as well as paracrine activation of passing platelets and their recruitment into the growing platelet aggregate. Compared to thrombin and collagen, ADP is a relatively weak agonist, but it is the most abundant constituent of platelet dense granules.[13]

Platelet aggregation

Although platelet activation is accompanied by the activation of a variety of distinct intracellular signaling pathways, the common goal of these signaling pathways is to increase the number of activated integrins $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa receptors) on the platelet surface. In this way, activated platelets are able to aggregate with each other by cross-linking their activated GPIIb/IIIa receptors via fibrinogen, and to a lesser extent via VWF.[14] Together, these platelet aggregates form a platelet plug, upon which coagulation and further thrombus growth can take place. Studies have shown that the ADP-receptor P2Y12 plays a central role in platelet aggregation and have suggested that the P2Y12 receptor is especially important in thrombus stability.[15-17]

ROLE OF P2Y12 RECEPTOR IN ARTERIAL THROMBOSIS AND RESTENOSIS

P2Y12 receptor

P2Y1 and P2Y12 are two purinergic G-protein coupled receptors on the platelet membrane that are activated upon binding of ADP.[18] When ADP binds to the P2Y1 receptor, it activates the phospholipase C pathway via the G_q-protein, which then leads to the secretion of calcium from internal stores and subsequent secretion of platelet granules.[12] In this way, P2Y1 receptor leads to platelet activation and platelet shape change.[19] ADP that binds to P2Y12 receptor inhibits the adenylyl cyclase via the G_i-protein. This leads to a lowering of

cyclic AMP (cAMP) in the cytosol and the inhibition of cAMP-dependent protein kinase A (PKA), which in turn cannot phosphorylate vasodilator-stimulated phosphoprotein (VASP). Together, this sequence of downstream events triggers the activation of GPIIb/IIIa receptor and enables platelet aggregation.[20] In addition to P2Y12 receptor, activation of P2Y1 receptor is necessary for full and irreversible aggregation.[21] On the other hand, P2Y12 receptor is able to potentiate the effect of P2Y1 stimulation via enhanced platelet degranulation and contributes in this way to platelet activation (Figure 2).[10]

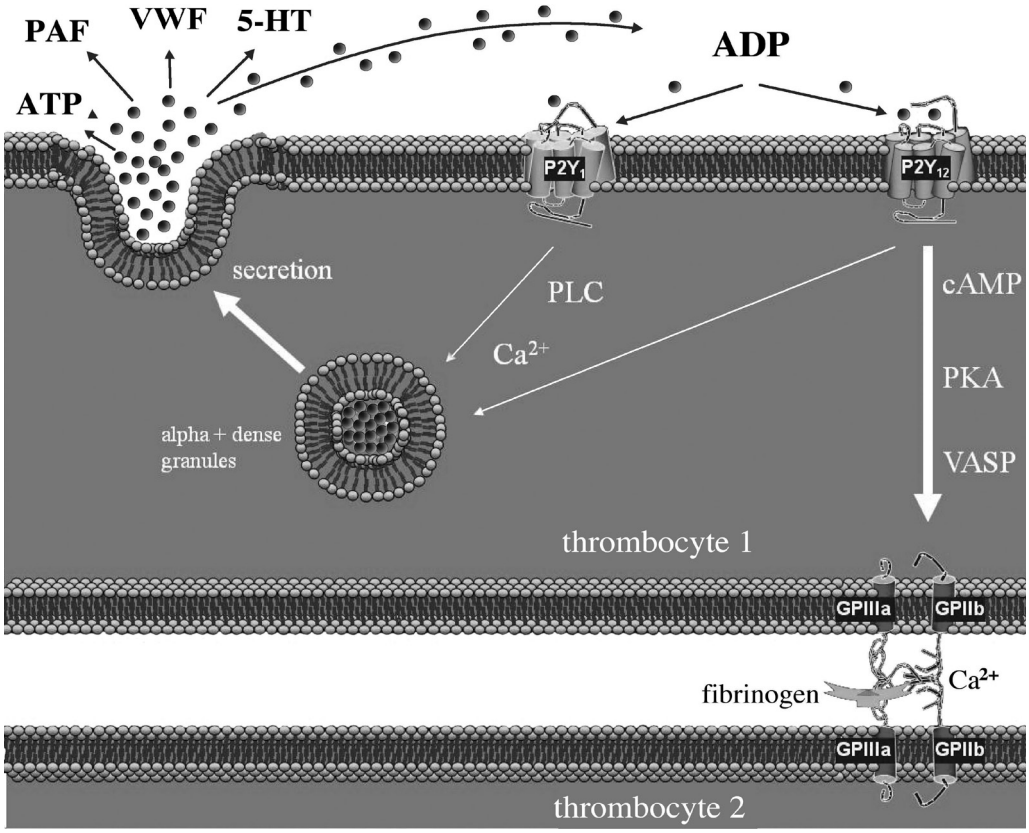


Figure 2. Platelet ADP receptors and their signaling pathways

P2Y1 and P2Y12 are two G-protein coupled ADP-receptors on platelet membranes. Stimulation of the P2Y1 receptor with ADP leads to the mobilization of internal calcium stores via phospholipase C (PLC) signaling and subsequent degranulation of alpha and dense granules. These granules contain a variety of stimulatory substances, among which ADP, adenosine 5'-triphosphate (ATP), platelet activating factor (PAF), von Willebrand factor (VWF) and serotonin (5-HT), which can activate the passing platelets and recruit them into a growing thrombus. Stimulation of the P2Y12 receptor with ADP leads to an inhibition of adenylyl cyclase, and concomitant lowering of cAMP levels. This results in the inhibition of cAMP-dependent protein kinase A (PKA), which in turn cannot phosphorylate vasodilator-stimulated phosphoprotein (VASP). Together, this sequence of downstream events triggers the activation of GPIIb/IIIa receptor and enables platelet aggregation through a calcium-dependent complex of two activated GPIIb/IIIa receptors that link two platelets via fibrinogen.

It has been suggested that the P2Y12 receptor can contribute to arterial thrombosis via increased platelet activation and aggregation.[22] In addition, studies have shown that vascular smooth muscle cells (VSMCs) express P2Y12 receptors, and that the stimulation of these P2Y12 receptors with ADP can evoke contractile responses in these cells.[23] Since VSMCs are key players in restenosis, and also platelet activation is suggested to be involved, this lead to a hypothesis that the P2Y12 receptor might also be important in restenosis.

Platelets in arterial thrombosis and restenosis

Platelets play a pivotal role in the pathogenesis of arterial thrombosis, and may also be involved in the development and progression of atherosclerosis, vascular inflammation and restenosis.[24, 25] Platelets are the major constituents of arterial thrombi that are formed under high shear stress in arterial vessels ("white thrombi").[26] In coronary arteries, these platelet-rich thrombi are formed upon locally ruptured atherosclerotic plaques. In ischemic stroke and PAD, on the other hand, platelet-rich thrombi are not always formed locally, but may also be the consequence of cardioembolism or, in case of ischemic stroke, may originate from upstream stenotic lesions of the carotid artery.[27]

Thrombus formation can also occur during or after percutaneous coronary interventions (PCI), which is currently the treatment of choice for many patients with established coronary artery disease. During PCI, a balloon-tipped catheter is guided to the coronary stenosis after which the stenosis is relieved by inflating the balloon. Nowadays, the majority of PCI treatments are accompanied by the implantation of a coronary stent for the prevention of re-occlusion of the treated vessel due to elastic recoil.[28] However, PCI treatment creates thrombogenic surfaces by inflicting damage to the local endothelium and by introducing a metal meshwork of a coronary stent.[29, 30] Platelets adhere to these thrombogenic surfaces, which in some patients results in an in-stent thrombosis during or shortly after percutaneous coronary interventions (PCI) ("early stent thrombosis"), or sometimes even months after the PCI ("late thrombosis").[31]

In most cases, however, a late re-occlusion of the treated coronary vessel is not the result of late in-stent thrombosis, but of restenosis due to neointimal proliferation.[32] Although restenosis mainly involves VSMCs, platelets are also suggested to play a role.[25, 33] Directly after the placement of a coronary stent, a thin layer of platelets rapidly covers the surface of the stent. These platelets provide a matrix for in-stent migration of VSMCs, which eventually form neointima. In addition, these activated platelets secrete the contents of their granules, thereby releasing a variety of cytokines that can stimulate VSMCs. Among the excreted cytokines are platelet-derived growth factor (PDGF), transforming

growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF), which can all promote neointimal proliferation.[7]

Antiplatelet therapy

Given the central role of platelets in arterial thrombosis, platelet inhibition has proven to be the most effective treatment strategy for the management and prevention of secondary ischemic events.[34] However, the complexity of biochemical pathways involved in the formation of an occlusive thrombus makes it difficult to implement a uniform antiplatelet therapy approach for each patient with arterial thrombosis. Depending on the type of patient, antiplatelet therapy is aimed at inhibiting one or more of these biochemical pathways, of which the TxA_2, ADP, GPIIb/IIIa, thrombin and collagen pathway are common targets for platelet inhibition. Current guidelines recommend lifelong aspirin as the standard antiplatelet therapy for most patients at risk of secondary cardiovascular events and as primary prophylaxis for high-risk patients.[35] Aspirin inhibits the platelet enzyme cyclo-oxygenase 1 (COX-1), which converts AA into TxA_2.[36] In combination with aspirin, the thienopyridine drug clopidogrel is prescribed for the secondary prevention of atherothrombotic events in patients with acute coronary syndromes (ACS) and those who undergo PCI, whereas clopidogrel alone is given to patients with PAD.[37, 38] Clopidogrel is a pro-drug that needs conversion into an active metabolite in the liver (predominantly by the cytochrome P450 3A5 isoenzyme) which irreversibly inhibits the P2Y12 receptor. This inhibition is achieved by the formation of a disulfide bridge between the active clopidogrel metabolite and the extracellular cysteine residues of the P2Y12 receptor, after which ADP cannot bind the receptor and the ADP-dependent platelet aggregation is reduced.[39] In patients with stroke, on the other hand, the recommended pharmacotherapy consists of the combination of aspirin and dipyridamole, which is another thienopyridine that blocks the P2Y12 receptor.[40] However, it is currently still not clear whether clopidogrel monotherapy in stroke is more efficient than the combination therapy of aspirin and dipyridamole.[41]

Despite the proven efficacy of dual antiplatelet therapy with aspirin and clopidogrel in patients with ACS and those who undergo PCI, considerable heterogeneity still exists in the response to this therapy. In approximately 5-30% of clopidogrel-treated patients the inhibition of platelet aggregation is insufficient.[42, 43] As a consequence, patients with high residual on-clopidogrel platelet reactivity may be at a higher risk of atherothrombotic events.[44, 45] Several underlying mechanisms have been proposed that account for this clopidogrel non-responsiveness, often popularly termed “clopidogrel resistance”.

Among these mechanisms are drug-drug interactions, underdosing and genetic factors.[46] Although the benefit of clopidogrel in the prevention of secondary atherothrombotic events is widely acknowledged, clopidogrel efficacy is compromised not only by variability in patients' response, but also by the delayed onset and cessation of clopidogrel's action and incomplete platelet inhibition.[40] This has led to the development of novel P2Y12 antagonists prasugrel (CS-747), AZD6140, cangrelor (ARC-669931MX), INC-50589 and CT-50547.[45] These novel drugs combine one or more improved features, such as more potent platelet inhibition than with clopidogrel, intravenous administration with rapid onset of action, reversible inhibition of the P2Y12 receptor with rapid cessation of action and fewer or none steps required for drug conversion. However, despite these promising features, the efficacy of these novel drugs may be hampered by a number of factors that are commonly associated with clopidogrel non-responsiveness, such as genetic variation in the P2Y12 receptor.

Common variation in the *P2RY12* gene

Since the P2Y12 receptor is crucial for platelet aggregation and, in addition, is the pharmacological target of clopidogrel, *P2RY12* is an apparent candidate gene for risk of arterial thrombosis and a potential determinant of clopidogrel efficacy. Proposed mechanisms that may underlie such an association are altered molecular structure and function of the P2Y12 receptor, or altered expression of P2Y12 receptors.[47] In case of altered molecular function, this would be caused by genetic variants that induce a change in the amino acid composition of the receptor, *e.g.* by non-synonymous single nucleotide polymorphisms (SNPs) in the coding region, or via SNPs that induce alternative splicing of the *P2RY12* mRNA. Recently, common variation in the *P2RY12* gene was found to be associated with platelet activation and aggregation[47] and the risk of cardiovascular disease[48, 49], but the results from these small studies are conflicting.[50, 51] In addition, these studies were limited by the fact that only the haplotype-tagging SNP (ht-SNP) rs2046934 ("i-T744C") was studied. Rs2046934 is in complete linkage disequilibrium (LD) with 3 other *P2RY12* SNPs and the haplotypes "H1" and "H2" that these SNPs determine cover only the 3' part of the *P2RY12* gene.

A comprehensive study of the common variation in a gene ideally includes ht-SNPs that cover all common haplotypes within the entire locus, *i.e.* the coding sequence and the regulatory regions, such as the promoter and the 3'untranslated region (UTR). To use this comprehensive approach for the *P2RY12* gene in this thesis, first a LD-map of the *P2RY12* locus was constructed based on the latest release of the International HapMap Project (phase II, October 2007; *http://www.hapmap.org/*) (Figure 3), or based on LD-data from other databases, such as the Seattle SNPs (*http://pga.mbt.washington.edu*).[52]

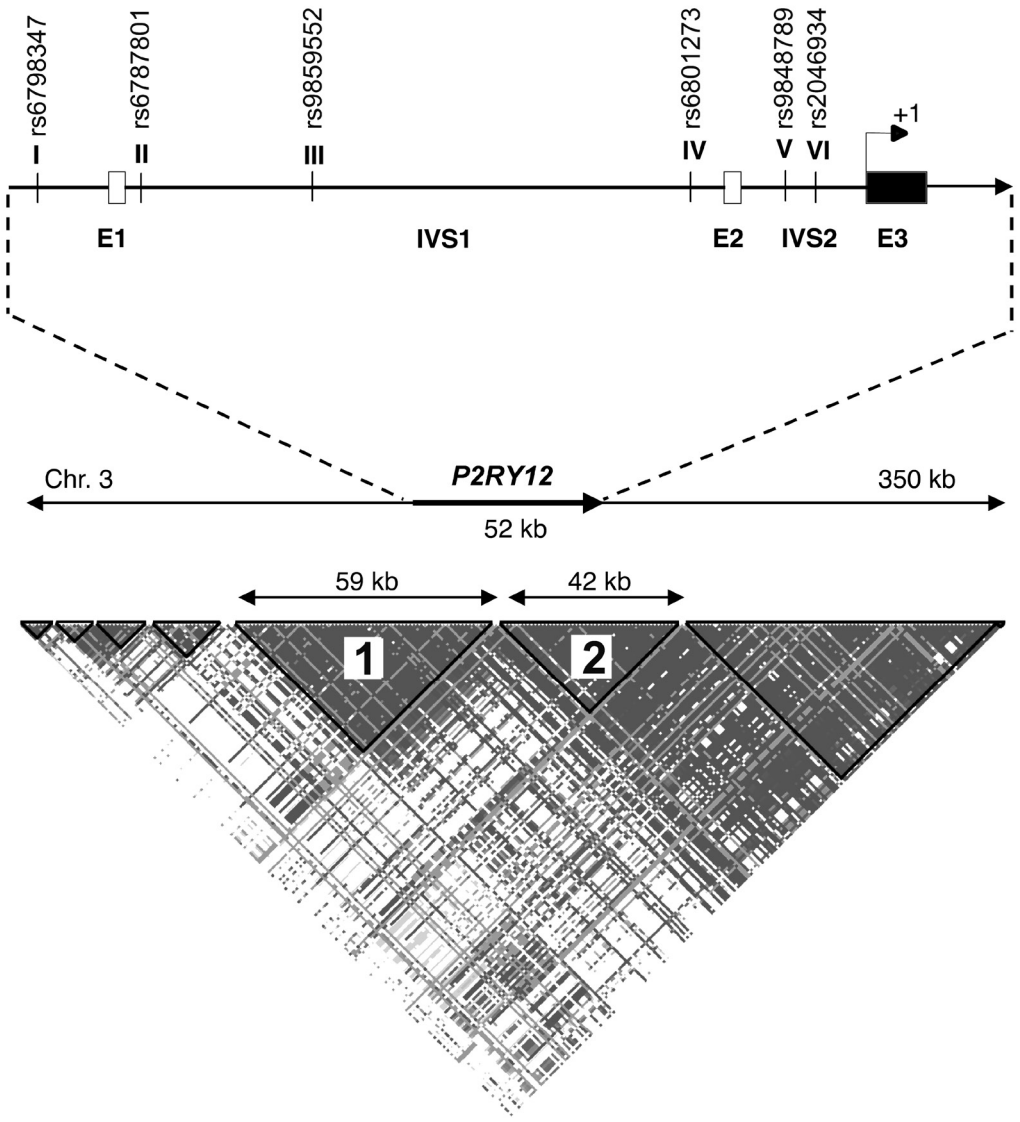


Figure 3. Schematic representation of the *P2RY12* gene and the linkage disequilibrium map of the *P2RY12* locus
The *P2RY12* gene consists of three exons (E1, E2 and E3, of which only E3 is coding) and two intervening sequences (IVS1 and IVS2). The gene spans 47kb of genomic DNA. The inverted pyramid-shaped figure depicts the LD-map for SNPs from the 350kb region on chromosome 3 surrounding the *P2RY12* gene that were genotyped in the International HapMap Project. In shades from white to grey are indicated increasing pairwise-LDs between these SNPs. Based on this map, two LD-blocks (59kb and 42kb) can be discerned that completely cover the *P2RY12* gene. Within these blocks there is little or no recombination between the SNPs, whereas the blocks are separated with recombination hot-spots. In order to tag all common combinations of SNPs (haplotypes with allele frequency >5 %) within the *P2RY12* gene and 2.5kb flanking regions (together 52kb), 6 haplotype-tagging SNPs (ht-SNPs) were selected for study and depicted I-VI in the figure with their corresponding rs-numbers.

Within this LD-map, blocks of haplotypes were defined from ht-SNPs selected within the Haploview software (*http://www.broad.mit.edu/mpg/haploview/index.php*). For the studies described in this thesis, only haplotypes with an allele frequency >5% were considered. Using this approach, the defined haplotypes together covered 88% of the total common DNA sequence variation in the *P2RY12* locus.

BIOLOGICAL VARIATION IN HEMOSTATIC AND INFLAMMATORY FACTORS

In addition to genetic factors, environmental factors may contribute to arterial thrombosis. These environmental factors may induce temporal variations in the biological factors that are important in the pathogenesis of this disease, and in this way influence an individual's risk to develop arterial thrombosis. In general, in studies where biological factors are measured, the corresponding data can be influenced by three types of variation: (i) the pre-analytical variation (*e.g.* the variation due to blood collection and processing), (ii) the analytical variation (*i.e.* the variation in the test method), and (iii) the biological variation (Table 2).[53]

Table 2. Sources of test result variation

Pre-analytical variation
Sample handling
Fasting state
Exercise
Posture
Analytical variation
Intra- and inter-assay
Random
Systematic = bias (*e.g.* different operators, lots of reagents, apparatus)
Biological variation
Within-subject fluctuation of a variable over time around a homeostatic setpoint
Random
Rhythmical
Daily
Monthly (women)
Seasonal
During a lifespan (*e.g.* puberty, adulthood, menopause, old age)

Biological variation

Biological variation is the within-subject variation over time due to natural biological factors, such as age, obesity or acute-phase reaction. When using biological factors as long-term risk indicators, however, we are interested in the differences between the habitual concentrations of the study participants (*i.e.* their true or homeostatic mean) and not in their short-term fluctuations. However, in most epidemiologic studies, only a single blood sample is taken from each subject and this is then accepted as representative of an individual's true mean. For some study variables, multiple sampling over time may be required to improve the estimate of an individual's homeostatic mean. Furthermore, the biological variation forms the basis for the analytical quality specifications regarding analytical precision and reference values that are required for diagnosis of patients or monitoring of subjects within population-based studies. In addition, biological variation is used as criteria in quality control of laboratory assays.

Epidemiologic studies on cardiovascular disease (CVD) have suggested that a number of inflammatory and haemostatic markers play an important role. Among these inflammatory markers are fibrinogen and C-reactive protein (CRP), which have been identified as independent risk factors for CVD.[54, 55] In addition, the hemostatic markers platelet aggregation, thrombin generation and prothrombin time (PT) have been shown to play a role in CVD.[8, 56, 57] Data on biological variation of these factors is only limited, or completely lacking.[58] Importantly, the plasma levels of fibrinogen and CRP are occasionally raised by an acute phase reaction (*e.g.* due to the common cold), and are also characterized by seasonal variation.[59] The latter observation has been linked to differences in rates of cardiovascular mortality, which are also characterized by seasonal variation.[60] Since biological variation is a source of test result variation, it may affect the diagnosis, monitoring and prognostic assessment of CVD that is based on tests of these inflammatory and hemostatic markers.[61]

Air pollution

One of the important environmental sources of biological variation in the industrialized world is air pollution. Air pollution has been consistently associated with increased morbidity and mortality due to respiratory disease and CVD.[62, 63] Underlying biological mechanisms are not entirely clear, but hemostasis and inflammation are suggested to be involved.[64-66] Air pollution can be roughly divided into particulate matter (PM) and gasses. It has been postulated that small particles, such as inhaled gasses and ultra-fine PM, are able to directly translocate into the blood stream.[67] There, they can directly

disturb the hemostatic balance, for instance via enhanced platelet activation and thrombin generation.[68-70] In contrast, larger particles that cannot pass the lung epithelium will cause local inflammation in the lung, which indirectly can alter hemostasis via systemic effects (Figure 4).[71, 72]

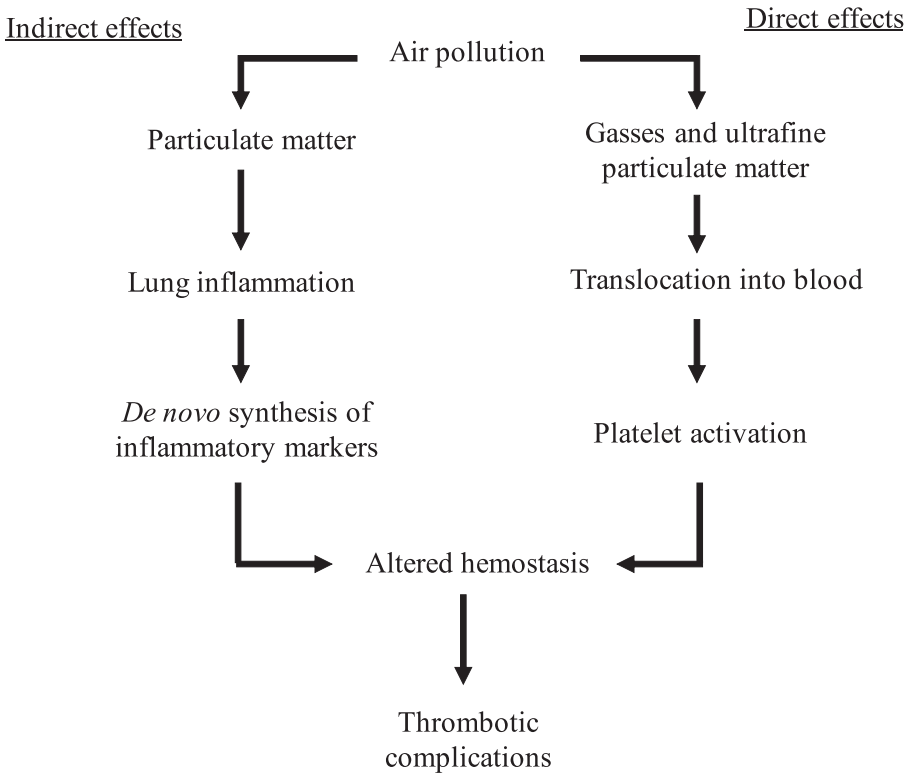


Figure 4. Possible mechanisms underlying the effects of air pollution on inflammation and hemostasis
The pathogenesis of CVD involving air pollution may be divided into direct and indirect effects. On the left, a hypothetical pathway is depicted for particulate matter that cannot pass the lung-blood barrier and is retained in the lung, which causes local inflammation and *de novo* synthesis of inflammatory markers. On the right, a hypothetical pathway is depicted for gasses and ultrafine particulate matter which are small enough to directly translocate from inhaled air of lung alveoli into the blood and cause direct systemic effects, such as increased platelet activation. Together, these indirect and direct effects of air pollutants may alter the hemostatic balance and lead to thrombotic complications.

AIM AND OUTLINE OF THE THESIS

The aim of this thesis is to address two main research questions:

1. What is the role of common variation in the *P2RY12* gene in arterial thrombosis at the level of:
    - *Predisposition*: Are *P2RY12* gene variants associated with the risk of arterial thrombosis?
    - *Treatment:* Do *P2RY12* gene variants compromise the efficacy of platelet inhibition by the P2Y12 antagonists clopidogrel and cangrelor? And is this effect on platelet inhibition different between clopidogrel and cangrelor, as well as across different dosing regimens?
    - *Outcome after treatment*: Does common variation in the *P2RY12* gene have an effect on the risk of restenosis after PCI?
2. What is the biological variability of hemostatic factors (platelet aggregation, thrombin generation and prothrombin time) and inflammatory factors (fibrinogen and CRP)?
    - Does air pollution contribute to this biological variability?
    - Is common variation in the *CRP* gene associated with a response in CRP serum levels after an inflammatory trigger?

Part I of this thesis provides insights into the first main research question. As previously described, the P2Y12 receptor plays a central role in platelet aggregation and is the pharmacological target of several thienopyridine drugs, of which clopidogrel is currently the most widely used for treatment of patients with arterial thrombosis. Therefore, we hypothesized that the common variation in the *P2RY12* gene increases the risk of arterial thrombosis via increased platelet aggregation and/or reduced platelet inhibition by P2Y12 receptor antagonists.

To investigate the association between common variation in the *P2RY12* gene and increased risk of arterial thrombosis, we conducted a case-control study in patients suffering from arterial thrombosis at young age. In general, the genetic contribution to the pathogenesis of a disease is stronger in younger individuals.[5, 73] In addition, we investigated whether this association with risk could be explained by increased platelet aggregation (**Chapter 2**).

Numerous studies in patients with arterial thrombosis have reported wide inter-individual variability in the patients' response to clopidogrel. Suboptimal platelet inhibition

by clopidogrel has been suggested to lead to an increased risk of adverse secondary events in these patients. To investigate whether common variation in the *P2RY12* gene is a determinant of this large inter-individual variability in the efficacy of clopidogrel treatment, we assessed the on-clopidogrel platelet reactivity in patients who underwent PCI and received clopidogrel. We performed platelet function measurements by means of ADP-induced light-transmittance aggregometry (the gold standard) and a point-of-care device, the Verify*Now* P2Y12 assay. We compared the corresponding results between different clopidogrel dosing regimens to seek for possible gene-dose effects of the *P2RY12* haplotypes (**Chapter 3**).

The large inter-individual variability in clopidogrel response is one of the limitations of clopidogrel that has led to the development of novel P2Y12 antagonists with potentially improved pharmacological (and/or practical) features. Cangrelor is one of these novel P2Y12 antagonists, which is intravenously administered and acts directly on the P2Y12 receptor. To study the inter-individual variability in response to cangrelor and the contribution to this variability by common *P2RY12* gene variants, we have conducted a study in a group of healthy subjects in whom platelet function was measured before and after administration of different doses of cangrelor (**Chapter 4**).

The efficacy of P2Y12 antagonists has been frequently linked to the risk of adverse secondary events, mainly due to the formation of an occlusive thrombus (*e.g.* stent thrombosis). This link has been frequently made given the central role of the P2Y12 receptor in platelets. However, since the P2Y12 receptor is also expressed on the VSMCs that are important in neointimal proliferation, we hypothesized that common *P2RY12* gene variants might also be associated with the risk of restenosis. We tested this hypothesis in a prospective study on clopidogrel-treated patients who underwent PCI with coronary stenting and were followed for 12 months after the intervention (**Chapter 5**).

Part II of this thesis addressed the second main research question. In a longitudinal study on 40 healthy subjects, we determined the biological variation during a period of one year for the hemostatic markers platelet aggregation, thrombin generation and prothrombin time, and the inflammatory markers fibrinogen and CRP. On the basis of this biological variation, we then calculated the recommended minimal number of repeated measurements required to assess an individual's true mean. Data on biological variation were also used to determine the quality specifications for the utilized assays by providing recommendations on the allowable analytical imprecision for diagnosis and monitoring of the studied variables, corresponding analytical bias and the contribution of analytical

imprecision to test result variability. Also, we determined the seasonal variation of the studied inflammatory and hemostatic markers (**Chapter 6**).

The latest literature on the effects of particles on hemostasis and inflammation was reviewed (**Chapter 7**).

Next, we investigated in the above mentioned population of 40 healthy subjects whether the temporal variations in the concentrations of various air pollutants are associated with the concentrations of the hemostatic and inflammatory markers measured in the previous study (**Chapter 8**).

CRP is an independent risk factor for CVD. Since CRP is a sensitive acute-phase protein, its serum level may rise up to 100-fold after an inflammatory trigger. We studied whether the common variation in the *CRP* gene is associated with the response in serum levels of CRP after a standardized inflammatory trigger. This was done in a population of 264 healthy subjects who underwent a well-controlled surgical procedure (**Chapter 9**).

REFERENCES

1. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, Haase N, Hailpern S, Ho M, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott M, Meigs J, Mozaffarian D, Nichol G, O'Donnell C, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Steinberger J, Thom T, Wasserthiel-Smoller S, Wong N, Wylie-Rosett J, Hong Y. Heart disease and stroke statistics-2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*. 2009;119:480-486.
2. Fuster V, Stein B, Ambrose JA, Badimon L, Badimon JJ, Chesebro JH. Atherosclerotic plaque rupture and thrombosis. Evolving concepts. *Circulation*. 1990;82:II47-59.
3. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med.* 2007;357:2482-2494.
4. Ajjan RA, Ariens RA. Cardiovascular disease and heritability of the prothrombotic state. *Blood Rev.* 2009;23:67-78.
5. Zdravkovic S, Wienke A, Pedersen NL, Marenberg ME, Yashin AI, De Faire U. Heritability of death from coronary heart disease: a 36-year follow-up of 20 966 Swedish twins. *J Intern Med.* 2002;252:247-254.
6. Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med.* 2008;359:938-949.
7. Gresele P, Page, C., Fuster, V., Vermylen, J. Platelets in thrombotic and non-thrombotic disorders. Cambridge: Cambridge University Press; 2002.
8. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med.* 2002;8:1227-1234.
9. Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med.* 2002;8:1175-1181.
10. Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol.* 2008;28:403-412.
11. Jackson SP. The growing complexity of platelet aggregation. *Blood.* 2007;109:5087-5095.
12. Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. *J Thromb Haemost.* 2003;1:1602-1612.
13. Woulfe D, Yang J, Brass L. ADP and platelets: the end of the beginning. *J Clin Invest.* 2001;107:1503-1505.
14. Lefkovits J, Plow EF, Topol EJ. Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med.* 1995;332:1553-1559.
15. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, Koller B, Phillips DR, Conley PB. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest.* 2003;112:398-406.
16. Goto S, Tamura N, Ishida H, Ruggeri ZM. Dependence of platelet thrombus stability on sustained glycoprotein IIb/IIIa activation through adenosine 5'-diphosphate receptor stimulation and cyclic calcium signaling. *J Am Coll Cardiol.* 2006;47:155-162.
17. Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood.* 2006;108:3045-3052.
18. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature.* 2001;409:202-207.
19. Kuwahara M, Sugimoto M, Tsuji S, Matsui H, Mizuno T, Miyata S, Yoshioka A. Platelet shape changes and adhesion under high shear flow. *Arterioscler Thromb Vasc Biol.* 2002;22:329-334.

20. Woulfe DS. Platelet G protein-coupled receptors in hemostasis and thrombosis. *J Thromb Haemost.* 2005;3:2193-2200.
21. Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP, Gachet C. The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood.* 1998;92:152-159.
22. Gachet C, Hechler B. The platelet P2 receptors in thrombosis. *Semin Thromb Hemost.* 2005;31:162-167.
23. Wihlborg AK, Wang L, Braun OO, Eyjolfsson A, Gustafsson R, Gudbjartsson T, Erlinge D. ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arterioscler Thromb Vasc Biol.* 2004;24:1810-1815.
24. Lindemann S, Kramer B, Seizer P, Gawaz M. Platelets, inflammation and atherosclerosis. *J Thromb Haemost.* 2007;5 Suppl 1:203-211.
25. Chandrasekar B, Tanguay JF. Platelets and restenosis. *J Am Coll Cardiol.* 2000;35:555-562.
26. Tan KT, Lip GY. Red vs white thrombi: treating the right clot is crucial. *Arch Intern Med.* 2003;163:2534-2535; author reply 2535.
27. Barnett HJ, Taylor DW, Eliasziw M, Fox AJ, Ferguson GG, Haynes RB, Rankin RN, Clagett GP, Hachinski VC, Sackett DL, Thorpe KE, Meldrum HE, Spence JD. Benefit of carotid endarterectomy in patients with symptomatic moderate or severe stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. *N Engl J Med.* 1998;339:1415-1425.
28. Silber S, Albertsson P, Aviles FF, Camici PG, Colombo A, Hamm C, Jorgensen E, Marco J, Nordrehaug JE, Ruzyllo W, Urban P, Stone GW, Wijns W. Guidelines for percutaneous coronary interventions. The Task Force for Percutaneous Coronary Interventions of the European Society of Cardiology. *Eur Heart J.* 2005;26:804-847.
29. Markovitz JH, Roubin GS, Parks JM, Bittner V. Platelet activation and restenosis after coronary stenting: flow cytometric detection of wound-induced platelet activation. *Coron Artery Dis.* 1996;7:657-665.
30. Vargova K, Toth-Zsamboki E, Beres BJ, Bencze J, Kerecsen G, Gulacsi-Bardos P, Kiss RG, Preda I. Circulating endothelial cell count, plasma vWF and soluble ICAM-1 levels following primary or elective percutaneous coronary intervention. *Atherosclerosis.* 2008;198:366-372.
31. Braunwald E, Angiolillo D, Bates E, Berger PB, Bhatt D, Cannon CP, Furman MI, Gurbel P, Michelson AD, Peterson E, Wiviott S. The problem of persistent platelet activation in acute coronary syndromes and following percutaneous coronary intervention. *Clin Cardiol.* 2008;31:I17-20.
32. Kipshidze N, Dangas G, Tsapenko M, Moses J, Leon MB, Kutryk M, Serruys P. Role of the endothelium in modulating neointimal formation: vasculoprotective approaches to attenuate restenosis after percutaneous coronary interventions. *J Am Coll Cardiol.* 2004;44:733-739.
33. Goel PK, Shahi M, Agarwal AK, Srivastava S, Seth PK. Platelet aggregability and occurrence of restenosis following coronary angioplasty. *Int J Cardiol.* 1997;60:227-231.
34. Phillips DR, Conley PB, Sinha U, Andre P. Therapeutic approaches in arterial thrombosis. *J Thromb Haemost.* 2005;3:1577-1589.
35. Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R, Buring J, Hennekens C, Kearney P, Meade T, Patrono C, Roncaglioni MC, Zanchetti A. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet.* 2009;373:1849-1860.
36. Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. *N Engl J Med.* 2005;353:2373-2383.
37. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. *Lancet.* 1996;348:1329-1339.

38. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *N Engl J Med.* 2001;345:494-502.
39. Pereillo JM, Maftouh M, Andrieu A, Uzabiaga MF, Fedeli O, Savi P, Pascal M, Herbert JM, Maffrand JP, Picard C. Structure and stereochemistry of the active metabolite of clopidogrel. *Drug Metab Dispos.* 2002;30:1288-1295.
40. Raju NC, Eikelboom JW, Hirsh J. Platelet ADP-receptor antagonists for cardiovascular disease: past, present and future. *Nat Clin Pract Cardiovasc Med.* 2008;5:766-780.
41. Leys D, Balucani C, Cordonnier C. Antiplatelet drugs for ischemic stroke prevention. *Cerebrovasc Dis.* 2009;27 Suppl 1:120-125.
42. Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation.* 2003;107:2908-2913.
43. Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL, Topol EJ. Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol.* 2005;45:246-251.
44. Snoep JD, Hovens MM, Eikenboom JC, van der Bom JG, Jukema JW, Huisman MV. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. *Am Heart J.* 2007;154:221-231.
45. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, Costa MA. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. *J Am Coll Cardiol.* 2007;49:1505-1516.
46. Nguyen TA, Diodati JG, Pharand C. Resistance to clopidogrel: a review of the evidence. *J Am Coll Cardiol.* 2005;45:1157-1164.
47. Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation.* 2003;108:989-995.
48. Fontana P, Gaussem P, Aiach M, Fiessinger JN, Emmerich J, Reny JL. P2Y12 H2 haplotype is associated with peripheral arterial disease: a case-control study. *Circulation.* 2003;108:2971-2973.
49. Ziegler S, Schillinger M, Funk M, Felber K, Exner M, Mlekusch W, Sabeti S, Amighi J, Minar E, Brunner M, Muller M, Mannhalter C. Association of a functional polymorphism in the clopidogrel target receptor gene, P2Y12, and the risk for ischemic cerebrovascular events in patients with peripheral artery disease. *Stroke.* 2005;36:1394-1399.
50. Hetherington SL, Singh RK, Lodwick D, Thompson JR, Goodall AH, Samani NJ. Dimorphism in the P2Y1 ADP receptor gene is associated with increased platelet activation response to ADP. *Arterioscler Thromb Vasc Biol.* 2005;25:252-257.
51. Schettert IT, Pereira AC, Lopes NH, Hueb WA, Krieger JE. Association between platelet P2Y12 haplotype and risk of cardiovascular events in chronic coronary disease. *Thromb Res.* 2006;118:679-683.
52. The International HapMap Project. *Nature.* 2003;426:789-796.
53. Fraser CG. Biological variation: from principles to practice. Washington, DC: AACC Press; 2001.
54. Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. *N Engl J Med.* 1984;311:501-505.
55. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med.* 2004;350:1387-1397.
56. Moss AJ, Goldstein RE, Marder VJ, Sparks CE, Oakes D, Greenberg H, Weiss HJ, Zareba W, Brown MW, Liang CS, Lichstein E, Little WC, Gillespie JA, Van Voorhees L, Krone RJ, Bodenheimer MM, Hochman J, Dwyer EM, Jr., Arora R, Marcus FI, Watelet LF, Case RB. Thrombogenic factors and recurrent coronary events. *Circulation.* 1999;99:2517-2522.

57. Freedman DS, Byers T, Barboriak JJ, Flanders WD, Duncan A, Yip R, Meilahn EN. The relation of prothrombin times to coronary heart disease risk factors among men aged 31-45 years. *Am J Epidemiol.* 1992;136:513-524.

58. Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. *www.westgard.com/biodatabase1.htm.* 2008.

59. Verschuur M, van der Beek MT, Tak HS, Visser LG, de Maat MP. Interindividual variation in the response by fibrinogen, C-reactive protein and interleukin-6 to yellow fever vaccination. *Blood Coagul Fibrinolysis.* 2004;15:399-404.

60. Woodhouse PR, Khaw KT, Plummer M, Foley A, Meade TW. Seasonal variations of plasma fibrinogen and factor VII activity in the elderly: winter infections and death from cardiovascular disease. *Lancet*. 1994;343:435-439.

61. Reynolds TM, Twomey P, Wierzbicki AS. Accuracy of cardiovascular risk estimation for primary prevention in patients without diabetes. *J Cardiovasc Risk.* 2002;9:183-190.

62. McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, Harrington R, Svartengren M, Han IK, Ohman-Strickland P, Chung KF, Zhang J. Respiratory effects of exposure to diesel traffic in persons with asthma. *N Engl J Med.* 2007;357:2348-2358.

63. Peng RD, Chang HH, Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F. Coarse particulate matter air pollution and hospital admissions for cardiovascular and respiratory diseases among Medicare patients. *Jama.* 2008;299:2172-2179.

64. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC, Jr., Tager I, Expert Panel on Population and Prevention Science of the American Heart A. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation.* 2004;109:2655-2671.

65. Peters A, Doring A, Wichmann HE, Koenig W. Increased plasma viscosity during an air pollution episode: a link to mortality? *Lancet.* 1997;349:1582-1587.

66. Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med.* 2006;173:432-441.

67. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation.* 2002;105:411-414.

68. Nemmar A, Hoylaerts MF, Hoet PH, Nemery B. Possible mechanisms of the cardiovascular effects of inhaled particles: systemic translocation and prothrombotic effects. *Toxicol Lett.* 2004;149:243-253.

69. Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet.* 1995;345:176-178.

70. Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, Radomski MW. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br J Pharmacol.* 2005;146:882-893.

71. Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environ Health Perspect.* 2001;109 Suppl 4:523-527.

72. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, Frew A. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med.* 1999;159:702-709.

73. Marenberg ME, Risch N, Berkman LF, Floderus B, de Faire U. Genetic susceptibility to death from coronary heart disease in a study of twins. *N Engl J Med.* 1994;330:1041-1046

I

Part I

2

Chapter 2

Goran Rudež
Emile de Bruijne
Tamara Bongers
Diederik Dippel
Jaap Deckers
Don Poldermans
Huub van Vliet
Moniek de Maat
Frank Leebeek

Common variation in the platelet receptor *P2RY12* gene is associated with the risk of arterial thrombosis

Submitted

ABSTRACT

Aim

There are indications that polymorphisms in the P2Y12 receptor contribute to pathogenesis of arterial thrombosis. We comprehensively investigated the association between the total common variation in the *P2RY12* locus and the risk of arterial thrombosis.

Methods and Results

In this study we included 374 young patients with arterial thrombosis (coronary heart disease (CHD), ischemic stroke (IS) or peripheral arterial disease (PAD)) and 332 controls. We selected five haplotype-tagging single nucleotide polymorphisms (ht-SNPs) to comprehensively study the total common variation in the *P2RY12* gene (ht-SNPs: rs6798347, rs10935842, rs6787801, rs6801273 and rs2046934). Haplotypes of these five ht-SNPs were inferred and haplotype H5 (TATGT, allele frequency 10%) was associated with an increased risk of an arterial thrombotic event (OR 1.7, 95% CI 1.1-2.8), especially CHD (OR 2.0, 95% CI 1.2-3.6), compared with the reference haplotype H1 (CATAC, allele frequency 17%).

Conclusions

P2RY12 haplotypes are associated with an increased risk of arterial thrombosis, especially CHD.

INTRODUCTION

Platelet aggregation plays a key role in arterial thrombosis.[1] The adenosine diphosphate (ADP) receptor P2Y12 is a central mediator of platelet activation and aggregation, and plays an important role in thrombus growth and stability.[2-4] Fontana *et al*. reported an association between a limited number of single nucleotide polymorphisms (SNPs) in the *P2RY12* gene and risk of peripheral arterial disease.[5] However, other studies on the role of *P2RY12* SNPs in atherothrombotic disease show conflicting results.[6-8] Although these discrepancies may be partly explained by the differences in study design and characteristics of the study population, it remains unclear whether *P2RY12* gene variations play a significant role in arterial thrombosis.

Previous studies on *P2RY12* gene variations covered only a small region of the *P2RY12* locus. However, to adequately determine the role of the common variation in the *P2RY12* gene, a comprehensive study of the total genetic variation in the *P2RY12* locus is required, including the regulatory regions, such as the promoter and the 3'untranslated region (UTR). Using this approach, we already reported that common variation in the *P2RY12* gene is associated with the risk of restenosis after percutaneous coronary interventions.[9] Since the genetic contribution to disease is stronger in younger individuals than in elderly subjects with clear atherosclerosis,[10,11] the optimal group to study the genetic component of pathogenesis of arterial thrombosis includes young patients. Therefore, the aim of this study was to comprehensively investigate the association between the total common genetic variation of the *P2RY12* locus and the risk of arterial thrombosis in a case-control study of young patients with arterial thrombosis.

METHODS

Study population

In this case-control study we have included consecutive patients with arterial thrombotic disease at young age. Patients were eligible for inclusion if they were 45 years or younger (males) or 55 years or younger (females) and suffered a first acute ischemic complication in either the cardiac, cerebral or peripheral vascular system. Patients were divided in three subgroups: (i) coronary heart disease (CHD), which included patients with acute myocardial infarction (AMI) or unstable angina pectoris (UAP); (ii) ischemic stroke (IS), which included patients with IS or transient ischemic attacks (TIA); and (iii) patients

who suffered from peripheral arterial disease (PAD). Patients were included one to three months after the event, to avoid the effects of an acute phase response. Controls were friends, neighbours or partners of the patients fulfilling the same age criteria but without a history of arterial thrombosis. Relatives of the patients were not permitted to participate in the study since gene polymorphisms were studied.

The study protocol is in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of Erasmus MC. Written informed consent was obtained from each participant.

Definitions

AMI was defined as typical chest pain, with elevated cardiac markers (CK-MB, troponin T) and/or characteristic electrocardiographic findings. UAP was defined as typical chest pain while at rest confirmed by characteristic electrocardiographic findings and normal levels of cardiac markers (CK-MB, troponin T). TIA was defined as focal cerebral deficit of sudden onset confirmed by a neurologist, which cannot be classified otherwise than as caused by local cerebral ischemia. Symptoms had to be temporary and last less than 24 hours after onset. IS was defined as suddenly occurring cerebral deficit, which cannot be explained otherwise than as local cerebral ischemia, and that lasted for longer than 24 hours after onset. Brain imaging by CT or MRI was required to confirm the initial diagnosis. The diagnosis PAD was defined as peripheral arterial stenosis resulting in ischemia, classified according to the Rutherford criteria.[12]

Clinical data were collected using a standard medical questionnaire and physical examination was performed by research physicians. Smoking was defined as previous or current smoking. Hypercholesterolemia was defined as blood total cholesterol >5.0 mmol/L or the use of lipid-lowering treatment on day of inclusion. Diabetes was defined as fasting plasma glucose >7 mmol/L or non-fasting plasma glucose >11 mmol/L, or the use of either anti-diabetic medication or insulin on day of inclusion. Hypertension was defined as a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg, or use of anti-hypertensive medication on day of inclusion.

Blood collection

Blood was drawn by venapuncture in the antecubital vein using the Vacutainer system (Beckton Dickinson, Plymouth, UK). For DNA isolation, blood was collected in tubes containing EDTA, and genomic DNA was extracted following standard salting-out procedures and stored at 4 °C for genetic analysis. DNA was not available from 10 persons. The characteristics of these

missing persons were similar to those who could be successfully genotyped. For platelet aggregation experiments, blood was collected in sodium citrate tubes (final concentration 3.2%) and immediately centrifuged for 15 min. at 150*g* to obtain platelet rich plasma (PRP).

Selection of SNPs in the *P2RY12* gene

The *P2RY12* gene spans 47kb and is located in the q25.1 region of chromosome 3 (Figure 1).

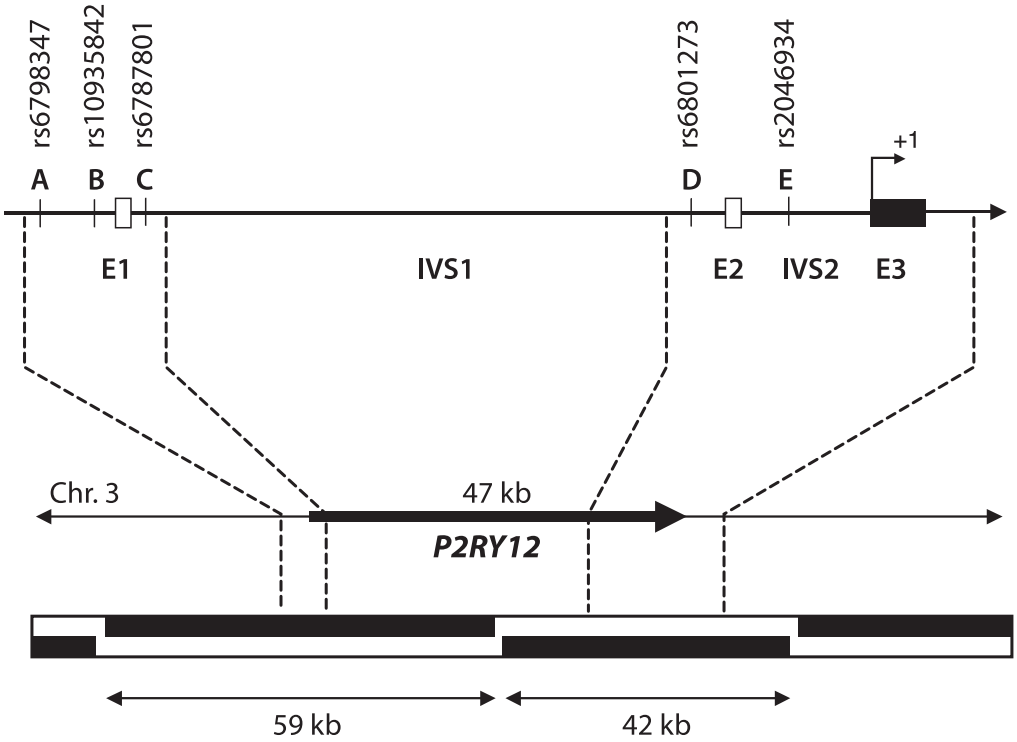


Figure 1. Schematic representation of *P2RY12* gene structure and linkage disequilibrium map of *P2RY12* locus

The *P2RY12* gene consists of three exons (E1, E2 and E3) and two intervening sequences (IVS1 and IVS2), which span 47kb of genomic DNA on chromosome 3. Only E3 is coding. Based on the genotype data of HapMap SNPs, a linkage disequilibrium map of the *P2RY12* locus (*P2RY12* gene with 125kb flanking DNA) can be derived. Black bars represent linkage disequilibrium blocks within which there is little or no recombination between SNPs. Blocks are separated by recombination hot-spots. Haplotypes tagged by SNPs A, B and C comprehensively represent the genetic variation in the promoter-E1 region localized in the 59kb linkage disequilibrium block, whereas haplotypes tagged by SNPs D and E represent the genetic variation in the E2-IVS2-E3 region present in the 43kb linkage disequilibrium block. The five selected SNPs also cover a significant portion of the genetic variation in IVS1, especially its 5' and 3'end.

The gene contains three exons, of which only exon 3 is coding. Over 120 SNPs have been detected in this genomic region, which are listed in the SNP database of NCBI (dbSNP, build 123). In our study, we considered only SNPs that were present in the Caucasian population with a minor allelic frequency of at least 5%. From these SNPs we selected tagging SNPs of haplotypes

that comprehensively represent the DNA sequence variation in the promoter-exon1 and most of intron 1 (from c.-281-4450 to c.-217+3350), and exon2-intron2-exon3 region (from c.-216-4850 to c.1257+1250) of the *P2RY12* gene, as these regions are likely to contain the main functional elements. This selection of ht-SNPs was performed on the basis of the linkage disequilibrium (LD) map of the *P2RY12* locus provided by the International HapMap Project (phase I, June 2005; *http://www.hapmap.org/*)(Figure 1). For the chosen *P2RY12* locus, which is comprised of the *P2RY12* gene (47kb) and 5'and 3'flanking regions (50kb each), blocks of haplotypes with frequency >5% were defined and their ht-SNPs selected using Haploview software (version 3.11, *http://www.broad.mit.edu/mpg/haploview/index.php*). For the selection of the ht-SNPs, we also took potential functionality into consideration, but none of the SNPs in the *P2RY12* gene results in an amino acid change, nor are there any SNPs located within consensus transcription factor binding sites in the 3 kb promoter region, exon-intron junctions, predicted splicing enhancer motifs, the 3 kb 3'UTR-region nor domains that are highly conserved among species.

The five selected ht-SNPs that were genotyped in this study are SNP A: 5'flanking region (c.-281-3614C>T) [rs6798347], SNP B: 5'flanking region (c.-281-1394A>T) [rs10935842] and SNP C: IVS1(c.-217+2739T>C) [rs6787801], which tag the promoter-exon1-intron1 region and SNP D: IVS1(c.-216-4445A>G) [rs6801273] and SNP E: IVS2(c.-15+742T>C) [rs2046934], which tag the intron1-exon2-intron2-exon3-3' UTR region (Figure 1 and Table 1). Annotation of the selected ht-SNPs is according to the nomenclature recommendations of the Human Genome Variation Society, using cDNA sequence available under GenBank accession number NM_022788.3 as reference, with nucleotide +1 being the A of the ATG-translation initiation codon.[13]

Table 1. Haplotype-tagging *P2RY12* SNPs

Region	SNP ID*	SNP ID†	SNP ID‡	SNP A§	SNP B§	SNP C§	SNP D§	SNP E§
5' flanking	SNP A	c.-281-3614C>T	rs6798347	-	0.98	0.91	0.37	0.88
5' flanking	SNP B	c.-281-1394A>T	rs10935842	0.14	-	0.98	0.21	0.38
IVS1	SNP C	c.-217+2739T>C	rs6787801	0.19	0.62	-	0.27	0.51
IVS1	SNP D	c.-216-4445A>G	rs6801273	0.06	0.04	0.04	-	0.99
IVS2	SNP E	c.-15+742T>C	rs2046934	0.04	0.02	0.06	0.13	-

*SNP annotation used for convenience in this paper. In previous publications SNP E has been annotated as i-T744C.[18]
†Nucleotide numbering of the SNPs according to the nomenclature recommendations of the Human Genome Variation Society using the cDNA reference sequence available under GenBank accession number NM_022788.3, with +1 being the A of the ATG translation initiation codon located in exon 3. ‡Reference ID according to NCBI dbSNP.
§Values of r^2 (under the diagonal axis) and D' (above the diagonal axis) for pairwise linkage disequilibrium analysis of the five *P2RY12* SNPs. High values of r^2 and D' represent high linkage disequilibrium indicating those SNP-pairs that are often co-inherited.

Genotyping and haplotype analysis

The five selected haplotype-tagging SNPs were genotyped using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, USA) under standard conditions.[14] The nucleotide sequences of the primers and probes used for each assay are available upon request. End-point fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, USA). A random selection of 10% of the samples was re-analysed, and the results were confirmed for more than 99%. Laboratory analyses were performed by research technicians who were blinded to the phenotype of the study subjects.

From the obtained unphased SNP genotype data, haplotypes were inferred using HaploStats software (*http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm*).[15] Haplotype alleles were coded with five letters (e.g. C<u>G</u>C<u>A</u>T) representing the nucleotides of SNPs A, B, C, D and E, respectively, with the minor alleles underlined. For convenience, the haplotype alleles were also coded H1 to H5 according to their descending allele frequency in the total study population.

Optical aggregometry

Optical platelet aggregometry was performed within 2 hours after blood collection in 116 healthy controls, as described previously.[16] To selectively study the contribution of ADP receptors to platelet aggregation, platelet-rich plasma was pre-incubated with aspirin (100 μM final) for at least 20 min. to inhibit thromboxane A2 synthesis. In addition, citrated platelet-rich plasma was brought at the physiological calcium concentration of 16.6 mM by the addition of $CaCl_2$ (Merck & Co, New York, NY, USA), after 2 min of pre-incubation with thrombin-inhibitor D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK)(40 μM final)(Merck & Co, Darmstadt, Germany). Platelet aggregation was induced by 5 and 2.5 μM ADP (Sigma-Aldrich, Missouri, USA) and recorded for 10 min on a 4-channel optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden, the Netherlands). The magnitude of the aggregation response (maximal aggregation), aggregation rate (slope) and aggregate stability (aggregation at 2, 4 and 6 minutes after maximal aggregation) were measured.

Statistical methods

Data are presented as means and standard deviations for continuous variables and as counts and percentages for categorical variables. To test whether the genotype distribution of the studied SNPs deviates from that expected for a population in Hardy-Weinberg equilibrium we used a Chi-squared test with one degree of freedom. The association between the

P2RY12 haplotypes and the risk of arterial thrombosis was determined by weighted logistic regression analysis using HaploStats software (*http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm*).[15] Briefly, this haplotype analysis calculates posterior probabilities for each possible haplotype of an individual and assigns an appropriate weight to the corresponding risk estimate. HaploStats accounts for missing genotype data by inducing in the analysis the likelihood for all possible genotypes for the incomplete loci. Individuals with missing genotype data for more than two SNPs, however, were excluded from the analysis to reduce the error of haplotype risk estimates. The characteristics of these individuals were similar to those who were included in the analysis. This statistical method improves the risk estimates that are calculated from phase-ambiguous genotype data. The association between the *P2RY12* haplotypes and the aggregation parameters was determined by linear regression analysis using HaploStats. Other statistical analyses were performed with SPSS for Windows, version 11.5 (SPSS Inc, Chicago, USA). The covariates included in the multivariable model are age and sex. A two-sided value of $P<0.05$ was considered statistically significant.

In case the number of homozygotes for the minor allele of a SNP was lower than 5, these genotypes were combined with the corresponding heterozygotes under assumption that the minor allele has additive effects on the risk. Statistical analyses were performed both within the total group of patients and within the subgroups of patients with CHD and IS. Subgroup analysis of PAD patients was not performed since the number of PAD patients was too small to yield sufficient statistical power, but these patients were included for the haplotype risk estimates of the total group of patients with arterial thrombosis.

RESULTS

Study population

A total of 374 patients and 332 controls were included in the study of whom the characteristics are listed in table 2. Of these 374 patients, 218 patients suffered from CHD, 109 from IS and 47 from PAD. As expected, the prevalence of known cardiovascular risk factors, *e.g.* hypertension and diabetes, was significantly higher in the patient group than in the healthy controls. The patients were slightly older than the controls (mean age: 43 and 39 years, respectively) (Table 2).

P2RY12 genotypes

The genotype distributions of the five *P2RY12* SNPs were in Hardy-Weinberg equilibrium. We studied the relationship of these individual *P2RY12* SNPs with arterial thrombosis and used homozygotes for the common alleles of these SNPs as our reference groups.

Table 2. Characteristics of the *ATTAC* study population

Demographics	Patients (n=374)	Controls (n=332)	p-value
Age (years)	43 (7)	39 (8)	<0.001
Male	162 (43%)	122 (37%)	0.08
Caucasian	299 (84%)	283 (88%)	0.08
Index event			
Coronary heart disease	218 (58%)	-	-
Cerebrovascular ischemia	109 (29%)	-	-
Peripheral arterial disease	47 (13%)	-	-
Risk factors			
BMI > 30 kg/m^2	78 (21%)	31 (9%)	<0.001
Hypertension	293 (79%)	62 (19%)	<0.001
Statin use	290 (78%)	4 (1%)	<0.001
Diabetes	33 (9%)	5 (2%)	<0.001
Smoking	297 (79%)	162 (49%)	<0.001

Values are given as n (%) for categorical variables and as mean (SD) for continuous variables. BMI indicates body mass index.

In this analysis, we observed the strongest risk association between CHD and SNP D in patients with the GG genotype (OR 2.6, 95% CI 1.4-4.9). Patients with the AG genotype of SNP D had an intermediate risk for CHD (OR 1.2, 95% CI 0.8-1.8) (Table 3). When SNP D was analysed in the total group of patients with arterial thrombosis, the OR for any arterial thrombotic event was 2.1 (95% CI 1.2-3.6) for patients with the GG genotype, whereas an intermediate OR of 1.1 (95% CI 0.8-1.6) was observed for patients with the AG genotype (table 3). In patients with IS, an OR of 1.7 (95% CI 0.8-3.6) was observed for the GG genotype of SNP D, while an intermediate OR of 1.1 (95% CI 0.7-1.8) was seen for the heterozygotes AG (Table 3).

For SNP A, there was a slightly higher risk of arterial thrombosis (OR 1.4, 95% CI 1.0-1.9) and CHD (OR 1.5, 95% CI 1.0-2.2) for the combined TC and CC genotype (Table 3). None of the other *P2RY12* SNPs was clearly associated with the risk of arterial thrombosis, CHD or IS (Table 3).

Table 3. *P2RY12* SNPs and the risk of arterial thrombosis

	Controls	Total		CHD		IS	
			OR		OR		OR
Genotype	(N=332)	(N=374)	(95% CI)	(N=218)	(95% CI)	(N=109)	(95% CI)
SNP A							
CC	59%	57%	1 (ref.)	57%	1 (ref.)	52%	1 (ref.)
CT & TT	33%	37%	1.4 (1.0-1.9)	39%	1.5 (1.0-2.2)	39%	1.4 (0.9-2.3)
SNP B							
AA	42%	39%	1 (ref.)	40%	1 (ref.)	34%	1 (ref.)
AT	39%	45%	1.2 (0.9-1.7)	43%	1.2 (0.8-1.8)	48%	1.5 (0.9-2.5)
TT	11%	10%	0.8 (0.5-1.4)	13%	1.0 (0.5-1.7)	5%	0.5 (0.2-1.5)
SNP C							
TT	27%	21%	1 (ref.)	24%	1 (ref.)	16%	1 (ref.)
TC	41%	44%	1.3 (0.9-2.0)	40%	1.1 (0.7-1.7)	53%	2.3 (1.3-4.3)
CC	22%	22%	1.2 (0.7-1.8)	23%	1.0 (0.6-1.8)	17%	1.3 (0.6-2.8)
SNP D							
AA	41%	36%	1 (ref.)	35%	1 (ref.)	35%	1 (ref.)
AG	42%	41%	1.1 (0.8-1.6)	40%	1.2 (0.8-1.8)	39%	1.1 (0.7-1.8)
GG	8%	14%	2.1 (1.2-3.6)	15%	2.6 (1.4-4.9)	13%	1.7 (0.8-3.6)
SNP E							
TT	59%	63%	1 (ref.)	63%	1 (ref.)	62%	1 (ref.)
TC & CC	35%	33%	0.8 (0.6-1.2)	33%	0.8 (0.6-1.3)	29%	0.8 (0.5-1.3)

Genotype frequencies are given as percentages of study (subgroup) population. Odds ratios are adjusted for age and sex. In case of SNP A and E, the number of homozygotes for the minor allele was lower than 5 in at least one of the table cells so that this group was pooled with the corresponding heterozygotes. CHD indicates coronary heart disease; IS, ischemic stroke.

P2RY12 haplotypes

By combining the genotypes of the five *P2RY12* SNPs, 32 haplotype alleles were inferred of which 5 had an allele frequency higher than 10% (Table 4 and Figure 2).

Table 4. *P2RY12* haplotypes and the risk of arterial thrombosis

Haplotype codes		Allele frequency		OR (95% CI)		
		Controls (n=310)	Patients (n=358)	Total (n=358)	CHD (n=211)	IS (n=101)
H1	CATAC	17%	14%	1 (ref.)	1 (ref.)	1 (ref.)
H2	CATAT	15%	14%	1.2 (0.8-1.9)	1.1 (0.7-2.0)	1.3 (0.7-2.5)
H3	CTCGT	15%	17%	1.4 (0.9-2.1)	1.4 (0.9-2.3)	1.3 (0.7-2.6)
H4	CTCAT	15%	14%	1.1 (0.7-1.8)	1.1 (0.7-1.9)	1.2 (0.6-2.3)
H5	TATGT	10%	12%	1.7 (1.1-2.8)	2.0 (1.2-3.6)	1.5 (0.7-3.0)

Odds ratios are adjusted for age and sex. Individuals with missing genotypes for more than two SNPs were excluded from the analysis after which the number of controls was 310, arterial thrombosis (total group) 358, CHD 211 and IS 101. CHD indicates coronary heart disease; IS, ischemic stroke.

Haplotype H1 (CATAC), which contains the minor allele of SNP E, was most frequently observed in our total study population (17%) and was associated with the lowest risk estimates in all analyses (Table 4 and Figure 2). Therefore, haplotype H1 was used as the reference in our weighted logistic regression analysis. Haplotype H5 (TATGT) was associated with an increased risk of an arterial thrombotic event when compared to the reference haplotype H1 (OR 1.7, 95% CI 1.1-2.8)(Table 4 and Figure 2A). In the subgroup analysis, haplotype H5 showed a two-fold higher risk of CHD (OR 2.0, 95% CI 1.2-3.6), while the risk increase in the IS subgroup was slightly weaker (OR 1.5, 95% CI 0.7-3.0) (Table 4 and Figure 2B). Additional adjustment for BMI >30 kg/m^2, hypertension, hypercholesterolemia, diabetes, smoking and race only slightly affected the risk estimates (data not shown).

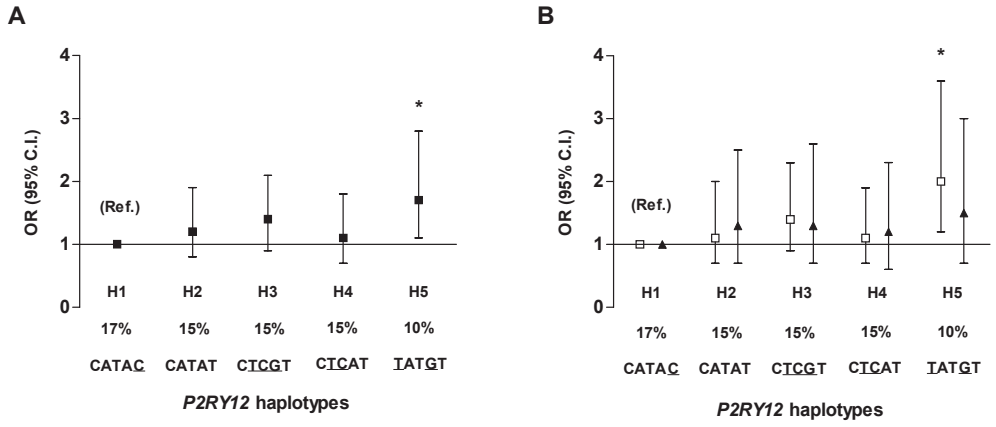


Figure 2. *P2RY12* haplotypes and the risk of arterial thrombosis
Odds ratios are adjusted for age and sex. Individuals with missing genotypes for more than two SNPs were excluded from the analysis after which the number of controls was 310, arterial thrombosis (total group) 358 (Panel A), coronary heart disease 211 and ischemic stroke 101 (Panel B: open squares and triangles, respectively).
Error bars represent 95% confidence intervals.
*p<0.05

When the risk estimates for the *P2RY12* haplotypes are compared, it is strongly suggested that SNPs D and E are the strongest contributors to the increased risks. Therefore, we have repeated our analysis with haplotypes comprising only SNPs D and E, which tag the region that contains the exon encoding the mature P2Y12 protein. In this analysis, haplotype GT (with G-allele of SNP D and T-allele of SNP E) was associated with a 45% higher risk of any arterial thrombotic event than the reference haplotype AC (OR 1.5, 95% CI 1.1-2.0). Similarly, haplotype GT showed a 54% higher risk of CHD (OR 1.5, 95% CI 1.1-2.3) and a 40% higher risk of IS than haplotype AC (OR 1.4, 95% CI 0.9-2.2). Overall, the risk estimates of arterial thrombosis, CHD and IS for haplotype GT were only slightly lower than for haplotype H5 (TATGT, which contains the same GT alleles).

To determine whether changes in platelet aggregation are a part of the underlying mechanism of the relationship between *P2RY12* haplotypes and risk of arterial thrombosis, we have studied platelet aggregation in 116 controls from our ATTAC study. Patients were not studied, because most of them were using platelet-function inhibiting medication. The effects of haplotypes on maximal aggregation, aggregation rate and aggregate stability were minimal (less than 5% difference compared to H1)(data not shown). We also performed the analysis of effects of individual SNPs and of haplotypes comprising only SNPs D and E on platelet aggregation, which gave no differences between haplotypes (data not shown).

DISCUSSION

In the present study we identified haplotype H5 (TATGT) of the platelet ADP receptor P2Y12 as an independent predictor of risk of arterial thrombosis. This association was predominantly observed in the subgroup analysis of patients with CHD, although the same trend was also seen for IS. The lack of statistically significant relation between haplotype H5 and IS may be due to the small sample size or the heterogeneity of the subgroup. Together, these results indicate that common variation in the *P2RY12* gene is associated with different cardiovascular endpoints. Previous studies support this view since they reported associations between *P2RY12* gene variants and PAD[5], cerebrovascular events after PAD[6] or restenosis after PCI.[17]

It is generally accepted that the contribution of the genetic component to the pathogenesis of arterial thrombosis is higher in young patients than in elderly patients in whom the contribution of the classical age-related cardiovascular risk factors is more important. For this reason, we studied a young population of patients with arterial thrombosis (males ≤45 years and females ≤55 years). A limitation of our case-control design is that we only looked at the survivors of CHD, IS and PAD, and we therefore expect may have underestimated the effects of *P2RY12* haplotypes on the risk of arterial thrombosis. We expect that the results of our study may also be valid for an elderly group of patients with arterial thrombosis, although additional studies are needed to asses the contribution of the *P2RY12* haplotypes to the overall risk of arterial thrombosis in the background of classical risk factors for this disease.

Our approach to study haplotypes enabled us to comprehensively investigate the common variations in the *P2RY12* gene, and this approach gave a more accurate risk prediction of arterial thrombosis than the analysis of individual SNPs. The haplotype analysis showed that a complex interaction exists between the SNPs. Therefore, not a single SNP could be identified that was responsible for all haplotype effects. However, our data indicate that predominantly SNPs D and E, located in the *P2RY12* gene region containing the coding exon 3, are responsible for the observed effects. This conclusion is strengthened by our finding that our risk estimates for haplotypes inferred from only SNPs D and E are similar to the risk estimates observed for haplotypes inferred from all five *P2RY12* SNPs.

SNPs D and E are located in another linkage disequilibrium block than SNPs A, B and C, which makes it likely that the coding region, and not the promoter region, contains a functional variant(s). Since SNPs D and E are localized in the region of the

P2RY12 gene containing the coding exon 3, one might expect that structural alterations of the P2Y12 receptor are involved in the underlying mechanism. However, no SNPs have been identified that result in a change in amino acid composition of the P2Y12 protein. On the other hand, it is possible that SNPs D and E affect splicing or enhancer elements located within the 42kb LD-block. Interestingly, SNP E was previously reported to be associated with an increased risk of peripheral arterial disease[5], but controversy still exists whether these findings also account for risk of other arterial thrombotic diseases.[6-8] *In vitro* studies need to be performed to confirm that SNPs D and E are indeed functional SNPs or whether they are in high LD with another, yet unidentified functional SNPs. Haplotype H5 (TATGT) also contains the minor allele of SNP A (the T allele), which was associated with a significant increase in risk of arterial thrombosis and CHD in the individual analysis of SNPs. However, the contribution of SNP A to the risk is minimal since we have not observed an effect of the haplotype TATAT (allele frequency 9%) in our study population (data not shown).

We investigated the effects of *P2RY12* haplotypes on platelet aggregation in healthy individuals to search for a possible underlying mechanism for the observed association. Overall, the effects of *P2RY12* haplotypes on platelet aggregation were very small, which suggests that the underlying mechanism for the observed association between *P2RY12* haplotypes and risk of arterial thrombosis is not reflected by optical platelet aggregometry. Future studies should focus on other measurements of platelet reactivity and molecular mechanisms underlying *P2RY12* gene regulation in order to reveal the biological processes that explain the effects of common variation in the *P2RY12* gene on the risk of arterial thrombosis.

In conclusion, our study shows that common variation in the *P2RY12* gene is associated with the risk of arterial thrombosis. *P2RY12* haplotyping may be a useful stratification tool for identification of patients who are genetically predisposed to arterial thrombosis and who may eventually benefit from additional or alternative drug treatment.

REFERENCES

1. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med*. 2002;8:1227-34.
2. Woulfe D, Yang J, Brass L. ADP and platelets: the end of the beginning. *J Clin Invest*. 2001;107:1503-5.
3. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, Koller B, Phillips DR, Conley PB. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. *J Clin Invest*. 2003;112:398-406.
4. Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Continuous signaling via PI3K isoforms beta and gamma is required for platelet ADP receptor function in dynamic thrombus stabilization. *Blood*. 2006;108:3045-52.
5. Fontana P, Gaussem P, Aiach M, Fiessinger JN, Emmerich J, Reny JL. P2Y12 H2 haplotype is associated with peripheral arterial disease: a case-control study. *Circulation*. 2003;108:2971-3.
6. Ziegler S, Schillinger M, Funk M, Felber K, Exner M, Mlekusch W, Sabeti S, Amighi J, Minar E, Brunner M, Muller M, Mannhalter C. Association of a functional polymorphism in the clopidogrel target receptor gene, P2Y12, and the risk for ischemic cerebrovascular events in patients with peripheral artery disease. *Stroke*. 2005;36:1394-9.
7. Schettert IT, Pereira AC, Lopes NH, Hueb WA, Krieger JE. Association between platelet P2Y12 haplotype and risk of cardiovascular events in chronic coronary disease. *Thromb Res*. 2006.
8. Zee RY, Michaud SE, Diehl KA, Chasman DI, Emmerich J, Gaussem P, Aiach M, Ridker PM. Purinergic receptor P2Y, G-protein coupled, 12 gene variants and risk of incident ischemic stroke, myocardial infarction, and venous thromboembolism. *Atherosclerosis*. 2007.
9. Rudez G, Pons D, Leebeek FWG, Monraats PS, Schrevel M, Zwinderman AH, de Winter RJ, Tio RA, Doevendans PA, Jukema JW, de Maat MP. Platelet Receptor P2RY12 Haplotypes Predict Restenosis after Percutaneous Coronary Interventions. *Hum Mutat*. 2008.
10. Marenberg ME, Risch N, Berkman LF, Floderus B, de Faire U. Genetic susceptibility to death from coronary heart disease in a study of twins. *N Engl J Med*. 1994;330:1041-6.
11. Zdravkovic S, Wienke A, Pedersen NL, Marenberg ME, Yashin AI, De Faire U. Heritability of death from coronary heart disease: a 36-year follow-up of 20 966 Swedish twins. *J Intern Med*. 2002;252:247-54.
12. Dormandy JA, Rutherford RB. Management of peripheral arterial disease (PAD). TASC Working Group. TransAtlantic Inter-Society Consensus (TASC). *J Vasc Surg*. 2000;31:S1-S296.
13. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat*. 2000;15:7-12.
14. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal*. 1999;14:143-9.
15. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet*. 2002;70:425-34.
16. van Gestel MA, Heemskerk JW, Slaaf DW, Heijnen VV, Reneman RS, oude Egbrink MG. In vivo blockade of platelet ADP receptor P2Y12 reduces embolus and thrombus formation but not thrombus stability. *Arterioscler Thromb Vasc Biol*. 2003;23:518-23.
17. Rudez G, Pons D, Leebeek FWG, Monraats PS, Schrevel M, Zwinderman AH, de Winter RJ, Tio RA, Doevendans PA, Jukema JW, de Maat MP. Platelet Receptor P2RY12 Haplotypes Predict Restenosis after Percutaneous Coronary Interventions. *Hum Mutat*. 2008;29:375-80.
18. Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation*. 2003;108:989-95

3

Chapter 3

Goran Rudež
Heleen Bouman
Wouter van Werkum
Frank Leebeek
Adrian Kruit
Hendrik Ruven
Jurriën ten Berg
Moniek de Maat
Christian Hackeng

Common variation in the platelet receptor *P2RY12* gene is associated with residual on-clopidogrel platelet reactivity in patients undergoing elective PCI

Circ Cardiovasc Genet, 2009 (In press)

ABSTRACT

Background

The clinical efficacy of clopidogrel is hampered by a large interindividual variability in platelet inhibition. Polymorphisms in the *P2RY12* receptor gene have been suggested to contribute to this variability, but previous studies included a relatively small number of patients and incompletely covered the common variation in the *P2RY12* gene. The aim of this study was to comprehensively investigate the possible association between common variation in the entire *P2RY12* locus and the magnitude of residual on-clopidogrel platelet reactivity measured by 2 commonly used platelet function assays in a large cohort of patients.

Methods and Results

A total of 1031 consecutive patients with coronary artery disease (CAD) who were scheduled for elective percutaneous coronary interventions (PCI) were enrolled. Platelet function was assessed by means of ADP-induced light-transmittance aggregometry (LTA) and the Verify*Now* P2Y12 assay. Six haplotype-tagging single nucleotide polymorphisms (ht-SNPs) were carefully selected to comprehensively cover the total common variation in the *P2RY12* gene and its flanking regulatory regions. Six common haplotypes were inferred from these ht-SNPs (denoted A to F). Haplotype F was associated with significantly lower residual on-clopidogrel platelet reactivity compared with the reference haplotype A. The size of this effect per haplotype allele was approximately 5% aggregation in the ADP-induced LTA ($p<0.05$) and 11 P2Y12 reaction units in the Verify*Now* P2Y12 assay ($p<0.05$).

Conclusions

Common variation in the *P2RY12* gene is a significant determinant of the interindividual variability in residual on-clopidogrel platelet reactivity in patients with CAD.

INTRODUCTION

Dual antiplatelet therapy with clopidogrel and aspirin is currently the treatment of choice for the secondary prevention of atherothrombotic events in patients with coronary artery disease (CAD) and in those undergoing coronary stent implantation.[1] In approximately 5% to 30% of clopidogrel-treated patients the inhibition of platelet aggregation is insufficient.[2,3] As a consequence, patients with a high residual on-clopidogrel platelet reactivity may be at a higher risk of atherothrombotic events.[4,5]

Common variation in the *P2RY12* gene has been suggested as one of the mechanisms underlying this large variability in clopidogrel response.[5] The *P2RY12* gene encodes the adenosine diphosphate (ADP) receptor P2Y12, the pharmacological target of clopidogrel. Previous investigations on the relationship between *P2RY12* single nucleotide polymorphisms (SNPs) and high residual on-clopidogrel platelet reactivity were limited by the fact that only the haplotype-tagging SNP (ht-SNP) rs2046934 ("i-T744C") was studied. Rs2046934 is in complete linkage disequilibrium (LD) with 3 other *P2RY12* SNPs and the haplotypes "H1" and "H2", which these SNPs determine, cover only the 3' part of the *P2RY12* gene.[6-14] In addition, most of the cited studies on *P2RY12* SNPs included a relatively small number of patients.

A comprehensive study of common variation in the *P2RY12* gene should include ht-SNPs that cover all common haplotypes within the entire *P2RY12* locus, *i.e.* the *P2RY1*2 gene and its regulatory regions, such as the promoter and the 3'untranslated region. Using this comprehensive approach, we previously demonstrated that common variation in the *P2RY12* gene is associated with the risk of restenosis after percutaneous coronary interventions (PCI).[15] Although ADP-induced light-transmittance aggregometry (LTA) is still considered the gold standard for the assessment of clopidogrel-induced platelet inhibition, methods have been recently introduced that are designed for a more standardized monitoring of the efficacy of clopidogrel, such as the Verify*Now* P2Y12 assay.[16] The aim of this study was to investigate a possible association between the common genetic variation of the entire *P2RY12* locus and residual on-clopidogrel platelet reactivity using several platelet function assays in a large population of patients who were scheduled to undergo elective PCI.

METHODS

Study population

One thousand thirty-one consecutive patients with established coronary artery disease (CAD) scheduled for elective PCI were included in this study. Because of different protocols of referring hospitals, patients had received a different, but adequate clopidogrel pretreatment: (A) maintenance therapy of 75 mg daily for >5 days (n=659), (B) a clopidogrel loading dose of 300 mg at least 24h before PCI (n=314) or (C) a clopidogrel loading dose of 600 mg at least 6h before PCI (n=58). Exclusion criteria were the use of GPIIb/IIIa antagonists in the past 7 days before the intervention and a platelet count $<150x10^9$ platelets/L. Ninety percent of the patients were on aspirin (80-100 mg daily) and 10% were on coumarins. Eighty-one percent of the patients were using statins (Table 1). Over 95% of the study population was of Caucasian origin.

The study protocol was approved by the institutional Medical Ethics Committee. Written informed consent was obtained from each participant.

Definitions

Smoking was defined as any cigarette smoking in the last month. Hypertension was defined as a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg. Diabetes mellitus was defined according to the World Health Organization criteria. Family history of cardiovascular disease was defined as having a first-degree relative with a history of CAD for women younger than 65 years and for men younger than 55 years.

Blood collection

Blood samples were drawn from the arterial sheath before heparinization into nonvacuum Sarstedt tubes containing 3.2% sodium citrate (Sarstedt, Nümbrecht, Germany). Haematocrit and platelet count were analysed in K_3-EDTA anticoagulated blood. Genomic DNA was extracted from K_3-EDTA anticoagulated whole-blood following standard salting-out procedures and stored at 4°C for genetic analysis.

Table 1. Characteristics of the study population

Demographics	n=1031
Age (yrs)	64 ± 11
Male	769 (75%)
Risk factors	
Body mass index (kg/m^2)	27 ± 4
Smoking	210 (20%)
Diabetes	197 (19%)
Hypertension	801 (78%)
Family history of cardiovascular disease	633 (61%)
Medication	
Clopidogrel – Total group	1031 (100%)
Dosing regimen A	659 (64%)
Dosing regimen B	314 (30%)
Dosing regimen C	58 (6%)
Aspirin	923 (90%)
Coumarins	105 (10%)
Statins	830 (81%)
Platelet count (x10^9/L)	272 ± 80
Haematocrit (%)	41.4 ± 4.3

Values are given as n (%) for categorical variables and as mean ± SD for continuous variables.

Light-transmittance aggregometry

Citrated whole-blood samples were centrifuged at 120*g* for 10 minutes to obtain platelet-rich plasma (PRP) and further centrifuged at 850*g* for 15 minutes to obtain platelet-poor plasma (PPP). Maximal and late aggregation (at 6 minutes) were measured in non-adjusted PRP after stimulation with different concentrations of the agonist ADP (final concentrations: 2, 5, 10 and 20 µmol/L) in an APACT 4004 four-channel aggregometer (LABiTec, Arensburg, Germany).[17]

Verify*Now* P2Y12 Assay

The Verify*Now* P2Y12 test cartridge system (Accumetrics, San Diego, CA) was used as described previously.[16,18] In brief, Verify*Now* P2Y12 is a rapid cartridge-based platelet-agglutination assay designed to directly measure the inhibitory effects of clopidogrel therapy. The results are reported in P2Y12 reaction units (PRU), where a higher PRU reflects greater on-clopidogrel ADP-induced platelet reactivity, and in percentage of inhibition, where a higher % reflects greater change of ADP-induced platelet agglutination from clopidogrel-independent (baseline) platelet agglutination that is induced with thrombin receptor activating peptide (TRAP) and protease-activated receptor 4 activating peptide (PAR4-AP).

Selection of SNPs in the *P2RY12* gene

Ht-SNPs were selected according to the approach described previously.[15] Based on the latest LD-map of the *P2RY12* locus (*i.e. P2RY12* gene with 2.5kb flanking sequence) provided by the International HapMap Project for a population of Utah residents with Northern and Western European ancestry from the CEPH collection (phase II, October 2007; *http://www.hapmap.org/*) (Figure 1), blocks of haplotypes with frequency >5% were defined from these ht-SNPs using Haploview software (version 3.3, http://www.broad.mit.edu/mpg/haploview/index.php). Together, the defined haplotypes cover 88% of the total common DNA sequence variation in the *P2RY12* locus. The 6 selected ht-SNPs that were genotyped are rs6798347 (c.-281-3614C>t), rs6787801 (c.-217+2739T>c) and rs9859552 (c.-217+11494C>a), which tag the promoter, exon 1 and a part of intron 1 region, and rs6801273 (c.-216-4445A>g), rs9848789 (c.-216-377G>a) and rs2046934 (c.-15+742T>c), which tag the remaining part of intron 1, as well as the entire exon 2, intron 2, exon 3, 3' UTR and flanking region (Figure 1).

Genotyping and haplotype analysis

The 6 selected haplotype-tagging SNPs were genotyped using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, USA) under standard conditions.[19] The nucleotide sequences of the primers and probes used for each assay are available upon request. End-point fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, USA) and clustered according to genotype using SDS 2.2.2 software (Applied Biosystems, Foster City, USA).

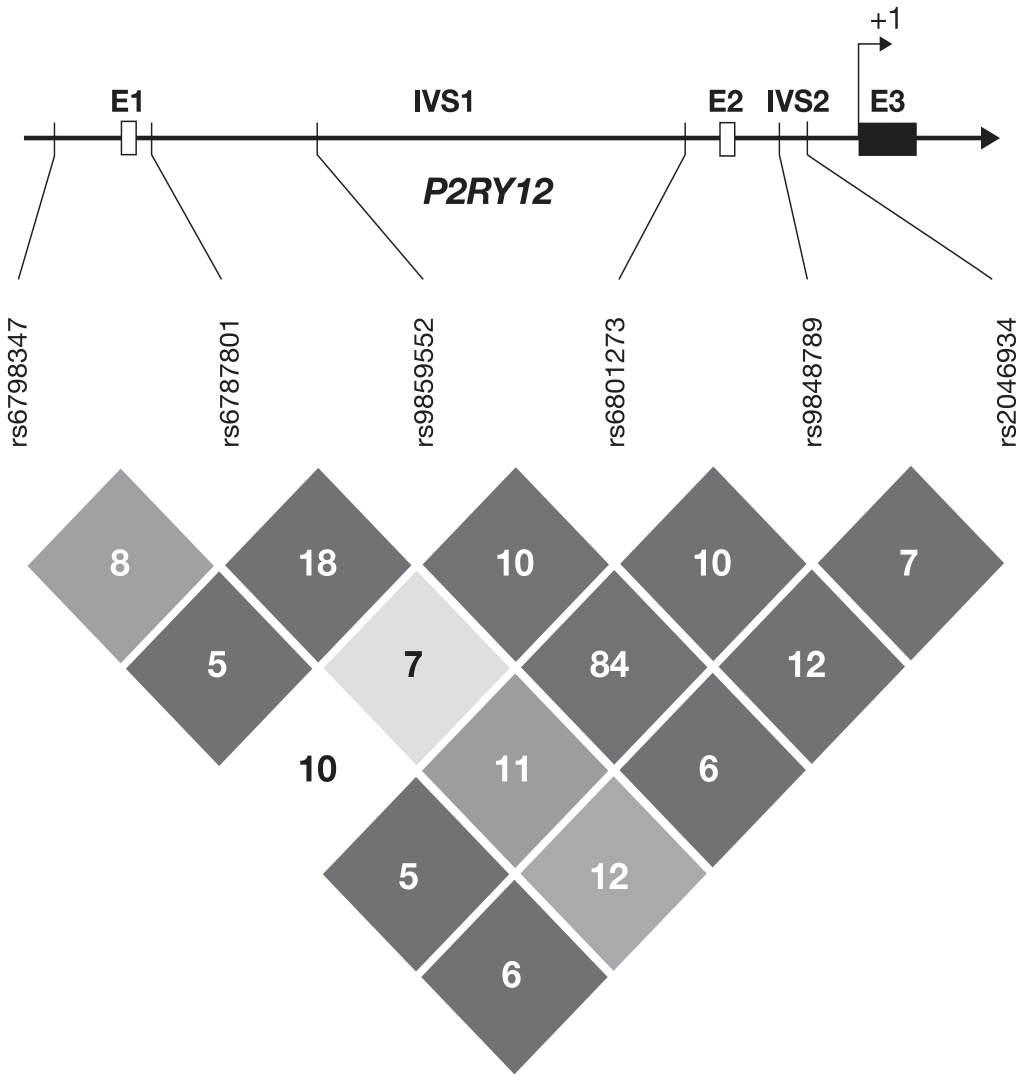


Figure 1. Schematic representation of the *P2RY12* gene and the linkage disequilibrium map of the 6 haplotype-tagging SNPs genotyped

The *P2RY12* gene consists of three exons (E1, E2 and E3, of which only E3 is coding) and two intervening sequences (IVS1 and IVS2), which span 47kb of genomic DNA on chromosome 3. The six ht-SNPs that are depicted in the figure with their corresponding rs-numbers tag all common combinations of SNPs (haplotypes with allele frequencies >5%) within the *P2RY12* gene and its 2.5kb flanking regions (52kb in total). The triangle-shaped figure depicts the linkage disequilibrium (LD)-map for these 6 ht-SNPs. In shades from white to grey are indicated increasing pairwise LDs between the ht-SNPs with the corresponding r^2 values given within the blocks.

A random selection of 10% of the samples was re-analysed, and the results were confirmed in 99.7%. Laboratory analyses were performed by research technicians who were blinded for the demographic data of the patients. All SNPs were in Hardy-Weinberg equilibrium when genotyped in a healthy population. Haplotypes were inferred using Haplo.Stats software (*http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm*) and coded from A to F, in the descending order of their effects on 20 μmol/L ADP-induced LTA, where A is defined as the reference haplotype.[20]

Statistical methods

Demographic data are presented as means and standard deviations for continuous variables and as counts and percentages for categorical variables. Results of the haplotype analysis are presented as mean effects per haplotype-allele with the corresponding standard errors. Haplotype analysis was performed with Haplo.Stats.[20] Briefly, this analysis calculates posterior probabilities for each possible haplotype of an individual and assigns an appropriate weight to the corresponding estimated effect on platelet function. Individuals with missing genotype data for more than two SNPs (n=26) were excluded from the analysis. Haplo.Stats assumes an additive effect of haplotype alleles, indicating that the net effect of a person's haplotype is the sum of the effects of its two haplotype alleles.

The associations between the *P2RY12* haplotype alleles and on-clopidogrel platelet reactivity were determined by weighted linear or logistic regression analysis, using haplo.glm function and results expressed as mean change from the reference haplotype allele ± SEM. The genetic analysis of the subgroup of patients who had received clopidogrel according to the dosing regimen C was not performed because of the small sample size (n=58).

Other statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc, Chicago, USA). ANCOVA was performed to study the differences between the mean on-clopidogrel platelet reactivity of the total study population and the three clopidogrel loading dose regimens, and to study the association between individual *P2RY12* SNPs and platelet function in the total study population. In each analysis, homozygotes for the common allele of these SNPs were used as the reference.

Analyses were adjusted for age, sex, BMI, diabetes and smoking. For the total group, additional adjustment for clopidogrel loading-dose was performed. In some analyses, adjustment was also made for the use of aspirin, coumadins and statins. A two-sided value of $p<0.05$ was considered statistically significant.

RESULTS

A large variability in on-clopidogrel platelet reactivity was found with 20 µmol/L ADP-induced LTA and Verify*Now* P2Y12 assay (Figure 2A and B). For 20 µmol/L ADP-induced LTA, on-clopidogrel platelet aggregation was 58% ± 14% (total group), 57% ± 14% (dosing regimen A), 62% ± 14% (dosing regimen B) and 56% ± 15% (dosing regimen C). For the Verify*Now* P2Y12 assay, PRU was 211 ± 76 (total group), 206 ± 74 (dosing regimen A), 228 ± 78 (dosing regimen B) and 187 ± 83 (dosing regimen C). Both for LTA and Verify*Now* P2Y12 assay, mean on-clopidogrel platelet reactivity was significantly higher in de dosing regimen B than the dosing regimens A and C (p<0.001). Similar variability in on-clopidogrel platelet reactivity was seen for the different concentrations of ADP in the LTA (data not shown).

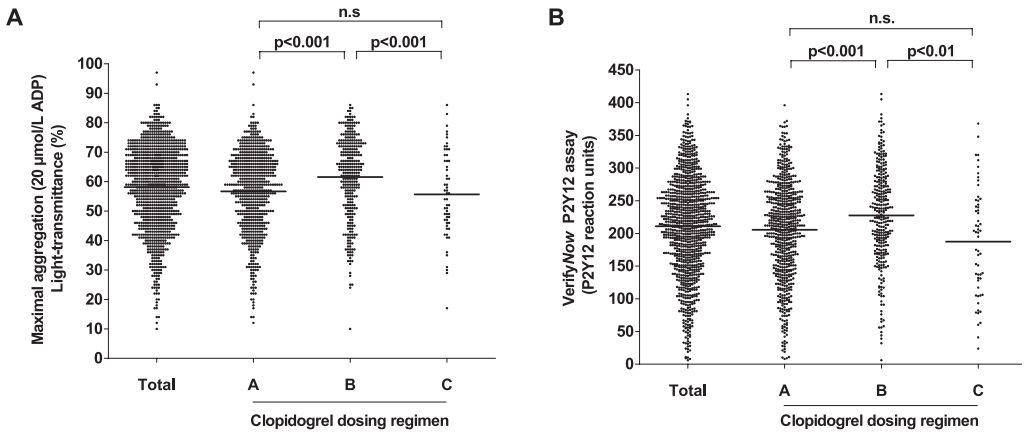


Figure 2. Maximal 20 µmol/L ADP-induced LTA and Verify*Now* P2Y12 assay in the total study population and according to the 3 clopidogrel dosing regimens

The total study population (n=1031) and the 3 different clopidogrel dosing regimens are presented. Depicted are the maximal 20 µmol/L ADP-induced LTA (panel A) and the Verify*Now* P2Y12 assay (panel B). Differences between mean platelet reactivity of the three dosing regimens were tested using ANCOVA with adjustment for age, sex, BMI, diabetes and smoking.

n.s., not significant.

P2RY12 haplotypes

By combining the 6 *P2RY12* ht-SNPs, 64 haplotype alleles were inferred of which 6 were common and had an allele frequency higher than 5%: (A) tTCgGT (10%), (B) tTCAGT (6%), (C) CTCAGc (15%), (D) CTaAaT (15%), (E) CcCgGT (21%) and (F) CcCAGT (22%)(Table 2).

Table 2. *P2RY12* haplotypes

Haplotype	SNP allele composition	Allele frequency (%)
A	tTCgGT	10
B	tTCAGT	6
C	CTCAGc	15
D	CTaAaT	15
E	CcCgGT	21
F	CcCAGT	22

Haplotype alleles were coded A - F, in the descending order of their effects on 20 µmol/L ADP-induced LTA, where A is the reference haplotype allele. SNP allele composition (*e.g.* CTaAaT) represents rs6798347, rs6787801, rs9859552, rs6801273, rs9848789 and rs2046934, respectively, with the minor alleles in lower case.

Light-transmittance aggregometry

In the analysis of the total study population of patients, haplotype F showed a significantly lower 5 µmol/L ADP-induced maximal and late aggregation (both -4% per haplotype allele, $p<0.05$, Figure 3A and B) compared with the reference haplotype A. Similar results were observed for 20 µmol/L ADP-induced maximal and late aggregation (-4% and –6% per haplotype allele; $p<0.001$ and $p<0.05$; Figure 3C and D, respectively) and for ADP concentrations 2 and 10 µmol/L (Figure 4). Similar differences between haplotype F and the reference haplotype A were seen in the subgroup-analysis of patients from the clopidogrel dosing regimens A and B (Figure 3). In addition, haplotype E was associated with a significantly lower 20 µmol/L ADP-induced maximal and late aggregation in the analysis of the total study population (both –3% per haplotype allele, $p<0.05$, Figure 3C and D). Haplotype E also showed a trend towards lower maximal and late aggregation in the subgroup-analysis of patients from the clopidogrel dosing regimens A and B (Figure 3C and D). Similar trends were seen for 2, 5 and 10 µmol/L ADP (Figure 3A and B, and Figure 4) and after additional adjustment for the use of aspirin, coumadins and statins (data not shown). Similar results were observed when non-Caucasians were excluded from the analyses (data not shown).

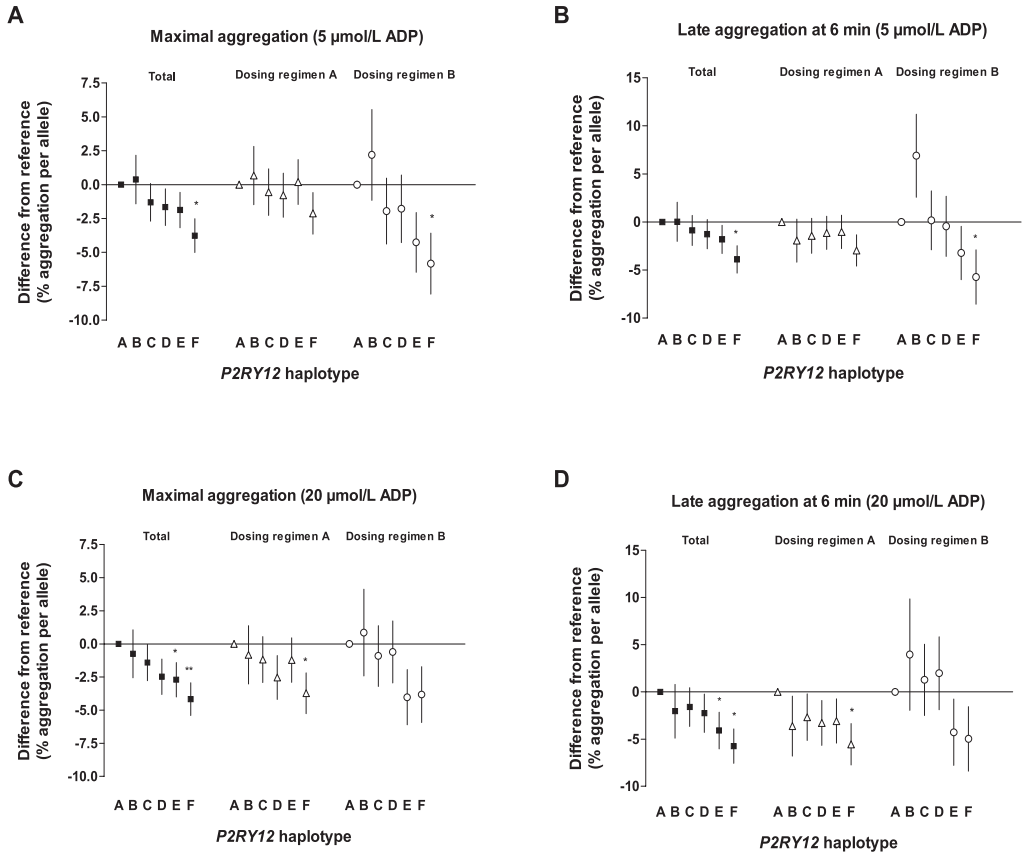


Figure 3. The effect of *P2RY12* haplotypes on platelet reactivity assessed by LTA

Results are shown for 5 µmol/L ADP-induced maximal aggregation (panel A) and late aggregation at 6 min (panel B), and 20 µmol/L ADP-induced maximal aggregation (panel C) and late aggregation at 6 min (panel D). Values are expressed as mean differences from the reference haplotype in percent of absolute aggregation per haplotype allele. Analyses were performed for the total study population (dosing regimens A+B+C), and for the subgroup of patients on dosing regimens A and B. Dosing regimen C was excluded from the analysis due to small sample size.
**p<0.001, *p<0.05

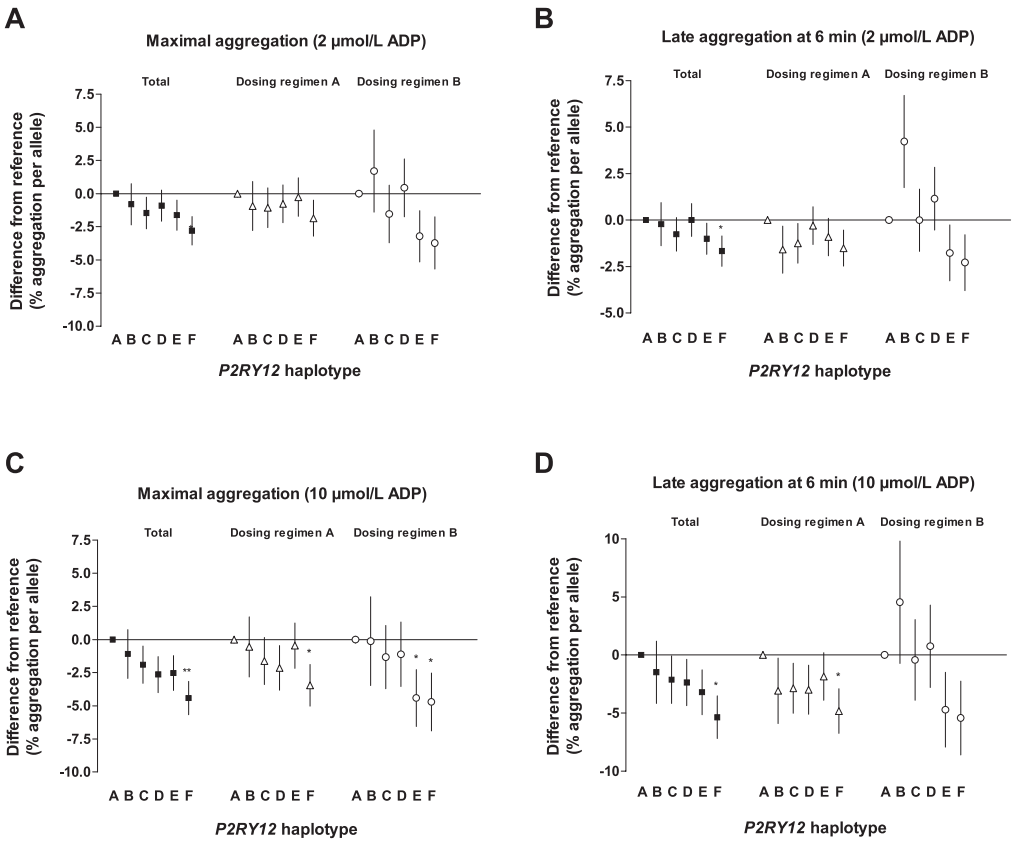


Figure 4. The effect of *P2RY12* haplotypes on platelet reactivity assessed by LTA

Results are presented for 2 µmol/L ADP-induced maximal aggregation (panel A) and late aggregation at 6 min (panel B), and 10 µmol/L ADP-induced maximal aggregation (panel C) and late aggregation at 6 min (panel D). In each panel, the results are shown for the analysis of the total study population (squares, dosing regimens A+B+C), the subgroup of patients who were on dosing regimen A (triangles) and dosing regimen B (circles). Dosing regimen C was excluded from the analysis due to small sample size. Values are expressed as mean differences from the reference haplotype in percent of absolute aggregation per haplotype allele, with error bars representing SEM.

**p<0.001, *p<0.05

Verify*Now P2Y12* Assay

Consistent with the results for LTA for the total study population, haplotype F was associated with a lower PRU as compared to the reference haplotype A (-11 PRU per haplotype allele, p<0.05, Figure 5). These differences were less pronounced and statistically not significant when clopidogrel dosing regimens A and B were analyzed separately (Figure 5). A stronger and significant association was observed for haplotype E, which was consistently associated with 27 less PRU in the Verify*Now* P2Y12 Assay for the total study population

and for the clopidogrel dosing regimens A and B (p<0.05, Figure 5). Similar results were obtained after additional adjustment for the use of aspirin, coumadins and statins, and when non-Caucasians were excluded from the analyses (data not shown). Also, similar results were obtained for the associations between haplotypes and percentage of inhibition from baseline platelet agglutination (data not shown).

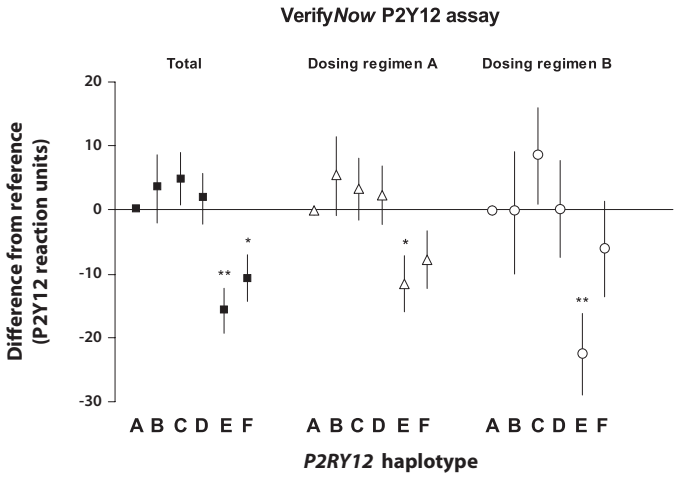


Figure 5. The effect of *P2RY12* haplotypes on platelet reactivity assessed by Verify*Now* P2Y12 assay

Results are expressed as mean differences from the reference haplotype in P2Y12 reaction units (PRU) per single haplotype allele. Analyses were performed for the total study population (dosing regimens A+B+C), and for the subgroup of patients on dosing regimens A and B. Dosing regimen C was excluded from the analysis due to small sample size. **p<0.001, *p<0.05

P2RY12 SNPs

Comparison of the SNP alleles within the haplotypes and their effects on platelet function suggested that SNP rs6787801 was responsible for most of the observed haplotype effects. This observation was confirmed in the single SNP analysis where homozygotes of the rare allele of SNP rs6787801 (CC genotype, n=261) had a significantly lower maximal and late aggregation (ranging from –2% to –7% across various concentrations of ADP, p<0.05 for all), as well as a significantly lower number of PRU in the Verify*Now* P2Y12 assay (-27 PRU, p<0.001, Table 3), when compared with homozygotes of the common allele of SNP rs6787801 (TT genotype, n=266). None of the other 5 ht-SNPs were associated with any of the platelet function assays, except for homozygotes of the rare allele of SNP rs6798347 (TT genotype, n=46), who showed 24 PRU less in the Verify*Now* P2Y12 assay as compared to homozygotes of the corresponding common allele (CC genotype, n=638, p<0.05) (data not shown).

Table 3. The effect of SNP rs6787801 on platelet function in total study population

Platelet function assay	Genotype			p-value
	TT (n=266)	TC (n=460)	CC (n=261)	TT *vs* CC
Maximal aggregation (%)				
2 µmol/L ADP	23 ± 12	23 ± 12	21 ± 12	0.046
5 µmol/L ADP	42 ± 14	40 ± 15	38 ± 13	0.012
10 µmol/L ADP	52 ± 15	50 ± 15	49 ± 14	0.020
20 µmol/L ADP	60 ± 14	59 ± 15	56 ± 14	0.005
Late aggregation at 6 min (%)				
2 µmol/L ADP	11 ± 10	11 ± 9	9 ± 9	0.023
5 µmol/L ADP	21 ± 19	20 ± 16	17 ± 15	0.006
10 µmol/L ADP	31 ± 22	30 ± 22	27 ± 21	0.016
20 µmol/L ADP	44 ± 23	41 ± 25	38 ± 24	0.003
Verify*Now* P2Y12 (PRU)	226 ± 74	209 ± 78	199 ± 71	<0.001

Platelet aggregation is expressed as percent absolute aggregation ± SD. Verify*Now* P2Y12 assay is expressed as P2Y12 reaction units (PRU) ± SD. P-values are for least significant difference post hoc test in ANCOVA with adjustment for age, sex, BMI, diabetes, smoking and clopidogrel loading dose.

DISCUSSION

In this study, we demonstrate that common variation in the ADP-receptor *P2RY12* gene is a significant determinant of the wide interindividual variability in on-clopidogrel platelet reactivity. Haplotype F (allele frequency 22%) was consistently associated with a lower on-clopidogrel platelet reactivity in the ADP-induced LTA and the Verify*Now* P2Y12 assay, which may give an adequate protection against atherothrombotic events. Haplotype E (21%) was also associated with lower on-clopidogrel platelet reactivity, especially in the Verify*Now* P2Y12 assay, but this association was less clear for the LTA. Interestingly, a single 300 mg loading dose of clopidogrel (regimen B) was less effective in inhibiting the platelet ADP response than the daily 75 mg dose (regimen A). However, although the overall mean on-clopidogrel platelet reactivity was significantly higher in clopidogrel dosing regimen B than dosing regimens A and C, these differences did not influence the associations between haplotypes across the regimens, neither did the adjustment for various covariates. In line with previous reports, the LTA and the Verify*Now* P2Y12 assay showed similar results.[16]

The haplotype-based approach enabled a comprehensive investigation of the common variation in the *P2RY12* gene. The results suggested that SNP rs6787801, or another SNP that is in high LD with this SNP, is responsible for the observed haplotype effects. SNP rs6787801 is located within the 59kb LD-block that contains the promoter region, but not the coding region of the *P2RY12* gene. This suggests that altered transcriptional activity of the *P2RY12* gene might be the underlying mechanism of the observed haplotype effects, rather than a structural change of the P2Y12 receptor. SNP rs6787801 and rs2046934 are located in different LD-blocks, which may explain why most of the previous studies on rs2046934 (the tagging-SNP of haplotype-alleles H1 and H2) have not found a similar association between rs2046934 and the response to clopidogrel.[6-14] Also in this study, we did not find any statistical differences between haplotypes H1 and H2, represented by our individual SNP analysis with rs2046934. Judging from the relative effects of the haplotypes, there is a complex interaction between the SNPs within a haplotype. Therefore, we cannot exclude the possibility that besides rs6787801 other SNPs, including rs2046934, might contribute to the observed haplotype effects. In addition, the effect of haplotype alleles may not be additive, as we assumed in our model.

Results were presented per single haplotype allele, which implies, under the assumption of an additive effect of the haplotype alleles, that the effect of a haplotype in a patient is the sum of the effects of its two single haplotype alleles. A single haplotype allele F was associated with 7% lower platelet aggregation compared with the reference haplotype allele A, indicating that the estimated net effect in patients homozygous for haplotype allele F might be a 14% lower platelet aggregation compared with patients homozygous for haplotype allele A. Smoking, polymorphisms of the cytochrome P450 C19 (CYP2C19) gene, diabetes mellitus, age and proton pump inhibitor treatment are reported to have a similar effect-size on clopidogrel-induced platelet inhibition.[21-24] It remains, however, to be established whether these differences are associated with clinical outcome and what the underlying biological processes are that explain the observed haplotype effects.

Our study confirmed a wide variability in residual on-clopidogrel platelet reactivity, as measured with ADP-induced LTA and the Verify*Now* P2Y12 assay. To date, a uniform definition of so-called clopidogrel "resistance" or clopidogrel "non-responsiveness" is lacking. This definition may vary depending on the type of platelet function assay used, or whether adverse clinical events occurred during clopidogrel therapy.[5,25] In our study, the absolute residual on-clopidogrel platelet reactivity was measured, and not the relative response to clopidogrel from baseline (*i.e.* the pharmacodynamic response). For this

reason, we cannot exclude the possibility that the observed differences between haplotypes may already be present at baseline and thus independent of clopidogrel treatment. In addition, we have not genotyped our patients for the *CYP2C19* loss-of-function alleles (*2, *3, *4 and *5), nor the *ABCB1* gene variants. *CYP2C19* variants have recently been shown to affect the hepatic bio-activation of the clopidogrel pro-drug and the concomitant platelet inhibition and risk of adverse ischemic cardiovascular events, whereas the *ABCB1* variants have been shown to affect clopidogrel absorption.[26-28] However, since *CYP2C19* and *ABCB1* genes are located on different chromosomes than the *P2RY12* gene, we expect that the *CYP2C19* and *ABCB1* alleles are independent of the *P2RY12* haplotypes; *i.e.* the *CYP2C19* and *ABCB1* alleles are randomly distributed over the different *P2RY12* haplotype-subgroups of our patients. In addition, *CYP2C19* and *ABCB1* genes do not have common biological pathways with the *P2RY12* gene, since the *P2RY12* gene encodes the target-receptor of clopidogrel, whereas *CYP2C19* and *ABCB1* genes are involved in clopidogrel bio-availability. Although these notions do not entirely exclude the possibility of interaction between the three genes, the actual effect of *P2RY12* gene variants (*i.e.* adjusted for any confounders) will probably be larger than the one observed in the present study. Although it would have been interesting to include additional pharmacokinetic and pharmacodynamic measurements of clopidogrel in our study, the absolute magnitude of the residual platelet reactivity during clopidogrel treatment is considered to be one of the most important end-stage determinants of risk of recurrent atherothrombotic events. Additional studies need to be performed to test whether our results may be generalized to other ethnicities than Caucasians.

In conclusion, common variation in the *P2RY12* gene is a significant determinant of the wide interindividual variability in residual on-clopidogrel platelet reactivity in patients with CAD.

REFERENCES

1. Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, Malmberg K, Rupprecht H, Zhao F, Chrolavicius S, Copland I, Fox KA. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet*. 2001;358:527-33
2. Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation*. 2003;107:2908-13.
3. Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL, Topol EJ. Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol*. 2005;45:246-51.
4. Snoep JD, Hovens MM, Eikenboom JC, van der Bom JG, Jukema JW, Huisman MV. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. *Am Heart J*. 2007;154:221-31.
5. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, Costa MA. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. *J Am Coll Cardiol*. 2007;49:1505-16.
6. Grossmann R, Sokolova O, Schnurr A, Bonz A, Porsche C, Obergfell A, Lengenfelder B, Walter U, Eigenthaler M. Variable extent of clopidogrel responsiveness in patients after coronary stenting. *Thromb Haemost*. 2004;92:1201-6.
7. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Cavallari U, Trabetti E, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Costa MA, Bass TA, Pignatti PF, Macaya C. Lack of association between the P2Y12 receptor gene polymorphism and platelet response to clopidogrel in patients with coronary artery disease. *Thromb Res*. 2005;116:491-7.
8. von Beckerath N, von Beckerath O, Koch W, Eichinger M, Schomig A, Kastrati A. P2Y12 gene H2 haplotype is not associated with increased adenosine diphosphate-induced platelet aggregation after initiation of clopidogrel therapy with a high loading dose. *Blood Coagul Fibrinolysis*. 2005;16:199-204.
9. Fontana P, Nolli S, Reber G, de Moerloose P. Biological effects of aspirin and clopidogrel in a randomized cross-over study in 96 healthy volunteers. *J Thromb Haemost*. 2006;4:813-9.
10. Smith SM, Judge HM, Peters G, Armstrong M, Fontana P, Gaussem P, Daly ME, Storey RF. Common sequence variations in the P2Y12 and CYP3A5 genes do not explain the variability in the inhibitory effects of clopidogrel therapy. *Platelets*. 2006;17:250-8.
11. Lev EI, Patel RT, Guthikonda S, Lopez D, Bray PF, Kleiman NS. Genetic polymorphisms of the platelet receptors P2Y(12), P2Y(1) and GP IIIa and response to aspirin and clopidogrel. *Thromb Res*. 2007;119:355-60.
12. Cuisset T, Frere C, Quilici J, Morange PE, Saut N, Lambert M, Camoin L, Vague IJ, Bonnet JL, Alessi MC. Role of the T744C polymorphism of the P2Y12 gene on platelet response to a 600-mg loading dose of clopidogrel in 597 patients with non-ST-segment elevation acute coronary syndrome. *Thromb Res*. 2007;120:893-9.
13. Staritz P, Kurz K, Stoll M, Giannitsis E, Katus HA, Ivandic BT. Platelet reactivity and clopidogrel resistance are associated with the H2 haplotype of the P2Y(12)-ADP receptor gene. *Int J Cardiol*. 2008.
14. Bura A, Bachelot-Loza C, Ali FD, Aiach M, Gaussem P. Role of the P2Y12 gene polymorphism in platelet responsiveness to clopidogrel in healthy subjects. *J Thromb Haemost*. 2006;4:2096-7.

15. Rudez G, Pons D, Leebeek F, Monraats P, Schrevel M, Zwinderman A, de Winter R, Tio R, Doevendans P, Jukema W, de Maat M. Platelet receptor P2RY12 haplotypes predict restenosis after percutaneous coronary interventions. *Hum Mutat*. 2008;29:375-80.

16. van Werkum JW, van der Stelt CA, Seesing TH, Hackeng CM, ten Berg JM. A head-to-head comparison between the VerifyNow P2Y12 assay and light transmittance aggregometry for monitoring the individual platelet response to clopidogrel in patients undergoing elective percutaneous coronary intervention. *J Thromb Haemost*. 2006;4:2516-8.

17. van Werkum JW, Kleibeuker M, Mieremet N, ten Berg JM, Hackeng CM. Evaluation of the platelet response to clopidogrel with light transmittance aggregometry: peak aggregation or late aggregation? *J Thromb Haemost*. 2007;5:884-6.

18. Price MJ, Endemann S, Gollapudi RR, Valencia R, Stinis CT, Levisay JP, Ernst A, Sawhney NS, Schatz RA, Teirstein PS. Prognostic significance of post-clopidogrel platelet reactivity assessed by a point-of-care assay on thrombotic events after drug-eluting stent implantation. *Eur Heart J*. 2008;29:992-1000.

19. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal*. 1999;14:143-9.

20. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet*. 2002;70:425-34.

21. Bliden KP, Dichiara J, Lawal L, Singla A, Antonino MJ, Baker BA, Bailey WL, Tantry US, Gurbel PA. The association of cigarette smoking with enhanced platelet inhibition by clopidogrel. *J Am Coll Cardiol*. 2008;52:531-3.

22. Trenk D, Hochholzer W, Fromm MF, Chialda LE, Pahl A, Valina CM, Stratz C, Schmiebusch P, Bestehorn HP, Buttner HJ, Neumann FJ. Cytochrome P450 2C19 681G>A polymorphism and high on-clopidogrel platelet reactivity associated with adverse 1-year clinical outcome of elective percutaneous coronary intervention with drug-eluting or bare-metal stents. *J Am Coll Cardiol*. 2008;51:1925-34.

23. Angiolillo DJ, Bernardo E, Sabate M, Jimenez-Quevedo P, Costa MA, Palazuelos J, Hernandez-Antolin R, Moreno R, Escaned J, Alfonso F, Banuelos C, Guzman LA, Bass TA, Macaya C, Fernandez-Ortiz A. Impact of platelet reactivity on cardiovascular outcomes in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol*. 2007;50:1541-7.

24. Gilard M, Arnaud B, Cornily JC, Le Gal G, Lacut K, Le Calvez G, Mansourati J, Mottier D, Abgrall JF, Boschat J. Influence of omeprazole on the antiplatelet action of clopidogrel associated with aspirin: the randomized, double-blind OCLA (Omeprazole CLopidogrel Aspirin) study. *J Am Coll Cardiol*. 2008;51:256-60.

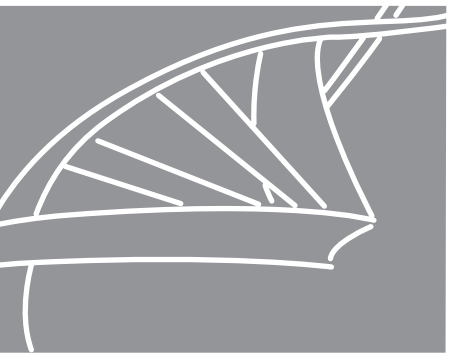
25. Frelinger AL, 3rd, Michelson AD. Clopidogrel linking evaluation of platelet response variability to mechanism of action. *J Am Coll Cardiol*. 2005;46:646-7.

26. Collet JP, Hulot JS, Pena A, Villard E, Esteve JB, Silvain J, Payot L, Brugier D, Cayla G, Beygui F, Bensimon G, Funck-Brentano C, Montalescot G. Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. *Lancet*. 2009;373:309-17.

27. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, Walker JR, Antman EM, Macias W, Braunwald E, Sabatine MS. Cytochrome p-450 polymorphisms and response to clopidogrel. *N Engl J Med*. 2009;360:354-62.

28. Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N, Steg PG, Ferrieres J, Danchin N, Becquemont L. Genetic determinants of response to clopidogrel and cardiovascular events. *N Engl J Med*. 2009;360:363-75.

Chapter 3



4

Chapter 4

Heleen Bouman
Wouter van Werkum
Goran Rudež
Frank Leebeek
Adrian Kruit
Hendrik Ruven
Jurriën ten Berg
Moniek de Maat
Christian Hackeng

The influence of common variation in the P2Y12 receptor gene on in vitro platelet inhibition with the direct P2Y12 antagonist cangrelor

Submitted

ABSTRACT

Background

Polymorphisms in the P2Y12 receptor gene have been proposed to contribute to the wide interindividual variability in clopidogrel responsiveness. Novel P2Y12 receptor inhibitors are currently in development to overcome this limitation.

Objectives

To determine the contribution of common variation in the P2Y12 receptor gene to platelet aggregation after *in vitro* P2Y12 receptor blockade with the direct antagonist cangrelor.

Methods

Light transmittance aggregometry was performed at baseline and after *in vitro* addition of 0.05 and 0.25 μmol/L cangrelor to the platelet-rich plasma of 254 healthy subjects. Subjects were genotyped for five haplotype-tagging (ht)-SNPs that cover the entire P2Y12 receptor gene and its flanking regions (rs6798347C>t, rs6787801T>c, rs9859552C>a, rs6801273A>g and rs2046934T>c [T744C]). Haplotypes with a frequency >5% were inferred from these ht-SNPs.

Results

The minor c allele of SNP rs6787801 was associated with a 5% lower peak of 20 μmol/L ADP-induced platelet aggregation (0.05 μmol/L cangrelor, $p<0.05$). Homozygotes for the minor allele of SNP rs9859552 showed 20% and 17% less inhibition of platelet aggregation with cangrelor when compared to the homozygotes for their common allele (0.05 and 0.25 μmol/L cangrelor, respectively; $p<0.05$). Results of the haplotype analyses were consistent with those of the single SNPs.

Conclusions

Polymorphisms of the P2Y12 receptor gene contribute significantly to the interindividual variability in platelet inhibition after partial *in vitro* blockade with the P2Y12 antagonist cangrelor.

INTRODUCTION

The efficacy of clopidogrel is seriously affected by a wide interindividual variability in response.[1, 2] Several mechanisms have been proposed to contribute to this phenomenon, such as the interindividual variation in gastro intestinal drug absorption, metabolic drug-conversion and pre-treatment platelet reactivity.[3-9] Genetic polymorphisms of the P2Y12 receptor have also been reported to increase ADP-induced platelet reactivity and to limit the magnitude of platelet inhibition by clopidogrel.[10-12]

Several novel P2Y12 receptor antagonists have been developed to overcome the problem of non-responsiveness to clopidogrel.[13] Cangrelor (formerly known as AR-C69931MX, The Medicines Company, Parsippany NJ USA) is a potent P2Y12 inhibitor that reaches a higher level of platelet inhibition than clopidogrel.[14] It reversibly binds to the P2Y12 receptor after intravenous administration and does not require conversion to an active metabolite for its antiplatelet action.[14, 15] These features allow a direct study of the effects of genetic polymorphisms of the P2Y12 receptor on platelet aggregation after *in vitro* P2Y12 receptor blockade with cangrelor.

The aim of the present study was to determine the contribution of polymorphisms in the P2Y12 receptor gene to variation in platelet aggregation after partial *in vitro* blockade of the P2Y12 receptor with the direct antagonist cangrelor.

METHODS

Study population

A total of 254 healthy volunteers without coronary artery disease were included. Due to technical failure of the aggregometer (n=5) and missing samples (n=7), 12 subjects were excluded from the analysis. The characteristics of the remaining 242 subjects are shown in Table 1. Subjects were ineligible if they had used any medication in the past 7 days known to affect platelet function. Other exclusion criteria were known platelet dysfunction or bleeding disorder, use of coumadins, a platelet count $<150x10^9$/L and age <18 years.

Data on risk factors and co-morbidities were carefully obtained using a standardized questionnaire. Physical examination of patients was performed by research physicians. Smoking was defined as any cigarette smoking in the last month. Diabetes mellitus was defined according to the World Health Organization criteria. All subjects gave informed consent and the study protocol complied with the declaration of Helsinki and was approved by the local Institutional Review Board.

Table 1. Characteristics of the study population

Demographics	n=242
Male	162 (64.1%)
Age (yrs)	49.7 ± 12.2
Caucasian	229 (94.6%)
Diabetes mellitus	8 (5.5%)
Current smoking	45 (18.5%)
Platelet Count (10^9/L)	252 ± 51
Platelet Count in PRP (10^9/L)	271 ± 21
Mean Platelet Volume (fL)	8.3 ± 0.9
Hemoglobine (g/dL)	8.9 ± 0.7

Values are presented as n (%) or mean ± sd.

Sample Collection

To avoid platelet activation during venapuncture, blood was drawn without a tourniquet from the antecubital vein into non-vacuum K_3-EDTA and citrate (3.2%) tubes (Sarstedt, Nümbrecht, Germany) after discarding the first 10 ml of blood. DNA isolation and measurements of platelet count, mean platelet volume and hemoglobin were performed in K_3-EDTA anticoagulated blood. Citrated blood was used for platelet function testing.

Platelet function testing

Citrated blood samples were centrifuged for 10 minutes at 120*g* to obtain platelet rich plasma (PRP), followed by an additional centrifugation for 15 minutes at 850*g* to recover platelet poor plasma (PPP). "Classical" light transmittance aggregometry (LTA) was performed on an APACT 4004 (LaBiTec, Ahrensburg, Germany), using PPP to set the aggregation to 100%. The PRP was adjusted with autologous PPP to achieve a final platelet count between 250 and 300x10^9/L. Two-hundred-and-fifty µl PRP was then pipetted into each cuvette, followed by incubation of the samples for 5 minutes with either 25 µl 0.9% NaCl (for baseline platelet aggregation) or cangrelor in final concentrations of 0.05 µmol/L and 0.25 µmol/L. These concentrations are located in the linear and the end phase of the S-shaped curve plotting the concentration of cangrelor against ADP induced platelet aggregation.[14] The final concentration of 0.25 µmol/L cangrelor corresponds to an infusion rate of 2 µg/kg/min, which has previously been used in a phase II dose-finding study.[14] This concentration corresponds to approximately half of the plasma concentration reached with the dosing regimen currently being evaluated in clinical trials.[16] Since a

lower level of P2Y12 inhibition is generally associated with an increased variability of platelet aggregation, a more pronounced effect of P2Y12 receptor gene polymorphisms might be expected with the concentrations 0.05 and 0.25 µmol/L cangrelor. After incubation with cangrelor, platelets were stimulated with adenosine diphosphate (ADP) in two final concentrations of 5 and 20 µmol/L. Both "peak" (maximal) and "late" (at 360 seconds) absolute % platelet aggregation were measured.[17] The absolute change in platelet aggregation from baseline (*i.e.* from the cangrelor naïve sample) was measured in order to determine the magnitude of inhibition of platelet aggregation (IPA).

Selection of Single Nucleotide Polymorphisms (SNPs) in the P2Y12 receptor gene

Haplotype-tagging SNPs (ht-SNPs) were selected according to the approach described previously.[18] Based on the linkage disequilibrium (LD) map of the P2Y12 locus provided by the International HapMap Project (phase II, October 2007; *http://www.hapmap.org/*), blocks of haplotypes with a frequency >5% and their ht-SNPs (n=6) were defined using Haploview software (version 3.3, *http://www.broad.mit.edu/mpg/haploview/index.php*). In the present study population, these haplotypes together cover 92% of the common DNA sequence variation in the entire P2Y12 receptor gene and its 5 kb flanking regions. None of the ht-SNPs results in an amino acid change, nor is located within consensus transcription factor binding sites in the 3kb promoter region, exon-intron junctions, predicted splicing enhancer motifs, the 3kb 3'UTR-region, or domains that are highly conserved among species. SNPs I-III tag the promoter, exon 1 and a part of the intron 1 region, while SNPs IV and V tag the remaining part of intron 1, as well as the entire exon 2, intron 2, exon 3 and the 3' UTR region. Since 97% linkage appeared to exist between rs9859552 and rs9848789, rs9848789 was excluded from the analysis in the present study. Further details on the 5 remaining ht-SNPs are shown in Table 2.

Table 2. Characteristics of the selected ht-SNPs of the P2Y12 receptor gene

SNP	NCBI dbSNP	Location	Allele†	Frequencies (genotype/n)		
I	rs6798347	Promoter c.-281-3614	C > t	CC / 150	Ct / 79	tt / 8
II	rs6787801	Intron c.-217+2739	T > c	TT / 59	Tc / 115	cc / 64
III	rs9859552	Intron c.-217+11494	C > a	CC / 174	Ca / 64	aa / 4
IV	rs6801273	Intron c.-216-4445	A > g	AA / 92	Ag / 125	gg / 25
V‡ T744C	rs2046934	Intron c.-15+742	T > c	TT / 152	Tc / 85	cc / 5

*Annotation of the selected ht-SNPs is according to the nomenclature recommendations of the Human Genome Variation Society, with nucleotide +1 being the A of the ATG-translation initiation codon[34]. †The common and minor alleles for each SNP are presented as common > minor, in upper and lower case respectively. The c allele for SNP II appeared to be the minor allele in a large cohort of patients from a previous performed study.[18] ‡SNP V was firstly described by Fontana et al and denoted [T744C].[10]

Genotyping of P2Y12 receptor polymorphisms

Genotyping was performed using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, USA) under standard conditions.[19] The nucleotide sequences of the primers and probes used for each assay are available upon request. End-point fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, USA) and clustered according to genotype using SDS 2.2.2 software (Applied Biosystems, Foster City, USA). A random selection of 10% of the samples was re-analysed, and the results were confirmed in 99.7%. All SNPs were in Hardy-Weinberg equilibrium. Haplo.Stats software (*http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm*) was used to infer haplotypes from the unphased SNP genotype data.

Statistical Analysis

Associations between single SNP genotypes and platelet aggregation as well as IPA, were tested using an ANCOVA (SPSS for Windows version 14.0; SPSS Inc, Chicago, USA). Based on the obtained platelet aggregation and IPA values, a dominant model was considered appropriate for testing the effects of SNP II genotypes (*i.e.* TT vs Tc + cc) and a recessive model for SNP II (*i.e.* CC + Ca vs aa). For the other SNPs, no obvious recessive or dominant effect could be observed and these SNPs were therefore tested in an additive model, comparing hetero- and homozygotes for the minor allele to

homozygotes for the common allele. Trend analysis was performed comparing the average effects of SNP genotypes across the range of cangrelor concentrations (represented by the combined p-value). Analyses of associations between haplotypes and platelet aggregation parameters were performed using the haplo.glm model in Haplo.Stats software. Effects of the haplotypes were calculated as mean difference ± SEM from the reference haplotype per single allele, using the most frequent haplotype as the reference (Href). In both SNP and haplotype analysis, results were adjusted for the known confounders age, gender, diabetes and smoking status. All platelet aggregation parameters are presented as absolute percentages and p values <0.05 were considered statistically significant.

RESULTS

Platelet Function

Individual values of 5 and 20 µmol/L ADP-induced peak and late aggregation at baseline and after the addition of 0.05 and 0.25 µmol/L cangrelor are depicted in Figure 1. Baseline mean peak and late platelet aggregation values were between 80% and 85% for 5 and 20 µmol/L ADP. Cangrelor greatly reduced the absolute magnitude of platelet aggregation, with an absolute IPA of up to 75% for late 5 µmol/L ADP induced platelet aggregation. Nonetheless, significant interindividual variability in cangrelor-inhibited platelet aggregation was evident, in particular with 20 µmol/L ADP induced platelet aggregation.

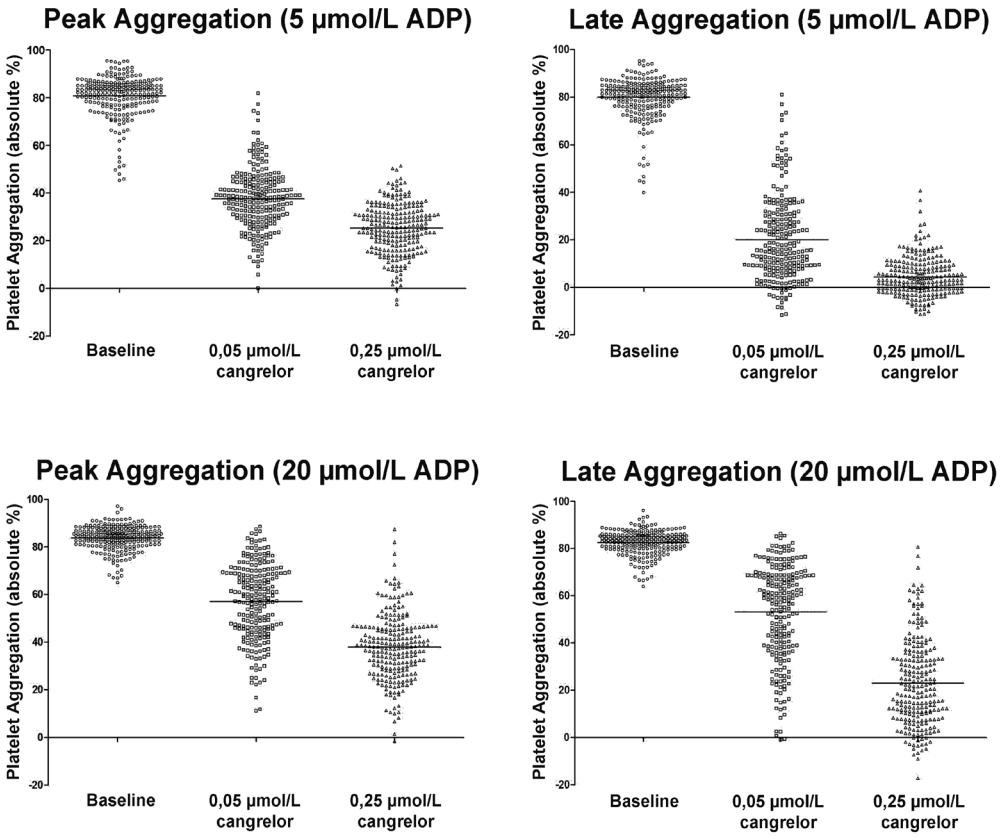


Figure 1. Individual values of 5 and 20 µmol/L ADP induced peak and late platelet aggregation at baseline and after incubation with 0.05 µmol/L and 0.25 µmol/L cangrelor

Data are presented as the absolute percentage of platelet aggregation, with lines indicating mean platelet aggregation values.

Single Nucleotide Polymorphisms and Platelet Function

Baseline 20 µmol/L ADP induced platelet aggregation was not associated with one of the SNP genotypes (Table 3).

Carriers of the minor c allele for SNP II exhibited significantly lower platelet aggregation values and a corresponding larger IPA after incubation with 0.05 µmol/L cangrelor (20 µmol/L ADP, both peak and late) as compared to TT homozygotes. The magnitude of the effect ranged from 4.5% to 7.0% of absolute aggregation ($p<0.05$ for all). This effect was substantially attenuated after stronger inhibition of platelet aggregation with 0.25 µmol/L cangrelor.

Table 3. Associations between SNP genotypes and platelet aggregation and cangrelor-induced platelet inhibition

		Peak aggregation (absolute %)				IPA (absolute %)		
			Cangrelor		*Combined p-value**	Cangrelor		*Combined p-value**
SNP	Genotype	Baseline	0.05 µmol/L	0.25 µmol/L		0.05 µmol/L	0.25 µmol/L	
I	CC	83 ± 0.5	59 ± 1.6	40 ± 1.4		24 ± 1.4	44 ± 1.3	
	Ct	84 ± 0.7	57 ± 2.1	38 ± 1.7	n.s.	27 ± 1.8	46 ± 1.6	n.s.
	tt	83 ± 1.9	53 ± 5.6	34 ± 4.8	n.s.	30 ± 5.1	49 ± 4.5	n.s.
p-value			n.s.	n.s.	n.s.		n.s.	n.s.
II	TT	85 ± 0.8	63 ± 2.3	41 ± 1.9		22 ± 2.0	43 ± 1.8	
	Tc + cc	83 ± 0.5	57 ± 1.5	38 ± 1.2	0.004	26 ± 1.3	45 ± 1.2	0.03
p-value			n.s.	0.02	n.s.		0.03	n.s.
III	CC +Ca	84 ± 0.5	58 ± 1.4	39 ± 1.2		26 ± 1.2	45 ± 1.1	
	aa	81 ± 2.6	72 ± 7.8	50 ± 6.6	0.04	10 ± 6.7	32 ± 6.1	0.002
p-value			n.s.	n.s.	n.s.		0.03	0.04
IV	AA	84 ± 0.6	61 ± 1.9	40 ± 1.6		24 ± 1.7	44 ± 1.5	
	Ag	83 ± 0.6	57 ± 1.7	38 ± 1.4	0.045	27 ± 1.5	45 ± 1.3	n.s.
	gg	83 ± 1.1	61 ± 3.5	40 ± 2.8	n.s.	23 ± 3.1	43 ± 2.6	n.s.
p-value			n.s.	n.s.	n.s.		n.s.	n.s.
V	TT	83 ± 0.5	57 ± 1.6	38 ± 1.4		26 ± 1.5	45 ± 1.3	
	Tc	84 ± 0.6	60 ± 2.0	40 ± 1.6	n.s.	24 ± 1.7	44 ± 1.5	n.s.
	cc	85 ± 2.4	71 ± 7.1	38 ± 6.0	n.s.	14 ± 6.3	47 ± 5.6	n.s.
p-value			n.s.	n.s.	n.s.		n.s.	n.s.

Table 3. continued

		Late aggregation (absolute %)				IPA (absolute %)		
			Cangrelor		*Combined p-value**	Cangrelor		*Combined p-value**
SNP	Genotype	Baseline	0.05 µmol/L	0.25 µmol/L		0.05 µmol/L	0.25 µmol/L	
I	CC	82 ± 0.5	55 ± 2.0	24 ± 1.8		27 ± 1.9	58 ± 1.7	
	Ct	83 ± 0.6	53 ± 2.6	24 ± 2.2	n.s.	30 ± 2.4	59 ± 2.1	n.s.
	tt	82 ± 1.8	48 ± 7.1	16 ± 6.3	n.s.	33 ± 6.6	65 ± 6.1	n.s.
p-value		n.s.	n.s.	n.s.		n.s.	n.s.	
II	TT	83 ± 0.7	60 ± 2.9	27 ± 2.5		23 ± 2.7	56 ± 2.4	
	Tc + cc	82 ± 0.5	53 ± 1.9	23 ± 1.6	0.002	29 ± 1.7	59 ± 1.5	0.009
p-value		n.s.	0.02	n.s.		0.03	n.s.	
III	CC +Ca	82 ± 0.5	54 ± 1.7	24 ± 1.5		28 ± 1.6	59 ± 1.4	
	aa	81 ± 2.6	73 ± 9.7	40 ± 8.6	0.01	8.3 ± 8.9	41 ± 8.2	0.002
p-value		n.s.	n.s.	n.s.		0.03	0.04	
IV	AA	83 ± 0.6	56 ± 2.4	25 ± 2.1		26 ± 2.2	57 ± 2.0	
	Ag	82 ± 0.5	53 ± 2.1	23 ± 1.8	n.s.	30 ± 1.9	60 ± 1.8	n.s.
	gg	82 ± 1.1	57 ± 4.3	27 ± 3.7	n.s.	26 ± 4.0	55 ± 3.5	n.s.
p-value		n.s.	n.s.	n.s.		n.s.	n.s.	
V	TT	82 ± 0.5	53 ± 2.1	22 ± 1.8		29 ± 1.9	60 ± 1.7	
	Tc	83 ± 0.6	56 ± 2.4	26 ± 2.1	0.03	27 ± 2.3	56 ± 2.1	n.s.
	cc	84 ± 2.3	67 ± 8.8	28 ± 7.8	n.s.	16 ± 8.1	55 ± 7.5	n.s.
p-value		n.s.	n.s.	n.s.		n.s.	n.s.	

Association between SNP genotypes and platelet aggregation and cangrelor-induced platelet inhibition (IPA). The mean ±SEM absolute % peak and late aggregation with their corresponding mean ± SEM IPA are presented per SNP genotype.
*The combined p-value represents the result of the additional statistical analysis comparing the average effect of SNP genotypes across the range of cangrelor concentrations.

Subjects carrying the aa genotype for SNP III showed a trend towards considerably higher 20 µmol/L ADP induced platelet aggregation after incubation with 0.05 and 0.25 µmol/L cangrelor as compared to the combined CC + Ca genotype (both peak and late, $p<0.1$ for all). This trend was confirmed in the analysis combining the effects of SNP III on platelet aggregation across the range of cangrelor concentrations (combined $p<0.05$ for aa vs CC + Ca). Correspondingly, the IPA was 16% and 20% smaller in aa homozygotes after incubation with 0.05 µmol/L cangrelor (peak and late respectively, $p<0.05$). This observation was consistent after stronger inhibition with 0.25 µmol/L cangrelor, with differences of 13% and 17% IPA between aa homozygotes and the combined CC + Ca genotype (peak and late respectively, $p<0.05$ and combined $p=0.002$ for both).

An overall trend towards higher platelet aggregation and a corresponding smaller IPA was observed in homozygote cc carriers for SNP V as compared to TT homozygotes after inhibition of 20 µmol/L ADP induced platelet aggregation with 0.05 µmol/L cangrelor. Absolute differences of 13% in peak aggregation and 12% in IPA (peak) were observed (cc vs TT; $p=0.06$ and $p=0.07$ respectively). However, this finding was not statistically significant and not evident after inhibition of platelet aggregation with 0.25 µmol/L cangrelor.

No clear associations were observed between SNP I and IV genotypes and absolute platelet aggregation values or IPA as measured with 20 µmol/L induced platelet activation after incubation with 0.05 and 0.25 µmol/L cangrelor. Similar results were observed for 5 µmol/L ADP-induced platelet aggregation (data not shown).

Haplotypes and Platelet Function

Of the inferred haplotypes, six had a frequency higher than 5% (Table 4).

Table 4. Characteristics of haplotypes A-F

	SNP					
Haplotype	I	II	III	IV	V	Frequency
A	t	T	C	g	T	10%
B	t	T	C	A	T	9%
C	C	T	C	A	c	15%
D	C	T	a	A	T	14%
E [Href]	C	c	C	g	T	24%
F	C	c	C	A	T	20%

Minor alleles are presented in lower case.
Href: reference haplotype.

For 20 µmol/L ADP induced baseline aggregation, no differences were observed between the five haplotypes and the reference haplotype (Figure 2).

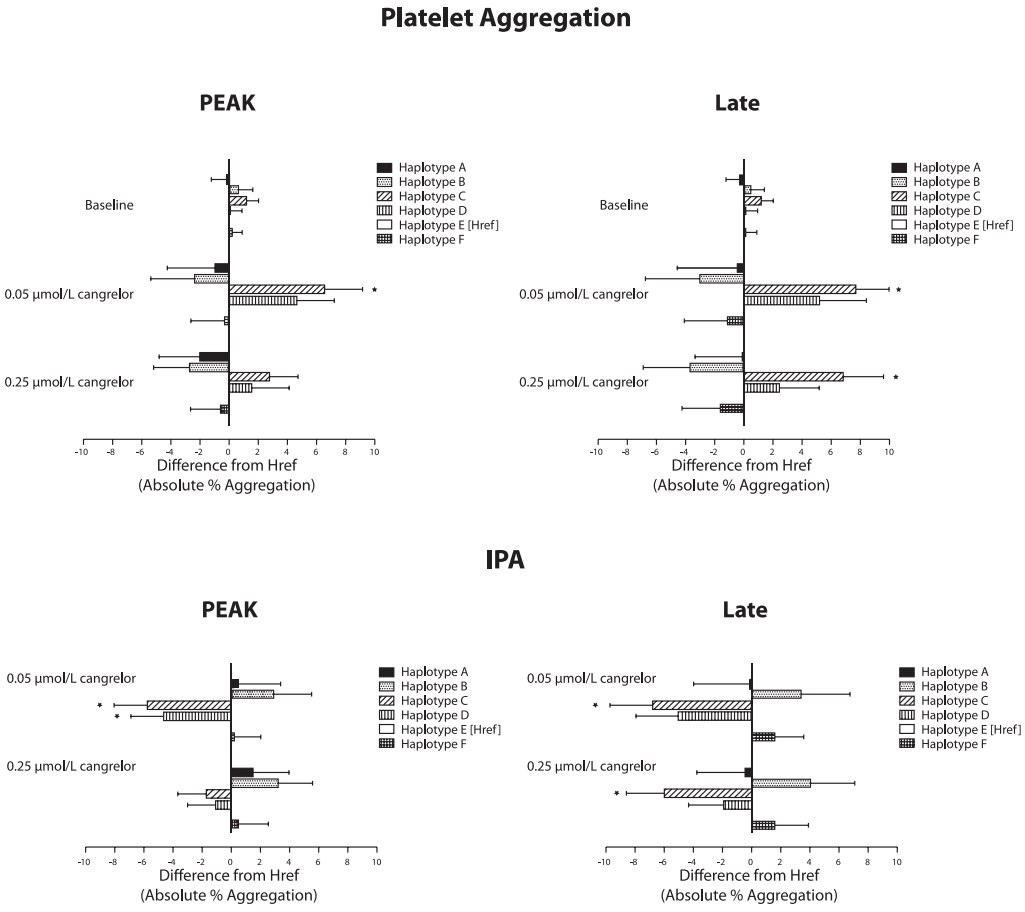


Figure 2. Absolute % difference in platelet aggregation and IPA between haplotypes A-F and the reference haplotype (20 µmol/L ADP induced, both peak and late) at baseline and after the addition of 0.05 and 0.25 µmol/L cangrelor

Results are presented as the mean difference ± SEM per single haplotype allele.

Haplotype C was associated with significantly higher peak and late aggregation after incubation with 0.05 µmol/L cangrelor (mean difference 6.6% and 7.7% per allele respectively, $p<0.05$). Correspondingly, IPA was smaller for haplotype C as compared to the reference haplotype (5.9% for peak and 7.0% for late, $p<0.05$ for both). Similar results for haplotype C were observed after 0.25 µmol/L cangrelor, but results were only statistically significant for the parameters late aggregation and IPA (late) (mean absolute difference 6.9% and 5.9% per allele respectively, $p<0.05$ for both).

Haplotype D also showed an overall trend towards higher platelet aggregation values and smaller IPA as compared to the reference haplotype. After incubation with 0.05 µmol/L cangrelor, peak and late aggregation were 4.7% and 5.3% higher as compared to Href ($p=0.06$ and $p=0.1$, respectively). A corresponding 4.7% and 5.2% smaller IPA as compared to the reference haplotype was observed ($p<0.05$ and $p=0.07$ for peak and late, respectively). Haplotype D was associated with neither platelet aggregation nor IPA after inhibition of platelet aggregation with 0.25 µmol/L cangrelor.

Haplotypes A, B and F showed no significant differences in platelet aggregation when compared to the reference haplotype. Similar results were observed for 5 µmol/L ADP induced platelet function results (data not shown).

DISCUSSION

The present study demonstrates that variation in the P2Y12 receptor gene is significantly associated with interindividual variability in platelet inhibition after partial *in vitro* blockade with the P2Y12 receptor antagonist cangrelor.

The minor c allele of SNP II increased the response to cangrelor, thereby exhibiting a small but protective effect on platelet function. In contrast, the aa genotype of SNP III was asso ciated with a substantially impaired platelet response to cangrelor. Of note, the effect of SNP III on P2Y12 inhibition appeared to be recessive, and therefore only displayed by the small subset of the population carrying the aa genotype for SNP III. As part of the H2 haplotype described by Fontana et al., the minor c allele of SNP V has previously been associated with a decreased response to clopidogrel.[10] In the present study, SNP V was likewise associated with a marked reduction of IPA with 0.05 µmol/L cangrelor. Statistical significance could however not be reached. Furthermore, SNP II and SNP V genotypes only affected platelet aggregation after inhibition with 0.05 µmol/L cangrelor. In contrast, the aa genotype for SNP III was consistently associated with impaired IPA, even after

incubation with the higher concentration of 0.25 μmol/L cangrelor.

The results of the haplotype analyses were consistent with the results from the single SNP association analyses. The reference haplotype contains the minor c allele of SNP II, which was associated with greater IPA in the single SNP analysis. Compared to the reference haplotype, haplotype C (containing the minor c allele for SNP V) was associated with a reduced response to platelet inhibition with cangrelor. Yet the magnitude of the effect of haplotype C was larger than for SNP V in the single SNP analysis. Adding up the single SNP effects of SNP II and V might explain the magnitude of the effect of haplotype C on platelet aggregation as compared to the reference haplotype. Apparently, the pronounced association between the aa genotype of SNP III and the magnitude of platelet inhibition was not observed for haplotype D, containing the minor a allele for SNP III. SNP III affects platelet aggregation in a recessive way, while haplotype analysis determines the effect of single haplotype alleles. Therefore, the effect of the aa genotype (n=4) is abolished by the large number of heterozygote carriers of the a allele for SNP III (n=64) in the haplotype analysis.

Haplotype analysis is useful in testing whether a combination of single SNPs exhibits either an additive, antagonistic or synergistic influence on the effect measure. In the present study, additive effects of the alleles associated with lower levels of IPA are observed for haplotype C (combining the T allele of SNP II with the a allele of SNP III) and haplotype D (combining the T allele of SNP II with the c allele of SNP V), resulting in a heightened magnitude of ADP-induced platelet aggregation as compared to the reference haplotype.

Previous studies investigating the possible relationship between genetic variation in the P2Y12 receptor and inhibition of platelet function with clopidogrel report conflicting results.[11, 20-27] This might in part be attributed to the multifactorial background of the interindividual variability seen in clopidogrel response. In addition to the effect of genetic polymorphisms of the P2Y12 receptor on the magnitude of platelet inhibition, variation in pharmacokinetics contribute significantly to variable plasma levels of clopidogrel and its active metabolite.[3, 4, 9] However, cangrelor is a direct-acting, intravenously administered P2Y12 inhibitor that does not exhibit the above mentioned pharmacokinetic limitations. Hence, the impact of genetic P2Y12 receptor polymorphisms on inhibition of platelet aggregation can directly be studied.

Several studies have shown that a high on-treatment platelet reactivity in clopidogrel pretreated patients is associated with the occurrence of atherothrombotic events and even death.[28-31] Furthermore, the results of the recently published TRial to Assess Improvement in

Therapeutic Outcomes by Optimizing Platelet InhibitioN with Prasugrel (TRITON)-TIMI 38 show that stronger P2Y12 inhibition with prasugrel compared to clopidogrel reduces early recurrent atherothrombotic events after PCI.[32, 33] This emphasizes the importance of sufficient P2Y12 inhibition during and in the first month following PCI. Patients carrying the aa genotype for SNP III that is associated with a more reactive platelet phenotype, might therefore be at an increased risk for atherothrombotic complications after PCI. Future studies assessing the impact of P2Y12 receptor gene polymorphisms on clinical outcome in cangrelor-treated patients may provide an answer to this hypothesis.

In conclusion, polymorphisms of the P2Y12 receptor gene significantly contribute to the interindividual variability in platelet inhibition after *in vitro* partial blockade with the P2Y12 antagonist cangrelor. The clinical relevance of this finding has yet to be determined.

REFERENCES

1. Serebruany VL, Steinhubl SR, Berger PB, et al. Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol* 2005;45:246-251.
2. Gurbel PA, Bliden KP, Hiatt BL, et al. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation* 2003;107:2908-2913.
3. Taubert D, Kastrati A, Harlfinger S, et al. Pharmacokinetics of clopidogrel after administration of a high loading dose. *Thromb Haemost* 2004;92:311-316.
4. Taubert D, von Beckerath N., grimberg G, et al. Impact of P-glycoprotein on clopidogrel absorption. *J Pharmacol Exp Ther* 2006;80:486-501.
5. Michelson AD, Linden MD, Furman MI, et al. Evidence that pre-existent variability in platelet response to ADP accounts for "clopidogrel resistance". *J Thromb Haemost* 2007;5:75-81.
6. Trenk D, Hochholzer W, Fromm MF, et al. Cytochrome P450 2C19 681G>A polymorphism and high on-clopidogrel platelet reactivity associated with adverse 1-year clinical outcome of elective percutaneous coronary intervention with drug-eluting or bare-metal stents. *J Am Coll Cardiol* 2008;51:1925-1934.
7. Mega JL, Close SL, Wiviott SD, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. *N Engl J Med* 2009;360:354-362.
8. Fontana P, Hulot JS, De Moerloose P, et al. Influence of CYP2C19 and CYP3A4 gene polymorphisms on clopidogrel responsiveness in healthy subjects. *J Thromb Haemost* 2007;5:2153-2155.
9. van Werkum JW, Heestermans AACM, Deneer VHM, et al. Clopidogrel resistance: Fact and Fiction. *Future Cardiology* 2006;2:215-228.
10. Fontana P, Dupont A, Gandrille S, et al. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation* 2003;108:989-995.
11. Staritz P, Kurz K, Stoll M, et al. Platelet reactivity and clopidogrel resistance are associated with the H2 haplotype of the P2Y(12)-ADP receptor gene. *Int J Cardiol* 2008 e-pub ahead of print.
12. Evans DJ, Jackman LE, Chamberlain J, et al. Platelet P2Y(12) receptor influences the vessel wall response to arterial injury and thrombosis. *Circulation* 2009;119:116-122.
13. Michelson AD. P2Y12 Antagonism. Promises and Challenges. *Arterioscler Thromb Vasc Biol* 2008;28:s33-8.
14. Storey RF, Wilcox RG, Heptinstall S. Comparison of the pharmacodynamic effects of the platelet ADP receptor antagonists clopidogrel and AR-C69931MX in patients with ischaemic heart disease. *Platelets* 2002;13:407-413.
15. Fugate SE, Cudd LA. Cangrelor for treatment of coronary thrombosis. *Ann Pharmacother* 2006;40:925-930.
16. A Clinical Trial to Demonstrate the Efficacy of Cangrelor (CHAMPION-PCI). Available at *http://www.clinicaltrials.gov/ct2/show/NCT00305162?term=cangrelor&rank=4.* Accessed April 2, 2009.
17. van Werkum JW, Kleibeuker M, Mieremet N, et al. Evaluation of the platelet response to clopidogrel with light transmittance aggregometry: peak aggregation or late aggregation? *J Thromb Haemost* 2007;5:884-886.
18. Rudez G, Pons D, Leebeek F, Monraats, et al. Platelet receptor P2RY12 haplotypes predict restenosis after percutaneous coronary interventions. *Hum Mutat* 2008;29:375-380.
19. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal* 1999;14:143-149.

20. Hetherington SL, Singh RK, Lodwick D, et al. Dimorphism in the P2Y1 ADP receptor gene is associated with increased platelet activation response to ADP. *Arterioscler Thromb Vasc Biol* 2005;25:252-257.
21. Giusti B, Gori AM, Marcucci R, et al. Cytochrome P450 2C19 loss-of-function polymorphism, but not CYP3A4 IVS10 + 12G/A and P2Y12 T744C polymorphisms, is associated with response variability to dual antiplatelet treatment in high-risk vascular patients. *Pharmacogenet Genomics* 2007;17:1057-1064.
22. von Beckerath N, von Beckerath O, Koch W, et al. P2Y12 gene H2 haplotype is not associated with increased adenosine diphosphate-induced platelet aggregation after initiation of clopidogrel therapy with a high loading dose. *Blood Coagul Fibrinolysis* 2005;16:199-204.
23. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, et al. Lack of association between the P2Y12 receptor gene polymorphism and platelet response to clopidogrel in patients with coronary artery disease. *Thromb Res* 2005;116:491-497.
24. Lev EI, Patel RT, Guthikonda S, et al. Genetic polymorphisms of the platelet receptors P2Y(12), P2Y(1) and GP IIIa and response to aspirin and clopidogrel. *Thromb Res* 2007;119:355-360.
25. Bura A, Bachelot-Loza C, Ali FD, et al. Role of the P2Y12 gene polymorphism in platelet responsiveness to clopidogrel in healthy subjects. *J Thromb Haemost* 2006;4:2096-2097.
26. Bernardo E, Angiolillo DJ, Ramirez C, et al. Lack of association between gene sequence variations of platelet membrane receptors and aspirin responsiveness detected by the PFA-100 system in patients with coronary artery disease. *Platelets* 2006;17:586-590.
27. Cuisset T, Frere C, Quilici J, et al. Role of the T744C polymorphism of the P2Y12 gene on platelet response to a 600-mg loading dose of clopidogrel in 597 patients with non-ST-segment elevation acute coronary syndrome. *Thromb Res* 2007;120:893-899.
28. Geisler T, Langer H, Wydymus M, et al. Low response to clopidogrel is associated with cardiovascular outcome after coronary stent implantation. *Eur Heart J* 2006;27:2420-2425.
29. Hochholzer W, Trenk D, Bestehorn HP, et al. Impact of the degree of peri-interventional platelet inhibition after loading with clopidogrel on early clinical outcome of elective coronary stent placement. *J Am Coll Cardiol* 2006;48:1742-1750.
30. Buonamici P, Marcucci R, Migliorini A, et al. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. *J Am Coll Cardiol* 2007;49:2312-2317.
31. Price MJ, Endemann S, Gollapudi RR, et al. Prognostic significance of post-clopidogrel platelet reactivity assessed by a point-of-care assay on thrombotic events after drug-eluting stent implantation. *Eur Heart J* 2008.
32. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med* 2007;357:2001-2015.
33. Wiviott SD, Trenk D, Frelinger AL, et al. Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. *Circulation* 2007;116:2923-2932.
34. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat* 2000;15:7-12.

5

Chapter 5

Goran Rudež
Douwe Pons
Frank Leebeek
Pascalle Monraats
Marlies Schrevel
Aeilko Zwinderman
Robbert de Winter
René Tio
Pieter Doevendans
Wouter Jukema
Moniek de Maat

Platelet receptor *P2RY12* haplotypes predict restenosis after percutaneous coronary interventions

Hum Mutat. 2008;29(3):375-80

ABSTRACT

The platelet receptor P2RY12 (alias P2Y12) is involved in several processes that contribute to restenosis after percutaneous coronary interventions (PCI). Therefore, common variation in the *P2RY12* gene may serve as a useful marker for risk stratification. We studied whether common variation in the platelet receptor *P2RY12* gene affects the risk of restenosis after PCI. Comprehensive coverage of common variation in the *P2RY12* gene was obtained by genotyping five haplotype-tagging single nucleotide polymorphisms (ht-SNPs) in 2062 PCI-treated patients who received a stent and participated in the GENetic DEterminants of Restenosis (GENDER) Study. Haplotypes were inferred and their association with target vessel revascularization (TVR) was studied. Seven *P2RY12* haplotypes were identified with an allele frequency above 5% (designated H1 to H7) of which two (H5 and H7) were associated with a higher risk of TVR (hazard ratios: 1.4, 95% C.I. 1.0-2.0 and 1.6, 95% C.I. 1.2-2.0, respectively) than the reference *P2RY12* haplotype (H1), which contains the common alleles of all five *P2RY12* ht-SNPs. Our study shows that common variation in the *P2RY12* gene predicts restenosis in PCI-treated patients.

INTRODUCTION

Despite all technological and pharmacological advances in percutaneous coronary interventions (PCI) in patients with coronary heart disease, in-stent restenosis is still a major problem requiring revascularization procedures.[1, 2] Platelets play a role in restenosis, which is a process characterized by neointimal proliferation.[3, 4] Directly after the coronary stent is placed, a thin layer of platelets will rapidly cover the surface of the stent. These platelets provide a matrix for in-stent migration of vascular smooth muscle cells (VSMCs), which eventually form neointima. In addition, these activated platelets secrete the contents of their granules thereby releasing a variety of cytokines that can stimulate VSMCs. Among the excreted cytokines are platelet-derived growth factor, transforming growth factor ß and vascular endothelial growth factor, which can all promote neointimal proliferation.[5]

The platelet membrane adenosine diphosphate (ADP) receptor P2RY12 (OMIM# 600515; alias P2Y12) plays a central role in platelet activation and aggregation.[6] Common variation in the *P2RY12* gene was found to be associated with platelet activation and aggregation,[7] and the risk of cardiovascular disease,[8, 9] but the results from these previous small studies are conflicting.[10, 11] Common variation of the *P2RY12* gene has been suggested to determine the function of the P2Y12 receptor via altered molecular structure or expression of P2Y12 receptors.[7] In addition, VSMCs have recently been reported to express P2Y12 receptors, which are able to evoke contractile response of VSMCs upon stimulation with ADP.[12] Since VSMCs are key players in restenosis, and also platelet activation and aggregation are contributing mechanisms to restenosis, we hypothesized that *P2RY12* gene variants are associated with the risk of restenosis.

We tested our hypothesis by selecting five haplotype-tagging single nucleotide polymorphisms (ht-SNPs), which comprehensively represent the common genetic variation of the *P2RY12* locus, and genotyping these five ht-SNPs in 2062 stented patients from a prospective multicenter study on clinical restenosis (the GENetic DEterminants of Restenosis (GENDER) Study).[1]

METHODS

Study Design

The GENetic DEterminants of Restenosis (GENDER) Study is a multicenter follow-up study of 3104 unselected consecutive patients undergoing PCI, who represent a clinical population practice. The GENDER study was designed to evaluate the association between various genetic risk factors and clinical restenosis. The study design has been described previously.[1] Briefly, patients were eligible for inclusion if they had been successfully treated with PCI for stable angina and non-ST-elevation acute coronary syndromes. Patients who had been treated for acute ST-elevation myocardial infarction (MI) were excluded. All patients were treated in four out of thirteen referral centers for interventional cardiology in the Netherlands (Academic Medical Center Amsterdam, University Medical Center Groningen, Leiden University Medical Center, and Academic Hospital Maastricht). The overall inclusion period lasted from March 1999 until June 2001.

The study protocol conforms to the Declaration of Helsinki and was approved by the medical ethics committees of the participating institutions. Written informed consent was obtained from each participant before the PCI procedure.

Stenting procedure

Standard angioplasty and stent placement were performed by experienced operators using a radial or femoral approach. Before the procedure, patients received 300 mg of aspirin and 7500 IU of heparin. The use of intracoronary stents and additional medication was at the discretion of the treating physician.

Definitions

A PCI procedure was considered successful if, upon visual inspection, the luminal stenosis of at least one lesion had been reduced to less than 50% of the luminal diameter. Hypertension was defined as a blood pressure of either above 160 mm Hg systolic or 90 mm Hg diastolic. Hypercholesterolemia was defined as total cholesterol concentration of at least 5 mmol/L. Current smokers were individuals who had smoked within the month preceding the index intervention. Past smokers were those individuals who had given up smoking in the preceding year. Individuals who had stopped smoking for more than one year were classified as non-smokers. A positive family history was noted if the patient had a first-degree relative with a history of coronary artery disease before the age of 60. Renal failure was defined as a serum creatinine concentration at least 150 μmol/L or as dialysis.

Patients using anti-diabetic medication or insulin at study entry were considered to be diabetics. Patients with a history of surgical treatment for non-cardiac vascular disease, such as aortic aneurysm or by-pass surgery of the peripheral arteries, were considered to have peripheral vessel disease. The pre-procedural lesions were classified according to the modified American College of Cardiology and American Heart Association Task Force classification.[13]

Follow-up and study endpoints

Follow-up lasted at least 9 months and at most 12 months, except when a coronary event occurred. TVR, either by PCI or CABG, was defined as the primary endpoint, because it is considered by regulatory agencies to be the most relevant for clinical practice and for the patient. The secondary combined endpoint was defined as death presumably from cardiac causes, MI not attributable to another coronary vessel than the target vessel or TVR.

Selection of SNPs in the *P2RY12* gene

The *P2RY12* gene spans 47kb and is located in the q25.1 region of chromosome 3 (Figure 1A). The gene contains three exons, of which only exon 3 is coding. Over 120 SNPs have been detected in this genomic region, which are listed in the SNP database of NCBI (dbSNP, build 123). In our study, we considered only SNPs that were present in a Caucasian population with a minor allele frequency of at least 5%. From these SNPs we selected tagging SNPs of haplotypes that comprehensively represent the DNA sequence variation in the promoter-exon1-intron1 region (from c.-281-4450 to c.-217+3350), and intron1-exon2-intron2-exon3 region (from c.-216-4850 to c.1257+1250) of the *P2RY12* gene, as these regions are likely to contain main functional elements. This selection of ht-SNPs was performed on the basis of the LD-map of *P2RY12* locus provided by the International HapMap Project (phase I, June 2005; *http://www.hapmap.org/*)(Figure 1B). For the chosen *P2RY12* locus, which is comprised of the *P2RY12* gene (47kb) and 5'and 3'flanking regions (50kb each), blocks of haplotypes with frequency >5% were defined and their ht-SNPs selected using Haploview software (version 3.11, *http://www.broad.mit.edu/mpg/haploview/index.php*). For the selection of the ht-SNPs, we also took potential functionality into consideration, but none of the SNPs in the *P2RY12* gene results in an amino acid change, nor are there any SNPs located within consensus transcription factor binding sites in the 3 kb promoter region, exon-intron junctions, predicted splicing enhancer motifs, 3' UTR, 3kb 3' flanking region nor domains that are highly conserved among species.

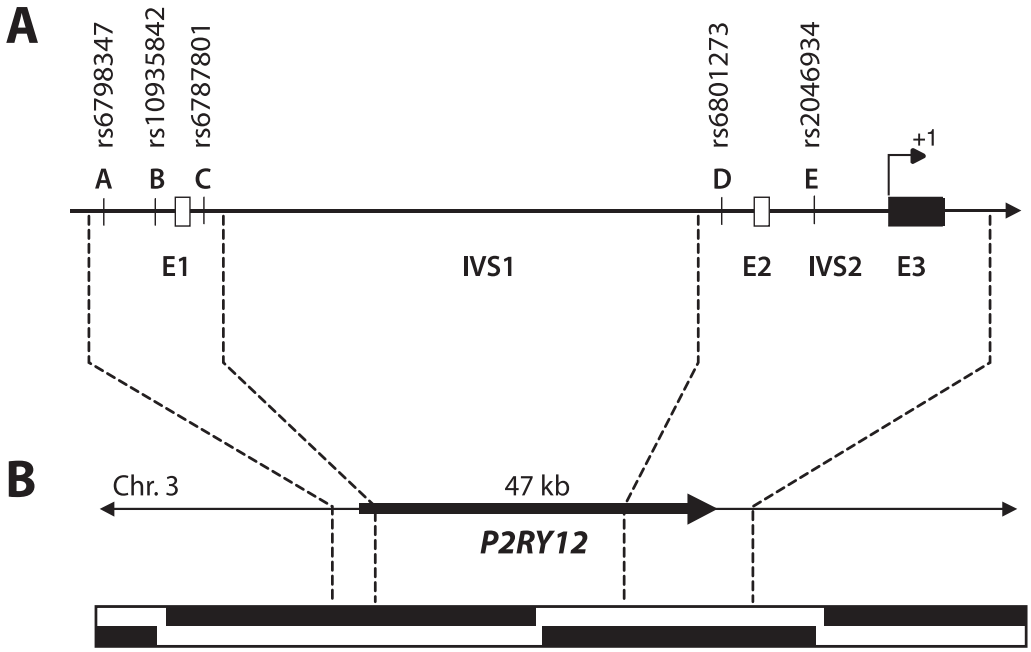


Figure 1. Schematic representation of *P2RY12* gene structure and LD map of *P2RY12* locus.
The *P2RY12* gene consists of three exons (E1, E2 and E3) and two intervening sequences (IVS1 and IVS2), which span 47kb of genomic DNA on chromosome 3. Only E3 is coding (Panel A). On the basis of the genotype data of HapMap SNPs (Phase I, June 2005), a LD map of the *P2RY12* locus (*P2RY12* gene (47kb) with 5'and 3' flanking regions 50kb each)) can be derived (Panel B). Black bars represent LD blocks of haplotypes (frequency >5%) within which there is little or no recombination between SNPs. Blocks are separated by recombination hot-spots. Haplotypes tagged by SNPs A, B and C comprehensively represent the genetic variation in the promoter-E1-intron1 region localized in the 59kb LD block, whereas haplotypes tagged by SNPs D and E represent the genetic variation in the E2-IVS2-E3 region present in the 42kb LD block

The five selected ht-SNPs that were genotyped in this study are SNP A: 5'flanking(c.-281-3614C>T) [rs6798347], SNP B: 5' flanking(c.-281-1394A>T) [rs10935842] and SNP C: IVS1(c.-217+2739T>C) [rs6787801], which tag the promoter-exon1-intron1 region and SNP D: IVS1(c.-216-4445A>G) [rs6801273] and SNP E: IVS2(c.-15+742T>C) [rs2046934], which tag the exon2-intron2-exon3 region (Figure 1 and Table 1). Annotation of the selected ht-SNPs is according to the nomenclature recommendations of the Human Genome Variation Society, using the cDNA sequence available under GenBank accession number NM_022788.3 as reference, with nucleotide +1 being the A of the ATG-translation initiation codon.[14]

Table 1. Haplotype-tagging *P2RY12* SNPs

Region	SNP ID*	SNP ID†	SNP ID‡	SNP A§	SNP B§	SNP C§	SNP D§	SNP E§
5' flanking	SNP A	c.-281-3614C>T	rs6798347	-	0.98	0.91	0.37	0.88
5' flanking	SNP B	c.-281-1394A>T	rs10935842	0.14	-	0.98	0.21	0.38
IVS1	SNP C	c.-217+2739T>C	rs6787801	0.19	0.62	-	0.27	0.51
IVS1	SNP D	c.-216-4445A>G	rs6801273	0.06	0.04	0.04	-	0.99
IVS2	SNP E	c.-15+742T>C	rs2046934	0.04	0.02	0.06	0.13	-

*SNP annotation used for convenience in this paper. In previous publications SNP E has been annotated as i-T744C.[7]
†Nucleotide numbering of the SNPs according to the nomenclature recommendations of the Human Genome Variation Society using the cDNA reference sequence available under GenBank accession number NM_022788.3, with +1 being the A of the ATG translation initiation codon located in exon 3.
‡Reference ID according to NCBI dbSNP.
§Values of r^2 (under the diagonal axis) and D' (above the diagonal axis) for pairwise analysis of the five studied *P2RY12* SNPs.

Blood collection and genotyping

Blood was collected in tubes containing EDTA at baseline, and genomic DNA was extracted following standard salting-out procedures. The five selected ht-SNPs were genotyped using Custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, USA).[15] The nucleotide sequences of the primers and probes used for each assay are available upon request. End-point fluorescence was measured on the ABI 7900HT instrument (Applied Biosystems, Foster City, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, USA). For each SNP, genotyping was possible, on average, in 94% of the 2062 subjects. Missing genotype data was primarily due to unavailability of patient DNA and was not assay-related. The baseline characteristics of missing patients were similar to those who could be successfully genotyped. A random selection of 10% of the samples was re-analysed, and the results were confirmed with the first genotyping analysis in more than 99% of the cases. Haplotype alleles were coded with five letters (*e.g.* C<u>GC</u>AT) representing the nucleotides of SNPs A, B, C, D and E, respectively, with the rare alleles underlined. For convenience, these haplotypes were also coded H1 to H7 according to their effects on the TVR risk relative to the reference haplotype H1 (CATAT).

Statistical methods

To test whether the genotype distribution of the ht-SNPs studied deviates from that expected for a population in Hardy-Weinberg equilibrium, we used a Chi-squared test with

one degree of freedom. The association of each of the five *P2RY12* ht-SNPs and TVR was determined using a Cox proportional regression model. From the obtained unphased SNP genotype data, the effect of haplotypes on risk of TVR was estimated using weighted Cox regression, incorporating the uncertainty about phase-ambiguous individuals as weights in the model, as described by Tanck *et al.*[16] The covariates included in the multivariable model were age, sex, smoking status, diabetes, hypertension, statin therapy, residual stenosis >20%, and stent length. These covariates have been previously found to be associated with TVR risk. Cumulative proportions of TVR-free patients were calculated by Kaplan-Meier survival analysis. A two-sided value of $P<0.05$ was considered statistically significant. Analyses were performed with SPSS for Windows, version 11.5 (SPSS Inc, Chicago, USA).

RESULTS

Characteristics of the patients

A total of 3509 patients were eligible for the GENDER study of whom 3177 were included and followed (Figure 2).

Of the 3146 patients with a complete follow-up, 42 had an event within one month of the index intervention due to acute thrombosis or occluding dissections. Excluding these early events, 3104 patients remained of whom 2062 were stented and were included in the present study. Baseline characteristics of these 2062 patients are listed in table 2. The majority of patients were Caucasian (97%). During a median follow-up of 9.6 months (interquartile range 3.9 months), 175 patients underwent TVR (8.4%), the primary endpoint. Additionally, 17 patients died (0.8%) and 13 patients had a MI (0.6%). At baseline, 21% of patients in the TVR group had diabetes mellitus, 33% had a previous MI and 59% used calcium antagonists, which was significantly higher than in the non-TVR group (13%, $P=0.009$, 41%, $P=0.035$ and 50%, $P=0.02$, respectively).

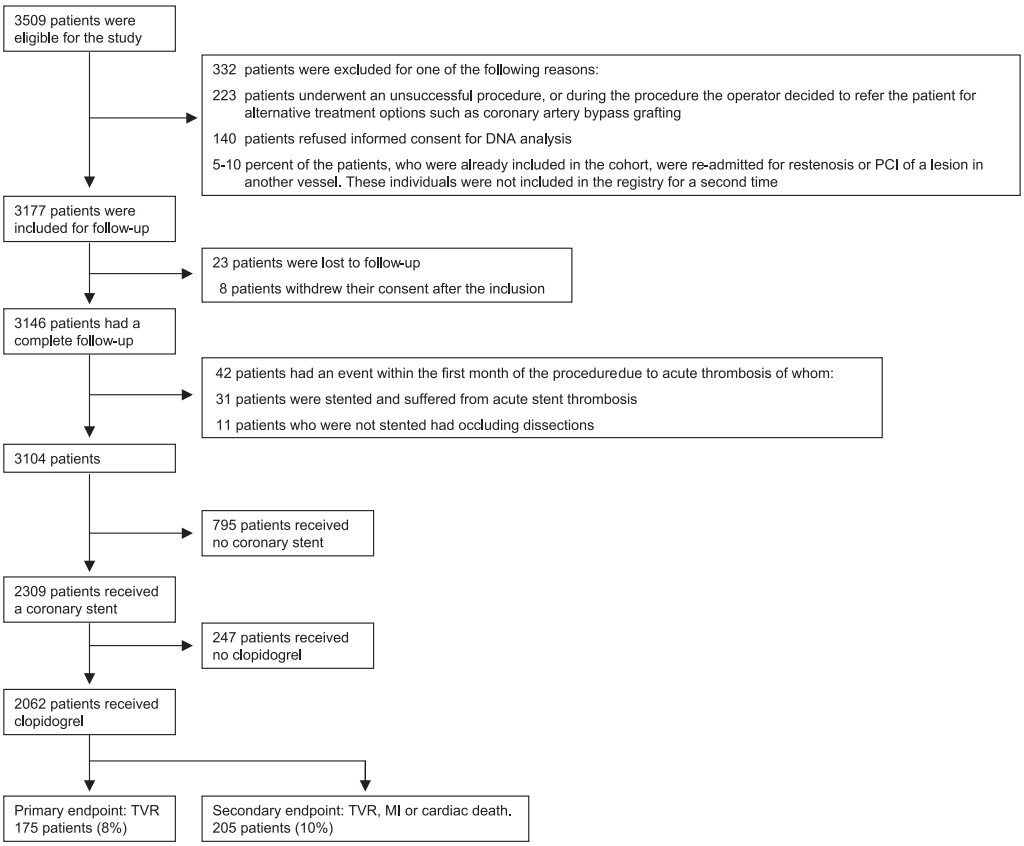


Figure 2. Flow-chart of the *GENDER* Study
Numbers of patients from the *GENDER* Study who were eligible for the study, the numbers who were included in the study, and the numbers for whom follow-up data was complete. Also, numbers of patients are given for those who had to undergo TVR (the primary endpoint) and those who underwent TVR, had a myocardial infarction or died from cardiac causes (the secondary endpoint).

Table 2. Baseline characteristics of 2062 patients after PCI

	TVR (n=175)	No TVR (n=1887)	p-value
Age (years)	62 (10)	62 (11)	0.58
Female Sex	51 (29%)	532 (28%)	0.83
BMI (kg/m^2)	26.9 (3.6)	27.1 (3.9)	0.65
Diabetes	36 (21%)	251 (13%)	0.009
Hypercholesterolemia	105 (60%)	1142 (61%)	0.82
Hypertension	75 (43%)	759 (40%)	0.54
Current smoking	39 (22%)	480 (26%)	0.34
Family history of MI	69 (39%)	624 (33%)	0.10
Previous MI	58 (33%)	775 (41%)	0.035
Previous PCI	27 (15%)	298 (16%)	0.87
Previous CABG	23 (13%)	219 (8%)	0.88
Baseline medication			
Beta-blockers	135 (77%)	1479 (78%)	0.60
Ca^{2+}-antagonists	104 (59%)	941 (50%)	0.020
ACE inhibitors	36 (21%)	377 (20%)	0.88
Statins	95 (54%)	1044 (55%)	0.73
Angiographic data			
Total stent length	22.8 (15.6)	21.3 (13.4)	0.16
Proximal LAD	42 (24%)	464 (25%)	0.83
Residual stenosis >20%	10 (6%)	78 (4%)	0.33

Values are given as n (%) except for age, BMI and total stent length.
BMI indicates body mass index; MI, myocardial infarction; CABG, coronary artery by-pass grafting; ACE, angiotensin converting enzyme; LAD, left anterior descending artery branch of the left coronary artery.

P2RY12 genotypes

The genotype distribution of all five *P2RY12* ht-SNPs was in Hardy-Weinberg equilibrium, and their allele frequencies were similar to those reported by NCBI dbSNP for Caucasian populations (Table 3).

Table 3. Cox regression analysis of TVR risk for five individual *P2RY12* SNPs

	Genotype	TVR	No TVR	HR TVR* (95% C.I.)
SNP A	CC	105 (65.6%)	1140 (64.6%)	
	CT	50 (31.3%)	552 (31.3%)	1.0 (0.7-1.4)
	TT	5 (3.1%)	74 (4.2%)	0.7 (0.3-1.8)
SNP B	AA	48 (31.0%)	699 (40.4%)	
	AT	82 (52.9%)	834 (48.2%)	1.4 (1.0-2.1)
	TT	25 (16.1%)	198 (11.4%)	1.9 (1.1-3.0)
SNP C	TT	31 (19.9%)	456 (26.4%)	
	TC	83 (53.2%)	906 (52.4%)	1.3 (0.9-2.0)
	CC	42 (26.9%)	368 (21.3%)	1.7 (1.1-2.7)
SNP D	AA	60 (38.5%)	719 (41.5%)	
	AG	73 (46.8%)	785 (45.3%)	1.1 (0.8-1.6)
	GG	23 (14.7%)	230 (13.3%)	1.2 (0.7-1.9)
SNP E	TT	103 (64.8%)	1096 (63.2%)	
	TC	48 (30.2%)	572 (33.0%)	0.9 (0.6-1.2)
	CC	8 (5.0%)	65 (3.8%)	1.4 (0.7-2.8)

Values are given as n (%).
*Hazard ratios are adjusted for age, sex, diabetes, hypertension, use of statins and residual stenosis >20%.

When analyzing *P2RY12* SNPs individually, patients with the TT genotype for SNP B had a significantly higher TVR risk than those with the AA genotype (hazard ratio (HR) 1.9, 95% C.I. 1.1-3.0), while the AT genotype had an intermediate effect on the risk (HR: 1.4, 95% C.I. 1.0-2.1)(Table 3). Similar results were observed for SNP C: TVR occurred significantly more often in CC homozygotes compared to the TT homozygotes (HR: 1.7, 95% C.I. 1.1-2.7) while the TC heterozygotes were associated with an intermediate, although not a significant effect on the risk of TVR (HR: 1.3, 95% C.I. 0.9-2.0)(Table 3).

No relationship between SNPs A, D and E and the primary endpoint was found. When the combined endpoint of TVR, MI or cardiac death was considered, similar results were obtained for both SNP B (TT vs AA, HR: 1.6, 95% C.I. 1.0-2.5; AT vs AA, HR: 1.3, 95% C.I. 1.0-1.8) and SNP C (CC vs TT, HR: 1.5, 95% C.I. 1.0-2.4; TC vs TT, HR: 1.3, 95% C.I. 0.9-1.8). No relationship between SNPs A, D and E and the combined endpoint was found.

P2RY12 haplotypes

By combining the genotypes of all five *P2RY12* SNPs, 32 haplotype alleles were inferred of which seven had an allele frequency above 5%. Haplotype H7 was most frequently found in our study population (18%)(Figure 3).

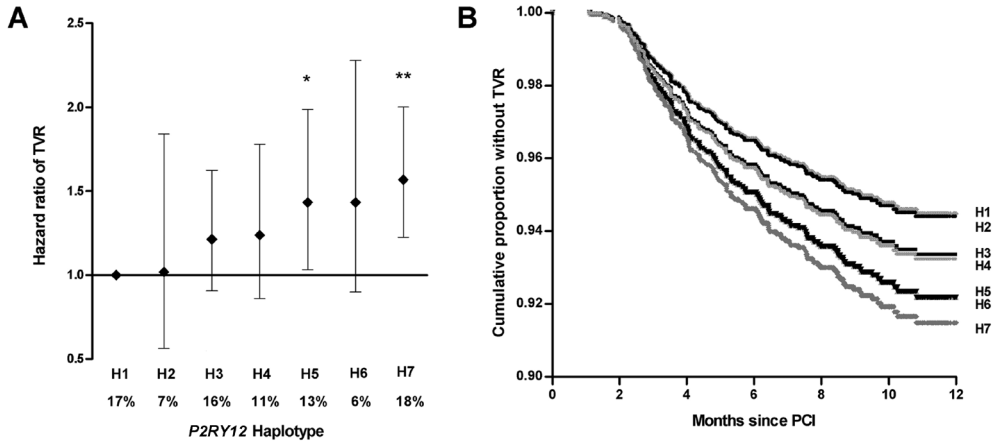


Figure 3. *P2RY12* haplotypes and the risk of TVR

Panel **A** shows the results of weighted multivariate Cox regression analysis of *P2RY12* haplotypes and TVR risk. As reference, haplotype H1 (CATAT, frequency 16%) is used, which is comprised of only common alleles of the *P2RY12* SNPs studied. Minor SNP alleles are underlined for the other haplotypes: H2 (TATAT)(7%), H3 (CATAC)(16%), H4 (TATGT)(11%), H5 (CTCAT)(13%), H6 (CACAT)(6%) and H7 (CTCGT)(18%). The haplotypes are sorted from left to right according to their relative effects on TVR risk found in the study population. Hazard ratios are adjusted for age, sex, diabetes, hypertension, use of statins and residual stenosis >20%. Panel **B** shows the Kaplan-Meier estimates of cumulative proportion of patients who did not need to undergo target vessel revascularization during the follow-up of 12 months. Patients were grouped according to their *P2RY12* haplotype. In both panels only haplotypes with obtained frequency of at least 5% were included in the analysis. * P<0.05 and ** P<0.001.

This haplotype H7 (CTCGT), which contains the minor alleles of SNPs B, C and D (alleles T, C and G, respectively) was associated with a significantly higher risk of TVR (HR: 1.6, 95% C.I. 1.2-2.0) than the reference haplotype H1 (CATAT, frequency 17%)

(Figure 3A). This reference haplotype contains only the common alleles of the five studied *P2RY12* SNPs. Similarly, the hazard ratio of TVR in the total population was significantly higher in patients with the haplotype H5 (CTCAT)(HR: 1.4, 95% C.I. 1.0-2.0) than in the H1 reference haplotype (Figure 3A). Furthermore, our Kaplan-Meier analysis of TVR-free survival revealed similar times of onset of TVR for these haplotypes (Figure 3B). When the secondary combined endpoint of cardiac death, MI or TVR was considered, the relationship between *P2RY12* haplotypes and risk was similar to that seen for the primary endpoint (H5: HR 1.4, 95% C.I. 1.0-1.9; H7: HR 1.5, 95% C.I. 1.2-1.9; other haplotypes: non-significant). Also, similar results were found when only Caucasians were analyzed, both for the primary and the secondary endpoint (data not shown).

DISCUSSION

In this study we identified genetic variation in the platelet ADP receptor *P2RY12* as a strong and independent predictor of risk of clinical restenosis in PCI-treated patients. Haplotypes H5 and H7 were associated with a significantly higher risk of TVR than the reference haplotype H1, which contains the common alleles of all five ht-SNPs. Haplotype H5 (CTCAT) contains the rare alleles of SNPs B and C, and haplotype H7 (CTCGT) the rare alleles of SNPs B, C and D. Since SNPs B and C are localized in the promoter region of the *P2RY12* gene, one might expect that transcriptional regulation of the *P2RY12* gene is the mechanism via which these SNPs have an effect on the pathogenesis of restenosis. At the time we selected our SNPs, no functional candidates had been present in the consensus transcription factor binding sites within the 3kb promoter region upstream of exon 1, nor in the predicted splicing-enhancer motifs. Our data, however, suggest the existence of such a site in these regions, but this site still needs to be identified.

In addition, our results suggest that SNP D, which tags the haplotypes present in the region containing the coding exon 3, contributes to the risk of restenosis. However, the effect of SNP D is probably not regulated by structural alteration of the *P2RY12* receptor, since no SNPs have been identified that result in a change in amino acid composition of the P2Y12 protein. In addition, no SNPs are located in exon-intron junctions, in the 3'UTR, 3'flanking region, nor in domains that are highly conserved among species. SNP D is located in another LD block than SNPs B and C, which makes it unlikely that SNP D affects the promoter region. Instead, it is possible that SNP D affects splicing or enhancer elements located within the 42kb LD-block. Interestingly, SNP E, which is in

high LD with SNP D, was previously found to be associated with increased ADP-induced platelet aggregation in healthy subjects and increased risk of peripheral arterial disease and ischemic stroke.[7-9] Overall, the results of this study warrant functional studies on SNPs in the investigated *P2RY12* gene regions.

Several underlying mechanisms for the observed relationship between *P2RY12* SNPs/haplotypes and restenosis can be hypothesized. Firstly, the *P2RY12* haplotypes may have an indirect effect on restenosis via altered platelet activation and aggregation (*e.g.* through increased expression of *P2RY12* gene or altered molecular structure and function of the P2Y12 receptor). This hypothesis is supported by the reports of Fontana *et al*. and our own group who showed in healthy individuals that platelet aggregation depends on *P2RY12* SNPs.[7, 17] However, not all studies were able to confirm these findings.[10] Secondly, *P2RY12* haplotypes may have a direct effect on restenosis by altering the number or the protein structure of the P2Y12 receptors that are expressed in VSMCs.[12] However, the function of P2Y12 receptors on VSMCs is yet rather unclear and needs to be studied further before we can elucidate the mechanism underlying the potential association between the VSMC-derived P2Y12 receptors and the pathogenesis of restenosis.

The observed effects of *P2RY12* haplotypes may not only apply for restenosis, but also for other biologically related cardiovascular disorders, such as ischemic stroke and acute coronary syndrome. Only a few studies have already tested this hypothesis, but they have not shown a consistent relationship between *P2RY12* SNPs and these clinical endpoints.[7, 9, 11] However, most of these studies were statistically underpowered and none of them covered the common variation of the *P2RY12* gene comprehensively. In contrast, the present study is the first large study, which has comprehensively investigated the common variation of most of the *P2RY12* locus. However, more large studies are needed that combine multiple relevant clinical endpoints, *P2RY12* haplotypes, several platelet function assays and SNP functionality, before we can reach a more effective approach to risk stratification and eventually improved (pharmacological) therapy.

In conclusion, this is the first study to show that common variation in the *P2RY12* gene predicts the risk of restenosis in patients treated with PCI and stenting. *P2RY12* haplotyping is potentially a useful stratification tool for identifying patients who are genetically predisposed to restenosis and who may eventually benefit from additional or alternative drug treatment.

REFERENCES

1. Agema WR, Monraats PS, Zwinderman AH, De Winter RJ, Tio RA, Doevendans PA, Waltenberger J, De Maat MP, Frants RR, Atsma DE, Van Der Laarse A, Van Der Wall EE, Jukema JW. Current PTCA practice and clinical outcomes in The Netherlands: the real world in the pre-drug-eluting stent era. *Eur Heart J.* 2004;25:1163-1170.
2. Pache J, Kastrati A, Mehilli J, Schuhlen H, Dotzer F, Hausleiter J, Fleckenstein M, Neumann FJ, Sattelberger U, Schmitt C, Muller M, Dirschinger J, Schomig A. Intracoronary stenting and angiographic results: strut thickness effect on restenosis outcome (ISAR-STEREO-2) trial. *J Am Coll Cardiol.* 2003;41:1283-1288.
3. Chandrasekar B, Tanguay JF. Platelets and restenosis. *J Am Coll Cardiol.* 2000;35:555-562.
4. Goel PK, Shahi M, Agarwal AK, Srivastava S, Seth PK. Platelet aggregability and occurrence of restenosis following coronary angioplasty. *Int J Cardiol.* 1997;60:227-231.
5. Gresele P, Page, C., Fuster, V., Vermylen, J. Platelets in thrombotic and non-thrombotic disorders. Cambridge: Cambridge University Press; 2002.
6. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature.* 2001;409:202-207.
7. Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation.* 2003;108:989-995.
8. Fontana P, Gaussem P, Aiach M, Fiessinger JN, Emmerich J, Reny JL. P2Y12 H2 haplotype is associated with peripheral arterial disease: a case-control study. *Circulation.* 2003;108:2971-2973.
9. Ziegler S, Schillinger M, Funk M, Felber K, Exner M, Mlekusch W, Sabeti S, Amighi J, Minar E, Brunner M, Muller M, Mannhalter C. Association of a functional polymorphism in the clopidogrel target receptor gene, P2Y12, and the risk for ischemic cerebrovascular events in patients with peripheral artery disease. *Stroke.* 2005;36:1394-1399.
10. Hetherington SL, Singh RK, Lodwick D, Thompson JR, Goodall AH, Samani NJ. Dimorphism in the P2Y1 ADP receptor gene is associated with increased platelet activation response to ADP. *Arterioscler Thromb Vasc Biol.* 2005;25:252-257.
11. Schettert IT, Pereira AC, Lopes NH, Hueb WA, Krieger JE. Association between platelet P2Y12 haplotype and risk of cardiovascular events in chronic coronary disease. *Thromb Res.* 2006.
12. Wihlborg AK, Wang L, Braun OO, Eyjolfsson A, Gustafsson R, Gudbjartsson T, Erlinge D. ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. *Arterioscler Thromb Vasc Biol.* 2004;24:1810-1815.
13. Ellis SG, Vandormael MG, Cowley MJ, DiSciascio G, Deligonul U, Topol EJ, Bulle TM. Coronary morphologic and clinical determinants of procedural outcome with angioplasty for multivessel coronary disease. Implications for patient selection. Multivessel Angioplasty Prognosis Study Group. *Circulation.* 1990;82:1193-1202.
14. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. *Hum Mutat.* 2000;15:7-12.
15. Livak KJ. Allelic discrimination using fluorogenic probes and the 5' nuclease assay. *Genet Anal.* 1999;14:143-149.
16. Tanck MW, Klerkx AH, Jukema JW, De Knijff P, Kastelein JJ, Zwinderman AH. Estimation of multilocus haplotype effects using weighted penalised log-likelihood: analysis of five sequence variations at the cholesteryl ester transfer protein gene locus. *Ann Hum Genet.* 2003;67:175-184.

17. Rudez G, de Bruijne, E.L.E., Bongers, T.N., Dippel, D.W.J., Deckers, P., Poldermans, D., de Maat, M.P.M., Leebeek, F.W.G. Genetic variation in ADP-receptor P2Y12 and the risk of arterial thrombosis. *British Journal of Haematology.* 2006;Volume 133, Supplement 1, Abstract 64.

Chapter 5

II

Part II

Chapter 6

Goran Rudež
Piet Meijer
Henri Spronk
Frank Leebeek
Hugo ten Cate
Cornelis Kluft
Moniek de Maat

Biological variation in inflammatory and hemostatic factors

J Thromb Haemost, 2009;7:1247-55

ABSTRACT

Background

Concentrations of inflammatory and hemostatic variables are influenced by biological variation, *i.e.* the natural within-subject variation over time.

Objectives

The aim of this study was to determine for fibrinogen, C-reactive protein (CRP), platelet aggregation, thrombin generation and prothrombin time (PT): (i) the number of repeated measurements needed to obtain the true habitual concentration of an individual, (ii) the recommended analytical imprecision for diagnosis and monitoring, (iii) the recommended analytical bias, (iv) the contribution of analytical imprecision to test result variability, (v) the index of individuality, (vi) the reference change value and (vii) the seasonal variation.

Subjects and Methods

We collected 520 blood samples over a one-year period from 40 healthy individuals, and determined the between-subject, within-subject and seasonal variation of fibrinogen, CRP, platelet aggregation, thrombin generation and PT.

Results

One or two repeated measurements were sufficient to establish the true habitual concentration, except for platelet aggregation and peak thrombin generation, where at least 4 and 9 repeated measurements were needed, respectively. For diagnosis, the maximal recommended coefficient of analytical variation (CV) was between 4% and 27%, except for CRP (77.7%). For monitoring, these CVs were on average 3% lower. Recommended analytical bias varied between 1.7% and 33.2%. Finally, seasonal variation was observed in concentrations of fibrinogen and thrombin generation, which could explain approximately 11% of their total variation.

Conclusion

This study provides insights into the biological variability of selected inflammatory and hemostatic markers, which can be used for sample size calculations and to determine the analytical quality specifications for their respective assays.

INTRODUCTION

Epidemiologic studies have identified the inflammatory markers fibrinogen and C-reactive protein (CRP) as independent risk factors for cardiovascular disease (CVD).[1, 2] In addition, the hemostatic markers platelet aggregation, thrombin generation and prothrombin time (PT) have been shown to play an important role in CVD.[3-5] Plasma concentrations of these variables are determined by a number of environmental factors which contribute to the biological variation, *i.e.* the natural within-subject variation over time. For instance, the plasma concentrations of fibrinogen and CRP are occasionally raised by an acute phase reaction, *e.g.* due to the common cold.[6] Since biological variation is a source of test variation, it may affect the diagnosis, monitoring and prognostic assessment of CVD that is based on tests of these inflammatory and hemostatic markers.[7]

When using markers as long-term risk indicators for cardiovascular disease, we are interested in the differences between the habitual concentrations of the study participants and not in their short-time fluctuations induced by transient (acute-phase) reactions. However, in most epidemiologic studies, only a single blood sample is taken from each subject and this is then accepted as representative of his/her habitual concentration. Multiple sampling improves the estimate of an individual's homeostatic mean and optimises the risk assessment, as each sampling provides information on the within-subject biological variation.

A limited number of previous studies have already gathered some information on the biological variation of inflammatory and hemostatic markers[8], but comprehensive studies are lacking that focus on the variability of these markers over a longer period of time. In addition, it is known that several inflammatory and hemostatic markers, like fibrinogen and CRP, are characterized by seasonal variation, which has been suggested to contribute to the seasonal variation in cardiovascular mortality.[9]

Therefore, the aim of this study was to investigate longitudinally (*i.e.* repeatedly over 1 year) the biological and seasonal variation of fibrinogen, CRP, platelet aggregation, thrombin generation and PT. Based on the obtained biological variation, we then aimed to provide a number of recommendations for diagnosis and monitoring of these inflammatory and hemostatic markers.

METHODS

Study population

A total of 40 apparently healthy individuals were included in the study. Exclusion criteria were symptoms of chronic infectious diseases, acute infections or any surgical procedure within the last three months. From each participant blood was collected at 13 visits during a one-year period. To minimize the effect of circadian variation on plasma concentrations of biomarkers, all blood samples were taken between 9 and 11 AM, and were spread throughout the study period. Data on demographics and cardiovascular risk factors were collected using a standardized questionnaire. Before each blood collection, the study participants were asked whether they had recently been using any medication or whether they had suffered from any recent inflammatory illness, *e.g.* common cold. The total study period was from January 2005 to December 2006. The study protocol is in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Erasmus University Medical Center. Written informed consent was obtained from each participant.

Blood collection

Blood was collected while the study participants were sitting and resting. They were allowed to have a light breakfast on the morning of blood collection. Blood was drawn by venepuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK) containing sodium citrate (final concentration 0.105 mol/L). Plasma was obtained by centrifugation at 1500*g* for 15 min at 4°C and stored in aliquots at –80ºC until further analysis. To exclude inter-assay analytical variation, all samples from each subject were analyzed in a single run, except for platelet aggregation which is performed on fresh plasma samples. For platelet aggregation experiments, blood was centrifuged at 150*g* for 15 min to obtain platelet-rich plasma (PRP) and subsequently at 1500*g* for 10 min to obtain platelet-poor plasma (PPP). PRP was adjusted with autologous PPP to $200x10^9$ platelets/L (P200), which was used in platelet aggregation experiments.

Laboratory measurements

Fibrinogen, CRP and prothrombin time

Fibrinogen concentrations were determined according to the von Clauss method on an ACL-300 (HemosIL (Fibrinogen-C), Instrumentation Laboratory, Breda, the Netherlands). CRP concentrations were measured by means of an in-house high-sensitivity ELISA with

polyclonal rat CRP antibodies for catching and tagging (DAKO A/S, Glostrup, Denmark) and a CRP-calibrator (DAKO). PT was measured on a Sysmex CA-1500 (Dade Thrombin Reagent, Siemens Diagnostics, Leusden, the Netherlands). George King pooled normal plasma was used as reference plasma in all three assays (George King Biomedical Inc., Overland Park, KS, USA).

Light-transmittance platelet aggregometry

Light-transmittance ADP-induced platelet aggregometry was performed within 2 hours after blood collection, as described previously.[10] Briefly, platelet aggregation was induced by 5 and 2.5 µmol/L ADP (Sigma-Aldrich, Saint Louis, MO, USA) in the presence of aspirin (100 µmol/L final), physiological calcium concentration (16.6 mmol/L final) and the thrombin-inhibitor D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK, 40 µmol/L, final, Merck & Co, Darmstadt, Germany), and recorded for 10 min on a 4-channel optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden, the Netherlands). Due to logistic reasons, platelet aggregation could only be performed in a subset of 155 plasma samples from 17 individuals. These individuals were unselected and their characteristics did not significantly differ from those in whom platelet aggregation was not measured.

Thrombin generation

Thrombin generation in tissue factor (TF)-triggered PPP was measured with the CAT method (Thrombinoscope BV, Maastricht, The Netherlands).[11, 12] Measurements were conducted on 80 µL plasma with final concentrations of 1 and 5 pmol/L TF (PPP Reagent Low and PPP Reagent, Thrombinoscope BV) and 4 µmol/L phospholipids. Thrombin Calibrator was obtained from Thrombinoscope BV. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 nm filter set. Thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV). Three parameters were derived from the thrombin generation curves: lag time (defined as the time to reach 1/6 of the peak height), endogenous thrombin potential (ETP), and peak height. Slope was calculated by dividing 5/6 of the peak height by the time to peak minus the lag time.

Statistical analysis

Data on population demographics are presented as means and standard deviations (SD) for continuous variables and as counts and percentages for categorical variables, except for age and BMI for which range is indicated. Variables are presented as arithmetic means

with their SDs. Components of variation are given as coefficients of variation (CV), *i.e.* (SD/mean) * 100%. Since CRP concentrations had a skewed distribution, they were first (natural) logarithmically transformed and their geometric mean with its 95% range presented. Corresponding CVs were calculated according to Euser *et al*..[13]

In the longitudinal study, sources of variation were calculated using an additive-variance component model in ANOVA[14]:

$$y(ij) = \mu + \alpha(i) + \varepsilon(ij)$$

whereby $y(ij)$ is the value of subject i at time j, μ is the true mean of the population, $\alpha(i)$ is the deviation from the true mean of the i^{th} person (i=1, 2, 3,…40) and $\varepsilon(ij)$ is the residual variance comprised of the within-subject variance and the intra-serial analytical variance. The random terms $\alpha(i)$ and $\varepsilon(ij)$ were assumed to be independent and normally distributed with zero expectations. The measurement number was not added as a factor because its deviations from the true mean were assumed to be independent of sampling time.

We have calculated the number of repeated measurements (m) that was needed to estimate with 95% probability a homeostatic setting point which does not deviate more than 20% from its true value. [14, 15] Since there are no strict criteria in the literature, 95% probability and 20% deviation were arbitrarily chosen:

$$m = (1.96 * (CV^2_{analytical} + CV^2_{within\text{-}subject})^{1/2}/20)^2$$

whereby $CV_{analytical}$ was the coefficient of analytical variation and $CV_{within\text{-}subject}$ the coefficient of within-subject biological variation.

Recommended analytical variation was calculated according to the criterion that the analytical imprecision of a diagnostic test should not exceed 58% of total variation of the study population. In this way, the analytical variation adds a maximum of 12% variation to the total test variability.[16] For monitoring (*e.g.* in epidemiologic studies) the recommended analytical imprecision was calculated as 50% of the within-subject biological variation, according to which an assay adds a maximum of 10% variation to the total test variability.[17] In addition, the recommended analytical bias was calculated, which indicated the difference between the expectation of measurement results and the true value of the measured quantity due to assay error[18]:

$$bias < 0.25 * (CV^2_{between\text{-}subject} + CV^2_{within\text{-}subject})^{1/2}$$

whereby $CV_{between\text{-}subject}$ was the coefficient of between-subject variation.

The contribution of the analytical imprecision to the overall test result variability was calculated using[19]:

$$[(CV_{analytical} + CV_{within\text{-}subject}) / CV_{within\text{-}subject}] - 1$$

We have calculated indices of individuality, which represent how unique each test result was within our total study population[19]:

$$(CV^2_{analytical} + CV^2_{within\text{-}subject})^{1/2} / CV_{between\text{-}subject}$$

Reference change values were calculated, which indicate how many percent a test result needs to deviate from the homeostatic setting point in order to be considered statistically different (*i.e.* outside of the 95% of the distribution)[19]:

$$2^{1/2} * 1.96 * (CV^2_{analytical} + CV^2_{within\text{-}subject})^{1/2}$$

Seasonal variation was modeled assuming that the outcome variable follows a sinusoidal curve with a period of one year.[20] The expected value for the outcome variable on day *t* was calculated with the corresponding regression formula:

$$a + b * \sin(2\pi(t-1)/365) + c * \cos(2\pi(t-1)/365)$$

whereby *a* is the annual mean of the variable, and *b* and *c* are the components of the seasonal variation. Using the Wald test[21], the null hypothesis was tested that there is no seasonal variation (*b* and *c* are zero). Seasonal variation was not calculated for platelet aggregation, since there were not enough values obtained to represent each season.

Statistical analyses were performed with SPSS for Windows, version 11.5 (SPSS Inc, Chicago, USA). A two-sided value of $p<0.05$ was considered statistically significant. Age, sex and BMI were added as covariates in the statistical model. Outliers were defined as values outside the 2.5-97.5% range. F-test was used to test the differences between corresponding CVs from the total study population and the following subgroups: non-smokers only, between men and women, after excluding periods of reported disease

(common cold), non-users of contraceptives, before and after adjustments for age and BMI, before and after exclusion of outliers, between 5 and 2.5 µmol/L ADP- induced platelet aggregation, and between 5 and 1 pmol/L TF-induced thrombin generation.

RESULTS

Forty apparently healthy volunteers with a mean age of 41 years (range: 21-70 years) were included in the study. Their mean body mass index (BMI) was 22.6 kg/m^2 (range: 18.3-27.2 kg/m^2). Twenty-six participants were women (65% of total) of whom 9 were using oral contraceptives throughout the follow-up period (23% of women). In total, there were 7 smokers (18%) (Table 1).

Table 1. Characteristics of the study population

Variable	Study population (n=40)
Age (years)	41 (21-70)
Females	26 (65%)
BMI (kg/m^2)	22.6 (18.3-27.2)
Smokers	7 (18%)
Oral contraceptives	9 (23%)

Values are given as n (%) for categorical variables and as means (range) for age and BMI.
BMI, body mass index

Individual concentrations of inflammatory and hemostatic markers

A total of 520 blood samples were collected on 197 different days that were spread throughout the study period. The mean fibrinogen plasma concentration in these samples was 2.8 g/L (Figure 1A, Table 2) with a coefficient of variation (CV) for the total, between-subject, within-subject and the analytical variation of 19.7%, 14.7%, 12.7% and 2.9%, respectively (Table 2). Geometric mean of the CRP plasma concentrations was 0.37 mg/L and the CVs for the total, between-subject, within-subject and the analytical variation were 132.7%, 107.6%, 77.6% and 3.0%, respectively (Table 2). These CVs were the highest amongst the studied variables, which reflects the sensitivity of CRP to react to inflammatory triggers (Figure 1B). In general, the temporary rises in CRP concentrations were accompanied by an increase in fibrinogen concentrations at the same sampling time-points (r=0.75, p=0.2).

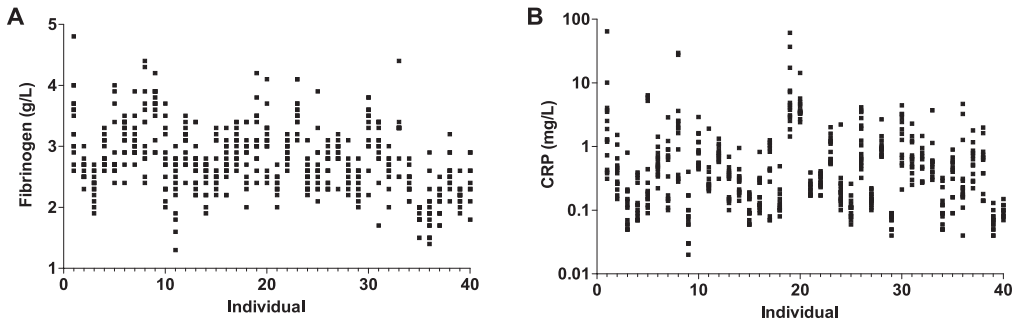


Figure 1. Individual plasma concentrations of fibrinogen and CRP
Fibrinogen plasma concentrations are given in panel A and CRP plasma concentrations in panel B. CRP-concentrations are depicted on a logarithmic scale.

Table 2. Components of variation in plasma concentrations of fibrinogen and CRP, and in concentrations of platelet aggregation, thrombin generation and prothrombin time

Variable	N		Mean ± SD	Coefficient of variation			
	Subjects	Observations		Total	Between-subject	Within-subject	Analytical**
Fibrinogen (g/L)	40	520	2.8 ± 0.5	19.7%	14.7%	12.7%	2.9%
CRP (mg/L)*	40	520	0.37 (0.03-5.08)*	132.7%*	107.6%*	77.7%*	3.0%*
Platelet aggregation (%)	17	155	59 ± 13	21.7%	6.8%	14.6%	14.6%
Thrombin generation							
ETP (nmol*min)	40	520	1012 ± 296	29.4%	23.8%	16.2%	6.1%
Peak (nmol)	40	520	148 ± 69	46.8%	34.4%	30.7%	7.9%
Lag-time (min)	40	520	4.3 ± 1.1	24.9%	21.3%	10.6%	7.4%
Prothrombin time (sec)	40	518	12.3 ± 0.9	7.0%	5.7%	3.3%	2.1%

*For CRP, the geometric mean is given with its 95% range, and the corresponding coefficients of variation are given for the logarithmically transformed data, which were normally distributed. **Analytical variation was obtained from our internal and external quality controls and was constant over time.
Mean ± SD, the overall mean ± standard deviation; ETP, endogenous thrombin potential.

Maximal platelet aggregation had a mean of 59% and CVs of 21.7%, 6.8%, 14.6% and 14.6%, respectively (Figure 2, Table 2).

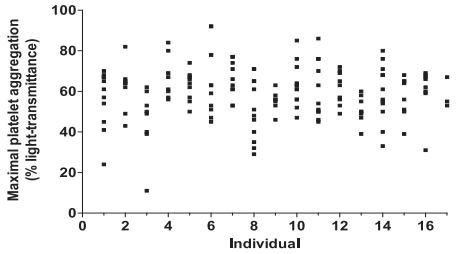


Figure 2. Individual concentrations of platelet aggregation

Light-transmittance aggregometry was induced by 5 µmol/L ADP and performed in 17 of the 40 healthy subjects. Maximal aggregation responses are depicted.

Thrombin generation had a mean ETP of 1012 nmol*min and CVs of 29.4%, 23.8%, 16.2% and 6.1%, respectively (Figure 3A, Table 2). Corresponding mean peak thrombin generation was 148 nmol with CVs of 46.8%, 34.4%, 30.7% and 7.9% (Figure 3B, Table 2), whereas the lag-time of thrombin generation had a mean of 4.3 min and CVs of 24.9%, 21.3%, 10.6% and 7.4%, respectively (Figure 3C, Table 2). The CVs of the PT, were the lowest (7.0%, 5.7%, 3.3% and 2.1%, respectively) with a mean PT of 12.3 sec (Figure 3D, Table 2).

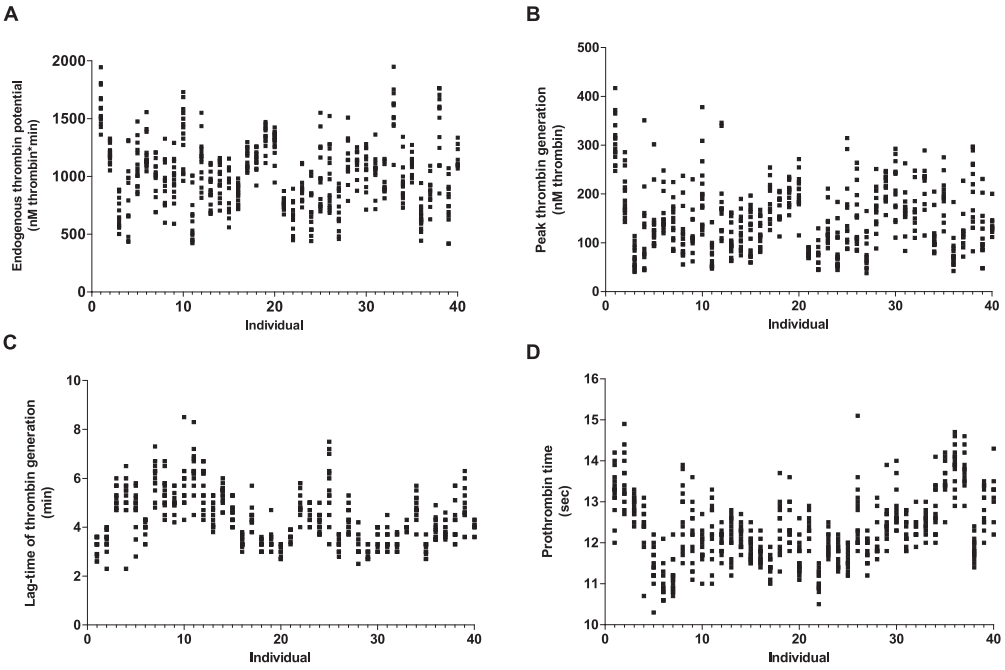


Figure 3. Individual concentrations of thrombin generation and prothrombin time

Thrombin generation was induced by 4 µmol/L phospholipids and 1 pmol/L TF. Values for endogenous thrombin potential are shown in panel A; those for the peak thrombin generation in panel B and the values for the lag-time of thrombin generation are shown in panel C.

The CVs were not statistically different when they were calculated for non-smokers only, in men and women separately, after excluding periods of reported disease (common cold), or for non-users of contraceptives. Also, adjustments for age and BMI, or the exclusion of outliers did not significantly affect the within-subject variation. In addition, CVs that were obtained when platelet aggregation was induced by 2.5 μmol/L ADP and thrombin generation was induced by 5 pmol/L TF, were not statistically different (data not shown). Furthermore, we have also calculated the CVs with the outliers defined according to the Cochran's test and Reed's criterion. Although according to these two criteria the number of outliers was lower than initially defined (at most 7 individual data-points for any of our datasets, instead of 5% of the distribution), the CVs have only changed slightly.

Repeated measurements

The number of repeated measurements was calculated that was needed to estimate with 95% probability a homeostatic setting point which does not deviate more than 20% from its true value. For fibrinogen, lag-time of thrombin generation and PT, one measurement was already sufficient to achieve this goal, whereas for CRP and ETP two repeated measurements were needed (Table 3). For maximal platelet aggregation and peak thrombin generation, we estimated that 4 and 9 repeated measurements were required, respectively (Table 3).

Maximal recommended analytical variation and bias

Based on the obtained within-subject biological variations, we calculated that the maximal analytical CVs that meet the recommendations for diagnosis were 11.4% for fibrinogen, 77.0% for ln[CRP], 12.6% for maximal platelet aggregation, 17.1% for ETP, 27.2% for peak thrombin generation, 14.4% for lag-time of thrombin generation and 4.0% for PT, whereas for monitoring purposes, these CVs were lower: 6.4%, 38.8%, 7.3%, 8.1%, 15.4%, 5.3%, and 1.7%, respectively (Table 3). The recommended analytical bias was calculated not to exceed 4.9% for fibrinogen, 33.2% for ln[CRP], 4.0% for maximal platelet aggregation, 7.2% for ETP, 11.5% for peak thrombin generation, 5.9% for lag-time of thrombin generation and 1.7% for PT (Table 3).

Table 3. Recommendations for the number of repeated measurements, analytical variation and analytical bias

	Number of repeated measurements	Coefficient of analytical variation		Analytical bias
		Diagnosis	Monitoring	
Fibrinogen	1	11.4%	6.4%	4.9%
ln[CRP]	2	77.0%	38.8%	33.2%
Platelet aggregation	4	12.6%	7.3%	4.0%
Thrombin generation				
ETP	2	17.1%	8.1%	7.2%
Peak	9	27.2%	15.4%	11.5%
Lag-time	1	14.4%	5.3%	5.9%
Prothrombin time	1	4.0%	1.7%	1.7%

Number of repeated measurements is given that is needed to estimate with 95% probability that the mean of the repeated measurements will not deviate more that 20% from the true homeostatic setting point.[15] Recommended analytical imprecision is given as coefficient of variation. Recommended analytical bias is given which is <25% of the total biological variation.
ln[CRP], logarithmically transformed CRP concentrations; ETP, endogenous thrombin potential.

Contribution of analytical imprecision to test result variability

In our study, the analytical imprecision contributed to the test result variability by 2.6% for fibrinogen, 0.1% for ln[CRP], 41.3% for maximal platelet aggregation, 6.9% for ETP, 3.2% for peak thrombin generation, 22.0% for lag-time of thrombin generation and 18.3% for PT (Table 4).

Indices of individuality and reference change values

Index of individuality, or the ratio of the total within-subject variation to between-subject variation, was calculated to be 0.89 for fibrinogen, 0.72 for ln[CRP], 3.02 for maximal platelet aggregation, 0.73 for ETP, 0.92 for peak thrombin generation, 0.61 for lag-time of thrombin generation and 0.68 for PT (Table 4). Reference change values were 36.1% for fibrinogen, 215.3% for ln[CRP], 57.3% for maximal platelet aggregation, 48.0% for ETP, 88.0% for peak thrombin generation, 35.8% for lag-time of thrombin generation and 10.9% for PT (Table 4).

Table 4. Contributions of analytical imprecision to test result variability, indices of individuality and reference change values

	Contribution of imprecision	Index of individuality	Reference change value
Fibrinogen	2.6%	0.89	36.1%
ln[CRP]	0.1%	0.72	215.3%
Platelet aggregation	41.3%	3.02	57.3%
Thrombin generation			
ETP	6.9%	0.73	48.0%
Peak	3.2%	0.92	88.0%
Lag-time	22.0%	0.61	35.8%
Prothrombin time	18.3%	0.68	10.9%

Contribution of imprecision indicates the percentage of variation that analytical imprecision has added to the overall test result variability. Index of individuality represents how unique each test result is within the total study population. Reference change value indicates how many percent a test result needs to deviate from the homeostatic setting point in order to be considered different (*i.e.* outside of the 95% of the distribution).
ln[CRP], logarithmically transformed CRP concentrations; ETP, endogenous thrombin potential.

Seasonal variation

Plasma concentrations of fibrinogen showed a significant seasonal variation over the year with higher concentrations in the summer and the first half of autumn (around 2.8 g/L) than for the rest of the year (around 2.6 g/L,) (P=0.03, Figure 4A). Similarly, a strong component of seasonal variation was observed for ETP, peak and lag-time of thrombin generation, with higher concentrations in the summer and autumn (around 1080 nmol*min, 170 nmol and 4.6 min, respectively), than for the rest of the year (around 950 nm*min, 135 nmol and 4.1 min; all P<0.001; Figure 4C, D and E, respectively). No significant seasonal trend was observed for ln[CRP] (P=0.53, Figure 4B), nor for PT (P=0.16, Figure 4F). When the analyses were performed for non-smokers only, in men and women separately, after excluding periods of reported disease (common cold), or for non-users of contraceptives, the seasonal variation was similar. Also, neither adjustments for age and BMI, nor the exclusion of outliers affected the seasonal variation. Similar results were also obtained for thrombin generation that was induced by 5 pmol/L TF (data not shown).

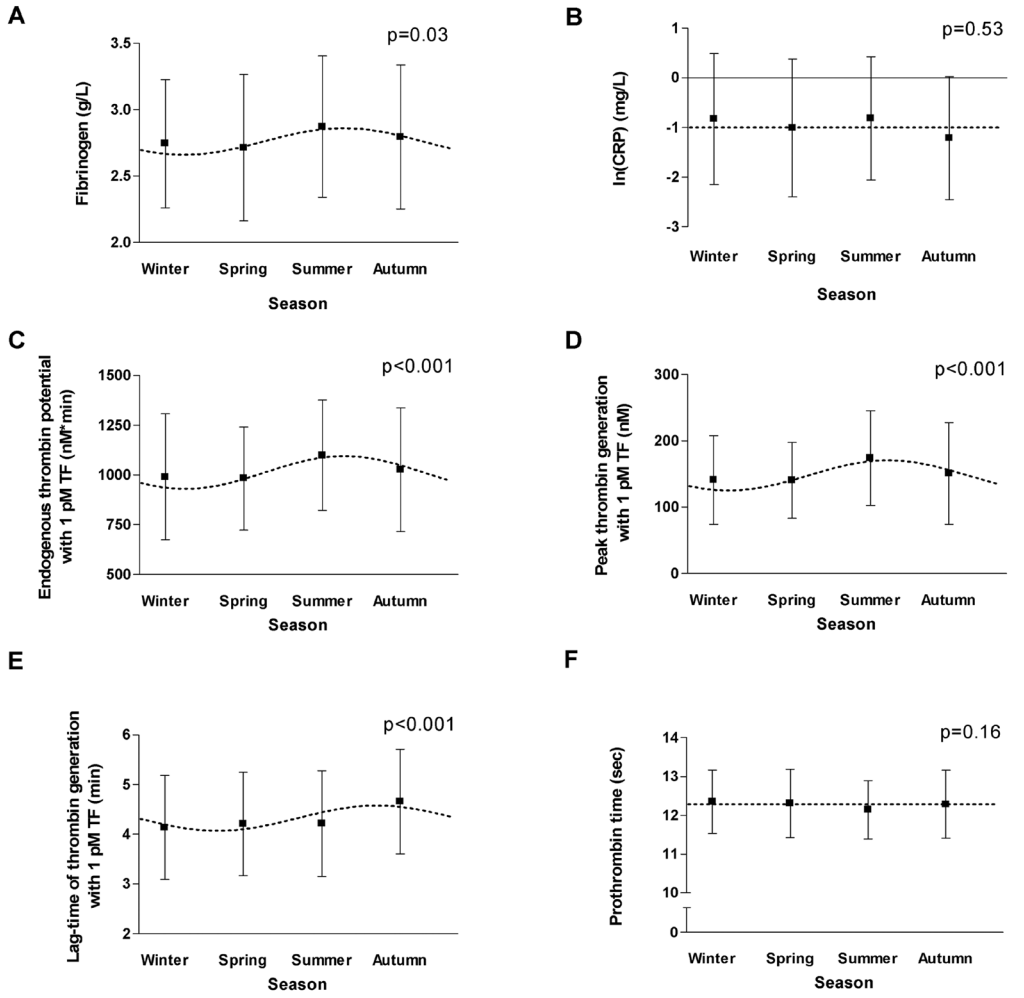


Figure 4. Seasonal variation in plasma concentrations of fibrinogen and CRP, and concentrations of platelet aggregation, thrombin generation and prothrombin time

P-values for the Wald test.

DISCUSSION

To gain insight into the biological variation, we documented in a longitudinal study the plasma concentrations of inflammatory markers fibrinogen and CRP, and the concentrations of hemostatic markers platelet aggregation, thrombin generation and PT. To our knowledge, this is the first report on biological variation of platelet aggregation and thrombin generation. The CVs for fibrinogen and PT that are reported in this study are similar to those that were previously obtained by others.[8, 22-27] We observed higher variations in CRP concentrations than in other studies. Our study population consisted of individuals with a relatively low median CRP, which in the literature is usually characterized by a larger between- and within-subject variation.[8, 22-27] For this reason, the contribution of the analytical imprecision to the overall test result variability of CRP concentrations was lower than for the other studied variables. Concomitantly, the highest reference change value in our study was calculated for CRP (215.3%), which indicates that a CRP measurement needs to deviate over twice the value of its mean in order to be considered statistically different. This observation is in line with the large temporal fluctuations that are frequently observed for CRP concentrations in both healthy and diseased individuals.

The temporary rises in CRP concentrations were on some, but not all samplings, accompanied by an increase in fibrinogen concentrations. Since both CRP and fibrinogen are acute-phase reactants, we expect that a concomitant increase in their concentrations probably represented acute-phase reactions, such as common cold.[9] Given these large temporal fluctuations in CRP concentrations, a general recommendation for the laboratory practice would be to take the lowest two of the three repeated values and discard a possible high outlier, as is now common practice for establishing the habitual concentration of blood cholesterol. In contrast to other studied variables, platelet aggregation was characterized by analytical and within-subject biological variation that was larger than its between-subject variation. The analytical imprecision alone contributed over 40% to the true test result variability. Interestingly, previous studies on platelet aggregation reported a wide between-subject variation.[28-30] However, in these studies only a single blood sample was taken from each individual, which suggests that a significant component of the large between-subject variation that was observed in these studies may actually be attributed to analytical and within-subject variation. This is strengthened by our observation that platelet aggregation has a relatively high index of individuality suggesting that, although platelet aggregation has a large total variation, all individuals' values span almost the entire reference interval. In other words, a single platelet aggregation measurement has very little discriminative power to distinguish between two healthy individuals.

Plasma concentrations of fibrinogen and thrombin generation varied significantly throughout the year according to a seasonal cycle, with relatively high concentrations during the summer and the autumn. This suggests an overall shift towards a more pro-coagulant condition during the summer and autumn than during the rest of the year. A number of previous reports on seasonal variation in fibrinogen concentrations have shown higher concentrations in the winter.[9, 20, 31] In the study by Woodhouse *et al*., but not in other studies, this correlated significantly with a higher incidence of infections and a concomitant increase in the acute-phase proteins. Seasonal variation in other acute phase proteins, such as alpha2-macroglobulin, has been reported with lowest concentrations in the winter.[32] When we took into consideration the data on inflammatory triggers, as reported by our study participants in the questionnaires, we could not find a statistically significant correlation between infections and rises in fibrinogen, nor CRP concentrations. To our knowledge, this is a first report of seasonal variation in concentrations of thrombin generation. Seasonal variation in a number of determinants of thrombin generation, *e.g.* FVII, protein C and fibrinogen, may explain part of this seasonal variation in thrombin generation.[9, 12, 33]

Limitations of our study were that the assays were not performed in duplicate to obtain the corresponding analytical variation. However, the analytical variation of these assays was determined previously in internal and external quality controls of our diagnostic laboratory and was consistently relatively low (CVs for fibrinogen: 2.9%, CRP: 3.0%, ETP: 6.1%, peak: 7.9%, lag-time: 7.4% and PT: 2.1%). For platelet aggregation, the previously determined analytical CV was relatively high (14.6%). In addition, platelet aggregation measurements were performed on a 4-channel aggregometer with a resulting technological limitation of a maximum of 4 measurements that could be performed in a single run. Therefore, the contribution of the analytical variation to the biological variation in platelet aggregation may be larger, as it also includes the between-run component of the analytical variation. This is in contrast to the other assays that were performed in a single run in this study. Since all samples were collected between 9-11h, we cannot make any conclusions on diurnal fluctuation. In addition, our interest in platelet aggregation arose during the last part of the study, and since this assay needs to be performed in fresh platelet-rich plasma, we could only perform platelet aggregation in those volunteers who were included last in the study. Furthermore, due to other logistics reasons, platelet aggregation data were not as complete as data from other assays. However, since these missing samples were random and the baseline characteristics of the corresponding subjects were similar to the remaining study participants, we do not expect that this difference in the percentage of

missing values could have had a major impact on our results.

In conclusion, this study provides insights into the biological variability of plasma concentrations of fibrinogen and CRP, and concentrations of platelet aggregation, thrombin generation and PT. In addition, this study shows that the concentrations of fibrinogen and thrombin generation are characterized by seasonal variation. Knowledge of these biological components of variation will improve the design of novel (epidemiologic) studies on the studied inflammatory and hemostatic markers and will aid the development of quality specifications for assays utilizing these markers for diagnosis and monitoring of CVD patients.

REFERENCES

1. Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. *N Engl J Med.* 1984;311:501-505.
2. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, Lowe GD, Pepys MB, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med.* 2004;350:1387-1397.
3. Ruggeri ZM. Platelets in atherothrombosis. *Nat Med.* 2002;8:1227-1234.
4. Moss AJ, Goldstein RE, Marder VJ, Sparks CE, Oakes D, Greenberg H, Weiss HJ, Zareba W, Brown MW, Liang CS, Lichstein E, Little WC, Gillespie JA, Van Voorhees L, Krone RJ, Bodenheimer MM, Hochman J, Dwyer EM, Jr., Arora R, Marcus FI, Watelet LF, Case RB. Thrombogenic factors and recurrent coronary events. *Circulation.* 1999;99:2517-2522.
5. Freedman DS, Byers T, Barboriak JJ, Flanders WD, Duncan A, Yip R, Meilahn EN. The relation of prothrombin times to coronary heart disease risk factors among men aged 31-45 years. *Am J Epidemiol.* 1992;136:513-524.
6. Verschuur M, van der Beek MT, Tak HS, Visser LG, de Maat MP. Interindividual variation in the response by fibrinogen, C-reactive protein and interleukin-6 to yellow fever vaccination. *Blood Coagul Fibrinolysis.* 2004;15:399-404.
7. Reynolds TM, Twomey P, Wierzbicki AS. Accuracy of cardiovascular risk estimation for primary prevention in patients without diabetes. *J Cardiovasc Risk.* 2002;9:183-190.
8. Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. *www.westgard.com/biodatabase1.htm.* 2008.
9. Woodhouse PR, Khaw KT, Plummer M, Foley A, Meade TW. Seasonal variations of plasma fibrinogen and factor VII activity in the elderly: winter infections and death from cardiovascular disease. *Lancet.* 1994;343:435-439.
10. van Gestel MA, Heemskerk JW, Slaaf DW, Heijnen VV, Reneman RS, oude Egbrink MG. In vivo blockade of platelet ADP receptor P2Y12 reduces embolus and thrombus formation but not thrombus stability. *Arterioscler Thromb Vasc Biol.* 2003;23:518-523.
11. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Beguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb.* 2003;33:4-15.
12. Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyak K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost.* 2008;6:125-131.
13. Euser AM, Dekker FW, le Cessie S. A practical approach to Bland-Altman plots and variation coefficients for log transformed variables. *J Clin Epidemiol.* 2008;61:978-982.
14. Harris EK. Statistical principles underlying analytic goal-setting in clinical chemistry. *Am J Clin Pathol.* 1979;72:374-382.
15. Cotlove E, Harris EK, Williams GZ. Biological and analytic components of variation in long-term studies of serum constituents in normal subjects. 3. Physiological and medical implications. *Clin Chem.* 1970;16:1028-1032.
16. Harris EK. Proposed goals for analytical precision and accuracy in single-point diagnostic testing. Theoretical basis and comparison with data from College of American Pathologists proficiency surveys. *Arch Pathol Lab Med.* 1988;112:416-420.

17. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci.* 1989;27:409-437.
18. Fraser CG, Hyltoft Petersen P, Libeer JC, Ricos C. Proposals for setting generally applicable quality goals solely based on biology. *Ann Clin Biochem.* 1997;34:8-12.
19. Fraser CG. Biological variation: from principles to practice. Washington, DC: AACC Press; 2001.
20. van der Bom JG, de Maat MP, Bots ML, Hofman A, Kluft C, Grobbee DE. Seasonal variation in fibrinogen in the Rotterdam Study. *Thromb Haemost.* 1997;78:1059-1062.
21. Rao CR. Linear statistical inference and its applications. 2nd ed. New York: Wiley. 1973:418-419.
22. de Maat MP, de Bart AC, Hennis BC, Meijer P, Havelaar AC, Mulder PG, Kluft C. Interindividual and intraindividual variability in plasma fibrinogen, TPA antigen, PAI activity, and CRP in healthy, young volunteers and patients with angina pectoris. *Arterioscler Thromb Vasc Biol.* 1996;16:1156-1162.
23. Marckmann P, Sandstrom B, Jespersen J. The variability of and associations between measures of blood coagulation, fibrinolysis and blood lipids. *Atherosclerosis.* 1992;96:235-244.
24. Kluft C, de Maat MP. Determination of the habitual low blood level of C-reactive protein in individuals. *Ital Heart J.* 2001;2:172-180.
25. Linnemann B, Schwonberg J, Mani H, Prochnow S, Lindhoff-Last E. Standardization of light transmittance aggregometry for monitoring antiplatelet therapy: an adjustment for platelet count is not necessary. *J Thromb Haemost.* 2008;6:677-683.
26. Devreese K, Wijns W, Combes I, Van kerckhoven S, Hoylaerts MF. Thrombin generation in plasma of healthy adults and children: chromogenic versus fluorogenic thrombogram analysis. *Thromb Haemost.* 2007;98:600-613.
27. Riese H, Vrijkotte TG, Meijer P, Kluft C, de Geus EJ. Diagnostic strategies for C-reactive protein. *BMC Cardiovasc Disord.* 2002;2:9.
28. Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood.* 2005;106:2723-2729.
29. Feng D, Lindpaintner K, Larson MG, Rao VS, O'Donnell CJ, Lipinska I, Schmitz C, Sutherland PA, Silbershatz H, D'Agostino RB, Muller JE, Myers RH, Levy D, Tofler GH. Increased platelet aggregability associated with platelet GPIIIa PlA2 polymorphism: the Framingham Offspring Study. *Arterioscler Thromb Vasc Biol.* 1999;19:1142-1147.
30. Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation.* 2003;108:989-995.
31. Crawford VL, Sweeney O, Coyle PV, Halliday IM, Stout RW. The relationship between elevated fibrinogen and markers of infection: a comparison of seasonal cycles. *Qjm.* 2000;93:745-750.
32. Kanikowska D, Grzymislawski M, Wiktorowicz K. Seasonal rhythms of "acute phase proteins" in humans. *Chronobiol Int.* 2005;22:591-596.
33. Stout RW, Crawford VL, McDermott MJ, Rocks MJ, Morris TC. Seasonal changes in haemostatic factors in young and elderly subjects. *Age Ageing.* 1996;25:256-258.

7

Chapter 7

Evren Kilinç
Goran Rudež
Henri Spronk
Hugo ten Cate
Abderrahim Nemmar
Moniek de Maat
Marc Hoylaerts

Particles, coagulation and thrombosis

Chapter from Cardiovascular Effects of Inhaled Ultrafine and Nano-sized Particles, RIVM, 2009 (In press)

Introduction

Epidemiologic, as well as experimental animal and human volunteer studies, suggest a close link between the risk for myocardial infarction, thrombosis, stroke and death and the inhalation of particulate matter (PM).[1-3] The mechanisms underlying cardiovascular complications executed by small particles are only partially understood. One potential effect of inhaled particles consists of its impact on the autonomic nervous system, leading to perturbations in heart rhythm.[4, 5] However, additional hypotheses have been formulated to explain the cardiovascular effects of PM. One hypothesis is that PM contribute to the occurrence of pulmonary inflammation, leading to the release of inflammatory mediators, which in turn cause platelet activation and upregulation of coagulation, thus generating or enhancing a protrombotic tendency[6, 7] or worsening of vascular dysfunction.[8, 9] Another hypothesis states that some particles can translocate from the lungs into the systemic circulation and thus, directly or indirectly, can influence hemostasis or cardiovascular integrity. Evidence for the existence of each mechanism is presented below, based on the findings from animal models and association studies.

In this chapter, the effects of PM on coagulation, platelet activation and thrombus formation will be discussed comprehensively.

Hemostasis

Hemostasis is a protective mechanism of the organism in response to vascular injury consisting of two major pathways: the activation and aggregation of thrombocytes or platelets (also called primary hemostasis) and the activation of the coagulation cascade (secondary hemostasis) resulting in an insoluble fibrin clot. The hemostatic system reacts quickly to stop blood loss from a damaged blood vessel wall and seals the wound site with a platelet-rich fibrin-stabilized clot. In primary hemostasis, circulating platelets bind to subendothelial collagen through their cell surface glycoprotein Ia/IIa receptors to form the primary hemostatic platelet plug. The adhesion of platelets is further stabilized by von Willebrand factor (VWF), which links platelets, glycoproteins and collagen fibrils. The action of a complex cascade of coagulation factors (a group of serine proteases) results in the formation of fibrin strands, which further strengthens the platelet plug.[10] Traditionally, the coagulation cascade has been divided into two distinct pathways: the intrinsic (contact) activation pathway and the extrinsic (tissue factor (TF)) pathway. In the current model of coagulation, TF, a membrane bound glycoprotein, is considered to be the single most relevant trigger of the blood coagulation system. The glycoprotein TF is expressed on a number of cells within the vasculature, including fibroblasts and smooth muscle cells. Circulating

blood cells such as monocytes only express this molecule upon induction, for instance by cytokines like Interleukin 6 (IL-6). The exposed TF forms a catalytic complex with factor VIIa (FVIIa) (TF-FVIIa complex) on a phospholipid membrane surface and activates both factor X (FX) and factor IX (FIX) resulting in the activation of prothrombin into thrombin.[11] All these reactions occur on negatively charged phospholipid membranes in the presence of calcium ions. Thrombin generation enhances its own formation through activation of factor XI (FXI) and the cofactors V (FV) and VIII (FVIII). The intrinsic pathway of the coagulation system can also be engaged when plasma coagulation factor XII (FXII), also known as Hageman factor, binds to negatively charged surfaces (note: not the previously mentioned negatively charged phospholipid membranes) involving the plasma proteins high molecular weight kininogen and plasma kallikrein (contact route). Although FXII is activated by a variety of poly-anions, including constituents of subendothelial matrix (glycosaminoglycans and collagens), sulfatides, nucleosomes and nonphysiological materials (glass, ellagic acid, kaolin, and silica), the *in vivo* contribution of these compounds to coagulation activity remains poorly understood.[12, 13] Ultimately, thrombin converts soluble fibrinogen into fibrin and activates factor XIII (FXIII) which in turn covalently cross-links the soluble fibrin strands into an insoluble fibrin clot. The formation of thrombin is downregulated through three pathways: (i) the tissue factor inhibitor pathway (TFPI) which inhibits the TF: activated FVII (FVIIa): activated FX (FXa) complex, (ii) antithrombin (AT) inhibiting mainly FXa and thrombin, and (iii) the protein C pathway. Protein C is activated by thrombin and utilizes protein S as a cofactor; activated protein C (APC) inhibits the activated forms of cofactors V (Va) and VIIIa through proteolytic inactivation.

Relationship between coagulation and inflammation

Inflammation and coagulation are closely associated and it has long been known that inflammation can lead to activation of the coagulation system. This interaction points to another evolutionary conserved role of blood coagulation, i.e. protection against invading bacteria. The generation of fibrin may serve to wall off bacteria protecting the organism against systemic dissemination of infection. Acute inflammation, as a response to infection or trauma, results in a systemic activation of blood coagulation[14, 15] through TF-mediated thrombin generation, down regulation of physiological anticoagulant mechanisms and inhibition of fibrinolysis. The resulting hypercoagulability (increased tendency to form a fibrin clot) increases the tendency to thrombosis, but also triggers intracellular signaling processes via activation of protease activated receptors (PARs) by several proteases including thrombin. PAR-mediated cellular effects comprise inflammation and angiogenesis and these effects may have important effects on complex processes like atherosclerosis.[16] PM can provoke an inflammatory response in the

lungs, which consequently results in hypercoagulability since prothrombotic and inflammatory cytokines are released into the circulation.

Relationship between PM10 particles and hemostasis

Air borne PM is currently divided in three types, that are classified according to diameter (PM10, 2.5-10 μm; PM2.5, 0.15-2.5 μm; ultrafine particles, <0.15 μm). These three types are all, although in different ways, involved in the mechanisms that lead towards alterations in hemostasis. The PM10 particles of 2.5-10 μm aerodynamic diameters affect the lungs in different ways, depending on their actual size. While PM10 particles cannot reach the most distal lung segments PM2.5 (0.15-2.5 μm), ultrafine particles (UFPs) of <0.15 μm aerodynamic diameter are able to penetrate the alveoli with far greater efficiency than the PM10 particles.[17] Studying the potential procoagulant effects of PM10, Baccarelli et al. reported that the prothrombin time (PT) shortens at elevated ambient air concentrations of PM10 and gaseous pollutants, mainly carbon monoxide (CO) and nitrogen dioxide (NO_2), in the 30 days before blood sampling in a cohort study (1218 healthy individuals). In this study, there were neither effects on activated partial thromboplastin time (aPTT), nor on antigen and/or functional levels of fibrinogen, AT, protein C, and protein S (free or total).[18] Shortening of the PT may be related to increased levels of coagulation proteins, particularly FVIIa, and /or increased activity of the extrinsic coagulation route. In another paper by the same authors, it was reported that exposure to PM10 increases the risk of venous thrombosis.[19] Depending on the ambient concentration of PM10, the relative risk of deep vein thrombosis was increased from 1.7 to more than 10 in patients living in the Lombardy region of Italy. In this study, a shortened PT was associated with increased PM exposure in the subjects suffering from a deep venous thrombosis, whereas the aPTT was not affected. In a recent longitudinal study including 40 healthy volunteers in whom platelet aggregation, thrombin generation and plasma levels of fibrinogen and C reactive protein (CRP) were measured 13 times over a period of 1 year, we observed a positive association between air pollution and platelet aggregation, as well as with the overall coagulation assessed by thrombin generation, without any evidence of systemic inflammation.[20] In this study, we investigated direct (within 24 hours before blood sampling), as well as indirect effects (within 24-96 hours before blood sampling) of environmental exposure to PM10, CO, nitrogen monoxide (NO), NO_2 and O_3 on blood hemostasis. Fibrinogen and CRP levels did not reveal an inflammatory effect of air pollution. Other studies failed to show clear pro-inflammatory or procoagulant effects of PM10 studying a range of markers including CRP, IL-6, fibrinogen, AT, factors II, VII, VIII and X.[21, 22] However, none of these observational studies explain the mechanisms that may be involved in the hypercoagulable response that occurs upon exposure to PM10.

Experimental studies provide some clues as to the mechanisms involved. Gilmour et al. showed that PM10 affects macrophages, epithelial and endothelial cell function to favor blood coagulation via induction of TF activity and inhibition of fibrinolysis pathways *in vitro*.[23] The animal studies performed with PM10 suggest that these particles cause a pulmonary inflammatory reaction with associated oxidative stress and tissue damage.[23, 24] Animal studies by our group suggest a dose dependent increase in TF and decrease in thrombomodulin (TM) in the lungs of spontaneously hypertensive rats at 4 and 48 hours after PM10 exposure.[25] Mutlu et al. reported a reduced bleeding time, reduced PT and aPTT and increased levels of FVIII, fibrinogen, prothrombin, FX and thrombin-antithrombin (TAT) complexes 24 hours after exposure of C57BL6/J mice to PM10.[26] Additionally, none of these changes were observed in IL-6 deficient mice, suggesting an inflammation driven disbalance in hemostasis. Depletion of macrophages by liposomal clodranate also resulted in similar null effects. Together, these data suggest that exposure to PM10 results in a dose- and time-dependent inflammation (IL-6) driven activation of coagulation by increasing TF and diminishing TM activities, possibly mediated through macrophages.

Relationship between PM2.5 and UFPs particles and hemostasis

In general, the studies in which humans and animals were exposed to PM2.5 and UFPs have not explored the same range of coagulation markers discussed for PM10 above. Most investigated parameters include VWF antigen and activity, D-dimer (a fibrin degradation product), protein C, fibrinogen and plasminogen activator inhibitor-1 (PAI-1). Carlsten et al. reported a decrease in VWF antigen with a lag of 7 hours and also a decrease in PAI-1 activity with a lag of 22 hours after exposure to diesel exhaust derived PM2.5 particles in individuals with the metabolic syndrome.[27] Additionally, there was no change in D-dimer. In another study, the same investigators found a decrease in D-dimer with a lag of 6 hours after exposure to filtered air only and a decrease in PAI-1 activity after exposure to filtered air and diesel exhaust derived PM2.5 particles in healthy human subjects by using the same experimental setup.[28] Both studies from the same group suggest no procoagulant effects, rather a reduced tendency to clot. However, exposure of healthy humans to wood smoke containing high concentrations of PM2.5 particles increases FVIII activity in plasma after 20 hours and FVIII/VWF ratio after 15 minutes, 3 and 20 hours. On the other hand, FVII, VWF activity, D-dimer, and fibrinogen levels were unaffected.[29] In comparison to these studies, Riediker et al. reported that PM2.5 originating from speed-changing traffic was associated with a slight increase in VWF activity and an 11% reduction in protein C concentrations in patrol officers by monitoring the pollutants in their cars during 4 days while working the 3 PM to midnight shift.[30] Taking into account

that VWF and FVIII are stored in endothelial cells and released into the circulation upon endothelial cell activation, one must assume that PM2.5 particles may cause endothelial cell activation. Exposure to UFPs also increases VWF activity with a lag of 2, 3 and 4 days after exposure in coronary heart disease patients.[31] On the other hand, another study did not show any procoagulant effects at 6 and 24 hours after exposure of patients with mild chronic obstructive pulmonary disease to diesel UFPs.[32] Finally, prothrombin activation peptide fragment 1 and 2 (F1+2) was increased in association with exposure to PM in a cohort of 57 patients with coronary heart disease. Activation of coagulation was mainly associated with elevated UFP levels, although no association with fibrinogen was found.[33] In all these studies one has to keep in mind that subjects with chronic inflammatory diseases like Chronic obstructive pulmonary disease (COPD) or coronary artery disease may already have upregulated antioxidant enzymes or other protective mechanisms that may in part blunt their inflammatory responses and protect against additional insults of PM exposure.

In general, experimental studies have been more straightforward in determining specific pro- and anticoagulant mechanisms after exposure to PM. In spite of that, there appears to be a number of differences in outcomes, most likely due to differences in PM source and dose used as well as a time dependency of the experimental setup.

The relationship between platelets and particles

One of the mechanisms underlying the prothrombotic effects of PM involves increased platelet activation and aggregation.[34] However, it is not yet clear what the exact pathways are that lead from inhalation of PM to platelet activation. Obviously, the PM-mediated effects on platelets are exerted across the lung-blood barrier, either directly by PM after translocation into blood, or indirectly via secondary mediators. It is important to consider the time-frame within which these effects take place. Previously, it has been shown that exposure to traffic, and concomitantly to combustion-generated nanoparticles, is associated with the onset of myocardial infarction already within 1h.[35] Given the role of platelets in hemostasis to rapidly detect sites of vascular injury, it has been postulated that these acute (short-term) effects of PM might reside in direct triggering of platelet receptors through binding to exogenous surfaces of translocated UFPs. Scanning electron microscopy examinations of human thrombi that were captured by vena cava filters have revealed that foreign nanosized particles are incorporated in these thrombi. This suggests that PM might actively participate in the formation of platelet aggregates serving as core components, a process termed platelet agglutination.[36] Nemmar *et al.* have demonstrated in a hamster model that already 1h after exposure to diesel exhaust particles (DEP), platelet activation and aggregation is increased. However, these observations were suggested to

involve the release of inflammatory intermediates histamine and neutrophil-derived elastase, rather than the direct triggering of platelets.[37, 38] In addition, Khandoga et al. demonstrated in mice that within 2h after intra-arterial infusion of UFPs, platelets accumulate on the endothelium.[39] Immunohistochemical evaluation of the treated vessels revealed fibrinogen and VWF-staining on these endothelial cells, which might explain platelet adhesion to the vessel wall and the local initiation of thrombus formation. Other mechanisms leading to platelet activation involve the effects of PM-induced lung inflammation. This inflammation is characterized by the release of TF-bearing microparticles from activated leucocytes and platelets.[40] In turn, these microparticles mediate the cross talk between platelets and other inflammatory cells and stimulate local coagulation by concentrating TF and other clotting factors on the surfaces of these platelets.[41, 42]

Based on the results of these previous studies, we also studied the relationship between air pollution and platelet aggregation in the biological variation study, that we mentioned previously.[20] We observed positive associations between local ambient concentrations of air pollutants PM10, CO, NO, NO_2 and O_3 that were measured within 1 to 4 days before blood sampling, and adenosine diphosphate (ADP)-induced platelet aggregation that was performed in plasma samples collected throughout the one-year study period. Also, indirect effects on thrombin generation were observed leading to increased endogenous thrombin potential and peak thrombin generation, which suggests that increased platelet aggregation is accompanied by hypercoagulability. No direct effects of air pollutants on platelet aggregation (association with air pollution levels in the 24 hr preceding the blood sampling) were observed, which we have confirmed *in vitro* by the absence of alterations in ADP-induced light-transmittance and whole-blood aggregometry after incubation (0-2h) of platelet-rich plasma or whole blood, respectively, with different doses and types of PM. As mentioned before, we did not find any effects of the studied air pollutants on the plasma levels of inflammatory markers fibrinogen and CRP, which suggests that the observed effects on platelet aggregation do not rely on changes in these inflammatory mediators. This however does not exclude the possibility that other inflammatory markers, such as IL-6 or tumor necrosis factor- α (TNF-α) might be involved.[26, 43]

Extrapulmonary translocation of UFPs

Intratracheally (i.t.) instilled UFPs can pass from the lungs into the blood circulation in hamsters.[44] This was demonstrated by using 80 nm albumin-nanocolloid particles labeled radioactively with technetium-99, as a model of UFPs, and via the study of their

distribution in the blood and other organs after their i.t. administration in the hamster. A substantial fraction of ^{99m}Tc-albumin was found to diffuse rapidly (within minutes and up to one hour) from the lungs into the systemic circulation.[44] In addition to our findings, others have also reported extrapulmonary translocation of UFPs after i.t. instillation or inhalation in other animal species.[45-47] The reported amount of UFPs translocating into blood and extrapulmonary organs differed, however, amongst these studies. Nevertheless, translocation was also found to occur to tissues in the systemic compartment, following intranasal delivery of polystyrene microparticles of 1.1 μm.[48] Recent morphological findings have illustrated that inhaled particles are transported into the pulmonary capillary space, presumably by transcytosis.[49-51] In *ex vivo* models of isolated perfused lungs of rat[52] and rabbit[37] , in the absence of lymph flow and inflammatory cell recruitment, UFPs can translocate from the lung into the circulation and *vice versa*, upon pharmacological modulation (H_2O_2 or vascularly administered histamine), increasing the pulmonary microvascular permeability.

Even though several recent animal exposure studies reported translocation of UFPs in humans, evidence for the existence of translocation of UFPs from the lungs into the blood circulation is limited and the data are conflicting. We studied the distribution of radiolabelled ultrafine carbon particles, commonly known as "Technegas", after their inhalation by non-smoking healthy human volunteers.[53] Individualized particles had a diameter in the order of 5 to 10 nm, as confirmed by electron microscopy. Radioactivity was detected in blood already after 1 minute, reaching a maximum between 10 and 20 minutes, remaining at this level up to 60 minutes. This radioactivity appeared to be largely particle-bound, as assessed by thin layer chromatography. Gamma camera images showed substantial radioactivity over the liver and other areas of the body. The presence of radioactivity in the liver is compatible with an accumulation of particles in Kupffer cells, known to occur with colloidal particles.[54] In agreement with these findings, Kawakami *et al*. reported the presence of radioactivity in blood immediately after inhalation of ^{99m}Tc-technegas in human volunteers.[55] In contrast, recent studies investigating the translocation of UFPs (10 nm in diameter, yet rapidly forming aggregates of 100 nm in diameter in the inhaled aerosol) administered as a technetium-99m–labelled aerosol in human volunteers reported radioactivity mainly localized in the lung (95.6 ± 1.7%), with no radioactivity found over the liver.[56, 57] Moreover, the nature of the radioactivity found in blood (4.4 %) consisted mainly of pertechnetate, as analyzed by thin layer chromatography. Further studies are required to resolve these discrepancies in man.

Effects of particles on thrombogenesis and platelet activation in experimental animal models

As outlined above, exposure to UFPs is associated with the rapid occurrence of myocardial infarction.[35, 58] In view of the important role of thrombosis in myocardial infarction, several animal studies were performed in hamsters and mice to assess the effects of particles on thrombosis, as a relevant cardiovascular endpoint. To mimic the existence of minimal vascular disease in man, in these models a mild local endothelial lesion was produced in blood vessels, by radicals formed upon decomposition of Rose Bengal, upon its irradiation with green light in a peripheral vessel. Hence, flow-controlled venous or arterial thrombi form, which can be followed online via transillumination and quantified by image analysis.[59] The intensity of thrombosis can be linked to the degree of vessel wall injury and to activation of hemostasis pathways.

Initially, well-characterized and chemically relatively inert polystyrene particles of 60 nm diameter were tested as a model of UFPs.[60] Moreover, polystyrene UFPs enabled easy surface modification, to allow for comparison between particles with neutral, negative or positive surface charges, mimicking the fact that most ambient particles are charged. Hemostasis was affected by the intravenous injection in hamsters of such UFPs, depending on their surface properties. Positively charged amine-modified particles led to a marked increase in prothrombotic tendency resulting, at least in part, from platelet activation.[60] Correspondingly, positively charged amine-modified polystyrene particles induced platelet aggregation *in vitro* and strongly increased the ADP-triggered aggregation in a dose-dependent manner, thus providing a mechanistic basis for the enhanced thrombogenicity observed *in vivo*. Confocal fluorescent microscopy analysis demonstrated that individual platelets were strongly stained by amine-polystyrene particles but not by the carboxylate-polystyrene particles. In subsequent experiments, using i.t. administered ultrafine polystyrene particles, in addition to causing neutrophil influx in the lung, the positively charged particles (60 nm) were also capable of enhancing the peripheral thrombogenicity, when analysed one hour after instillation.[61] In parallel, we found that 400-nm positive particles also caused pulmonary inflammation at one hour, but they did not enhance *in vivo* thrombosis at all, one hour after i.t. instillation. These findings clarified that the enhanced peripheral thrombogenicity at 1h could not be explained solely by pulmonary inflammation, implying that direct passage of UFPs (but not the larger 400 nm particles) and activation of circulating platelets contributed to the development of an extrapulmonary and peripheral prothrombotic tendency.

Subsequently, also Silva *et al*.[62] assessed in a rat model of ear vein thrombosis the effects of i.v. and i.t. administration of amine- and carboxylate-modified polystyrene particles

of similar diameter. These authors also found a dose-dependent enhanced thrombogenicity for positively charged UFPs but not for negatively charged UFPs of the same size. Likewise, Khandoga *et al*.[39] observed that intra-arterial injection of ultrafine carbon particles led to prothrombotic changes in mice.

The same hamster model of photochemically induced vascular thrombosis was also applied to study the effects of real pollutant particles, i.e. DEP on thrombosis, when given i.t.. In this model, DEP caused a dose-dependent enhanced peripheral venous and arterial thrombogenicity, one hour after their deposition in the lungs.[38] As flow-dependent thrombosis in the model depends on primary hemostatic activation (platelet adhesion and aggregation), the role of platelets during thrombus formation was assessed *ex vivo*, in time-wise blood samples from hamsters exposed to DEP. Platelet function was tested in the platelet function analyzer (PFA-100), which device measures platelet activation and aggregation in a shear stress–dependent way[63], by recording a flow pattern and a closure time. In blood samples from hamsters i.t. instilled with DEP, platelet activation was apparent already 30 minutes after instillation. Additional controls showed that the direct addition of DEP to hamster blood *in vitro* already caused platelet activation within 5 min, with as little as 0.5 µg of DEP/ml.[38]

Elevation of histamine levels has been correlated with the onset of myocardial infarction[64-66], but the evidence linking exposure to DEP and release of histamine is more abundant in the context of airway allergic and inflammatory processes. Thus, an increase in mast cell numbers in the submucosa and elevated bronchoalveolar lavage (BAL) histamine levels were observed, in humans, 6 hours after exposure to diesel exhaust particles (DEP).[67] DEP have also been demonstrated to directly degranulate mast cells and to increase histamine levels and symptom severity in humans.[68, 69] Therefore histamine was measured at different time points (1, 6 and 24h) after i.t. instillation of DEP, both in the respiratory tract and in the peripheral circulation and the effects of pre-treatment with a histamine receptor-1 blocking agent (diphenhydramine) were investigated, on pulmonary inflammation and vascular thrombogenicity. Pre-treatment with diphenhydramine attenuated both pulmonary inflammation and the prothrombotic effect at 6h and 24h. At one hour, only the pulmonary inflammation was diminished, whereas platelet activation and the enhanced thrombogenicity were not affected. In agreement with the statements made above, these early effects are compatible with direct platelet activation by DEP (or their constituents) having penetrated into the circulation.[70] In subsequent experiments, we found, 24h after DEP exposure, that pre-treatment with dexamethasone or with cromoglycate blocked the DEP-induced pulmonary inflammation, prothrombotic events and histamine release in BAL and plasma. Therefore, the systemic inflammatory and prothrombotic effects observed 24h after DEP administration seem

secondary to lung inflammation; they can be prevented by mast cell stabilization.[71] Of note, we did not find increased levels of plasma VWF, which is a marker of endothelial activation, after DEP administration.[71]

The relationship between pulmonary inflammation and thrombotic complications has been studied further, using the established model of sustained pulmonary inflammation induced by silica particles. In the hamster, we demonstrated that the i.t. instillation of silica particles leads to significant dose-dependent increases of macrophage and neutrophil numbers in BAL and the development of a prothrombotic tendency in circulating blood.[72] By specifically depleting lung macrophages with clodronate-liposomes, both the influx of PMN in BAL and the peripheral thrombotic tendency were abrogated. The depletion of circulating neutrophils and monocytes by cyclophosphamide also abolished both the cellular influx in BAL and the peripheral thrombotic tendency, despite normal numbers of lung macrophages. Moreover, silica caused an increase in neutrophil elastase activity in plasma. These findings uncover pulmonary macrophage–neutrophil cross-talk releasing neutrophil elastase into the blood circulation. Elastase, by participating in the activation of circulating platelets, may then predispose platelets to initiate thrombotic events on mildly damaged vasculature.

The more recent application of photochemically induced thrombosis in the carotid artery in the mouse opened the possibility of studying biological interactions more precisely, owing to the larger number of reagents available for the mouse and the availability of genetically modified mice. During i.t. instillation of carbon nanotubes in the mouse, we established a critical role for platelet P-selectin in mediating systemic inflammatory reactions, accelerating thrombus formation.[73] Lung inflammation was found to lead to enhanced heteroconjugate formation between circulating platelets and monocytes, respectively granulocytes, in turn resulting in leukocyte activation, and production of TF positive microvesicles in the circulation. Neutralizing P-selectin with a monoclonal antibody abrogated heteroconjugate formation and the production of microvesicles. At the same time the peripheral thrombogenicity was eliminated. These findings illustrated the critical role of P-selectin in transmission of pulmonary inflammation to the systemic circulation.

Conclusions

The majority of experimental and clinical (epidemiologic) studies point to an effect of different sources of PM on the blood coagulation system. These effects include alterations of platelet function, coagulation activity and to a lesser effect of fibrinolysis. Two mechanistic scenarios may be operational, and these may not be mutually exclusive:

Inflammation driven scenario

In this scenario, exposure to PM provokes an inflammatory response in the lung with consequent release of pro inflammatory cytokines into the circulation. PM predominantly triggers IL-6 production by alveolar macrophages. The procoagulant response to PM is characterized by increased cellular expression of TF and probably loss of vascular endothelial TM activity. The perturbation of the balance between pro- and anticoagulant activity in organs exposed to PM may thus be a contributing factor to development of cardiovascular disease.

Direct effect scenario

This hypothesis proposes that inhaled, insoluble, PM2.5 or nanoparticles could rapidly translocate into the circulation, with the potential for direct effects on hemostasis and adverse cardiovascular endpoints. Translocation of inhaled nanoparticles across the alveolar–blood barrier has been demonstrated in animal studies for a range of nanoparticles delivered by inhalation or instillation. Translocation of these particles may also directly activate the contact system of blood coagulation via factor XII activation (Kilinc et al, unpublished work 2009). This is currently under further investigation in our own studies.

In conclusion, a large variety of both *in vivo* and *in vitro* studies have demonstrated the association between exposure to PM in air pollution and activation or alterations of the haemostatic system. Epidemiologic studies have clearly shown the association between PM exposure and the risk for both venous and arterial thrombosis. Platelet activation and hypercoagulability have been shown in human volunteer and animal studies and both effects appear to be inflammation driven, although the study data are not consistent.

In general however, it seems prudent to conclude that PM may through different mechanisms enhance blood coagulation activity which may contribute to the risk of developing thrombosis. In addition to an effect on venous thrombosis, PM may either by accelerating atherosclerosis or by stimulating a platelet dependent clotting response, also induce the risk of arterial thrombosis. These prothrombotic effects of PM are likely to contribute to the health hazards involved in environmental exposure to PM.

REFERENCES

1. Delfino RJ, Sioutas C, Malik S. Potential role of ultrafine particles in associations between airborne particle mass and cardiovascular health. *Environ Health Perspect.* 2005;113:934-946.
2. Kunzli N, Tager IB. Air pollution: from lung to heart. *Swiss Med Wkly.* 2005;135:697-702.
3. Mills NL, Tornqvist H, Robinson SD, Gonzalez MC, Soderberg S, Sandstrom T, Blomberg A, Newby DE, Donaldson K. Air pollution and atherothrombosis. *Inhal Toxicol.* 2007;19 Suppl 1:81-89.
4. Gold DR, Litonjua A, Schwartz J, Lovett E, Larson A, Nearing B, Allen G, Verrier M, Cherry R, Verrier R. Ambient pollution and heart rate variability. *Circulation.* 2000;101:1267-1273.
5. Riediker M, Cascio WE, Griggs TR, Herbst MC, Bromberg PA, Neas L, Williams RW, Devlin RB. Particulate matter exposure in cars is associated with cardiovascular effects in healthy young men. *Am J Respir Crit Care Med.* 2004;169:934-940.
6. Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet.* 1995;345:176-178.
7. Seaton A, Soutar A, Crawford V, Elton R, McNerlan S, Cherrie J, Watt M, Agius R, Stout R. Particulate air pollution and the blood. *Thorax.* 1999;54:1027-1032.
8. O'Neill MS, Veves A, Sarnat JA, Zanobetti A, Gold DR, Economides PA, Horton ES, Schwartz J. Air pollution and inflammation in type 2 diabetes: a mechanism for susceptibility. *Occup Environ Med.* 2007;64:373-379.
9. O'Neill MS, Veves A, Zanobetti A, Sarnat JA, Gold DR, Economides PA, Horton ES, Schwartz J. Diabetes enhances vulnerability to particulate air pollution-associated impairment in vascular reactivity and endothelial function. *Circulation.* 2005;111:2913-2920.
10. Rittirsch D, Flierl MA, Ward PA. Harmful molecular mechanisms in sepsis. *Nat Rev Immunol.* 2008;8:776-787.
11. Spronk HM, Govers-Riemslag JW, ten Cate H. The blood coagulation system as a molecular machine. *Bioessays.* 2003;25:1220-1228.
12. Muller F, Renne T. Novel roles for factor XII-driven plasma contact activation system. *Curr Opin Hematol.* 2008;15:516-521.
13. Schousboe I. Pharmacological regulation of factor XII activation may be a new target to control pathological coagulation. *Biochem Pharmacol.* 2008;75:1007-1013.
14. Levi M, Keller TT, van Gorp E, ten Cate H. Infection and inflammation and the coagulation system. *Cardiovasc Res.* 2003;60:26-39.
15. ten Cate H, Falanga A. Overview of the postulated mechanisms linking cancer and thrombosis. *Pathophysiol Haemost Thromb.* 2008;36:122-130.
16. Borissoff JI, Spronk HM, Heeneman S, Ten Cate H. Is thrombin a key player in the 'coagulation-atherogenesis' maze? *Cardiovasc Res.* 2009.
17. Harrison RM, Yin J. Particulate matter in the atmosphere: which particle properties are important for its effects on health? *Sci Total Environ.* 2000;249:85-101.
18. Baccarelli A, Zanobetti A, Martinelli I, Grillo P, Hou L, Giacomini S, Bonzini M, Lanzani G, Mannucci PM, Bertazzi PA, Schwartz J. Effects of exposure to air pollution on blood coagulation. *J Thromb Haemost.* 2007;5:252-260.
19. Baccarelli A, Martinelli I, Zanobetti A, Grillo P, Hou LF, Bertazzi PA, Mannucci PM, Schwartz J. Exposure to particulate air pollution and risk of deep vein thrombosis. *Arch Intern Med.* 2008;168:920-927.

20. Rudež G, Janssen, NAH., Kilinc, E., Leebeek, FWG., Gerlofs-Nijland, ME., Spronk, HMH., Ten Cate, H., Cassee, FR., de Maat, MPM. Effects of ambient air pollution on hemostasis and inflammation: *Environ Health Perspect.* 2009;117:995-1001.

21. Brauner EV, Forchhammer L, Moller P, Barregard L, Gunnarsen L, Afshari A, Wahlin P, Glasius M, Dragsted LO, Basu S, Raaschou-Nielsen O, Loft S. Indoor particles affect vascular function in the aged: an air filtration-based intervention study. *Am J Respir Crit Care Med.* 2008;177:419-425.

22. Scharrer E, Hessel H, Kronseder A, Guth W, Rolinski B, Jorres RA, Radon K, Schierl R, Angerer P, Nowak D. Heart rate variability, hemostatic and acute inflammatory blood parameters in healthy adults after short-term exposure to welding fume. *Int Arch Occup Environ Health.* 2007;80:265-272.

23. Gilmour PS, Morrison ER, Vickers MA, Ford I, Ludlam CA, Greaves M, Donaldson K, MacNee W. The procoagulant potential of environmental particles (PM10). *Occup Environ Med.* 2005;62:164-171.

24. Brown DM, Hutchison L, Donaldson K, Stone V. The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. *Am J Physiol Lung Cell Mol Physiol.* 2007;292:L1444-1451.

25. Frederix K, Kooter IM, van Oerle R, Fens D, Hamulyak K, Gerlofs-Nijland ME, Ten Cate H, Spronk HM. A new method to determine tissue specific tissue factor thrombomodulin activities: endotoxin and particulate air pollution induced disbalance. *Thromb J.* 2008;6:14.

26. Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest.* 2007;117:2952-2961.

27. Carlsten C, Kaufman JD, Trenga CA, Allen J, Peretz A, Sullivan JH. Thrombotic markers in metabolic syndrome subjects exposed to diesel exhaust. *Inhal Toxicol.* 2008;20:917-921.

28. Carlsten C, Kaufman JD, Peretz A, Trenga CA, Sheppard L, Sullivan JH. Coagulation markers in healthy human subjects exposed to diesel exhaust. *Thromb Res.* 2007;120:849-855.

29. Barregard L, Sallsten G, Gustafson P, Andersson L, Johansson L, Basu S, Stigendal L. Experimental exposure to wood-smoke particles in healthy humans: effects on markers of inflammation, coagulation, and lipid peroxidation. *Inhal Toxicol.* 2006;18:845-853.

30. Riediker M, Devlin RB, Griggs TR, Herbst MC, Bromberg PA, Williams RW, Cascio WE. Cardiovascular effects in patrol officers are associated with fine particulate matter from brake wear and engine emissions. *Part Fibre Toxicol.* 2004;1:2.

31. Yue W, Schneider A, Stolzel M, Ruckerl R, Cyrys J, Pan X, Zareba W, Koenig W, Wichmann HE, Peters A. Ambient source-specific particles are associated with prolonged repolarization and increased levels of inflammation in male coronary artery disease patients. *Mutat Res.* 2007;621:50-60.

32. Blomberg A, Tornqvist H, Desmyter L, Deneys V, Hermans C. Exposure to diesel exhaust nanoparticles does not induce blood hypercoagulability in an at-risk population. *J Thromb Haemost.* 2005;3:2103-2105.

33. Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med.* 2006;173:432-441.

34. Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, Sandstrom T, Blomberg A, Newby DE. Adverse cardiovascular effects of air pollution. *Nat Clin Pract Cardiovasc Med.* 2009;6:36-44.

35. Peters A, von Klot S, Heier M, Trentinaglia I, Hormann A, Wichmann HE, Lowel H. Exposure to traffic and the onset of myocardial infarction. *N Engl J Med.* 2004;351:1721-1730.

36. Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, Radomski MW. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br J Pharmacol.* 2005;146:882-893.
37. Nemmar A, Hamoir J, Nemery B, Gustin P. Evaluation of particle translocation across the alveolo-capillary barrier in isolated perfused rabbit lung model. *Toxicology.* 2005;208:105-113.
38. Nemmar A, Hoet PH, Dinsdale D, Vermylen J, Hoylaerts MF, Nemery B. Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. *Circulation.* 2003;107:1202-1208.
39. Khandoga A, Stampfl A, Takenaka S, Schulz H, Radykewicz R, Kreyling W, Krombach F. Ultrafine particles exert prothrombotic but not inflammatory effects on the hepatic microcirculation in healthy mice in vivo. *Circulation.* 2004;109:1320-1325.
40. Hrachovinova I, Cambien B, Hafezi-Moghadam A, Kappelmayer J, Camphausen RT, Widom A, Xia L, Kazazian HH, Jr., Schaub RG, McEver RP, Wagner DD. Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A. *Nat Med.* 2003;9:1020-1025.
41. Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. *Blood.* 2000;95:1317-1323.
42. Namba M, Tanaka A, Shimada K, Ozeki Y, Uehata S, Sakamoto T, Nishida Y, Nomura S, Yoshikawa J. Circulating platelet-derived microparticles are associated with atherothrombotic events: a marker for vulnerable blood. *Arterioscler Thromb Vasc Biol.* 2007;27:255-256.
43. Ruckerl R, Greven S, Ljungman P, Aalto P, Antoniades C, Bellander T, Berglind N, Chrysohoou C, Forastiere F, Jacquemin B, von Klot S, Koenig W, Kuchenhoff H, Lanki T, Pekkanen J, Perucci CA, Schneider A, Sunyer J, Peters A, Group AS. Air pollution and inflammation (interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. *Environ Health Perspect.* 2007;115:1072-1080.
44. Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med.* 2001;164:1665-1668.
45. Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *J Toxicol Environ Health A.* 2002;65:1531-1543.
46. Takenaka S, Karg E, Roth C, Schulz H, Ziesenis A, Heinzmann U, Schramel P, Heyder J. Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. *Environ Health Perspect.* 2001;109 Suppl 4:547-551.
47. Kreyling WG, Semmler M, Erbe F, Mayer P, Takenaka S, Schulz H, Oberdorster G, Ziesenis A. Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J Toxicol Environ Health A.* 2002;65:1513-1530.
48. Eyles JE, Bramwell VW, Williamson ED, Alpar HO. Microsphere translocation and immunopotentiation in systemic tissues following intranasal administration. *Vaccine.* 2001;19:4732-4742.
49. Kato T, Yashiro T, Murata Y, Herbert DC, Oshikawa K, Bando M, Ohno S, Sugiyama Y. Evidence that exogenous substances can be phagocytized by alveolar epithelial cells and transported into blood capillaries. *Cell Tissue Res.* 2003;311:47-51.
50. Kapp N, Kreyling W, Schulz H, Im Hof V, Gehr P, Semmler M, Geiser M. Electron energy loss spectroscopy for analysis of inhaled ultrafine particles in rat lungs. *Microsc Res Tech.* 2004;63:298-305.

51. Geiser M, Rothen-Rutishauser B, Kapp N, Schurch S, Kreyling W, Schulz H, Semmler M, Im Hof V, Heyder J, Gehr P. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ Health Perspect.* 2005;113:1555-1560.
52. Meiring JJ, Borm PJ, Bagate K, Semmler M, Seitz J, Takenaka S, Kreyling WG. The influence of hydrogen peroxide and histamine on lung permeability and translocation of iridium nanoparticles in the isolated perfused rat lung. *Part Fibre Toxicol.* 2005;2:3.
53. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation.* 2002;105:411-414.
54. Simon BH, Ando HY, Gupta PK. Circulation time and body distribution of 14C-labeled amino-modified polystyrene nanoparticles in mice. *J Pharm Sci.* 1995;84:1249-1253.
55. Kawakami K, Iwamura A, Goto E, Mori Y, Abe T, Hirasawa Y, Ishida H, Shimada T, Tominaga S. [Kinetics and clinical application of 99mTc-technegas]. *Kaku Igaku.* 1990;27:725-733.
56. Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel D, de la Fuente JM, Cassee FR, Boon NA, Macnee W, Millar AM, Donaldson K, Newby DE. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *Am J Respir Crit Care Med.* 2006;173:426-431.
57. Moller W, Felten K, Sommerer K, Scheuch G, Meyer G, Meyer P, Haussinger K, Kreyling WG. Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. *Am J Respir Crit Care Med.* 2008;177:426-432.
58. Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction. *Circulation.* 2001;103:2810-2815.
59. Kawasaki T, Kaida T, Arnout J, Vermylen J, Hoylaerts MF. A new animal model of thrombophilia confirms that high plasma factor VIII levels are thrombogenic. *Thromb Haemost.* 1999;81:306-311.
60. Nemmar A, Hoylaerts MF, Hoet PH, Dinsdale D, Smith T, Xu H, Vermylen J, Nemery B. Ultrafine particles affect experimental thrombosis in an in vivo hamster model. *Am J Respir Crit Care Med.* 2002;166:998-1004.
61. Nemmar A, Hoylaerts MF, Hoet PH, Vermylen J, Nemery B. Size effect of intratracheally instilled particles on pulmonary inflammation and vascular thrombosis. *Toxicol Appl Pharmacol.* 2003;186:38-45.
62. Silva VM, Corson N, Elder A, Oberdorster G. The rat ear vein model for investigating in vivo thrombogenicity of ultrafine particles (UFP). *Toxicol Sci.* 2005;85:983-989.
63. Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer--PFA-100. *Semin Thromb Hemost.* 1995;21 Suppl 2:106-112.
64. Zaca F, Benassi MS, Ghinelli M, Trianni M, Vaccarino R, Malavolta E, Muratori M, Lenzi S. Myocardial infarction and histamine release. *Agents Actions.* 1986;18:258-261.
65. Clejan S, Japa S, Clemetson C, Hasabnis SS, David O, Talano JV. Blood histamine is associated with coronary artery disease, cardiac events and severity of inflammation and atherosclerosis. *J Cell Mol Med.* 2002;6:583-592.
66. Laine P, Kaartinen M, Penttila A, Panula P, Paavonen T, Kovanen PT. Association between myocardial infarction and the mast cells in the adventitia of the infarct-related coronary artery. *Circulation.* 1999;99:361-369.
67. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, Frew A. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med.* 1999;159:702-709.

68. Devouassoux G, Saxon A, Metcalfe DD, Prussin C, Colomb MG, Brambilla C, Diaz-Sanchez D. Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J Allergy Clin Immunol.* 2002;109:847-853.

69. Diaz-Sanchez D, Penichet-Garcia M, Saxon A. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J Allergy Clin Immunol.* 2000;106:1140-1146.

70. Nemmar A, Nemery B, Hoet PH, Vermylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. *Am J Respir Crit Care Med.* 2003;168:1366-1372.

71. Nemmar A, Hoet PH, Vermylen J, Nemery B, Hoylaerts MF. Pharmacological stabilization of mast cells abrogates late thrombotic events induced by diesel exhaust particles in hamsters. *Circulation.* 2004;110:1670-1677.

72. Nemmar A, Nemery B, Hoet PH, Van Rooijen N, Hoylaerts MF. Silica particles enhance peripheral thrombosis: key role of lung macrophage-neutrophil cross-talk. *Am J Respir Crit Care Med.* 2005;171:872-879.

73. Nemmar A, Hoet PH, Vandervoort P, Dinsdale D, Nemery B, Hoylaerts MF. Enhanced peripheral thrombogenicity after lung inflammation is mediated by platelet-leukocyte activation: role of P-selectin. *J Thromb Haemost.* 2007;5:1217-1226.

8

Chapter 8

Goran Rudež
Nicole Janssen
Evren Kilinç
Frank Leebeek
Miriam Gerlofs-Nijland
Henri Spronk
Hugo ten Cate
Flemming Cassee
Moniek de Maat

Effects of air pollution on hemostasis and inflammation

Environ Health Perspect, 2009;117(6):995-1001

ABSTRACT

Background

Air pollution has consistently been associated with increased morbidity and mortality due to respiratory and cardiovascular disease. Underlying biological mechanisms are not entirely clear, and hemostasis and inflammation are suggested to be involved.

Objectives

Our aim was to study the association between the variation in local concentrations of airborne particulate matter (PM) with aerodynamic diameter <10μm, carbon monoxide, nitrogen monoxide, nitrogen dioxide and ozone, and platelet aggregation, thrombin generation, fibrinogen and C-reactive protein (CRP) levels in healthy individuals.

Methods

From 40 healthy volunteers we collected 13 consecutive blood samples within a 1-year period and measured light-transmittance platelet aggregometry, thrombin generation, fibrinogen and CRP. Regression analysis using generalized additive models was performed to study the association between the hemostatic and inflammatory variables, and local environmental concentrations of air pollutants for time lags within 24h before blood sampling or 24-96h before blood sampling).

Results

In general, air pollutants were associated with platelet aggregation (average +8% per interquartile range (IQR), $p<0.01$) and thrombin generation (average +1% per IQR, $p<0.05$). Platelet aggregation was not affected by in vitro incubation of plasma with PM. No relationship was observed between any of the air pollutants and fibrinogen or CRP levels.

Conclusions

Air pollution increased platelet aggregation as well as coagulation activity, but had no clear effect on systemic inflammation. These prothrombotic effects may partly explain the relationship between air pollution and the risk of ischemic cardiovascular disease.

INTRODUCTION

Epidemiologic studies have linked elevated levels of both gaseous and (ultra-)fine particulate matter (PM) ambient air pollutants to increased morbidity and mortality due to respiratory and cardiovascular disease.[1, 2] Underlying biological mechanisms are unclear, but inflammation and hemostasis are suggested to be involved.[3-5] It has been postulated that inhaled gasses, and also ultrafine PM because of its very small particle size (<0.1μm), can readily cross the lung epithelium into the bloodstream.[6] There they can have direct, transient systemic effects leading to a prothrombotic state, such as enhanced platelet activation and thrombin generation.[7-9] In contrast, larger particles that cannot pass the alveolar blood barrier will perturb the lung epithelium, where they may give rise to local inflammation.[10, 11] Under experimental conditions, human or animal exposure to a controlled high-dose of air pollutants has been shown to cause pulmonary inflammation that leads to a systemic release of cytokines. This in turn induces *de novo* synthesis of inflammatory biomarkers in the liver, such as fibrinogen, which also plays a major part in blood clotting, and C-reactive protein (CRP). In general, at least 24h elapses from the onset of this protein synthesis to a clear increase in plasma levels of inflammatory markers.[12] We therefore hypothesized that air pollution has both direct and indirect effects on platelet aggregation and coagulation, but only indirect effects on plasma levels of the inflammatory variables fibrinogen and CRP.

Previous studies have aimed primarily at finding epidemiologic associations between concentrations of air pollution and health effects, including mortality, or associations between experimentally-controlled exposures to air pollution and various biological variables in human and animal models.[13, 14] However, studies are still lacking that focus on the effect of air pollution on hemostasis and inflammation in a real-life urban situation over a longer period of time, especially because continuous long-term monitoring shows large variations within each year in local concentrations of air pollutants (Figure 1).[3] Therefore, the aim of this study was to investigate longitudinally (*i.e.* repeatedly over 1 year) the associations between local urban concentrations of ambient air pollution and plasma markers of hemostasis and inflammation. In addition, we aimed to investigate *in vitro* whether PM can have an effect on platelet aggregation.

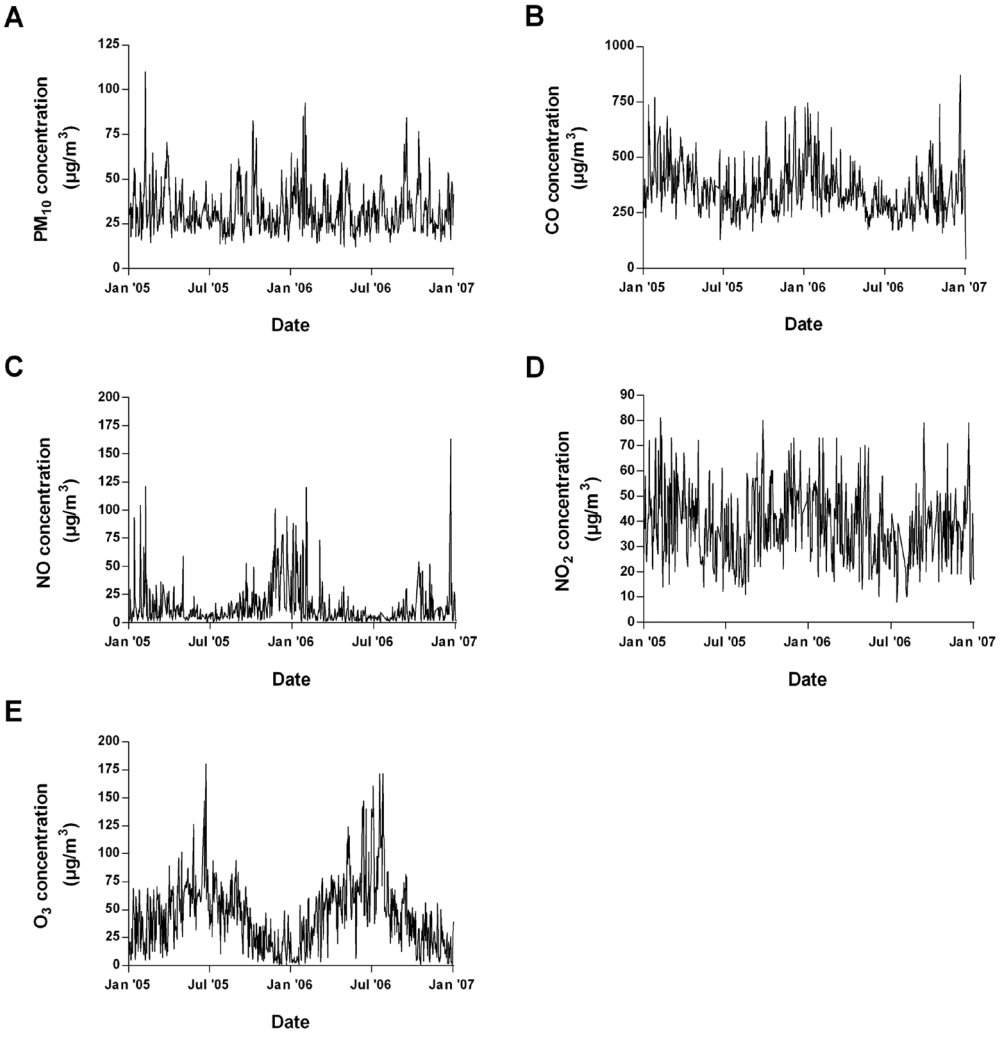


Figure 1. Concentration profiles of air pollutants during the study period

The 24h mean concentrations are depicted for PM_{10} (panel A), CO (panel B), NO (panel C) and NO_2 (panel D). For O_3 (panel E) 8-hour mean concentrations (1200 hours till 2000 hours) are depicted.

MATERIALS AND METHODS

Study population

Between January 2005 and December 2006, we included 40 healthy individuals who were living or working in the city-center of Rotterdam (the Netherlands), a city agglomerate with almost 1 million inhabitants. Exclusion criteria were symptoms of chronic infectious diseases, acute infections or any surgical procedure within the preceding 3 months. From each participant blood was collected at 11-13 (mean 12.5) different visits throughout a 1-year period. In total 498 blood samples were collected on 197 days. For each subject, the minimal interval between successive blood collections was 3 days and the maximal interval was 6 months, with a similar pattern of distribution in each subject. Data on demographics and cardiovascular risk factors were collected using a standardized questionnaire. To minimize the effect of circadian variation on plasma levels of biomarkers, blood samples were taken between 0900 hours and 1100 hours. The study protocol is in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Erasmus University Medical Center. Written informed consent was obtained from each participant.

Air pollution monitoring data

Concentrations of PM with aerodynamic diameter <10μm (PM_{10}), carbon monoxide (CO), nitrogen monoxide (NO), nitrogen dioxide (NO_2) and ozone (O_3) were obtained from the Dutch National Air Quality Monitoring Network (*http://www.lml.rivm.nl/data/smog/index.html*), which measured these air pollutants hourly at monitoring station no. 418 (Schiedamse Vest, Rotterdam, the Netherlands). This monitoring site is located in the Rotterdam city-center and is subject to frequent quality control to ensure its ability to represent urban background air pollution. For data analysis, 6h means were calculated and then combined into 12h and 24h means. If there were more than 2 missing hourly concentrations for a 6h mean, they were imputed using data from five other monitoring stations of the Dutch National Air Quality Monitoring Network that were all within 25 km of Rotterdam.

Blood collection

Blood was drawn by venapuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK) containing sodium citrate (final concentration 3.2%). Plasma was obtained by centrifugation at 1500*g* for 10 min at 4°C and stored in aliquots at –80ºC until further analysis. For platelet aggregation, blood was centrifuged at 150*g*

for 15 min to obtain platelet-rich plasma (PRP) and subsequently at 1500*g* for 10 min to obtain platelet-poor plasma (PPP). PRP was adjusted with autologous PPP to 200 x 10^9 platelets/L (P200), which was used in platelet aggregation experiments.

Laboratory measurements

Light-transmittance platelet aggregometry

Adenosine diphosphate (ADP-)induced light-transmittance platelet aggregometry was performed as described previously.[15] ADP was chosen as the agonist, because the ADP-pathway in platelets plays an important role in atherothrombosis.[16] P200 was preincubated with aspirin (100 µmol/L final) for 20 min. and brought to a physiologic calcium concentration of 16.6 mM by adding calcium chloride ($CaCl_2$) (Merck & Co, New York, NY, USA) after pre-incubation with the thrombin-inhibitor D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK, 40 µmol/L, final; Merck & Co., Darmstadt, Germany). Platelet aggregation was induced by 5 and 2.5 µmol/L ADP (Sigma Chemical Co, St. Louis, MO, USA) and recorded for 10 min on a 4-channel optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden, the Netherlands). Maximal aggregation and late aggregation (residual aggregation at 6 minutes after the maximum representing platelet aggregate stability) were determined. Because of logistic reasons, platelet aggregation could be performed in only a subset of 139 plasma samples from 16 individuals.

Direct *in vitro* effects of PM on platelet aggregation were studied by adding different types of diluted PM (reference PM with diameter size <0.1, 2.5, or 10 µm, diesel soot collected with a diesel generator, urban background dust collected from a local baghouse filter extract, or EHC-93 reference dust that was collected in Ottawa, Ontario, Canada) to P200 or whole blood in various concentrations (range, 0-100 µg/mL) for different incubation periods (range, 0-2h) and performing ADP-induced light-transmittance, or ADP-induced impedance whole-blood platelet aggregation experiments (Chrono-log), respectively. Results were compared with those obtained with aliquot samples without incubation with PM.

Thrombin generation

Thrombin generation in tissue factor (TF)-triggered PPP was measured with the Calibrated Automated Thrombogram (CAT)-method (Thrombinoscope, Maastricht, The Netherlands).[17] Measurements were conducted on 80 µL plasma with final concentrations of 1 and 5 pM TF (PPP Reagent Low and PPP Reagent, Thrombinoscope) and 4 µM phospholipids. Thrombin Calibrator was obtained from Thrombinoscope.

Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460-nm filter set. Thrombin generation curves were calculated with the Thrombinoscope software. Three parameters were derived from the thrombin generation curves: lag time (defined as the time to reach one-sixth of the peak height), endogenous thrombin potential (ETP), and peak height. A thrombin generation curve is characterized by the initial burst of thrombin formation and the lag time, which depends on the amount of TF present in the sample or added to the plasma to trigger coagulation. Furthermore, the lag time is negatively associated with the plasma levels of factors VII and IX, antithrombin, free protein S, and free TF pathway inhibitor.[18] The other two main parameters, ETP and peak height, reflect the potential of plasma to generate thrombin and have been suggested to indicate a state of hypercoagulability when elevated.[19] Both the ETP and peak height are determined by plasma levels of fibrinogen, factor XII, antithrombin, and free TF pathway inhibitor.[18]

Fibrinogen and CRP

Fibrinogen levels were determined according to von Clauss (Instrumentation Laboratory, IJsselstein, the Netherlands) and the prothrombin (PT)-derived method (Dade Thrombin Reagent, Siemens Diagnostics, Leusden, the Netherlands) on a Sysmex CA-1500 automated coagulation analyzer (Siemens Diagnostics, Leusden, the Netherlands). CRP levels were measured by means of an in-house high-sensitivity ELISA with polyclonal rat anti-human CRP antibodies (DAKO, Glostrup, Denmark) and a CRP-calibrator (DAKO).

Statistical analysis

Data are presented as means and standard deviations for continuous variables and as counts and percentages for categorical variables. Linear regression analysis was performed between plasma levels and air pollution concentration at different periods before each blood sampling. Data were analyzed in R software (version 2.5.1; R Foundation for Statistical Computing, Vienna, Austria) using Generalized Additive Models (GAM) with individual intercepts for each subject, day of the week as a an indicator variable and penalized spline-smoothers for date (to adjust for trend and seasonality) and meteorological parameters (temperature, pressure and relative humidity). Degrees of freedom used for the splines were optimized by the software, according to the procedure described by Wood (2001).[20] We used 10 knots as a starting point. The effective degrees of freedom for trend ranged from 1 to 8 for the different models.

A time lag corresponds to a mean concentration of an air pollutant that was

calculated from concentrations hourly measured within the corresponding time window preceding each blood sampling, whereby the time of the blood sampling was set to 0000 hours. Time lags that represent direct effects are $D_{0\text{-}6}$, $D_{0\text{-}12}$ and $D_{0\text{-}24}$, indirect effects $I_{24\text{-}48}$, $I_{48\text{-}72}$ and $I_{72\text{-}96}$, and both direct and indirect effects $D+I_{0\text{-}96}$ (Figure 2). In addition, for O_3 the maximum concentration that was measured within the 24 hours preceding each blood sampling was added to study the effect of peak exposures. The longitudinal study-design included repeated measures analysis, whereby subjects served as their own references. In this analysis, plasma levels in 13 blood samples of each subject were associated with the corresponding local concentrations of air pollutants. Effects of air pollution on plasma variables were normalized and presented as percentage change of the variable of interest for one interquartile range (IQR) of an air pollutant (%/IQR). In this model, effects can be compared among all air pollutants and all plasma variables. A two-sided value of $p<0.05$ was considered statistically significant. For CRP and fibrinogen, only indirect effects of air pollution were determined (time lags $I_{24\text{-}48}$, $I_{48\text{-}72}$ and $I_{72\text{-}96}$).[12] All analyses were also performed excluding smokers (n=7) or women using oral contraceptives (n=9). The correlation coefficients between the concentrations of different air pollutants were calculated by means of Pearson's correlation test.

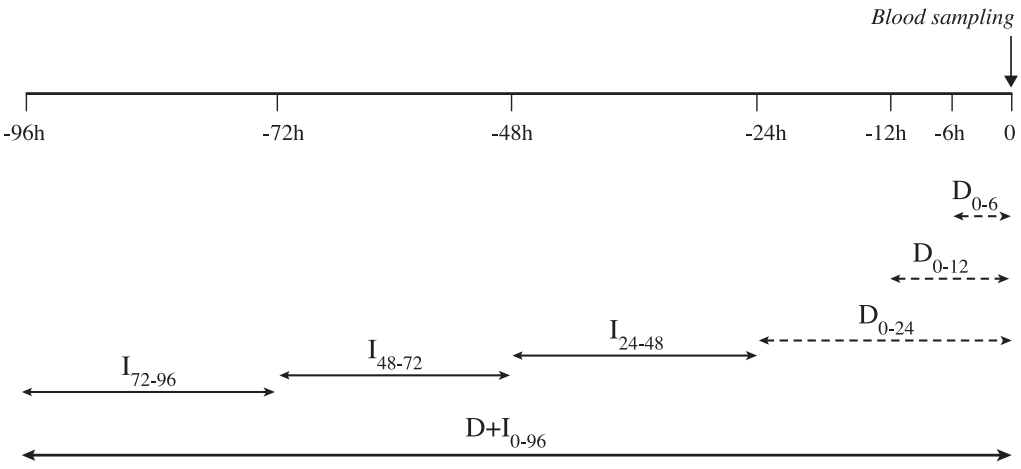


Figure 2. Time lags of estimated exposure to air pollution before blood sampling
The time of each blood sampling was set to 0 hours. Time lags represent means of air pollution concentrations that were determined hourly within the corresponding time-window preceding each blood sampling. Direct effects of air pollution are represented with dashed arrows ($D_{0\text{-}6}$, $D_{0\text{-}12}$ and $D_{0\text{-}24}$) and indirect effects with solid arrows ($I_{24\text{-}48}$, $I_{48\text{-}72}$ and $I_{72\text{-}96}$). Time lag $D+I_{0\text{-}96}$ represents the mean concentration within 4 days before blood sampling.

RESULTS

Study population and concentration profiles of air pollutants

Forty healthy subjects with a mean age of 41 years were included in the study (Table 1). Twenty-six women participated in the study (65% of total). In total, there were seven current smokers (18%).

Table 1. Characteristics of the study population

Characteristic	(n=40)
Age (years)	41 ± 15
Females	26 (65%)
BMI (kg/m^2)	22.6 ± 2.0
Smokers	7 (18%)
Oral contraceptives	9 (23%)
Blood parameters	
Fibrinogen (g/L)	2.6 ± 0.5
CRP (mg/L)	0.6 ± 1.2
Platelet aggregation (n=16)	
Maximal aggregation (%)	65 ± 13
Late aggregation (%)	46 ± 20
Thrombin generation	
ETP (nM*min)	999 ± 317
Peak (nM)	141 ± 71
Lag-time (min)	4.2 ± 0.9

Values are no. (%) for categorical variables and mean ± SD for continuous variables.
BMI, body mass index; CRP, C-reactive protein; ETP, endogenous thrombin potential.

The profile of air pollution concentrations throughout the study period shows quite variable levels of air pollutants (Table 2 and Figure 1). The correlation coefficients between the concentrations of different air pollutants were >0.6 per each of the studied time lags, and were negative between O_3 and the other air pollutants (–0.4 to –0.6).

Table 2. Concentration of air pollutants during the study period

Air pollutant	Median	p25-75	Max
PM_{10}	29.3	23.8 – 39.2	110.1
CO	333	276 – 412	1283
NO	7	4 – 15	163
NO_2	37	27 – 48	81
O_3	44	21 – 63	180

Data are presented as $\mu g/m^3$. For each air pollutant, median, 25^{th} to 75^{th} percentile and maximal concentration are given.

Platelet aggregation

The characteristics of the subset of subjects in whom platelet aggregation was performed (n=16) were similar to those of the remaining 24 subjects, except that there were no users of oral contraceptives in this subset of 16 subjects (data not shown). We observed indirect effects of air pollution on platelet aggregation, represented by a positive significant association between 5 µmol/L ADP-induced maximal aggregation and PM_{10} concentrations for time lag I_{72-96} (+8%/IQR, $p<0.01$) (Figure 3A).

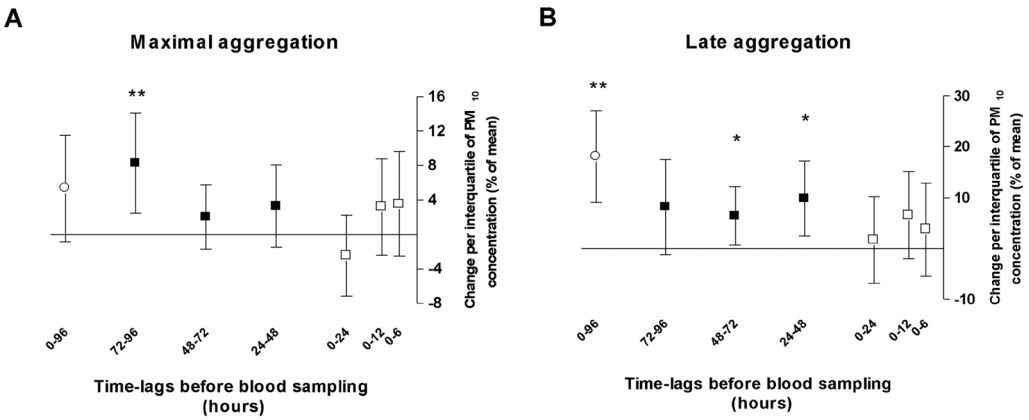


Figure 3. Effects of PM_{10} on platelet aggregation
Estimated effects of PM_{10} on maximal aggregation (panel A) and late aggregation (panel B) are given as percentage change from the mean of each individual per increase in interquartile of PM_{10} concentration. Direct effects of PM_{10} on platelet aggregation are represented by time lags D_{0-6}, D_{0-12} and D_{0-24} (open squares), whereas indirect effects are represented by time lags I_{24-48}, I_{48-72} and I_{72-96} (closed squares). The effect of 4-day mean concentration of PM_{10} on platelet aggregation is represented by the time lag $D+I_{0-96}$ (open circle). *$p<0.05$, **$p<0.01$

In addition, late aggregation was significantly associated with PM_{10} for time lags I_{24-48} and I_{48-72} (+10 and +6%/IQR, respectively, both $p<0.05$) and for time lag $D+I_{0-96}$ (+18%/IQR, $p<0.01$) (Figure 3B). Significant associations were also observed between maximal aggregation and CO, NO and NO_2 for time lag I_{48-72} (+8, +6 and +6%/IQR, respectively, all $p<0.01$) and for time lag $D+I_{0-96}$ (+9%/IQR of CO, $p<0.05$, and +8%/IQR of NO, $p<0.01$) (Table 3). Similarly, late aggregation was significantly associated with CO, NO, and NO_2 for these time lags (I_{48-72}: +18, +8 and +9%/IQR, respectively; $D+I_{0-96}$: +20, +13 and +16%/IQR, respectively, all $p<0.01$) (Table 3). In addition, a significant association was observed between maximal daily concentration of O_3 and late aggregation (-26 and -16%/IQR, $p<0.05$, Table 3). Similar results were obtained when platelet aggregation was induced with 2.5 µmol/L ADP, instead of 5 µmol/L ADP, and when the analyses were performed in non-smokers only or after exclusion of women using oral contraceptives.

No direct effect of PM_{10} on platelet aggregation was observed, because no associations between the PM_{10} concentration and maximum platelet aggregation or late aggregation for the direct-effect time lags D_{0-6}, D_{0-12} and D_{0-24} were noted (Figure 3). We confirmed this absence of direct effects *in vitro*, because the addition of various types of PM to P200 or whole blood did not lead to any changes in light-transmittance or impedance whole-blood platelet aggregation, compared with an aliquot P200 sample to which no PM was added. However, a direct *in vivo* effect was suggested for CO, because a significant positive association with late aggregation was observed (+11, +12 and +11%/IQR for D_{0-6}, D_{0-12} and D_{0-24}, respectively; all $p<0.05$) (Table 3).

Table 3. Estimated changes of platelet aggregation parameters associated with mean air pollutant levels at various time lags preceding blood samplings

	PM_{10}	CO	NO	NO_2	O_3
Maximum					
D_{0-6}	3.5 (-2.5, 9.6)	-3.6 (-9.3, 2.1)	1.3 (-4.4, 7.1)	-2.3 (-7.3, 2.7)	7.0 (-1.7, 15.7)
D_{0-12}	3.2 (-2.4, 8.8)	-4.7 (-11.0, 1.5)	0.7 (-5.4, 6.8)	-2.6 (-8.4, 3.3)	4.1 (-4.6, 12.8)
D_{0-24}	-2.5 (-7.2, 2.3)	-2.6 (-7.9, 2.7)	1.9 (-3.0, 6.9)	-3.0 (-10.3, 4.3)	4.9 (-6.6, 16.3)
I_{24-48}	3.3 (-1.5, 8.1)	-1.1 (-7.2, 4.9)	1.2 (-4.1, 6.5)	-0.6 (-6.6, 5.3)	-5.7 (-20.3, 9.0)
I_{48-72}	2.0 (-1.7, 5.8)	8.4 (2.5, 14.3)**	6.1 (2.4, 9.7)**	5.6 (1.5, 9.7)**	-8.1 (-18.8, 2.7)
I_{72-96}	8.3 (2.5, 14.1)**	-0.1 (-5.1, 5.0)	-0.4 (-5.5, 4.8)	1.2 (-4.5, 6.9)	-1.6 (-10.4, 7.3)
$D+I_{0-96}$	5.4 (-0.8, 11.6)	9.5 (1.6, 17.4)*	8.5 (2.8, 14.1)**	3.0 (-3.8, 9.8)	-7.2 (-22.4, 8.1)
max					1.1 (-9.5, 11.7)
Late aggregation					
D_{0-6}	3.7 (-5.4, 12.9)	10.5 (0.8, 20.3)*	8.1 (-1.2, 17.3)	3.3 (-5.3, 11.8)	-15.0 (-30.4, 0.5)
D_{0-12}	6.6 (-2.0, 15.1)	11.6 (1.2, 21.9)*	8.5 (-0.7, 17.6)	7.5 (-2.3, 17.2)	-14.1 (-29.0, 0.8)
D_{0-24}	1.7 (-6.8, 10.2)	11.2 (1.4, 21.0)*	8.9 (1.12, 16.6)*	9.9 (-2.5, 22.3)	-17.3 (-35.2, 0.6)
I_{24-48}	9.8 (2.4, 17.2)*	7.5 (-2.2, 17.1)	5.5 (-1.5, 12.4)	1.9 (-9.0, 12.7)	-18.4 (-39.0, 2.2)
I_{48-72}	6.4 (0.7, 12.2)*	18.1 (8.4, 27.8)**	7.9 (2.3, 13.4)**	8.9 (2.6, 15.2)**	-26.0 (-44.1, -7.8)**
I_{72-96}	8.2 (-1.2, 17.6)	4.2 (-5.5, 13.9)	3.4 (-5.7, 12.6)	4.8 (-4.3, 13.9)	1.2 (-14.8, 17.3)
$D+I_{0-96}$	18.1 (9.1, 27.1)**	20.4 (8.4, 32.4)**	13.0 (4.9, 21.1)**	16.1 (5.0, 27.2)**	-17.1 (-40.8, 6.7)
max					-16.4 (-31.0, -1.8)*

Data are percent change of 5 µmol/L ADP-induced maximal platelet aggregation and late aggregation (6 min after maximum), with 95% C.I. in parentheses. Values are based on hourly measurements from a monitor located within the city center of Rotterdam. Blood was drawn from all subjects between 0900 and 1100 hours. *$p<0.05$; **$p<0.01$

Thrombin generation

A significant increase in ETP was observed for the gaseous pollutants CO, NO and NO_2 at time lags representing indirect effects of air pollution (I_{24-48}: +2%/IQR of NO and +4%/IQR of NO_2; I_{72-96}: +3%/IQR of CO and +2%/IQR of NO, all $p<0.05$) and a significant increase in peak thrombin generation (I_{24-48}: +4%/IQR of NO and +8%/IQR of NO_2, both $p<0.01$; I_{72-96}: +4%/IQR of NO, $p<0.05$) (Table 4). In addition, peak thrombin generation was significantly increased by 6% per IQR of maximal daily concentration of O_3 (Table 4).

Table 4. Estimated changes of thrombin generation associated with mean air pollutant levels at various time lags preceding blood samplings

	PM_{10}	CO	NO	NO_2	O_3
ETP					
D_{0-6}	-0.2 (-2.6, 2.1)	-1.5 (-3.7, 0.8)	-0.4 (-2.3, 1.5)	-1.2 (-3.6, 1.2)	3.2 (-0.3, 6.7)
D_{0-12}	-0.2 (-2.2, 1.8)	-1.1 (-3.4, 1.1)	-0.4 (-2.0, 1.1)	-0.2 (-2.8, 2.4)	0.8 (-2.8, 4.4)
D_{0-24}	-0.7 (-2.7, 1.4)	-1.5 (-3.9, 0.9)	-0.3 (-2.2, 1.6)	0.3 (-2.5, 3.1)	0.3 (-3.8, 4.5)
I_{24-48}	-0.2 (-2.3, 1.9)	-0.7 (-3.4, 2.0)	1.9 (0.1, 3.7)*	3.5 (0.2, 6.8)*	-0.9 (-5.4, 3.6)
I_{48-72}	1.9 (-0.2, 4.0)	0.8 (-1.9, 3.4)	0.8 (-0.8, 2.5)	2.1 (-1.0, 5.2)	-0.6 (-4.5, 3.3)
I_{72-96}	1.5 (-1.1, 4.2)	3.5 (0.8, 6.2)*	2.1 (0.2, 4.0)*	0.8 (-1.9, 3.5)	-0.6 (-4.1, 3.0)
$D+I_{0-96}$	0.7 (-2.3, 3.8)	0.8 (-2.7, 4.3)	1.7 (-1.1, 4.5)	1.1 (-1.7, 4.0)	1.0 (-3.2, 5.2)
max					2.3 (-1.2, 5.8)
Peak					
D_{0-6}	-0.8 (-4.8, 3.1)	-2.5 (-6.3, 1.3)	-0.4 (-3.6, 2.8)	-1.5 (-5.5, 2.5)	5.7 (-0.2, 11.7)
D_{0-12}	-0.7 (-4.1, 2.6)	-1.9 (-5.7, 1.9)	-0.4 (-3.1, 2.3)	-0.7 (-5.1, 3.7)	2.8 (-3.4, 8.9)
D_{0-24}	-1.6 (-5.0, 1.8)	-3.3 (-7.3, 0.7)	-0.6 (-3.9, 2.6)	-0.6 (-5.3, 4.1)	2.6 (-4.8, 9.9)
I_{24-48}	-0.2 (-3.7, 3.3)	-1.3 (-6.1, 3.6)	4.1 (1.1, 7.2)**	8.0 (2.4, 13.6)**	0.2 (-7.3, 7.8)
I_{48-72}	1.9 (-1.6, 5.4)	-0.5 (-5.0, 4.0)	1.2 (-1.6, 4.0)	3.7 (-1.5, 9.0)	1.4 (-5.1, 8.0)
I_{72-96}	0.2 (-4.2, 4.6)	3.8 (-0.8, 8.4)	3.5 (0.4, 6.7)*	-0.2 (-4.8, 4.4)	2.6 (-3.3, 8.5)
$D+I_{0-96}$	-1.9 (-6.9, 3.2)	-1.7 (-7.5, 4.2)	3.1 (-1.7, 7.8)	1.0 (-4.1, 6.0)	6.6 (-0.7, 13.8)
max					6.3 (0.3, 12.3)*
Lag-time					
D_{0-6}	0.1 (-1.4, 1.7)	1.0 (-0.5, 2.5)	-0.1 (-1.4, 1.1)	0.0 (-1.6, 1.6)	-0.3 (-2.6, 2.0)
D_{0-12}	0.2 (-1.2, 1.5)	1.0 (-0.5, 2.5)	0.0 (-1.1, 1.0)	0.0 (-1.8, 1.7)	-0.4 (-2.7, 2.0)
D_{0-24}	1.1 (-0.2, 2.4)	1.6 (0.1, 3.1)*	0.6 (-0.6, 1.8)	0.2 (-1.6, 2.0)	-0.3 (-2.9, 2.3)
I_{24-48}	0.1 (-1.3, 1.5)	0.4 (-1.3, 2.2)	-1.8 (-2.9, -0.7)**	-3.1 (-5.1, -1.0)**	3.0 (0.4, 5.7)*
I_{48-72}	0.0 (-1.4, 1.4)	-1.0 (-2.7, 0.7)	-0.8 (-1.8, 0.3)	-2.5 (-4.3, -0.6)*	1.2 (-1.1, 3.6)
I_{72-96}	0.6 (-1.2, 2.3)	-1.5 (-3.2, 0.2)	-1.4 (-2.6, -0.2)*	0.0 (-1.8, 1.7)	0.6 (-1.6, 2.8)
$D+I_{0-96}$	1.2 (-0.7, 3.2)	0.1 (-2.1, 2.2)	-1.1 (-2.8, 0.5)	-0.7 (-2.5, 1.1)	0.6 (-2.0, 3.2)
max					-1.2 (-3.5, 1.0)

Percent change of thrombin with their 95% C.I. in parentheses. Thrombin generation was induced with 1 pM tissue factor and 4 µmol/L phospholipids. Values are based on hourly measurements from a monitor located within the city center of Rotterdam. Blood was drawn from all subjects between 0900 and 1100 hours. *p<0.05; **p<0.01

The associations with PM_{10} levels were less clear and not statistically significant, although the estimates for time lags that represent indirect effects on ETP were mainly positive (Figure 4A). The lag-time of thrombin generation was significantly lower when the concentrations of gaseous pollutants were increased at time lags representing indirect

effects (I_{24-48}: -2%/IQR of NO and –3%/IQR of NO_2, both $p<0.01$; I_{48-72}: -2%/IQR of NO_2, $p<0.05$; I_{72-96}: -1%/IQR of NO, $p<0.05$), except for the time lag D_{0-24} (+2%/IQR of CO, $p<0.05$)(Table 4). We observed no clear associations between PM_{10} and peak height or lag-time of thrombin generation (Figure 4B,C). The associations between air pollutants and parameters of thrombin generation that was induced by 5 pM TF showed similar associations (data not shown). Similar results for thrombin generation parameters were also obtained when the analyses were performed in non-smokers only or after exclusion of women using oral contraceptives (data not shown).

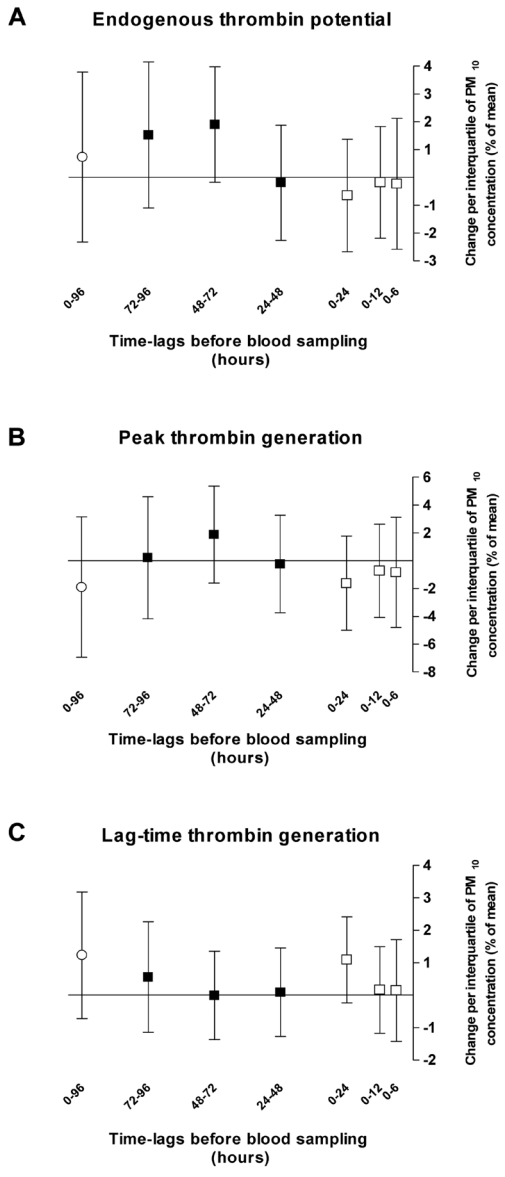


Figure 4. Effects of PM_{10} on thrombin generation
Estimated effects of PM_{10} on endogenous thrombin potential (panel A), peak height (panel B) and lag-time (panel C) are given as percent change from the mean of each individual per increase in interquartile of PM_{10} concentration. Direct effects of PM_{10} on thrombin generation are represented by time lags D_{0-6}, D_{0-12} and D_{0-24} (open squares), whereas indirect effects by time lags I_{24-48}, I_{48-72} and I_{72-96} (closed squares). The effect of 4-day mean concentration of PM_{10} on thrombin generation is represented by time lag $D+I_{0-96}$ (open circle).

Inflammation

Our data suggest that there are no indirect effects of PM_{10} on inflammation, since there were no statistically significant associations between PM_{10} concentrations and neither CRP nor fibrinogen levels (Figure 5). Similar results were obtained for CO, NO, NO_2 and O_3 (Table 5), and when the analyses were performed in nonsmokers only or after excluding women using oral contraceptives (data not shown).

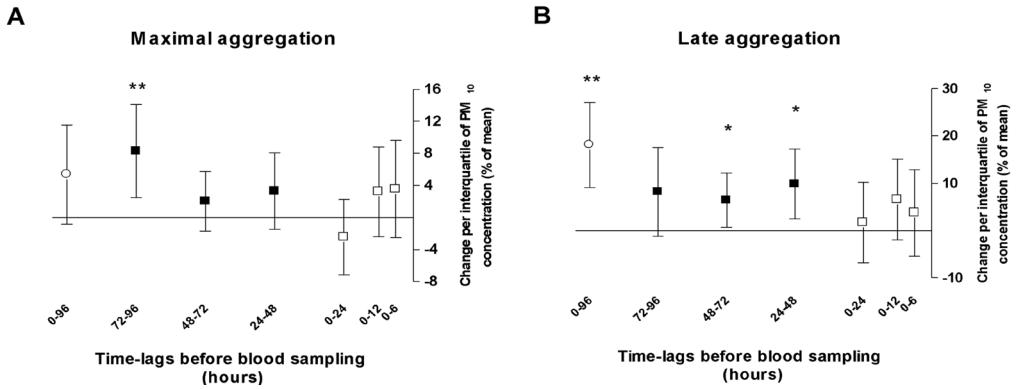


Figure 5. Indirect effects of PM_{10} on inflammation
Estimated indirect effects of PM_{10} on fibrinogen (panel A) and CRP (panel B) are given as percent change from the mean of each individual per increase in interquartile of PM_{10} concentration. These indirect effects of PM_{10} on inflammation are represented by time lags I_{24-48}, I_{48-72} and I_{72-96} (closed squares). The effect of 4-day mean concentration of PM_{10} on inflammation is represented by time lag $D+I_{0-96}$ (open circle).

Table 5. Estimated changes of inflammatory markers associated with mean air pollutant levels time lags representing indirect effects preceding blood samplings

	PM_{10}	CO	NO	NO_2	O_3
Fibrinogen					
I_{24-48}	0.4 (-1.1, 1.8)	0.0 (-1.7, 1.8)	0.1 (-1.0, 1.3)	0.4 (-1.7, 2.5)	-0.6 (-3.2, 2.1)
I_{48-72}	1.0 (-0.5, 2.4)	0.0 (-1.8, 1.9)	0.3 (-0.8, 1.4)	1.4 (-0.6, 3.4)	-1.4 (-3.8, 1.0)
I_{72-96}	0.2 (-1.6, 2.0)	-0.1 (-1.9, 1.7)	0.1 (-1.1, 1.4)	-0.4 (-2.3, 1.4)	0.5 (-1.7, 2.8)
CRP					
I_{24-48}	1.9 (-5.6, 9.4)	3.2 (-6.4, 12.8)	3.6 (-2.9, 10.0)	6.5 (-4.9, 17.8)	-0.5 (-14.7, 13.8)
I_{48-72}	5.8 (-2.0, 13.5)	-1.9 (-12.5, 8.7)	0.1 (-6.5, 6.7)	-0.1 (-11.0, 10.8)	3.7 (-9.7, 17.2)
I_{72-96}	3.4 (-6.2, 12.9)	-4.5 (-15.3, 6.3)	-4.6 (-12.0, 2.9)	-6.9 (-17.2, 3.5)	5.9 (-6.8, 18.7)

Percent change with 95% C.I. in parentheses. Values are based on hourly measurements from a monitor located within the city center of Rotterdam. Blood was drawn from all subjects between 0900 and 1100 hours.

DISCUSSION

The main observation of this study was that air pollution (except O_3) was associated with increased platelet aggregation and increased thrombin generation, but not with the inflammatory markers fibrinogen and CRP. The significant associations between various air pollutants and increased maximal aggregation and late aggregation for time lags within 48-96h before blood sampling, suggest that exposure to air pollution indirectly increases blood thrombogenicity. These indirect effects may be the result of air pollution-induced synthesis of TF[21], which

can increase *in vivo* platelet reactivity.[22] TF-bearing microparticles have also been suggested to contribute to these effects on platelets, either directly, or indirectly via increased blood coagulability.[23] Another possible mechanism of indirect platelet activation might reside in pulmonary oxidative stress and the activation of subsets of white blood cells that lead to a systemic lowering of endothelial- and platelet-derived nitrogen oxide and concomitant platelet activation.[24] Our results also indicate that it is unlikely that blood platelets are directly activated by contact with artificial surfaces of PM since we observed no association between platelet aggregation and PM concentrations for time lags representing direct effects. We confirmed these findings with our *in vitro* experiments, in which we saw no direct effects (0-2h) of the addition of PM on platelet aggregation.

Results for thrombin generation suggest that air pollution leads to an overall tendency towards a hypercoagulable state, since both ETP and peak thrombin generation were increased after exposure to higher levels of gaseous air pollutants. Again, these indirect effects on thrombin generation may be caused by elevated levels of TF. Notably, only gaseous pollutants, and not PM_{10}, were associated with these indirect effects. The gaseous air pollutants, especially NO_2 and CO, can be considered markers for motor vehicle traffic and have been shown to be highly correlated with ultrafine particles.[25] It is mainly this subset of ultrafine particles from the overall particulate matter air pollution, which has an effect on thrombin generation (Spronk et al., unpublished data). This could explain why only the gaseous pollutants, and not PM_{10} mass concentration, was associated with thrombin generation in this study. Another mechanism may involve altered synthesis of pro- and anticoagulant proteins. However, previous studies on air pollution-related changes in plasma levels of prothrombin, Factor VII, antithrombin, Protein C and S are conflicting.[4, 26-28] In our study changes in thrombin generation were independent of the changes in fibrinogen concentrations. Since thrombin generation dependss not only on fibrinogen, but also on other pro- and anticoagulant proteins, this suggests that air pollution can induce changes in hemostatic balance that are not inflammation-driven.

In our study, air pollution was not associated with systemic inflammation. We measured fibrinogen and CRP, two sensitive inflammatory markers that are consistently associated with cardiovascular risk. The results from epidemiologic studies on air pollution and inflammation are conflicting, as are those from laboratory studies on the inflammatory responses in human volunteers or animals after experimentally controlled exposure to air pollution.[26, 28-31] Since fibrinogen and CRP do not reflect all aspects of inflammation, measuring other inflammatory markers, such as interleukin 6 and tumor necrosis factor alpha, may still reveal associations between air pollution and inflammation.[4, 32]

Most of the significant associations observed in the present study concern more than one air pollutant, especially in the case of late aggregation, which was associated with all studied air pollutants. The correlation coefficients between the concentrations of air pollutants were moderate and mainly positive (>0.6), except between O_3 and the other air pollutants (–0.4 to –0.6). This probably explains the observed opposite effects of O_3 on most of the plasma variables, compared with the effects of PM_{10}, CO, NO and NO_2. However, when these effects of O_3 were analyzed in a two-pollutant model with PM_{10}, only the effects of PM_{10} remained statistically significant with similar estimates (data not shown), suggesting that PM_{10}, rather than O_3, is responsible for the observed effects on platelet aggregation and thrombin generation. This was in contrast to the results of the two-pollutant models that combined PM_{10} with CO, NO or NO_2, which indicated that effects of these gases are mainly independent of the effects of PM_{10} (data not shown). Nevertheless, it remains difficult to completely discern causal air pollutant(s) from their surrogate markers.

The present study was designed to represent a real-life urban situation. Our approach combines several strengths. First, we have evaluated the effects of air pollution on blood parameters over a period of 1 year. In this longitudinal study design, participants are their own controls, which ensures the most reliable estimates of acute effects of exposure to air pollution. Second, we selected subjects with a similar exposure to local air pollution. Third, several studies have shown that central site measurements correlate well with personal exposures for longitudinal acute effects.[33-35] Although the study participants have spent a significant amount of time living or working in Rotterdam during the study period, their estimated exposure to air pollution may vary. However, our study was not designed to correct for these possible variations. Another limitation of the study is that the PM_{10} mass concentration is a poor measure of its biologic activity, since the corresponding particles are heterogeneous in composition (*e.g.* endotoxins and metals), and may therefore trigger different biological responses.[3] All subjects in this study were healthy volunteers. The observed effects of air pollution on hemostasis may differ (being possibly more pronounced) in subjects with coronary artery disease or subjects at higher risk for this disease.

In conclusion, our data show a significant association between exposure to air pollution and systemic prothrombotic tendency of the blood via increased platelet aggregation and thrombin generation in a healthy population. This association may point to a relevant biological mechanism that contributes to the risk association between air pollution and cardiovascular disease.

REFERENCES

1. Peng RD, Chang HH, Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F. Coarse particulate matter air pollution and hospital admissions for cardiovascular and respiratory diseases among Medicare patients. *Jama.* 2008;299:2172-2179.
2. McCreanor J, Cullinan P, Nieuwenhuijsen MJ, Stewart-Evans J, Malliarou E, Jarup L, Harrington R, Svartengren M, Han IK, Ohman-Strickland P, Chung KF, Zhang J. Respiratory effects of exposure to diesel traffic in persons with asthma. *N Engl J Med.* 2007;357:2348-2358.
3. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC, Jr., Tager I, Expert Panel on Population and Prevention Science of the American Heart A. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation.* 2004;109:2655-2671.
4. Ruckerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A. Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. *Am J Respir Crit Care Med.* 2006;173:432-441.
5. Peters A, Doring A, Wichmann HE, Koenig W. Increased plasma viscosity during an air pollution episode: a link to mortality? *Lancet.* 1997;349:1582-1587.
6. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation.* 2002;105:411-414.
7. Nemmar A, Hoylaerts MF, Hoet PH, Nemery B. Possible mechanisms of the cardiovascular effects of inhaled particles: systemic translocation and prothrombotic effects. *Toxicol Lett.* 2004;149:243-253.
8. Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet.* 1995;345:176-178.
9. Radomski A, Jurasz P, Alonso-Escolano D, Drews M, Morandi M, Malinski T, Radomski MW. Nanoparticle-induced platelet aggregation and vascular thrombosis. *Br J Pharmacol.* 2005;146:882-893.
10. Donaldson K, Stone V, Seaton A, MacNee W. Ambient particle inhalation and the cardiovascular system: potential mechanisms. *Environ Health Perspect.* 2001;109 Suppl 4:523-527.
11. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, Frew A. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med.* 1999;159:702-709.
12. Ruckerl R, Greven S, Ljungman P, Aalto P, Antoniades C, Bellander T, Berglind N, Chrysohoou C, Forastiere F, Jacquemin B, von Klot S, Koenig W, Kuchenhoff H, Lanki T, Pekkanen J, Perucci CA, Schneider A, Sunyer J, Peters A, Group AS. Air pollution and inflammation (interleukin-6, C-reactive protein, fibrinogen) in myocardial infarction survivors. *Environ Health Perspect.* 2007;115:1072-1080.
13. Gong H, Jr. Health effects of air pollution. A review of clinical studies. *Clin Chest Med.* 1992;13:201-214.
14. Brook RD. Is air pollution a cause of cardiovascular disease? Updated review and controversies. *Rev Environ Health.* 2007;22:115-137.
15. van Gestel MA, Heemskerk JW, Slaaf DW, Heijnen VV, Reneman RS, oude Egbrink MG. In vivo blockade of platelet ADP receptor P2Y12 reduces embolus and thrombus formation but not thrombus stability. *Arterioscler Thromb Vasc Biol.* 2003;23:518-523.

16. Woulfe D, Yang J, Brass L. ADP and platelets: the end of the beginning. *J Clin Invest.* 2001;107:1503-1505.

17. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Beguin S. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb.* 2003;33:4-15.

18. Dielis AW, Castoldi E, Spronk HM, van Oerle R, Hamulyak K, Ten Cate H, Rosing J. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost.* 2008;6:125-131.

19. ten Cate-Hoek AJ, Dielis AW, Spronk HM, van Oerle R, Hamulyak K, Prins MH, ten Cate H. Thrombin generation in patients after acute deep-vein thrombosis. *Thromb Haemost.* 2008;100:240-245.

20. Wood SN. GAMs and Generalized Ridge Regression for R. *R News.* 2001;1:20-25.

21. Sun Q, Yue P, Kirk RI, Wang A, Moatti D, Jin X, Lu B, Schecter AD, Lippmann M, Gordon T, Chen LC, Rajagopalan S. Ambient air particulate matter exposure and tissue factor expression in atherosclerosis. *Inhal Toxicol.* 2008;20:127-137.

22. Kuijpers MJ, Munnix IC, Cosemans JM, Vlijmen BV, Reutelingsperger CP, Egbrink MO, Heemskerk JW. Key Role of Platelet Procoagulant Activity in Tissue Factor-and Collagen-Dependent Thrombus Formation in Arterioles and Venules In Vivo Differential Sensitivity to Thrombin Inhibition. *Microcirculation.* 2008;15:269-282.

23. Morel O, Toti F, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol.* 2006;26:2594-2604.

24. Brook RD. Air pollution: what is bad for the arteries might be bad for the veins. *Arch Intern Med.* 2008;168:909-911.

25. Cyrys J, Stolzel M, Heinrich J, Kreyling WG, Menzel N, Wittmaack K, Tuch T, Wichmann HE. Elemental composition and sources of fine and ultrafine ambient particles in Erfurt, Germany. *Sci Total Environ.* 2003;305:143-156.

26. Seaton A, Soutar A, Crawford V, Elton R, McNerlan S, Cherrie J, Watt M, Agius R, Stout R. Particulate air pollution and the blood. *Thorax.* 1999;54:1027-1032.

27. Pekkanen J, Brunner EJ, Anderson HR, Tiittanen P, Atkinson RW. Daily concentrations of air pollution and plasma fibrinogen in London. *Occup Environ Med.* 2000;57:818-822.

28. Baccarelli A, Zanobetti A, Martinelli I, Grillo P, Hou L, Giacomini S, Bonzini M, Lanzani G, Mannucci PM, Bertazzi PA, Schwartz J. Effects of exposure to air pollution on blood coagulation. *J Thromb Haemost.* 2007;5:252-260.

29. Maier KL, Alessandrini F, Beck-Speier I, Hofer TP, Diabate S, Bitterle E, Stoger T, Jakob T, Behrendt H, Horsch M, Beckers J, Ziesenis A, Hultner L, Frankenberger M, Krauss-Etschmann S, Schulz H. Health effects of ambient particulate matter--biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhal Toxicol.* 2008;20:319-337.

30. Nemmar A, Nemery B, Hoet PH, Vermylen J, Hoylaerts MF. Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. *Am J Respir Crit Care Med.* 2003;168:1366-1372.

31. Chuang KJ, Chan CC, Su TC, Lee CT, Tang CS. The effect of urban air pollution on inflammation, oxidative stress, coagulation, and autonomic dysfunction in young adults. *Am J Respir Crit Care Med.* 2007;176:370-376.

32. Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christman JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J Clin Invest.* 2007;117:2952-2961.

33. Janssen NA, Lanki T, Hoek G, Vallius M, de Hartog JJ, Van Grieken R, Pekkanen J, Brunekreef B. Associations between ambient, personal, and indoor exposure to fine particulate matter constituents in Dutch and Finnish panels of cardiovascular patients. *Occup Environ Med.* 2005;62:868-877.

34. Janssen NA, Hoek G, Brunekreef B, Harssema H, Mensink I, Zuidhof A. Personal sampling of particles in adults: relation among personal, indoor, and outdoor air concentrations. *Am J Epidemiol.* 1998;147:537-547.

35. WHO. Particulate matter, ozone, nitrogen dioxide and sulfur dioxide. In: Air quality guidelines: Global update 2005. *World Health Organization (Regional Office for Europe)*. 2006:76-100.

Chapter 8

9

Chapter 9

Goran Rudež
Niels Kok
Frank Leebeek
Kim van Gaalen
Carla Baan
Jan IJzermans
Moniek de Maat

C-reactive protein gene haplotypes determine the acute phase response of serum CRP concentration in healthy individuals

Submitted

ABSTRACT

Objectives

We investigated the association between C-reactive protein (CRP) gene haplotypes that represent the total common variation across the *CRP* gene, and the increase of the inflammatory marker CRP after a standardized surgical intervention.

Background

CRP is a sensitive acute phase marker and it is known that its levels are determined by genetic factors. However, the effect of total common variation in the *CRP* gene on the acute phase response is unknown.

Methods

264 consecutive healthy kidney donors were included and CRP levels were measured before and 1, 2 and 3 days after the standardized removal of a kidney. DNA was isolated and *CRP* haplotypes were analyzed and their association with CRP increase was determined using linear regression analysis.

Results

CRP haplotypes are strong determinants of the liver-induced CRP-increase: haplotypes TCC and CCG (H3 and H4 respectively) were associated with a significantly stronger rise in CRP serum levels (average 9 mg/L and 14 mg/L per allele, respectively) relative to the reference CTC (H1) on day 1 after the intervention. Similar but nonsignificant effects of the haplotypes on CRP levels were seen on baseline, while the differences had disappeared on day 2 and day 3.

Conclusions

This study demonstrates that the common variation in the *CRP* gene strongly predicts the CRP-increase during an acute phase response in healthy individuals.

INTRODUCTION

C-reactive protein (CRP) is a very sensitive acute phase protein, and its serum concentration can rise from 1 mg/L to more than 100 mg/L after an acute phase trigger. There is a substantial genetic component involved in the variation in steady-state CRP levels, as shown by family and twin studies.[1-4] The genetic component includes common variations in the *CRP* gene, as demonstrated by the associations between single nucleotide polymorphisms (SNPs) in the *CRP* gene and CRP levels.[5-14] In populations of European descent, four haplotypes represent the total common variation across the *CRP* gene and two recent studies have reported associations of these haplotypes with basal CRP levels.[15, 16]

The genetic variation is also a major determinant of the increase of CRP levels during an acute phase reaction.[4] Although information on the relationship between genetic variation and response is still limited, several small studies with single SNPs have already shown that promoter SNPs predict the extent of the acute phase response.[5, 7, 17] A major problem in studying the response to acute phase triggers is that there are no broadly applicable standardized acute phase triggers available for the use in population studies. Triggers that have been studied in small studies are vaccination against common flu and yellow fever, but the acute phase response to these triggers is weak and variable.[17, 18] An alternative approach would be to use a well-controlled, well-standardized surgical procedure. In our hospital, the removal of a kidney from a healthy donor fulfills this criterion. Therefore, in this study, we determined the effect of haplotypes representing the total common variation in the *CRP* locus on the response of serum CRP levels after an acute phase trigger, *i.e.* trauma as a result of a well-controlled surgical procedure in a healthy, homogeneous population.

SUBJECTS AND METHODS

Study population

Two hundred and sixty four healthy kidney donors were included in this study. In these donors a kidney with normal anatomy was removed using a minimal invasive procedure according to a detailed, standardized protocol[19] (Table 1).

Table 1. Characteristics of the study population

Variable	Total (n=264)
Age (years)	49.6 ± 13.2
Women (%)	155 (60%)
Body mass index (kg/m^2)	26.3 ± 4.2
Baseline hypertension	14 (5%)
Procedure	
Open	80 (31%)
Laparoscopic	180 (69%)
Left kidney	122 (46%)
Postoperatively developed hypertension	6 (2%)
Blood loss during procedure (ml)	100 (50-300)
CRP levels (mg/L)	
Baseline	1 (1-4)
Day 1	59 (25-80)
Day 2	93 (35-133)
Day 3	75 (44-106)

Categorical variables are expressed as percentages. Values of continuous variables are expressed as mean ± standard deviation. CRP levels are given as median and interquartile range.

Blood collection

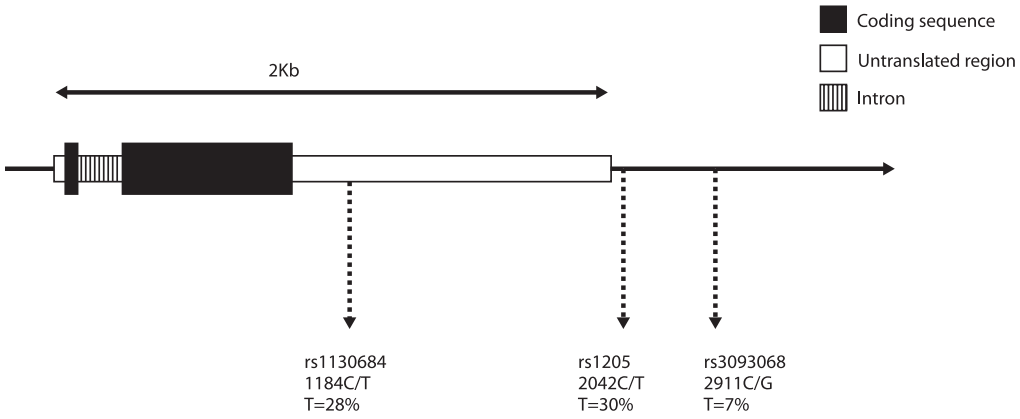
Non-fasting blood was collected from donors before and 1, 2 and 3 days after the operation in tubes without anticoagulant. From the donors at least on one of these time points blood was collected, before the operation from 237 donors, on day 1 from 244, on day 2 from 228 and on day 3 from 178. After clotting of the blood, tubes were centrifuged at 3500*g* for 15 min. and serum was used immediately for CRP analysis. For SNP analysis, genomic DNA was isolated from blood according to standard salting-out procedures.

Measurement of CRP

CRP was determined in serum using an immunoturbidimetric method according to the instructions of the manufacturer (Hitachi 917, Roche).

Genotyping

On the basis of data on 23 unrelated individuals of European descent, the "Seattle SNPs" database (*http://pga.gs.washington.edu/*) identified 31 SNPs in the *CRP* gene. From these SNPs, 4 common haplotypes can be inferred, which are tagged by the SNPs 1184C>T (rs1130864), 2042C>T (rs1205) and 2911C>G (rs3093068) (Figure 1).[20]



Halotype	11B4C/T	2042C/T	2911C/G	Frequency
1	C	T	C	32%
2	T	C	C	35%
3	C	C	C	27%
4	C	C	G	7%
Other				<0.001%

Figure 1. Schematic representation of the *CRP* gene
Schematic representation of the *CRP* gene (2 kb) with its 2 exons (black boxes) and untranslated regions (white boxes). The single intron is depicted with the arced white box. Location of the 3 studied haplotype-tagging SNPs is indicated with vertical arrows together with the SNP rs-number, its Seattle-SNPs code and the minor allele with its allele frequency.

All participants in our study were genotyped for these 3 haplotype-tagging SNPs (ht-SNPs) and genotypes could be determined from 259 donors for rs1130864, 250 donors for rs1205 and 257 donors for rs3093068. The polymorphisms are denoted in relation to the start of the coding sequence of exon 1 using the Human May 2004 (hg 17) assembly (*http://genome.ucsc.edu)*. DNA was processed according to standard techniques. Genotypes were determined in 2 ng genomic DNA with the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimized using the SNP Assay-by-Design service of Applied Biosystems *(http://store.appliedbiosystems.com)* and are available upon

request. End-point fluorescence was measured on an ABI Prism 7900HT (Applied Biosystems, Foster City, California) and genotype calls performed within the SDS 2.2.2 software (Applied Biosystems, Foster City, California). Haplotypes were inferred using the haplo.em function of the Haplo.Stats program *(http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html)*[21] and coded as haplotypes 1 through 4 : haplotype 1 CTC, haplotype 2 CCC, haplotype 3 TCC and haplotype 4 CCG (from left to right within each haplotype: 1184C/>T, 2042C>T, and 2911 C>G) with the minor allele underlined.

Data analysis

Deviations from the Hardy-Weinberg equilibrium were tested for each of the three *CRP* ht-SNPs using a Chi-square test. Differences in serum CRP levels between the three SNPs were examined using analysis of covariance with adjustment for age and sex. Before this analysis, baseline CRP serum levels were log-transformed to normalize their skewed distribution. No conversion was performed for CRP levels at the other time points because the CRP levels were normally distributed.

To investigate the association between *CRP* haplotypes and serum CRP levels we used an allele-based approach, where the effects are calculated for haplotype alleles, rather than for subjects. Analysis of covariance was used within the haplo.glm function of Haplo.Stats. Haplo.glm is based on a generalized linear model and computes the regression of a trait of haplotypes and other covariates.[21] H1 had the lowest CRP levels at all time points and served as the reference category. Using haplo.score function, we computed simulation p-values for each haplotype to account for multiple testing. Haplo.em, haplo.score and haplo.glm were all implemented in the Haplo.Stats software using the R language. The number of simulations was set to 1,000. All analyses were adjusted for age and sex, and also for a full model, including BMI, site of kidney removal, procedure. All tests were two-sided and a p-value <0.05 was considered statistically significant.

RESULTS

In our study population the frequency of the minor 1184T-allele was 28%, of the 2042T-allele 30% and of the 2911G-allele 7%. We observed no significant differences in baseline serum CRP levels between the genotypes of the three individual *CRP* ht-SNPs (Table 2). There were no clear associations of the three individual polymorphisms with the increase in serum CRP levels 1, 2 and 3 days after the surgery (Table 2).

Table 2. CRP serum levels for individual genotypes of *CRP* haplotype-tagging polymorphisms at baseline and 1, 2 and 3 days after removal of the donor kidney

	1184C>T			2042C>T			2911C>G		
	genotype	n	CRP (mg/L)	genotype	n	CRP (mg/L)	genotype	n	CRP (mg/L)
Baseline	CC	113	1 (1-4)	CC	107	2 (1-6)	GG	202	1 (1-4)
	CT	111	2 (1-5)	CT	100	1 (1-3)	GC	24	2 (1-6)
	TT	9	3 (2-5)	TT	18	1 (1-1)	CC	4	5 (2-7)
	p-value		0.19	p-value		0.007	p-value		0.41
Day 1	CC	115	56 (41-73)	CC	107	58 (41-94)	GG	210	57 (68-79)
	CT	116	59 (37-89)	CT	103	57 (36-78)	GC	23	58 (45-96)
	TT	8	72 (41-97)	TT	21	45 (36-62)	CC	4	85 (73-105)
	p-value		0.49	p-value		0.10	p-value		0.09
Day 2	CC	105	95 (60-130)	CC	100	98 (60-148)	GG	195	90 (59-130)
	CT	115	91 (56-135)	CT	98	88 (54-130)	GC	23	95 (66-142)
	TT	7	84 (34-135)	TT	17	84 (60-110)	CC	4	137 (105-149)
	p-value		0.91	p-value		0.36	p-value		0.21
Day 3	CC	88	88 (42-105)	CC	81	85 (43-105)	GG	148	69 (42-105)
	CT	78	84 (46-111)	CT	75	77 (43-111)	GC	22	83 (52-105)
	TT	9	84 (34-135)	TT	14	69 (40-98)	CC	4	98 (92-103)
	p-value		0.59	p-value		0.56	p-value		0.77

We then combined the polymorphisms and performed a haplotype analysis. The haplotype alleles were present in the following frequencies: H1 (C<u>T</u>C) in 32%, H2 (CCC) in 35%, H3 (<u>T</u>CC) in 27% and H4 (CC<u>G</u>) in 6%. At baseline, the mean CRP level was 1 mg/L (Figure 2) and H3 and H4 showed somewhat, but not significantly, higher mean serum CRP levels than the reference H1 (Figure 3) in a model that is adjusted for age and sex.

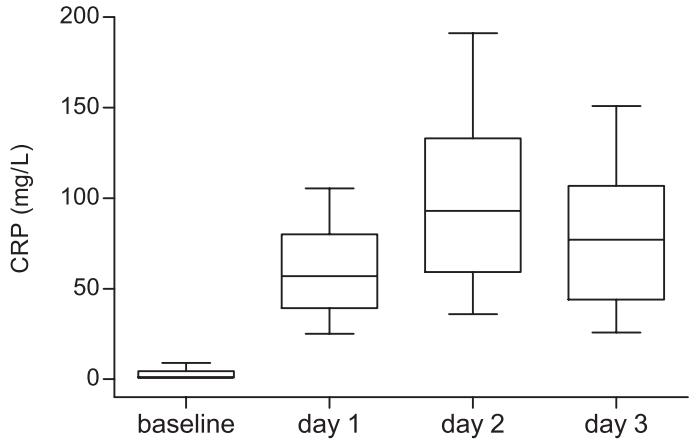


Figure 2. Change in CRP levels after the surgical procedure
Boxplots indicate 10%, 25%. 50%, 75% and 90% of the distribution of data

On day 1 after the surgery, the mean CRP level was 59 mg/L (SD 33 mg/L) (Figure 2) and the smallest rise of CRP levels was seen in H1. Significantly higher post-operational rises in CRP were seen for H3 and H4, and were 14% (95% CI 1-28%) and 23% (95% CI 3-44%) higher per allele, respectively) (Figure 3). When we calculated the association between *CRP* haplotypes and the change in CRP level between day 1 and baseline, similar results were observed, since the baseline levels of CRP were very low (data not shown). Additional adjustment for BMI, side of the kidney and type of procedure had no effect on the results (data not shown).

On day 2, the CRP levels has raised further (mean 102 mg/L with SD 56 mg/L) Figure 2), but this rise was no longer associated with the haplotype. On day 3 after the operation, the CRP levels were going down in 83% of the population and no association with genotype was observed. We also observed no relation between CRP genetic variation or increase in CRP with postoperative course, which was in all cases uneventful.

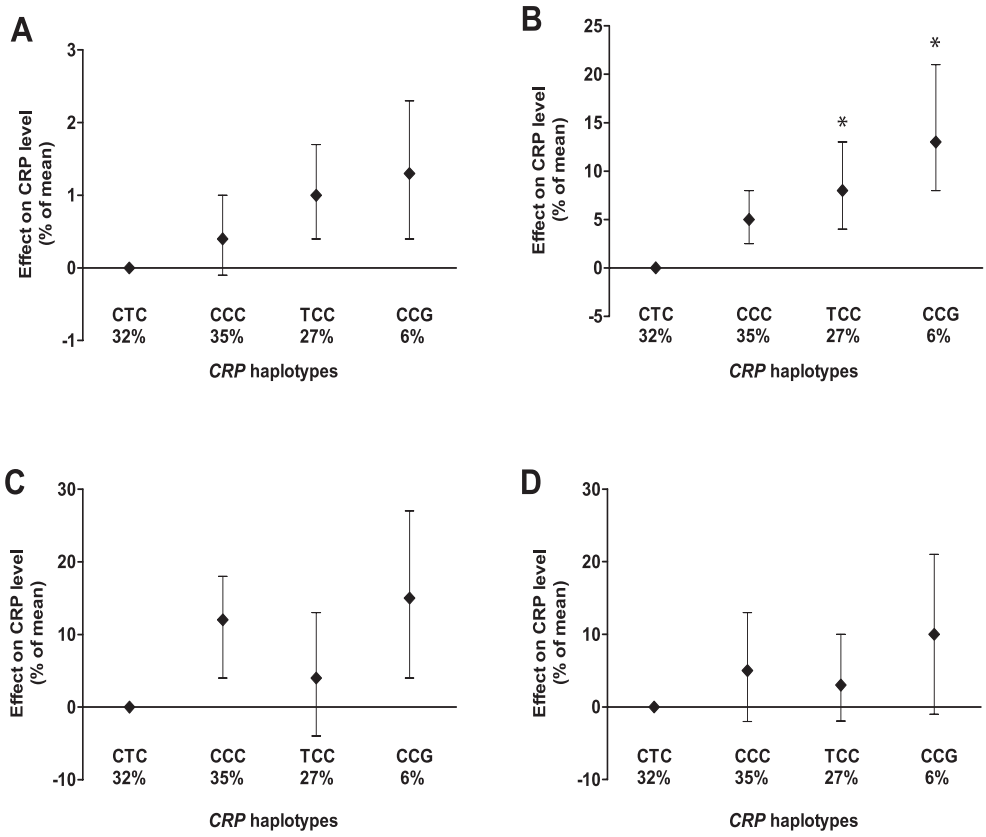


Figure 3. Effect of *CRP* haplotypes on serum CRP levels before and after surgery
The effects of *CRP* haplotypes (x-axis) on CRP levels at baseline (panel A), and 1 (panel B), 2 (panel C) and 3 days (panel D) after kidney transplantation, with adjustment for age and sex, are given. Effects of CRP levels (y-axis) are expressed as changes from the geometric mean of the CRP levels. H3 was used as the reference haplotype. *p<0.05

DISCUSSION

In this study, *CRP* haplotypes were strongly associated with the response of CRP serum levels to a standardized acute phase trigger. Overall, there was a clear contribution of the *CRP* haplotypes to the increase of CRP levels on day1 after surgery. The present study uses haplotypes to describe the total common variation of the *CRP* gene. We and others have previously used this approach to study the relationship between genetic variation in CRP and baseline CRP levels. Indeed, H3 and H4 (according to the nomenclature used in this study) were consistently associated with higher CRP levels than the reference H1.[15, 16]

Other studies on *CRP* haplotypes and risk of cardiovascular events have been conducted mostly in studies on relatively small numbers of high-risk patients[8, 9, 13, 22-25] and the results of these studies were conflicting. Also, several, mostly small studies have demonstrated associations between various individual *CRP* SNPs and CRP levels.[5-14, 26] Although these studies were different in design and were conducted in various populations, their findings support our haplotyping results.

High CRP levels may contribute to a number of pathogenic mechanisms, such as complement activation, up-regulation of cell-adhesion molecules and interaction with monocytes via FcRγIIa receptor. Therefore, high CRP levels may exert these harmful effects in the acute phase. It may be expected that subjects who are susceptible to inflammation (hyper-responsive to acute phase triggers) may have an increased risk of diseases in which CRP plays a role, such as cardiovascular disease. One hypothesis is that increased levels of these acute phase markers are especially seen in individuals who are hyperresponsive to environmental acute phase stimuli. It was our intention to study how the CRP genetic variation determines the elevation of CRP after an acute phase trigger. It has already been shown that enhanced acute phase responsiveness may help explain the association between inflammation and cardiovascular disease.[27] For example, it has been shown that mononuclear cells of unstable angina patients with recurrent phases of instability exhibit an enhanced production of IL-6 in response to low-dose of LPS.[27]

We saw a clear association of haplotypes with CRP levels on day 1, and a similar trend at baseline. However, on day 2 this association was no longer seen. Also on day 3, when CRP levels already started to normalize, no association was seen (data not shown) This suggests that the CRP genetic variation is mainly associated with the initial rate of CRP increase. Further studies with more frequent blood collected would be needed to test this hypothesis.

In this study we evaluated a standardized trigger which was supported by the fact that most patients went home on day 3 after the operation, the observed range of CRP during the acute phase reaction is still quite pronounced (50-90 mg/L) and not in the range where CRP is associated with the risk for cardiovascular disease, i.e. 0.1-10 mg/L.

In conclusion, this study is the first to demonstrate that the common variation in the *CRP* gene is a strong determinant of the increase of serum CRP levels during acute phase in healthy individuals.

REFERENCES

1. MacGregor AJ, Gallimore JR, Spector TD, Pepys MB. Genetic effects on baseline values of C-reactive protein and serum amyloid a protein: a comparison of monozygotic and dizygotic twins. *Clin Chem.* 2004;50:130-134.
2. Pankow JS, Folsom AR, Cushman M, Borecki IB, Hopkins PN, Eckfeldt JH, Tracy RP. Familial and genetic determinants of systemic markers of inflammation: the NHLBI family heart study. *Atherosclerosis.* 2001;154:681-689.
3. Retterstol L, Eikvar L, Berg K. A twin study of C-Reactive Protein compared to other risk factors for coronary heart disease. *Atherosclerosis.* 2003;169:279-282.
4. de Maat MP, Bladbjerg EM, Hjelmborg JB, Bathum L, Jespersen J, Christensen K. Genetic influence on inflammation variables in the elderly. *Arterioscler Thromb Vasc Biol.* 2004;24:2168-2173.
5. Brull DJ, Serrano N, Zito F, Jones L, Montgomery HE, Rumley A, Sharma P, Lowe GD, World MJ, Humphries SE, Hingorani AD. Human CRP gene polymorphism influences CRP levels: implications for the prediction and pathogenesis of coronary heart disease. *Arterioscler Thromb Vasc Biol.* 2003;23:2063-2069.
6. Zee RY, Ridker PM. Polymorphism in the human C-reactive protein (CRP) gene, plasma concentrations of CRP, and the risk of future arterial thrombosis. *Atherosclerosis.* 2002;162:217-219.
7. D'Aiuto F, Casas JP, Shah T, Humphries SE, Hingorani AD, Tonetti MS. C-reactive protein (+1444C>T) polymorphism influences CRP response following a moderate inflammatory stimulus. *Atherosclerosis.* 2005;179:413-417.
8. Kovacs A, Green F, Hansson LO, Lundman P, Samnegard A, Boquist S, Ericsson CG, Watkins H, Hamsten A, Tornvall P. A novel common single nucleotide polymorphism in the promoter region of the C-reactive protein gene associated with the plasma concentration of C-reactive protein. *Atherosclerosis.* 2005;178:193-198.
9. Russell AI, Cunninghame Graham DS, Shepherd C, Roberton CA, Whittaker J, Meeks J, Powell RJ, Isenberg DA, Walport MJ, Vyse TJ. Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. *Hum Mol Genet.* 2004;13:137-147.
10. Davey Smith G, Lawlor DA, Harbord R, Timpson N, Rumley A, Lowe GD, Day IN, Ebrahim S. Association of C-reactive protein with blood pressure and hypertension: life course confounding and mendelian randomization tests of causality. *Arterioscler Thromb Vasc Biol.* 2005;25:1051-1056.
11. Suk HJ, Ridker PM, Cook NR, Zee RY. Relation of polymorphism within the C-reactive protein gene and plasma CRP levels. *Atherosclerosis.* 2005;178:139-145.
12. Szalai AJ, McCrory MA, Cooper GS, Wu J, Kimberly RP. Association between baseline levels of C-reactive protein (CRP) and a dinucleotide repeat polymorphism in the intron of the CRP gene. *Genes Immun.* 2002;3:14-19.
13. Obisesan TO, Leeuwenburgh C, Phillips T, Ferrell RE, Phares DA, Prior SJ, Hagberg JM. C-reactive protein genotypes affect baseline, but not exercise training-induced changes, in C-reactive protein levels. *Arterioscler Thromb Vasc Biol.* 2004;24:1874-1879.
14. Eklund C, Lehtimaki T, Hurme M. Epistatic effect of C-reactive protein (CRP) single nucleotide polymorphism (SNP) +1059 and interleukin-1B SNP +3954 on CRP concentration in healthy male blood donors. *Int J Immunogenet.* 2005;32:229-232.
15. Carlson CS, Aldred SF, Lee PK, Tracy RP, Schwartz SM, Rieder M, Liu K, Williams OD, Iribarren C, Lewis EC, Fornage M, Boerwinkle E, Gross M, Jaquish C, Nickerson DA, Myers RM, Siscovick DS, Reiner AP. Polymorphisms within the C-Reactive Protein (CRP) Promoter Region Are Associated with Plasma CRP Levels. *Am J Hum Genet.* 2005;77.

16. Miller DT, Zee RY, Suk Danik J, Kozlowski P, Chasman DI, Lazarus R, Cook NR, Ridker PM, Kwiatkowski DJ. Association of common CRP gene variants with CRP levels and cardiovascular events. *Ann Hum Genet.* 2005;69:623-638.
17. Verschuur M, van der Beek MT, Tak HS, Visser LG, de Maat M. Interindividual variation in the response by fibrinogen, C-reactive protein and interleukin-6 to yellow fever vaccination. *Blood Coagul Fibrinolys.* 2004;15:399-404.
18. Posthouwer D, Voorbij HA, Grobbee DE, Numans ME, van der Bom JG. Influenza and pneumococcal vaccination as a model to assess C-reactive protein response to mild inflammation. *Vaccine.* 2004;23:362-365.
19. Kok NF, Lind MY, Hansson BM, Pilzecker D, Mertens zur Borg IR, Knipscheer BC, Hazebroek EJ, Dooper IM, Weimar W, Hop WC, Adang EM, van der Wilt GJ, Bonjer HJ, van der Vliet JA, IJzermans JN. Comparison of laparoscopic and mini incision open donor nephrectomy: single blind, randomised controlled clinical trial. *BMJ.* 2006;333:221.
20. Kardys I, de Maat MP, Uitterlinden AG, Hofman A, Witteman JC. C-reactive protein gene haplotypes and risk of coronary heart disease: the Rotterdam Study. *Eur Heart J.* 2006;27:1331-1337.
21. Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet.* 2002;70:425-434.
22. Zee RY, Hegener HH, Fernandez-Cruz A, Lindpaintner K. C-reactive protein gene polymorphisms and the incidence of post-angioplasty restenosis. *Atherosclerosis.* 2004;176:393-396.
23. Zee RY, Hegener HH, Cook NR, Ridker PM. C-reactive protein gene polymorphisms and the risk of venous thromboembolism: a haplotype-based analysis. *J Thromb Haemost.* 2004;2:1240-1243.
24. Chen J, Zhao J, Huang J, Su S, Qiang B, Gu D. -717A>G polymorphism of human C-reactive protein gene associated with coronary heart disease in ethnic Han Chinese: the Beijing atherosclerosis study. *J Mol Med.* 2005;83:72-78.
25. Szalai AJ, Wu J, Lange EM, McCrory MA, Langefeld CD, Williams A, Zakharkin SO, George V, Allison DB, Cooper GS, Xie F, Fan Z, Edberg JC, Kimberly RP. Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. *J Mol Med.* 2005.
26. Szalai AJ, Alarcon GS, Calvo-Alen J, Toloza SM, McCrory MA, Edberg JC, McGwin G, Jr., Bastian HM, Fessler BJ, Vila LM, Kimberly RP, Reveille JD. Systemic lupus erythematosus in a multiethnic US Cohort (LUMINA). XXX: association between C-reactive protein (CRP) gene polymorphisms and vascular events. *Rheumatology (Oxford).* 2005.
27. Liuzzo G, Angiolillo DJ, Buffon A, Rizzello V, Colizzi C, Ginnetti F, Biasucci LM, Maseri A. Enhanced response of blood monocytes to in vitro lipopolysaccharide-challenge in patients with recurrent unstable angina. *Circulation.* 2001;103:2236-2241.

Chapter 9

10

Chapter 10

Summary and general discussion

SUMMARY

Although the pathogenesis of arterial thrombosis has been studied intensively in the past, a number of aspects remain unclear, thereby preventing an optimal management and therapy of patients with arterial thrombosis. Given the multifactorial nature of this disease, we sought to unravel the role of a number of genetic and environmental factors that are known to contribute to the complex pathogenesis of arterial thrombosis. Part I of this thesis addresses the first main objective, which is to investigate whether common variation in the *P2RY12* gene is a risk factor for arterial thrombosis and whether it plays a role in the efficacy of antiplatelet treatment. Part II of this thesis deals with the second main objective which is to study the biological variability of several important hemostatic factors and to investigate the role of air pollution.

Part I

Previous studies have shown that increased platelet aggregation is associated with an increased risk of arterial thrombosis. Since the P2Y12 receptor is a central mediator of platelet aggregation, it has been hypothesized that genetic variation in the P2Y12 receptor may increase platelet aggregation and in this way lead to an increased risk of arterial thrombosis. A number of recent studies have addressed this hypothesis, but their results were conflicting. However, these studies were relatively small and they covered only the 3' part of the *P2RY12* gene. Therefore, in **Chapter 2** we used a comprehensive approach to investigate, the association between the risk of arterial thrombosis and the total common variation in the *P2RY12* locus, *i.e.* the *P2RY12* gene and the regulatory regions, such as the promoter and the 3' untranslated region (UTR). For this purpose, we performed a case-control study (the *ATTAC* Study), in which we included 374 young patients between one to three months after their first arterial thrombotic event (coronary artery disease (CAD), ischemic stroke (IS) or peripheral arterial disease (PAD)) and 332 healthy controls. Male study participants were 45 years or younger, and females 55 years or younger. Previous studies suggest that in this relatively young study population the genetic contribution to arterial thrombosis is stronger than in an elderly population with clear atherosclerosis. We selected five haplotype-tagging single nucleotide polymorphisms (ht-SNPs) to represent the total common variation in the *P2RY12* gene (rs6798347 C>T, rs10935842 A>T, rs6787801 T>C, rs6801273 A>G and rs2046934 T>C). This selection of ht-SNPs was made on the basis of the linkage disequilibrium map of the SNPs from the *P2YR12* gene region that were genotyped for the International HapMap Project (phase I,

June 2005, *http://www.hapmap.org/*). Five common haplotypes were inferred from these five ht-SNPs and denoted H1: CATAc (17%), H2: CATAT (15%), H3: CtcgT (15%), H4: CtcAT (15%) and H5: tATgT (11%), with minor alleles given in lower case and allele frequencies between parentheses. Haplotype H5 was associated with an increased risk of arterial thrombotic events (odds ratio (OR) 1.7; 95% CI, 1.1-2.8) compared with the most common haplotype H1, which was selected as the reference haplotype. This association was most pronounced in the subgroup of patients with CAD (OR 2.0, 95% CI 1.2-3.6). To study whether increased platelet aggregation could explain the association between the haplotype H5 and the increased risk of arterial thrombosis, adenosine diphosphate (ADP)-induced light-transmittance aggregometry (LTA) was performed in 116 subjects from the healthy control group. However, the differences between the *P2RY12* haplotypes and maximal platelet aggregation, aggregation rate or late aggregation were minimal (at most 5% light transmittance), and were not statistically different. In conclusion, P2Y12 haplotypes are associated with an increased risk of a first arterial thrombotic event at young age, with CAD in particular. However, we did not find a significant difference between these haplotypes by studying light-transmittance aggregometry as a measure of ADP-dependent platelet aggregation.

Given the central role of the P2Y12 receptor in platelet aggregation, inhibition of the P2Y12-dependent platelet aggregation has proven to be an effective pharmacological strategy for the treatment of arterial thrombosis. Clopidogrel is currently the most widely used P2Y12 antagonist which, in combination with aspirin, significantly reduces the risk of recurrent atherothrombotic events in patients with acute coronary syndromes (ACS) and those who undergo percutaneous coronary interventions (PCI). However, there is a wide inter-individual variability in response of these patients to clopidogrel. Studies have suggested that patients with a sub-optimal platelet inhibition may be at higher risk of arterial thrombotic events. Conflicting results are seen in different studies that tested the hypothesis that the *P2RY12* gene variants are causative for this large between-subject variability in the efficacy of clopidogrel treatment. However, also these studies were relatively small and they focused only on the 3' part of the *P2RY12* gene. Therefore, we have performed in **Chapter 3** a more comprehensive study (the *POPular* Study) on the role of *P2RY12* gene variants in on-clopidogrel platelet reactivity. For this purpose, we included 1031 consecutive patients with CAD who were scheduled for elective PCI. To assess the residual on-clopidogrel platelet reactivity, platelet function measurements were performed by means of ADP-induced LTA (using 2, 5, 10 and 20 µmol/L ADP) and the Verify*Now* P2Y12 assay. Six ht-SNPs were selected based on the phase II release

(October 2007) of the HapMap Project to cover the common variation in the *P2RY12* gene (88% of total variation, remaining 12% are rare variants) and its flanking regions: rs6798347 C>T, rs6787801 T>C, rs9859552 C>A, rs6801273 A>G, rs9848789 G>A and rs2046934 T>C. Six common haplotypes with allele frequencies >10% were inferred from these ht-SNPs in our study population and denoted A: tTCgGT (10%), B: tTCAGT (6%), C: CTCAGc (15%), D: CTaAaT (15%), E: CcCgGT (21%) and F: CcCAGT (22%), in the descending order of their effects on LTA, with minor alleles in lower case and allele frequencies between parentheses. We observed a large inter-individual variability in on-clopidogrel platelet reactivity across all clopidogrel dosing-regimens. Haplotype F was associated with approximately 5% lower maximal and late ADP-induced LTA per haplotype allele, as compared to the reference haplotype A ($p<0.05$). In the Verify*Now* P2Y12 assay this difference was 11 P2Y12 reaction units (PRU) ($p<0.05$). Haplotype E was also associated with lower Verify*Now* P2Y12 assay (27 PRU less than haplotype A, $p<0.05$), but this association was less clear in the LTA and statistically not significant. The individual SNP analysis suggested that most of the observed haplotype effects were caused by rs6787801, or possibly another SNP that is in high LD with rs6787801. The observed associations were similar across the three clopidogrel dosing regimens and different ADP concentrations used in the LTA. In conclusion, common variation in the *P2RY12* gene is a significant determinant of the inter-individual variability in residual on-clopidogrel platelet reactivity in patients with CAD.

The large inter-individual variability in clopidogrel-induced platelet inhibition is one of the reasons that novel P2Y12 antagonists with potentially improved pharmacological features are being developed. Cangrelor (formerly known as AR-C69931MX) is one of these novel P2Y12 antagonists, which is intravenously administered and acts directly and reversibly on the P2Y12 receptor. Although cangrelor has a number of improved features compared to clopidogrel (*e.g.* no need for drug metabolism into an active metabolite), we hypothesized that common *P2RY12* gene variants might affect the efficacy of cangrelor via alterations of the structure and/or the expression of P2Y12 receptors. In **Chapter 4**, we have tested this hypothesis in 254 healthy subjects (the *Cangrelor* Study). Response to cangrelor in these subjects was assessed by measuring platelet function with ADP-induced LTA (using 5 and 20 μmol/L ADP) before and after addition of 0.05 and 0.25 μmol/L cangrelor to platelet-rich plasma. Study participants were genotyped for the six ht-SNPs that were selected in the *POPular* study (Chapter 3). Ht-SNP rs9848789 G>A was excluded from the analysis since in this healthy population there was 97% linkage disequilibrium between rs9848789 and another ht-SNP, rs9859552 C>A. Haplotypes

that were inferred from these five ht-SNPs were A: tTCgT (10%), B: tTCAT (9%), C: CTCAc (15%), D: CTaAT (14%), E: CcCgT (24%) and F: CcCAT (20%). We observed a large inter-individual variability in cangrelor-inhibited platelet aggregation, in particular with 20 μmol/L ADP-induced LTA. At baseline, there were no differences between the haplotypes or the individual ht-SNPs. After addition of 0.05μmol/L cangrelor, haplotype C was associated with higher maximal and late aggregation than the reference haplotype E (7% and 8% higher, respectively; both $p<0.05$). A similar trend was observed for haplotype D, although statistically not significant. In the individual SNP analysis, the rare alleles of SNPs rs6787801 and rs9859552 were consistently associated with lower LTA in the presence of 0.05 μmol/L cangrelor, as compared to their common alleles (between 5% to 7% lower, $p<0.05$). Associations between genetic variation and platelet function were less pronounced with 0.25 μmol/L cangrelor. Similar associations were observed for the haplotypes and individual ht-SNPs when cangrelor-induced platelet inhibition from baseline was considered. In conclusion, common variation in the *P2RY12* gene significantly contributes to the inter-individual variability in cangrelor-induced platelet inhibition in healthy subjects.

Sub-optimal platelet inhibition by clopidogrel has been suggested to increase the risk of secondary atherothrombotic events in PCI-treated patients (*e.g.* stent thrombosis). In addition to thrombotic events, a significant portion of these patients (5%-30%, depending on the type of treatment) suffers from in-stent restenosis within months after PCI. Platelets have been suggested to play a role in neointimal proliferation which is the most important mechanism underlying restenosis. Since the P2Y12 receptor is essential in platelet aggregation and is also expressed on the vascular smooth muscle cells (VSMCs) that are important in neointimal proliferation, we hypothesized that common *P2RY12* gene variants might also be associated with the risk of restenosis. We tested this hypothesis in **Chapter 5** in a prospective study (the *GENDER* Study) in 2062 clopidogrel-treated patients who underwent PCI with coronary stenting. Patients were followed for 12 months after the intervention for target vessel revascularization (TVR) as the primary endpoint. The secondary combined endpoint was defined as death presumably from cardiac causes, myocardial infarction due to occlusion of the target vessel, or TVR. Patients were genotyped for the same five ht-SNPs as in the *ATTAC* study (Chapter 2), to comprehensively cover the common genetic variation in the *P2RY12* locus. Seven haplotypes were identified designated H1: CATAT (16%), H2: tATAT (7%), H3: CATAc (16%), H4: tATgT (11%), H5: CtcAT (13%), H6: CAcAT (6%) and H7: CtcgT (18%). Haplotypes H5 and H7 were associated with a higher risk of TVR (hazard ratios (HR): 1.4, 95% C.I. 1.0-2.0 and 1.6,

95% C.I. 1.2-2.0, respectively) than the reference haplotype H1. In the individual SNP analysis, homozygotes for the minor alleles of SNPs rs10935842 A>T and rs6787801 T>C were associated with a higher risk of TVR than the homozygotes for the corresponding common alleles (HR: 1.9, 95% C.I. 1.1-3.0 and 1.7, 95% C.I. 1.1-2.7, respectively). The haplotype effects suggested that there is a complex interaction between individual SNPs within the haplotype. This study showed that common variation in the P2Y12 gene predicts restenosis in PCI-treated patients.

Part II

Risk of arterial thrombosis has frequently been associated with levels of hemostatic and inflammatory factors. However, in the majority of epidemiologic studies these levels are determined by single measurements which are then regarded as the true mean of an individual. However, levels may fluctuate around this true mean over time due to biological variation. Knowledge of the biological variation of a marker is important because it provides the basis for the calculation of the minimal number of measurements that are needed to assess an individual's true mean. In addition, biological variation is used to determine the analytical quality specifications for diagnosis, monitoring and prognosis of patients. For a number of hemostatic factors, biological variation has been previously determined, but for others no data were available. In **Chapter 6**, we determined in a longitudinal study on 40 healthy subjects the biological variation of platelet aggregation, thrombin generation and prothrombin time and the inflammatory markers fibrinogen and CRP. For this purpose, we collected 520 blood samples over a one-year period from these 40 individuals, and determined the between-subject, within-subject and seasonal variation of the studied factors. Based on these components of variation, we calculated that one or two repeated measurements were sufficient to establish the true habitual concentration, except for platelet aggregation and peak thrombin generation, where at least 4 and 9 repeated measurements were needed, respectively. For diagnosis, the maximal recommended coefficient of analytical variation (CV) was 4%-27%, except for CRP (77.7%). For monitoring, these CVs were on average 3% lower. Recommended analytical bias varied between 1.7% and 33.2%. Finally, we observed seasonal variation in concentrations of fibrinogen and thrombin generation, which could explain approximately 11% of their total variation.

Air pollution has consistently been associated with cardiovascular disease, including arterial thrombosis, and hemostasis and inflammation are suggested to be involved. In **Chapter 7** we reviewed the latest literature on the effects of particles on hemostasis and inflammation.

In **Chapter 8**, our study is described in which we measured throughout the study period of the biological variation study (Chapter 6) the environmental concentrations of the following air pollutants: particulate matter with aerodynamic diameter <10μm (PM_{10}), carbon monoxide, nitrogen monoxide, nitrogen dioxide and ozone. These measurements were performed at a monitoring site that covers the area where our study participants worked and/or lived. Exposure to these air pollutants was represented by average concentrations of air pollutants measured within different time-lags before blood sampling. Time-lags within 24h before blood sampling were considered to represent direct effects of air pollution on studied variables, whereas time-lags within 24-96h before blood sampling represented indirect effects. In general, PM_{10} concentrations were associated with indirect effects on platelet aggregation (average +8% per interquartile range (IQR) of PM_{10} concentration, $p<0.01$). There were no direct effects of PM_{10} (or other air pollutants) on platelet aggregation, which we confirmed *in vitro* by incubating plasma with different types of PM and in different concentrations. In addition, concentrations of gases were associated with indirect effects on thrombin generation (average +1% per IQR of endogenous thrombin potential and peak thrombin generation, and –1% per IQR of lag-time of thrombin generation, all $p<0.05$). No relationship was observed between air pollution and fibrinogen or CRP levels. We showed in this study that increased concentrations of ambient air pollution were associated with increased platelet aggregation as well as coagulation activity, but had no clear effect on systemic inflammation. This finding could provide a biological explanation for the association between air pollution and cardiovascular disease.

Biological variation of CRP, a sensitive acute-phase marker and an established independent predictor of CVD, is partly determined by the response of CRP to inflammatory triggers. In turn, the magnitude of this acute-phase reaction is dependent upon other factors, such as the strength of the inflammatory trigger and the rate of CRP synthesis. It has already been shown that steady-state CRP levels are determined by genetic factors, such as the common variation in the *CRP* gene. In **Chapter 9**, we tested the hypothesis that this common variation in the *CRP* gene is also a determinant of the response in serum levels of CRP after a standardized inflammatory trigger. This study was performed in a population of 264 healthy individuals who were exposed to a standardized inflammatory trigger in the form of a well-controlled surgical procedure of a kidney removal. Serum CRP levels were measured before and 1, 2 and 3 days after the surgery. Study participants were genotyped for three ht-SNPs that represent the total common variation in the *CRP* gene: rs1130864C>T, rs1205C>T and rs3093068C>G. Four haplotypes were inferred: CtC (32%), CCC (35%), tCC (27%) and CCg (6%), with rare alleles in lower cases.

On day 1 after the intervention, haplotypes tCC and CCC were associated with a stronger rise in CRP serum levels (average 9 mg/L and 14 mg/L per allele, respectively, $p<0.05$) relative to the reference haplotype CtC. At baseline, similar differences between haplotypes were observed, but these were statistically not significant. On day 2 and 3 after the surgery, the differences between the haplotypes had disappeared. In conclusion, the common variation in the *CRP* gene is a determinant of the CRP-response to a standardized inflammatory trigger in healthy individuals.

GENERAL DISCUSSION

The focus of this thesis is on the role of variability in hemostatic risk factors for arterial thrombosis. This chapter aims to integrally discuss the overall implications of the results that are described in previous chapters. Additionally, this chapter provides future recommendations based on the results from this thesis and other recent studies.

Part I – Role of common *P2RY12* gene variation in arterial thrombosis

Results of the studies that were performed in Part I of this thesis suggest an important role for the common variation in the *P2RY12* gene in arterial thrombosis. This role concerns predisposition to arterial thrombosis (*ATTAC* study, Chapter 2), the efficacy of platelet inhibition with P2Y12 receptor antagonists clopidogrel (*POPular* study, Chapter 3) and cangrelor (*Cangrelor* study, Chapter 4), as well as risk of secondary adverse outcome after clopidogrel treatment (*GENDER* study, Chapter 5).

In our *ATTAC* case-control study, *P2RY12* gene variation was associated with the risk of arterial thrombosis. However, we did not find statistically significant differences in ADP-induced LTA measurements between *P2RY12* haplotypes in our healthy controls. Similarly, in the *Cangrelor* study (Chapter 4), we did not find significant differences between *P2RY12* haplotypes for ADP-induced LTA measurements that were performed in an independent group of healthy volunteers at baseline, *i.e.* prior to the administration of cangrelor. Although in these two studies different sets of ht-SNPs were genotyped yielding different haplotypes with slightly different coverage of the *P2RY12* locus, the results from both studies suggest that there is no relationship between *P2RY12* gene variation and platelet aggregation in healthy subjects. It therefore remains unclear what the underlying mechanism is for the association between common *P2RY12* gene variation and increased risk of arterial thrombosis. Future studies are needed to validate the association between

common *P2RY12* gene variation and risk of arterial thrombosis observed in the *ATTAC* study and to reveal the mechanism underlying this risk association.

Our results from the *POPular* and *Cangrelor* study strongly suggest that the common variation in the *P2RY12* gene is a determinant of the efficacy of antiplatelet treatment with both the P2Y12 receptor antagonists clopidogrel and cangrelor. Previous studies suggest that "clopidogrel resistance" is multi-factorial, with various extrinsic and intrinsic underlying mechanisms (Table 1).[1-4]

Table 1. Potential mechanisms of clopidogrel resistance

Extrinsic mechanisms
Patient non-compliance
Under-dosing or inappropriate dosing of clopidogrel
Poor intestinal absorption of clopidogrel
Drug-drug interactions involving cytochrome P450 isoenzymes
Intrinsic mechanisms
Genetic variation
P2RY12 gene
Genes encoding cytochrome P450 isoenzymes (*e.g. CYP2C19*)
ABCB1 gene
Platelet hyper-reactivity (*e.g.* due to smoking or diabetes mellitus)
Increased release of ADP
Shortened platelet half-life
Alternate pathways of platelet activation
Failure to inhibit catecholamine-mediated platelet activation (epinephrine)
Greater extent of P2Y1-dependent platelet aggregation
Up-regulation of P2Y12-independent pathways (thrombin, thromboxane A2, collagen)

Potential mechanisms of clopidogrel resistance can be divided in extrinsic and intrinsic. Among the intrinsic mechanisms is common variation in the *P2RY12* gene. Table adapted from Nguyen *et al*.[1]

Among these mechanisms is the common variation in the *P2RY12* gene, which was described in this thesis.[5, 6] Both *POPular* and *Cangrelor* study were not designed to investigate the role of additional factors which have been suggested to contribute to clopidogrel resistance. Therefore, it remains difficult to speculate about the relative contribution of the common *P2RY12* gene variation to clopidogrel efficacy, as compared to these other potential determinants. Also, we cannot exclude the possibility that there is an interaction between the *P2RY12* gene variants and other (genetic) factors that are associated with clopidogrel efficacy. A specific interest in the

CYP2C19 variants was recently raised by a number of studies that have clearly shown that these *CYP2C19* variants affect the hepatic bio-activation of clopidogrel pro-drug and concomitant magnitude of platelet inhibition, as well as the risk of adverse ischemic cardiovascular events.[7-9] One of these studies also investigated the role of *P2RY12* variants, but found no association with clopidogrel efficacy, possibly due to an incomplete coverage of the gene.[9] Although it is unlikely that there is an interaction between the *P2RY12* and *CYP2C19* gene (both are located on different chromosomes and they do not share a single biological pathway), these recent findings warrant future studies on clopidogrel efficacy that include variants from both the *P2RY12* and *CYP2C19* gene. In addition, it would be interesting to see in a follow-up of the *POPular* study whether the common *P2RY12* gene variants are also associated with secondary adverse outcome, such as late stent thrombosis, and whether this association can then be explained by the observed effects of these variants on clopidogrel efficacy. Given the promising results of clinical trials on novel P2Y12 receptor antagonists, such as prasugrel, it would be interesting to study whether common variation in the *P2RY12* gene is also associated with the efficacy of these novel P2Y12 antagonists.[10-12] The haplotype effects observed in the *POPular* study were consistent across the three clopidogrel dosing regimens. This suggests that simply increasing the dosage of clopidogrel to overcome sub-optimal platelet inhibition in certain patients may not be sufficient, and that it may be advisable to reconsider current guidelines for clopidogrel therapy.

Restenosis is one of the secondary (long-term) complications associated with PCI. The results from our *GENDER* study clearly suggest that common variation in the *P2RY12* gene is associated with the risk of restenosis. Platelets play a role in the pathogenesis of restenosis, which is characterized by neointimal proliferation and VSMC migration.[13, 14] Potential effects of the *P2RY12* gene variation on this VSMC migration may involve altered release of platelet-derived cytokines, which normally stimulate VSMCs to migrate and reduce the in-stent lumen.[15] As the *GENDER* study did not include any measurements of platelet function or other platelet-derived factors that are involved in the underlying mechanisms of restenosis, it remains unclear what is causal for the observed association between *P2RY12* gene variation and risk of restenosis. The *GENDER* study population also included a subgroup of patients who had not received clopidogrel (n=548). Interestingly, in this subgroup of patients, the associations between *P2RY12* haplotypes and the risk of restenosis were more pronounced (unpublished results), suggesting a causal role for clopidogrel. However, these observations were not conclusive, as clopidogrel was not randomized over the two subgroups. Moreover, the patients who did not receive clopidogrel, neither received a stent. Therefore, the observed differences between the two subgroups (clopidogrel with stent *vs* no clopidogrel and no stent)

could also be caused by differences in treatment modalities and/or patient characteristics, and not only by clopidogrel treatment. Still, it seems plausible that clopidogrel treatment plays a major role in the relationship between the studied *P2RY12* gene variants and the risk of restenosis since the P2Y12 receptor is the exclusive target of clopidogrel. The *GENDER* study included patients who received a bare metal stent during the PCI, because at that time bare metal stents were most commonly used during the PCI procedure. Nowadays, bare metal stents have been mostly replaced by drug-eluting stents. The drugs that these novel stents elute inhibit neointimal proliferation and concomitantly reduce the risk of in-stent restenosis.[16] Therefore, it would be of interest to study whether the common *P2RY12* gene variation is also associated with the risk of restenosis when these drug-eluting stents are used. This might be of particular interest since several recent studies have shown that the relative risk reduction of restenosis for drug-eluting stents compared to bare metal stents is (partly) compensated for by an increase in the risk of late stent thrombosis due to delayed endothelialization of the drug-eluting stent and/or premature discontinuation of clopidogrel treatment.[17-19]

Part I of this thesis describes a number of significant associations between *P2RY12* haplotypes, platelet function and various cardiovascular endpoints. Over the time that the corresponding studies were successively designed, the information from online databases on the LD patterns across the *P2RY12* locus has increased. Consequently, we selected different sets of ht-SNPs with improved coverage of the *P2RY12* locus to genotype in different studies from this thesis. In addition, in each study different reference haplotypes were selected in the analyses making it difficult to compare the haplotype effects observed across these studies (Table 2). Interestingly, haplotypes C, D, E and F, which were all associated with lower ADP-induced platelet aggregation in clopidogrel-treated patients from the *POPular* study, were also associated with a lower risk of arterial thrombosis in the *ATTAC* study (haplotypes H1, H2, H3 and H4). The association between these haplotypes and the risk of restenosis in the *GENDER* study was less consistent, with haplotypes H7 and H5+H6 associated with higher risk of restenosis, whereas H3 and H1 were associated with lower risk. In addition, haplotypes E and F from the *Cangrelor* study were associated with lower on-cangrelor platelet reactivity, similarly to their analogous haplotypes E and F from the *POPular* study. For haplotypes C and D, on the other hand, the observed effects were opposite. If haplotype effects are compared within each study, there appears to be a complex interaction between ht-SNPs within the studied *P2RY12* haplotypes, rather than the existence of a single functional polymorphism that is responsible for all associations found between common *P2RY12* gene variation and platelet function and cardiovascular endpoints. The results from Chapter 3, 4 and 5 do, however, suggest the importance of ht-SNP rs6787801 that is located in intron 1 of the *P2RY12* gene.

Table 2. Comparative analysis of *P2RY12* haplotype effects from different studies described in this thesis

	ATTAC study (Chapter 2)	Odds ratio for arterial thrombosis		*POPular* study (Chapter 3)	Effect on 20µM ADP aggregation (total group)		*Cangrelor* study (Chapter 4)	Effect on 20µM ADP aggregation (0.05 µM cangrelor)		*GENDER* study (Chapter 5)	Hazard ratio for restenosis
Hapl. code	**A,B,C,-,D,-,E**	**Converted / Original**	**Hapl. code**	**A,-,C,C_2,D,D_2,E**	**Original**	**Hapl. code**	**A,-,C,C_2,D,-,E**	**Converted / Original**	**Hapl. code**	**A,B,C,-,D,-,E**	**Converted / Original**
H5	tAT-g-T (10%)	1.0 / 1.7*	**A (ref)**	t-TCgGT (10%)	0%	**A**	t-TCg-T (10%)	0% / +2%	**H4**	tAT-g-T (11%)	1.0 / 1.2
-	-	- / -	**B**	t-TCAGT (6%)	-1%	**B**	t-TCA-T (9%)	-6% / -4%	**H2**	tAT-A-T (7%)	0.8 / 1.0
H1(ref)	CAT-A-c (17%)	0.3 / 1.0	**C**	C-TCAGc (15%)	-1.5%	**C**	C-TCA-c (15%)	+5% / +7%*	**H3**	CAT-A-c (16%)	1.0 / 1.2
H2	CAT-A-T (15%)	0.5 / 1.2	**D**	C-TaAaT (15%)	-2.5%	**D**	C-TaA-T (20%)	+3% / +5%	**H1 (ref)**	CAT-A-T (16%)	0.8 / 1.0
H3	Ctc-g-T (15%)	0.7 / 1.4	**E**	C-cCgGT (21%)	-3%*	**E (ref)**	C-cCg-T (24%)	-2% / 0%	**H7**	Ctc-g-T (18%)	1.3 / 1.5*
H4	Ctc-A-T (15%)	0.4 / 1.1	**F**	C-cCAGT (22%)	-4%*	**F**	C-cCA-T (14%)	-4% / -2%	**H5+H6**	Ctc-A-T (19%)	1.2 / 1.4*

Each haplotype is listed with the code that was used in the corresponding study. The reference haplotype is indicated by (ref). Ht-SNPs: A,B,C,C2,D,D2,E, as they are located in the *P2RY12* locus from 5' to 3'. In each row, analogous haplotypes are listed. Since the *POPular* study had the best coverage of the *P2RY12* locus, its reference haplotype A is also used as the reference haplotype for the overall comparative analysis in this table.

Converted: haplotype that is corresponding to A (the reference haplotype from the *POPular* study) is set to 0% (or OR=1.0 and HR=1.0 in case of the *ATTAC* and the *GENDER* study, respectively), and the effects of other haplotypes from that study were adjusted consequently.
Original: haplotype effects as they are reported in their own study.

The haplotype from the *ATTAC* study which corresponds to haplotype B from the *POPular* study had a too low count and was excluded from the analysis. Allele percentages of haplotypes from the *ATTAC* study are given as they were observed for healthy controls. Haplotype effects on platelet aggregation are expressed per haplotype allele and given as percentage change from reference. *$p<0.05$ for the comparison with the haplotype that was used as a reference in the corresponding study.

This is strengthened by our most recent findings from an *in silico* analysis of the *P2RY12* locus, which indicated that several *P2RY12* promoter SNPs, which are in high LD with rs6787801, may indeed affect transcription (unpublished results). The reason for this is that they are located in, or near, regulatory elements of the *P2RY12* promoter, such as putative transcription factor binding sites (TFBS) and a CpG-island, which is potentially important for transcriptional regulation via DNA-methylation (Figure 1). Taken together, these observations warrant studies on the molecular mechanism of *P2RY12* SNPs underlying their association with platelet function and the risk of arterial thrombosis.

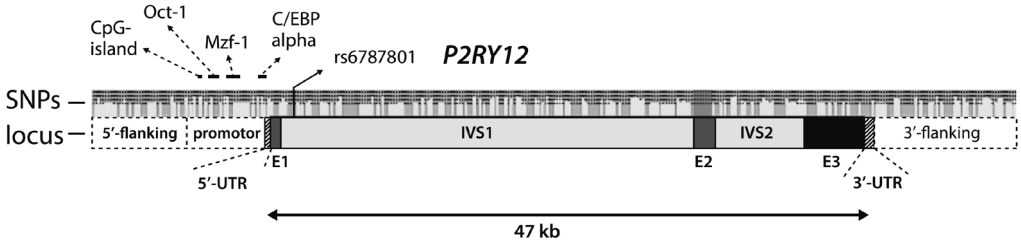


Figure 1. The *P2RY12* locus containing the *P2RY12* gene (47 kb) and its regulatory elements
Depicted regulatory elements are putative transcription factor binding sites (Oct-1, Mzf-1 and C/EBP alpha) and a CpG-island (important in DNA-methylation). SNPs which are located in, or in the vicinity of these regulatory elements may alter transcriptional regulation of the *P2RY12* gene and explain the effects that were observed for *P2RY12* haplotypes in the corresponding studies from this thesis. Position of SNP rs6787801 is indicated.

In conclusion, the efficiency and cost-effectiveness of treatment of arterial thrombosis, and CVD in general, can be significantly increased with improved models for identification of subjects at risk of CVD and of patients in need of additional or alternative (pharmaco)therapy. Results from this thesis strongly suggest that *P2RY12* haplotyping is such a useful stratification tool for identifying patients who are genetically predisposed to primary or secondary arterial thrombosis, and who may eventually benefit from additional or alternative antiplatelet treatment.

Part II – Biological variation of hemostatic and inflammatory factors involved in arterial thrombosis

In Part II of this thesis, several research questions have been addressed concerning the biological variation of hemostatic and inflammatory markers involved in arterial thrombosis. Our study described in Chapter 6 showed that the magnitude of between-subject variation and the biological within-subject variation can vary from as little as 3% for prothrombin time to as much as 108% for CRP. It is therefore important to take these

various components of test result variation into account when designing novel studies and when interpreting the results from existing studies.[20, 21] If we consider LTA, which was utilized in several studies described in this thesis, we calculated in Chapter 6 that in order to estimate the biological variation of LTA with less than 5% error, 9 repeated measurements are needed. The reason for this large number of samplings was a large within-subject variation, whereas the between-subject variation was relatively small. Many pre-analytical factors may contribute to this large within-subject biological variation. For instance, variation in blood collection and handling may lead to higher variation, although in our study blood handling was done according to a standardized protocol. Furthermore, the analytical variation itself is considerable for LTA, as witnessed by a within-run analytical CV of 14.1% that we have determined in our laboratory on a 4-channel aggregometer. Notably, a recommendation for 9 repeated measurements for LTA seems quite impractical for any guideline within a diagnostic laboratory. However, in practice, LTA may still be considered as one of the most useful assays for diagnosis of platelet disorders. The reason for this is that abnormal platelet disorders are often diagnosed by platelet aggregometry that is performed on a single platelet-rich plasma specimen using a set of different platelet agonists (*e.g.* thrombin, collagen, ADP), whereby each agonist provides information on one or several distinct platelet signaling pathways. The results from this set of platelet aggregations are then combined and interpreted in a mainly categorical manner (*i.e.* normal aggregation *vs* abnormal aggregation), whereby the differences between normal and abnormal aggregation are mostly quite obvious. Therefore, the detection of platelet disorders by means of LTA is not compromised by a large analytical and/or biological test variation.

However, these implications from our biological variation study for a diagnostic laboratory may be different from those for mechanistic studies, such as for studies that focus on *P2RY12* genetics. In the *ATTAC*, *POPular* and *Cangrelor* studies, LTA measurements showed a large between-subject variation, both in drug-naïve and cangrelor-treated healthy subjects, as well as in clopidogrel-treated CVD patients. In these studies, platelet aggregation was assessed by means of LTA, as it is considered the most optimal method available for measurements of platelet function.[22, 23] However, given the large analytical variability of LTA, novel, more improved platelet function tests are needed to overcome the methodological limitations of LTA for population- and patient-based studies. The benefit of such improved platelet function assays would be especially valuable for monitoring of the efficacy of antiplatelet therapy in the treatment of secondary arterial thrombosis.[24-27] Despite the limitations of LTA and the notion that only a single blood sample was taken in

the *POPular* and *Cangrelor* study, we were still able to detect significant differences in LTA between *P2RY12* haplotypes in these studies. This suggests that the observed haplotype effects on LTA were strong enough to be detected by an otherwise highly variable assay. Furthermore, in a large study population, such as the *POPular* study, a single blood sample is sufficient to estimate the between-subject variation of interest, as individual within-subject variations are averaged among the large number of study participants. In general, the estimates of between-subject variation improve with increasing sample size.[21]

To our knowledge, the study on biological variation (Chapter 6) is the first to report data on biological variation for ADP-induced platelet aggregation and thrombin generation. In this study, the biological variation of fibrinogen and prothrombin time was similar to that reported previously, whereas for CRP it was somewhat higher, probably due to differences in population's characteristics.[28-31] Knowledge of biological variation in the studied variables is crucial for the development of quality specifications and reference values for assays that measure these variables.[32] In turn, improved assay quality specifications will also aid the design of novel studies on corresponding hemostatic and inflammatory markers that are involved in CVD.[33-35]

In Chapter 8, we have demonstrated that increased levels of ambient air pollutants are associated with increased levels of platelet aggregation and thrombin generation in healthy volunteers. This association might provide a biological explanation for the association between increased levels of air pollution and increased risk of CVD that was frequently reported in previous epidemiologic studies.[36] Therefore, it would be informative to perform a similar longitudinal study in CVD patients as well. The aim of this study would then be to investigate whether the levels of air pollutants are indeed associated with the risk of CVD, and whether this association is explained by increased levels of CVD risk factors. Such a study would ideally include measurements of the actual exposure of study participants to air pollutants, for instance, by means of a portable device that records the levels of air pollutants throughout the study period. In this way, possible differences in exposure can be detected among study participants from otherwise "homogeneous" study population. Such differences in exposure could be the result of, for example, the way a person travels daily from home to work (*e.g.* bike *vs* car *vs* public transportation).[37]

In Chapter 9, we have shown that common variation in the *CRP* gene is a determinant of the acute phase response, as characterized by sharp rises in plasma CRP levels after a standardized trigger. We chose to study CRP levels since CRP is one of the most sensitive markers of acute phase. Further studies are needed to confirm our findings and to investigate whether the acute phase response that is evoked by other triggers is also

under genetic control. Interestingly, several family and twin studies in healthy subjects have shown that heritable factors account for around 50% of the overall between-subject variation in baseline CRP levels.[38-41] If CVD patients with a genetic predisposition to increased baseline CRP levels are considered in this model, their estimates for heritability would be even higher. Since increases in CRP levels from baseline are associated with the risk of CVD, it would be interesting to study the genetic component of an acute-phase response in CVD patients who are susceptible to inflammation (*i.e.* hyper-responsive to environmental acute phase stimuli), and in this way may be at increased risk of cardiovascular complications associated with inflammation.[42, 43]

REFERENCES

1. Nguyen TA, Diodati JG, Pharand C. Resistance to clopidogrel: a review of the evidence. *J Am Coll Cardiol.* 2005;45:1157-1164.
2. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, Costa MA. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. *J Am Coll Cardiol.* 2007;49:1505-1516.
3. Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. *Circulation.* 2003;107:2908-2913.
4. Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL, Topol EJ. Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol.* 2005;45:246-251.
5. Fontana P, Dupont A, Gandrille S, Bachelot-Loza C, Reny JL, Aiach M, Gaussem P. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation.* 2003;108:989-995.
6. Hetherington SL, Singh RK, Lodwick D, Thompson JR, Goodall AH, Samani NJ. Dimorphism in the P2Y1 ADP receptor gene is associated with increased platelet activation response to ADP. *Arterioscler Thromb Vasc Biol.* 2005;25:252-257.
7. Collet JP, Hulot JS, Pena A, Villard E, Esteve JB, Silvain J, Payot L, Brugier D, Cayla G, Beygui F, Bensimon G, Funck-Brentano C, Montalescot G. Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. *Lancet.* 2009;373:309-317.
8. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, Walker JR, Antman EM, Macias W, Braunwald E, Sabatine MS. Cytochrome p-450 polymorphisms and response to clopidogrel. *N Engl J Med.* 2009;360:354-362.
9. Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N, Steg PG, Ferrieres J, Danchin N, Becquemont L. Genetic determinants of response to clopidogrel and cardiovascular events. *N Engl J Med.* 2009;360:363-375.
10. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, Neumann FJ, Ardissino D, De Servi S, Murphy SA, Riesmeyer J, Weerakkody G, Gibson CM, Antman EM, Investigators T-T. Prasugrel versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med.* 2007;357:2001-2015.
11. Jernberg T, Payne CD, Winters KJ, Darstein C, Brandt JT, Jakubowski JA, Naganuma H, Siegbahn A, Wallentin L. Prasugrel achieves greater inhibition of platelet aggregation and a lower rate of non-responders compared with clopidogrel in aspirin-treated patients with stable coronary artery disease. *Eur Heart J.* 2006;27:1166-1173.
12. Raju NC, Eikelboom JW, Hirsh J. Platelet ADP-receptor antagonists for cardiovascular disease: past, present and future. *Nat Clin Pract Cardiovasc Med.* 2008;5:766-780.
13. Chandrasekar B, Tanguay JF. Platelets and restenosis. *J Am Coll Cardiol.* 2000;35:555-562.
14. Goel PK, Shahi M, Agarwal AK, Srivastava S, Seth PK. Platelet aggregability and occurrence of restenosis following coronary angioplasty. *Int J Cardiol.* 1997;60:227-231.
15. Gresele P, Page, C., Fuster, V., Vermylen, J. Platelets in thrombotic and non-thrombotic disorders. Cambridge: Cambridge University Press; 2002.
16. Fattori R, Piva T. Drug-eluting stents in vascular intervention. *Lancet.* 2003;361:247-249.

17. Roukoz H, Bavry AA, Sarkees ML, Mood GR, Kumbhani DJ, Rabbat MG, Bhatt DL. Comprehensive meta-analysis on drug-eluting stents versus bare-metal stents during extended follow-up. *Am J Med.* 2009;122:581 e581-510.

18. Shuchman M. Trading restenosis for thrombosis? New questions about drug-eluting stents. *N Engl J Med.* 2006;355:1949-1952.

19. Akbulut M, Ozbay Y, Karaca I, Ilkay E, Gundogdu O, Arslan N. The effect of long-term clopidogrel use on neointimal formation after percutaneous coronary intervention. *Coron Artery Dis.* 2004;15:347-352.

20. Reynolds TM, Twomey P, Wierzbicki AS. Accuracy of cardiovascular risk estimation for primary prevention in patients without diabetes. *J Cardiovasc Risk.* 2002;9:183-190.

21. Fraser CG. Biological variation: from principles to practice Washington, DC: AACC Press; 2001.

22. Michelson AD, Frelinger AL, 3rd, Furman MI. Current options in platelet function testing. *Am J Cardiol.* 2006;98:4N-10N.

23. Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood.* 2005;106:2723-2729.

24. Bonello L, Camoin-Jau L, Arques S, Boyer C, Panagides D, Wittenberg O, Simeoni MC, Barragan P, Dignat-George F, Paganelli F. Adjusted clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation index decrease rate of major adverse cardiovascular events in patients with clopidogrel resistance: a multicenter randomized prospective study. *J Am Coll Cardiol.* 2008;51:1404-1411.

25. Jakubowski JA, Payne CD, Li YG, Brandt JT, Small DS, Farid NA, Salazar DE, Winters KJ. The use of the VerifyNow P2Y12 point-of-care device to monitor platelet function across a range of P2Y12 inhibition levels following prasugrel and clopidogrel administration. *Thromb Haemost.* 2008;99:409-415.

26. Labarthe B, Theroux P, Angioi M, Ghitescu M. Matching the evaluation of the clinical efficacy of clopidogrel to platelet function tests relevant to the biological properties of the drug. *J Am Coll Cardiol.* 2005;46:638-645.

27. Price MJ, Endemann S, Gollapudi RR, Valencia R, Stinis CT, Levisay JP, Ernst A, Sawhney NS, Schatz RA, Teirstein PS. Prognostic significance of post-clopidogrel platelet reactivity assessed by a point-of-care assay on thrombotic events after drug-eluting stent implantation. *Eur Heart J.* 2008;29:992-1000.

28. Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M. *www.westgard.com/biodatabase1.htm.* 2008.

29. de Maat MP, de Bart AC, Hennis BC, Meijer P, Havelaar AC, Mulder PG, Kluft C. Interindividual and intraindividual variability in plasma fibrinogen, TPA antigen, PAI activity, and CRP in healthy, young volunteers and patients with angina pectoris. *Arterioscler Thromb Vasc Biol.* 1996;16:1156-1162.

30. Marckmann P, Sandstrom B, Jespersen J. The variability of and associations between measures of blood coagulation, fibrinolysis and blood lipids. *Atherosclerosis.* 1992;96:235-244.

31. Kluft C, de Maat MP. Determination of the habitual low blood level of C-reactive protein in individuals. *Ital Heart J.* 2001;2:172-180.

32. Buttner J. Reference materials and reference methods in laboratory medicine: a challenge to international cooperation. *Eur J Clin Chem Clin Biochem.* 1994;32:571-577.

33. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci.* 1989;27:409-437.

34. Fraser CG, Hyltoft Petersen P, Libeer JC, Ricos C. Proposals for setting generally applicable quality goals solely based on biology. *Ann Clin Biochem.* 1997;34:8-12.

35. Harris EK. Statistical principles underlying analytic goal-setting in clinical chemistry. *Am J Clin Pathol.* 1979;72:374-382.

36. Peng RD, Chang HH, Bell ML, McDermott A, Zeger SL, Samet JM, Dominici F. Coarse particulate matter air pollution and hospital admissions for cardiovascular and respiratory diseases among Medicare patients. *Jama.* 2008;299:2172-2179.

37. Janssen NA, Hoek G, Brunekreef B, Harssema H, Mensink I, Zuidhof A. Personal sampling of particles in adults: relation among personal, indoor, and outdoor air concentrations. *Am J Epidemiol.* 1998;147:537-547.

38. MacGregor AJ, Gallimore JR, Spector TD, Pepys MB. Genetic effects on baseline values of C-reactive protein and serum amyloid a protein: a comparison of monozygotic and dizygotic twins. *Clin Chem.* 2004;50:130-134.

39. Pankow JS, Folsom AR, Cushman M, Borecki IB, Hopkins PN, Eckfeldt JH, Tracy RP. Familial and genetic determinants of systemic markers of inflammation: the NHLBI family heart study. *Atherosclerosis.* 2001;154:681-689.

40. Retterstol L, Eikvar L, Berg K. A twin study of C-Reactive Protein compared to other risk factors for coronary heart disease. *Atherosclerosis.* 2003;169:279-282.

41. de Maat MP, Bladbjerg EM, Hjelmborg JB, Bathum L, Jespersen J, Christensen K. Genetic influence on inflammation variables in the elderly. *Arterioscler Thromb Vasc Biol.* 2004;24:2168-2173.

42. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Gallimore JR, Pepys MB. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. *BMJ.* 2000;321:199-204.

43. De Servi S, Mariani M, Mariani G, Mazzone A. C-reactive protein increase in unstable coronary disease cause or effect? *J Am Coll Cardiol.* 2005;46:1496-1502.

NEDERLANDSE SAMENVATTING

NEDERLANDSE SAMENVATTING

Het ziekteproces van arteriële trombose is nog niet volledig opgehelderd wat een optimale behandeling van patiënten met deze ziekte in de weg staat. Aangezien arteriële trombose een aandoening met meervoudige oorzaken is, is er in dit proefschrift getracht te onderzoeken wat de rol is van zowel erfelijke als omgevingsfactoren die betrokken zijn bij het ontstaan van arteriële trombose. De studies die beschreven zijn in Deel I van dit proefschrift hadden als doel te onderzoeken of de veelvoorkomende variaties in het *P2RY12* gen een risicofactor zijn voor arteriële trombose en of deze genetische variaties de effectiviteit van de bloedplaatjesremmende medicaties kunnen beïnvloeden. In Deel II van dit proefschrift worden de studies beschreven die als doel hadden om de biologische variatie van enkele belangrijke hemostase en ontstekingsfactoren te beschrijven en om de rol van de luchtverontreiniging hierbij te bestuderen.

DEEL I

Voorgaande studies hebben aangetoond dat een verhoogde mate van bloedplaatjesaggregatie geassocieerd is met een verhoogd risico op arteriële trombose. Gezien het gegeven dat de P2Y12 receptor een centrale rol speelt bij deze plaatjesaggregatie, is er verondersteld dat de veelvoorkomende variaties in het *P2RY12* gen zouden kunnen leiden tot een verhoogde plaatjesaggregatie en op die manier het risico op arteriële trombose zouden verhogen. Enkele voorgaande studies hebben deze hypothese al getoetst, maar hun resultaten waren tegenstrijdig. Dit zou mogelijk kunnen worden verklaard door hun kleine studieomvang en het gegeven dat in deze studies alleen werd gekeken naar de variaties in het 3'-uiteinde van het *P2RY12* gen. In **Hoofdstuk 2** werden de resultaten beschreven van een studie naar de variaties in het hele *P2RY12* gen, inclusief de regulerende genregio's. In de betreffende studie, genaamd de *ATTAC* studie, werden 374 patiënten onderzocht die op relatief jonge leeftijd arteriële trombose hadden doorgemaakt in de vorm van een coronaire hartziekte, een ischemisch herseninfarct of perifeer arteriëel vaatlijden. Deze patiënten werden vervolgens vergeleken met 332 gezonde vrijwilligers die als controle personen dienden. Mannen van 45 jaar en jonger, en vrouwen van 55 jaar en jonger werden geïncludeerd. 5 SNPs (veelvoorkomende puntmutaties) werden bestudeerd die samen 5 veelvorkomende haplotypes uit het *P2RY12* gengebied beschrijven. Een van deze haplotypen (allelfrequentie 11%) was geassocieerd met een 70% hogere kans op arteriële trombose

dan het referentie-haplotype dat in deze studie het meeste voorkwam (17%). De gevonden associatie bleek het sterkst te zijn in de subgroep van patiënten met coronaire hartziekte, die een twee keer zo hoge risico hadden. Vervolgens werd in 116 van de gezonde controles onderzocht of de gevonde relatie met het risico kon worden verklaard door een verhoogde mate van plaatjesaggregatie. Echter, de gevonden verschillen tussen de *P2RY12* haplotypen en maximale licht-transmissie aggregatie (LTA), aggregatie snelheid danwel aggregatie stabiliteit waren statistisch niet significant. Concluderend kunnen we vaststellen dat de *P2RY12* haplotypes geassocieerd zijn met het risico op het krijgen van arteriële trombose op jonge leeftijd, vooral in de vorm van coronaire hartziekte. De onderzochte *P2RY12* haplotypes zijn verder niet geassocieerd met de mate van ADP-geïnduceerde plaatjesaggregatie.

Gezien de centrale rol die de P2Y12 receptor speelt bij plaatjesaggregatie, is deze receptor een effectief aangrijpingspunt gebleken van de plaatjesremmende medicatie die wordt toegepast bij de behandeling van arteriële trombose. Clopidogrel is momenteel het meest gebruikte P2Y12 remmer die, in combinatie met aspirine, het risico op recidief arteriële trombose verlaagt. Er bestaat een aanzienlijke variatie in de respons op clopidogrel, en patiënten met een suboptimale plaatjesremming hebben een verhoogd risico op secundaire arteriële trombose. In **Hoofdstuk 3** wordt de *POPular* studie beschreven waarin de hypothese werd getoetst dat *P2RY12* genvarianten een rol spelen bij plaatjesreactiviteit onder clopidogrel gebruik. In totaal werden in de *POPular* studie 1031 patiënten geïncludeerd die wegens hun coronaire hartziekte een geplande dotterbehandeling moesten ondergaan en hiervoor clopidogrel kregen toegediend. Plaatjesreactiviteit werd bij deze patiënten gemeten met LTA en de Verify*Now* P2Y12 methode. Patiënten werden gegenotypeerd voor 6 ht-SNPs die geselecteerd werden op basis van de HapMap database (fase II, oktober 2007) en die een dekking gaven van 88% van alle veelvoorkomende variaties in het *P2RY12* gen en de flankerende regio's. Van deze 6 ht-SNPs werden in totaal 6 haplotypes afgeleid met een allelfrequentie >10%. Net als in voorgaande studies werd ook hier een grote variatie in plaatjesreactiviteit gevonden. In vergelijking met het referentie haplotype (10%), waren twee haplotypes (beide met allelfrequentie >20%) geassocieerd met lagere plaatjesreactiviteit, gemeten met zowel LTA als de Verify*Now* P2Y12 assay. De resultaten van de individuele SNP analyse suggereerden dat de meeste waargenomen haplotype effecten veroorzaakt waren door rs6787801, of een andere SNP die in hoge mate gecorreleerd is met rs6787801. Concluderend, veelvoorkomende variaties in het *P2RY12* gen dragen bij patiënten met coronaire hartziekte in significante mate bij aan de variatie in plaatjesreactiviteit onder clopidogrel gebruik.

De grote variatie in clopidogrel-gemedieerde plaatjesremming is een van de redenen voor de ontwikkeling van nieuwe P2Y12 remmers met verbeterde farmacologische kenmerken. Cangrelor is een van deze nieuwe P2Y12 antagonisten die intraveneus wordt toegediend en die direct en irreversibel de P2Y12 receptor remt. In **Hoofdstuk 4** werd de hypothese getoetst dat *P2RY12* genvariaties de effectiviteit van cangrelor zouden kunnen beïnvloeden ondanks deze betere farmacologische kenmerken, bijvoorbeeld via een verandering in de structuur en/of de expressie van P2Y12 receptoren. Deze *Cangrelor* studie werd uitgevoerd onder 254 gezonde vrijwilligers bij wie plaatjesrijk plasma werd afgenomen en plaatjesfunctie werd gemeten met ADP-geïnduceerde LTA vóór en na toediening van 0.05 en 0.25 µmol/L cangrelor. De studiepopulatie werd gegenotypeerd voor de 6 ht-SNPs die ook voor de *POPular* studie werden geselecteerd (Hoofdstuk 3). Er werd een grote variatie in cangrelor-gemedieerde plaatjesremming gevonden. Bij metingen in monsters waaraan geen congrelor was toegevoegd waren er geen significante verschillen tussen de haplotypes waar te nemen, evenmin tussen de individuele SNPs. Echter de mate van plaatjesremming door cangrelor was wel geassocieerd met P2Y12 haplotypes. In de analyse van de individuele SNPs waren de zeldzame allelen van SNPs rs6787801 en rs9859552 consistent geassocieerd met een lagere LTA in de aanwezigheid van cangrelor, in vergelijking met hun wild-type allelen. Concluderend, veelvoorkomende variaties in het *P2RY12* gen dragen significant bij aan de variatie in cangrelor-gemedieerde plaatjesremming bij gezonde mensen.

Sub-optimale plaatjesremming door clopidogrel bij patiënten die een dotterbehandeling hebben ondergaan wordt vaak in verband gebracht met een verhoogd risico op secundaire atherothrombotische events, zoals stent trombose. Naast deze trombotische complicaties krijgt een significant deel van deze patiëntengroep (5%-30%, afhankelijk van het type behandeling) binnen enkele maanden te maken met in-stent restenose. P2Y12 receptor wordt tot expressie gebracht op de vasculaire gladde spiercellen die een belangrijke rol spelen bij neointima proliferatie. In **Hoofdstuk 5** wordt de *GENDER* studie beschreven waarin de hypothese werd getoetst dat *P2RY12* varianten geassocieerd zijn met het risico op restenose. In deze studie werden 2062 patiënten geïncludeerd die een dotterbehandeling met coronaire stent-implantatie ondergingen en hiervoor clopidogrel kregen toegediend. De patiënten werden gevolgd gedurende 12 maanden na de behandeling voor het primair eindpunt van revascularisatie van het behandelde vat (TVR). Het secundaire eindpunt werd gedefiniëerd as dood door een waarschijnlijk cardiale oorzaak, myocardinfarct door occlusie van een ander dan behandeld vat, of TVR. De patiënten werden gegenotypeerd voor dezelfde set van 5 ht-SNPs als in de *ATTAC* studie (Hoofdstuk 2).

Van deze ht-SNPs werden in totaal 7 haplotypen afgeleid waarvan 2 haplotypen geassocieerd bleken te zijn met een 40%, respectivelijk 60% hoger risico op TVR dan het referentie haplotype. In de individuele SNP analyse waren de zeldzame allelen van SNPs rs10935842 en rs6787801 geassocieerd met een 90%, respectivelijk 70% hoger risico op TVR in vergelijking met hun wild-type allelen. De waargenomen haplotype effecten suggereerden dat er een complexe interactie bestaat tussen de individuele SNPs binnen een haplotype. Deze studie liet zien dat de veelvoorkomende variaties in het *P2RY12* gen restenose kunnen voorspellen in patiënten die een dotterbehandeling hebben ondergaan.

DEEL II

Het risico op arteriële trombose wordt vaak in verband gebracht met de spiegels van hemostase en ontstekingsfactoren in bloed. Echter, in de meeste studies worden deze spiegels bepaald aan de hand van één enkele bloedafname dat vervolgens als representatief wordt beschouwd voor het ware gemiddelde van een individu. Dit is vaak niet correct aangezien de betreffende spiegels in de tijd kunnen fluctueren rondom dit ware gemiddelde door de biologische variatie. Kennis van deze biologische variatie is dus belangrijk, omdat het de basis vormt voor de berekening van het minimaal aantal herhaalde metingen dat vereist is om met goede precisie het ware gemiddelde van een persoon te kunnen vaststellen. Daarnaast wordt biologische variatie gebruikt voor de kwaliteitscontrole van assays die voor de diagnostiek, het monitoren en de prognose van patiënten worden toegepast. In **Hoofdstuk 6** wordt een longitudinale studie beschreven waarin bij 40 gezonde vrijwilligers de biologische variatie werd bepaald van plaatjesaggregatie, trombine generatie en protrombine tijd, en de ontstekingsmarkers fibrinogeen en CRP. In totaal werden er 520 bloedmonsters afgenomen gedurende een vervolgperiode van 1 jaar waarin vervolgens de tussenpersoons-, binnenpersoons en seizoensvariatie van deze factoren werden bepaald. Op basis van deze variatiecomponenten hebben we berekend dat 1 of 2 herhaalde metingen voldoende waren om de “ware” concentratie vast te kunnen stellen. Uitzonderingen hierop vormden de plaatjesaggregatie en de piek van de trombine generatie waarvoor minimaal 4, respectievelijk 9 herhaalde meting waren vereist. Er werd berekend dat de bestudeerde assays in de diagnostiek een maximale coefficiënt van analytische variatie (CV) dienen te hebben tussen 4% en 27%, behalve voor de CRP bepalingen (77.7%). Voor het monitoren vielen deze CVs gemiddeld 3% lager uit. De maximaal toegestane analytische bias variëerde tussen 1.7% en 33.2%. Verder namen

we een seizoensvariatie waar in de concentraties van fibrinogeen en trombine generatie die ongeveer 11% van de totale variatie in de spiegels van deze factoren kon verklaren.

Luchtverontreiniging wordt veelvuldig in verband gebracht met hart- en vaatziekten, waaronder arteriële trombose. Verstoorde hemostase en ontsteking worden hierbij vaak als oorzakelijk aangeduid. In **Hoofdstuk 7** wordt recente literatuur samengevat en besproken waarin de effecten van luchtverontreiniging op hemostase en ontsteking werden onderzocht.

In **Hoofdstuk 8** werden gedurende de gehele periode van de biologische variatie studie (Hoofdstuk 6) de omgevingsconcentraties gemeten van partikels met een luchtdiameter <µm (PM_{10}), koolstofmonoxide, stikstofmonoxide, stikstofdioxide en ozon. De meetlocatie waarop deze metingen werden verricht was representatief voor het hele gebied waarin de studiepersonen hadden gewerkt en/of gewoond. De mate van blootstelling binnen deze studiepopulatie werd geschat aan de hand van de gemiddelde concentraties aan luchtverontreinigingscomponenten die binnen verschillende tijdsperiodes (genaamd lags) voorafgaand aan de bloedafnames werden gemeten. Er werd aangenomen dat lags die binnen de 24 uurs periode voor een bloedafname lagen de directe effecten van luchtverontreiniging op de gemeten variabelen voorstellen, terwijl de lags die binnen 24-96h voor een bloedafname lagen de indirecte effecten voorstellen. Over het algemeen waren de PM_{10} concentraties geassocieerd met indirecte effecten op plaatjesaggregaties (gemiddeld +8% per interkwartiel range (IQR) van PM_{10} concentraties). We hebben geen directe effecten van PM_{10} of andere vormen van luchtverontreiniging op plaatjesaggregaties waargenomen. Deze bevinding hebben we ook bevestigd *in vitro* door plasma te incuberen met verschillende typen partikels en in verschillende concentraties. De concentraties aan gasvormige componenten van luchtverontreiniging waren geassocieerd met indirecte effecten op trombine generatie (gemiddeld +1% per IQR voor endogene trombine potentiaal en de piek van trombine generatie, en -1% per IQR voor de lag-time van trombine generatie). Er werd geen relatie waargenomen tussen de luchtverontreiniging en fibrinogeen, danwel CRP concentraties. In deze studie is aangetoond dat verhoogde concentraties van luchtverontreiniging in de leefomgeving geassocieerd zijn met verhoogde plaatjesaggregatie en bloedstolling, zonder een duidelijk effect op systemische ontsteking. De bevindingen uit deze studie vormen een mogelijke biologische verklaring voor de relatie tussen de luchtverontreiniging en hart- en vaatziekten.

CRP is een gevoelige marker van acute fase en een onafhankelijke voorspeller van hart- en vaatziekten. Biologische variatie van CRP wordt deels bepaald door de respons van CRP op een ontsteking. De mate waarin deze acute fase reactie plaatsvindt,

is afhankelijk van andere factoren, zoals sterkte van de ontsteking en de mate van CRP vorming. In **Hoofdstuk 9** werd de hypothese getoetst dat *CRP* genvarianten de respons in serum CRP spiegels tijdens ontsteking kunnen bepalen. Dit werd onderzocht in 264 gezonde vrijwilligers die een ontstekingsreactie ondergingen na een chirugische ingreep waarbij een nier werd verwijderd ten behoeve van een donatie. Serum CRP spiegels werden in deze studiepersonen gemeten voor de ingreep, en 1, 2 en 3 dagen na de ingreep. De studiepopulatie werd gegenotypeerd voor 3 ht-SNPs die representatief zijn voor de totale veelvoorkomende variatie in het *CRP* gen. Van deze ht-SNPs werden 4 haplotypes afgeleid. Op dag 1 na de ingreep waren 2 haplotypes geassocieerd met een sterke stijging van de CRP serum spiegels (respectievelijk gemiddeld 9 mg/L en 14 mg/L per haplotype allel) in vergelijking met het referentie haplotype. Vergelijkare verschillen werden ook waargenomen tussen deze haplotypes bij serum monsters die voor de ingreep werden afgenomen, maar deze verschillen waren statistisch niet significant. Op dag 2 en 3 na de ingreep waren de verschillen nagenoeg verdwenen. Concluderend, de veelvoorkomende variaties in het *CRP* gen zijn een determinant van een ontstekingsgemedieerde CRP respons in gezonde individuen.

DANKWOORD

Na al die keren dat ik andermans dankwoord heb mogen lezen, is het nu dan eindelijk aan mij de beurt om er zelf een te mogen schrijven. Gaandeweg het tikken van deze eerste woorden realiseer ik me hoezeer mijn promotietijd één grote reis is geweest die ik met velen om me heen heb mogen maken. Een reis met dit proefschrift als eindbestemming, maar ook een die enkele waardevolle vriendschappen en samenwerkingen heeft voortgebracht, die ongetwijfeld ook in de toekomst nog meer leuke momenten gaan brengen. Hieronder doe ik een poging om alle mensen te bedanken die op enigerlei wijze hebben bijgedragen aan de totstandkoming van dit proefschrift, hopende dat degenen die ik hier oversla weten dat ik ze minstens evenveel dank ben verschuldigd.

Allereerst wil ik mijn promotor prof.dr. **Frank Leebeek** bedanken. Beste Frank, dit is dan eindelijk het moment dat ik je veelgestelde vraag: “En, is het al af?” volmondig met “Ja!” kan beantwoorden. Bedankt voor het in mij gestelde vertrouwen dat ik dit meerjarentraject tot een succesvol einde zou brengen. Je vertrouwen in mij waardeer ik des te meer wetende dat niet iedereen in jouw positie de gok had durven nemen met een werknemer die een spierziekte heeft. Ik bewonder jouw grote drive in de wetenschap en in de kliniek, maar tevens je kenmerkend gevoel voor humor waarmee je net zo goed de zaken weet te relativeren. Je hebt een benijdenswaardig geheugen voor namen en wetenschappelijke publicaties (altijd handig om een wandelende PubMed in de buurt te hebben). Het was voor mij een zeer leerzame en prettige ervaring om met jou te mogen samenwerken. Ik wens jou, je echtgenote en je leuke kinderen al het goede toe!

Dr. **Moniek de Maat**: beste Moniek, vanaf dag 1 was jij naast Frank betrokken bij mijn promotie. Ook aan jou ben ik enorm veel dank verschuldigd, te beginnen bij de kans die je mij hebt gegeven om mij binnen de werkgroep te kunnen ontplooien en uiteindelijk om mezelf te kunnen bewijzen. Je stond altijd voor me klaar met je advies, je tips en je correcties op al die versies manuscripten, abstracts en presentaties. Onze vele emails en telefoontjes (ook in het weekend, op je vakanties en tijdens congresreizen) zijn daar getuige van. Ik bewonder je mensenkennis en je netwerk skills. Je vriendelijke manier van interactie zorgde er altijd voor dat ik me bij jou op mijn gemak voelde en mijn geest de vrije loop kon laten tijdens onze brainstormsessies. Die fijne omgang hield mij ook in minder plezierige fasen van mijn promotietraject gemotiveerd om er toch tegenaan te blijven gaan. Hartelijk dank daarvoor. Ik wens jou en Dick al het geluk toe, en ik kijk uit naar onze toekomstige samenwerking!

De overige leden van mijn promotiecommissie, prof.dr. **Hugo ten Cate**, prof.dr. **Maarten Simoons**, prof.dr. **Andre Uitterlinden**, prof.dr. **Bob Löwenberg**, prof.dr. **Jan-Willem Akkerman** en dr. **Eric Sijbrands** wil ik bedanken voor hun bereidwilligheid om mijn proefschrift te beoordelen.

Drs. (soon to become dr.) Bongers: Beste **Tamara**, waar moet ik beginnen? Wij zijn zo'n beetje tegelijkertijd begonnen aan ons avontuur binnen de wetenschap en hebben ontzettend veel samen meegemaakt. Ik heb het erg leuk en motiverend gevonden om vanaf dat begin bij elkaar betrokken te zijn, vanaf het opzetten van onze projecten tot en met het schrijven van onze boekjes. Bedankt dat je al die tijd mijn klaagmuur wilde zijn wanneer ik weer eens de behoefte had om mijn frustraties te ventileren (ook al compenseerde je dat ruimschoots met je talloze verhalen over Italië). En dan al die mooie congresreizen die we samen hebben beleefd (ik denk alleen al aan ISTH 2005 in Sydney). Je bent mijn onbetwiste nr. 1 rolstoelduwer. Eentje die niet terugdeinst om met een volle sprint de helling naar Victoria Building te nemen. Ik heb veel van je geleerd, in het bijzonder je kijk op de dingen des levens (my personal Dr. Phil!), en heb bewondering voor je positivisme en je vermogen om nooit contactschuw te zijn wanneer je de zaken snel geregeld wilt krijgen. Bedankt dat je mijn paranimf wilt zijn en bedankt dat je mij gevraagd hebt "to return the favours". Succes met de laatste loodjes rondom je eigen boekje en je toekomstige dokterscarrière! Ik wens je het allerbeste toe, evenals je gastvrije ouders en je zusje. We houden contact!

Dr. Guimarães, beste **Ana**: mijn wijze roommate uit Portugal, die samen met nog een wijze postdoc (Eric van den Akker) in 2004 opeens opgescheept kwam te zitten met die nieuwe AIO op jullie kamer. Het was voor mij in het begin even wennen, maar al gauw werd het supergezellig. Wat hebben we toch veel afgelachen. Over muziek, over onze vakanties, ons gebrekkig Nederlands en al die rare woorden (kikiriki, kokoš...). Ik vond het gaaf om Ee-1330d te mogen delen met een stoere surfchick zoals jij, die jaarlijks zonder angst de golven van Bretagne trotseert. Je bent onze koningin van GraphPad (lees Graf-pad) en Origin; ik blijf me nog steeds verwonderen over de hoeveelheid grafieken die jij op één dag weet te produceren. Petje af voor je kookkunsten en je enorme kennis van goede restaurants in Rotterdam en daarbuiten (met jou heeft niemand nog een Michelin gids nodig). Ook leuk om nog een Audi-fan in mijn vriendenkring te hebben (wanneer komt die RS6 Avant nou 'ns een keer?). Je hebt al een tijdje geleden de academische wereld verlaten

en bent de flitsende zakenwereld ingegaan. Desondanks hield je nog steeds contact met je stoffige collega uit Erasmus MC, want zo ben je ook wel weer. Sterker nog, je hebt je drukke agenda van 3 november opzij gezet om die dag mijn paranimf te kunnen zijn. Daarvoor, en voor al het andere, bedankt! En graag tot weer een gezellige borrel of een lekker etentje met je trouwe oud-collega's. Moge je zeilen, en die van Win(d)frie(n)d, altijd in de wind staan!

De rest van de stollingsdames wil ik ook hartelijk danken voor al hun geestelijke en lichamelijke hulp (dus ook voor al dat duwen, hijsen, schouder bieden, etc.), maar uiteraard ook voor hun enorme gezelligheid (het is altijd weer een feest met ze), een luisterend oor en op zijn tijd een wijze raad. Beste **Marianne**, bedankt voor je empathie en minstens zoveel vrolijkheid. Ik bewonder je doorzettingsvermogen (je komt er echt wel!) en koester onze diepgaande gesprekken in crisistijden. Succes met je VWF quest! (Wanneer komt nou dat dagje A'dam met Jeroen?). **Eva**, bedankt dat je over mij en Jasper wilde waken. Dat soort boys hebben ook op hun werk een moederfiguur nodig, en jij was er altijd voor ons. Als ik aan jou denk, denk ik aan je betoverend pianospel. Veel gezondheid en geluk voor jou en je gezin! **Joyce**, het was leuk om een prettig gestoorde suikerverslaafde Helmondse op het lab te hebben gekend. Bedankt nog voor al je labassistenties door de tijd heen, en ik zou zeggen: *blijf lachen totdat oewen boks op skuppe zeuve hangt* (ofzoiets). **Simone**, mijn enige hemostase mede-medisch-bioloog; wat was het fijn om een studiesoortgenote in mijn midden te hebben tussen al die artsen en analisten (die zijn ook wel leuk, maar op een andere golflengte, zeg maar). Bedankt voor je gezelligheid en succes met je proefjes en al die ingewikkelde pipetopstellingen. **Janine**, ondanks dat ik jou het kortste ken binnen onze hemostase groep, was het met jou minstens zoveel lachen geblazen en er was gewoon een leuke interactie. Ook jou wens ik veel succes met je onderzoek. **Marjolein**, bedankt voor al je hulp op het lab. Thumbs up voor het feit dat je toch nog al die P2Y12 mutanten hebt weten te kloneren voor je vertrek naar een andere afdeling. Ik vond het erg leuk om zo af en toe naast je te kunnen pipetteren. All the best! Altijd lachende **Kim**, bedankt voor de vrolijkheid die je steeds met je meebracht. Jammer dat ik er pas zo laat achterkwam hoe goed je kennis van Apple-gadgets is, anders hadden we ongetwijfeld nog meer gespreksstof gehad. Je hebt mijn waardering voor je persoonlijke hulp aan weeskindjes in Azië. Veel geluk toegewenst! **Elim**, good luck with the last phase of your PhD internship and all the best to you and your family. **Elisabeth**, bedankt voor al je steun in de begindagen van mijn promotie en graag tot binnenkort.

En dan de stollingsjongens; wat waren wij in de minderheid, zeg, vergeleken met al die dames op het lab! Maar zo nu en dan leek het of we dik in de meerderheid waren, als we wederom om onze inside-jokes moesten lachen, terwijl niemand ze door had. Beste **Steffen**, eerlijk gezegd had ik in het begin van mijn promotietraject niet verwacht dat ik bevriend zou kunnen raken met een collega die op het eerste gezicht een pimp-my-Prelude Wu-Tang Clan homie leek te zijn. Maar al gauw ontdekte ik wat een goeie gast je bent. Eentje waarop ik kon bouwen en waar ik veel gemeenschappelijke gespreksthema's mee had. Ik ben onze vriendschap door de jaren heen steeds meer gaan koesteren. Bedankt dat je op mijn promotie de fotograaf wilt uithangen. Ik moet zeggen dat die hobby van jou ondertussen iets heel moois heeft opgeleverd waarvoor je mijn bewondering hebt. Ik wens jou, Albertina en de kleine op komst veel geluk toe! **Lamberto**, velen hebben je niet kunnen ontcijferen, je blijft een aparte vogel. Maar wel een waarmee ik erg veel heb kunnen lachen. Bedankt voor al je labsteun en leuke tijd met Mojito Racing. Laat me weten als je ooit nog rolstoel-salsalessen denkt te gaan geven. Voor nu heel veel succes met je studie en als je daadwerkelijk die stap maakt om terug te keren naar je tropische Curaçao, dan hoop ik dat je daar je gezochte succes weet te vinden. Van het L-vleugel, **Jasper**, the ladykiller. Het was altijd lachen met jou tijdens onze pauzes en kroegtochten. Je bent de enige die mijn afkeer tegen boeken hebt weten in te perken en me aan het lezen heeft gebracht met je titels die bedoeld zijn om wat "levenswijsheid" op te doen. Succes als je straks zelf het AIO-pad gaat bewandelen. **Dick Rijken**, onze fibrinolyse goeroe. Ik noem je naam bewust in deze paragraaf over jonge honden, want ondanks het verschil in rang (en een beetje in leeftijd) ging je op congressen altijd mee met de AIO's wat reuze gezellig was. Ik ben je daarnaast zeer dankbaar voor alle steun tijdens onze gezamenlijke reizen. Ik vond het een eer om samen met jou mijn eerste (hopelijk niet de laatste) business class vliegreis mee te maken. Veel geluk! **Emile de Bruijne**, onze ATTAC man, wil ik danken voor zijn nuttige (klinische) input tijdens de beginmaanden van mijn promotietraject, maar vooral ook voor de gezelligheid. Ik denk met weemoed terug aan onze gesprekken over first person shooters. Jammer dat het er door tijdgebrek nooit van kwam om elkaar online in het vizier te krijgen. Succes met je eigen boekje en ik wacht nog steeds met spanning op je eigen promotie. Veel succes in Harderwijk!

Mijn studenten **Marlies**, **Femke** en **Narcisse** wil ik bedanken voor hun ondersteuning bij mijn projecten en ze succes wensen in hun verdere carrière.

Alle andere collega's van de 13^{e} wil ik bedanken voor de leuke tijd en hun constante

bereidwilligheid om me zo nu en dan te helpen met iets van een hoge plank te halen of iets zwaars te verplaatsen. Bedankt voor de ontelbare lunchuurtjes aan gezelligheid in onze bieb en de maffe tijd tijdens onze labuitjes en weekenden weg (ik ben nog steeds mijn trauma niet te boven van die nachtelijke chocoladegemaskerde terroristen uit Lochem). In het bijzonder wil ik mijn kamergenoten bedanken: **Eric van den Akker**, bedankt voor de gezellige tijd en je muzikale educatie (je lijst met Top-10 bands allertijden kan ik nog steeds niet onthouden, te exotisch voor mijn smaak). Succes bij het RIVM en veel geluk met je gezin! En 'tuurlijk zal ik niet vergeten om je mijn boekje te sturen! **Stefan Erkeland**, onze kleine grote microRNA man: ook jou wil ik bedanken voor de leuke tijd op onze kamer (ook al ben je bijna altijd aan het pipetteren) en tijdens de BBQs bij je thuis. Ik hoop dat je succes blijft boeken ook nu met je eigen werkgroep. Het allerbeste toegewenst aan jou, Sabine en je leuke kids! **Arturo Sanz-Sanz**, muchas gracias for our pleasant conversations and your support. Good luck with all those difficult calculations you are doing! **Saman Abbas**, the rebel. Je was dan geen kamergenoot van me, maar wel een goede maat op het lab en daarbuiten. Ik ben jaloers op je enorme filmkennis; je bent een wandelende IMDB. Ik wens jou en je familie veel geluk toe!

Alle mensen van het **hemostase- en transfusielab** op de L-vleugel van de 4e wil ik bedanken voor hun ondersteuning rondom al het prikken, afdraaien en stickeren, maar bovenal dat jullie me zo snel hebben opgenomen in jullie gezellige groep, toen ik daar dagenlang aggregaties aan het meten was. Mag ik nog steeds af en toe bij jullie op de taart komen? In het bijzonder wil ik **Huub van Vliet** danken voor het delen van zijn onuitputtelijke kennis van biochemische pathways die mij hebben geholpen om "het P2Y12 gebeuren" zoveel mogelijk te doorgronden.

Ik wil de mensen van de 5e verdieping bedanken (**Pascal**, **Lisette**, **Rowena** en **Saskia**) voor hun waardevolle ondersteuning met al mijn TaqMan genotyperingen, evenals onze gezellige praatjes.

I would also like to thank **Aaron Isaacs** from the Epidemiology department for his valuable input in genetic data-analysis and for the pleasant talks. I am looking forward to continue our collaboration.

Verder wil ik alle coauteurs van mijn publicaties bedanken voor de aangename samenwerking. In het bijzonder wil ik de volgende namen noemen: **Wouter van Werkum**,

je hebt mijn bewondering voor je gigantische drive in de wetenschap. Ik werd af en toe wel een beetje gek van al je telefoontjes of de paper al af was, maar waardeerde je betrokkenheid in deze des te meer. Ik denk dat we allemaal enorm trots kunnen zijn op het uiteindelijke resultaat. **Heleen Bouman**, ook jou wil ik bedanken voor de fijne samenwerking. Ik had niet gedacht dat het uitleggen van haplo.stats aan iemand toch nog zo leuk kon zijn. Succes met je promotieonderzoek, je gaat nu al als een speer. **Chris Hackeng** wil ik vooral bedanken voor de relaxte sfeer waarin ik met hem en zijn groep heb mogen samenwerken. Daar hebben die avonden aan de bar in Bormio zeker aan bijgedragen. Alle betrokkenen uit de groep van prof.dr. ten Cate uit Maastricht wil ik bedanken, waaronder **Henri Spronk** voor zijn Limburgse gezelligheid tijdens onze ontmoetingen en **Evren Kilinç** voor onze inspirerende gesprekken en zijn betrokkenheid. Niet te vergeten in mijn bedankje zijn **Nicole Janssen** en **Miriam Gerlofs-Nijland** van het RIVM voor hun waardevolle input en in het bijzonder Nicole voor haar enorme brei aan statistische analyses met fijnstof en andere "luvo's", zelfs toen ze door overmacht een tijdje eruit was.

Bart Pompe (UWV arbeidsdeskundige), **Karin Alderliesten-Visser** (ergotherapeut) en **Wim Janssen** (revalidatiearts) wil ik speciaal bedanken voor hun continue ondersteuning achter de schermen en het meedenken over werkplekaanpassingen die het mij mogelijk hebben gemaakt om probleemloos te kunnen functioneren op het lab.

Al mijn **vrienden** buiten de wetenschap (teveel om op te noemen), wil ik bedanken voor hun begrip die ze tijdens mijn promotietijd hebben opgebracht voor het feit dat ik niet meer zo vaak met ze kon afspreken, en de keren dat we wel bij elkaar konden komen, voor de quality-time en de broodnodige ontspanning. En jongens, voor de laatste keer: ik ben straks wel een dr., maar ook dan geen arts, dus ik kan jullie helaas weinig helpen met gezondheidskwaaltjes. Tevens voor de duidelijkheid, ik heb tijdens mijn promotieonderzoek geen "ratjes gemarteld". Zo, nu staat het ook op papier! ☺

Mijn **nieuwe collega's van de 14e** wil ik bedanken dat ze mij zo snel een thuisgevoel gaven. Ik kijk er naar uit om met jullie nieuwe dingen te gaan beleven.

Lieve **oma Milka** (baba šusterica), je weet niet half hoe trots ik ben dat je mijn promotie nog kunt meemaken. Helaas kan jij op je 90-jarige leeftijd vanwege je gezondheid niet zomaar afreizen naar Nederland om op mijn grote dag aanwezig te zijn, maar ik weet wel dat je aan me zult denken die dag, zoals je dat eigenlijk altijd al hebt gedaan. Opa kijkt

dan gewoon mee vanaf boven. Veliko hvala od tvog najmlađeg unuka za tvoju bezuvjetnoj podršku i Blagoslove! Također hvala ostaloj rodbini koja mi je davala podrške da istrajem.

Ten slotte wil ik mijn **ouders** bedanken voor hun onvoorwaardelijke steun die ze mij dag in dag uit door al die jaren heen hebben gegeven. Alleen jullie weten hoe het voor mij in het echt was om zover te kunnen komen en wat de nodige offers waren. Zonder jullie hulp was het me niet gelukt. Daarom draag ik mijn proefschrift aan jullie op. Od srca vam hvala! Vaš jedinac.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 29 maart 1978 geboren in Zagreb (Kroatië), toenmalige Joegoslavië. Aan het begin van de burgeroorlog in 1991 verliet hij zijn geboorteland en heeft zich sindsdien in Rotterdam gevestigd. In 1997 behaalde hij zijn Atheneum diploma aan de O.S.G. Wolfert van Borselen te Rotterdam. In datzelfde jaar startte hij met de studie Biomedische Wetenschappen aan de Universiteit Utrecht. Zijn doctoraal behaalde hij in 2002. Als doctoraalstudent deed hij in 2000 zes maanden onderzoek naar het lipidenmetabolisme in het laboratorium van dr.ir. Geesje Dallinga-Thie (afdeling Vasculaire Geneeskunde, UMC Utrecht). Daarna deed hij een onderzoeksstage van 12 maanden naar de fundamentele aspecten van het humane kopermetabolisme in het laboratorium van dr. Leo Klomp (afdeling Metabole Ziekten, UMC Utrecht). In 2004 begon hij als promovendus op de afdeling Hematologie (Erasmus MC) in de onderzoeksgroep van prof.dr. Frank Leebeek en dr. Moniek de Maat. De resultaten van dat onderzoek staan beschreven in dit proefschrift. Vanaf 2009 is hij werkzaam als postdoc aan een project betreffende de farmacogenetica van antihypertensiva bij prof.dr. Jan Danser (afdeling Farmacologie, Inwendige Geneeskunde, Erasmus MC) en prof.dr. Maarten Simoons (afdeling Cardiologie, Erasmus MC).

PUBLICATIONS

Goran Rudež, Heleen J. Bouman, Jochem W. van Werkum, Frank W.G. Leebeek, Adrian Kruit, Hendrik J.T. Ruven, Jurriën M. ten Berg, Moniek P.M. de Maat, Christian M. Hackeng. Common variation in the platelet receptor *P2RY12* gene is associated with residual on-clopidogrel platelet reactivity in patients undergoing elective PCI. *Circ Cardiovasc Genet.* 2009 (in press)

Goran Rudež, Piet Meijer, Henri M.H. Spronk, Frank W.G. Leebeek, Hugo ten Cate, Cornelis Kluft, Moniek P.M. de Maat. Biological variation in inflammatory and hemostatic markers. *J Thromb Haemost.* 2009;7:1247-55.

Goran Rudež, Nicole A.H. Janssen, Evren Kilinc, Frank W.G. Leebeek, Miriam E. Gerlofs-Nijland, Henri M.H. Spronk, Hugo ten Cate, Flemming R. Cassee, Moniek P.M. de Maat. Effects of ambient air pollution on hemostasis and inflammation. *Environ Health Perspect.* 2009;117(6):995-1001.

Evren Kilinç, **Goran Rudež**, Henri M.H. Spronk, Hugo ten Cate, Abderrahim Nemmar, Moniek P.M. de Maat, Marc F. Hoylaerts. Particles, coagulation and thrombosis. Chapter 20 from Cardiovascular Effects of Inhaled Ultrafine and Nano-sized Particles, RIVM. 2009 (in press)

Goran Rudež, Nicole A.H. Janssen, Evren Kilinc, Frank W.G. Leebeek, Miriam E. Gerlofs-Nijland, Henri M.H. Spronk, Hugo ten Cate, Flemming R. Cassee, Moniek P.M. de Maat. Luchtverontreiniging en hemostase. *Tromnibus.* 2008;36(3):53-6.

Goran Rudež, Pascalle Monraats, Frank Leebeek, Marlies Schrevel, Aeilko Zwinderman, Robbert de Winter, Rene Tio, Pieter Doevendans, Wouter Jukema, Moniek de Maat. Platelet receptor *P2RY12* haplotypes predict restenosis after percutaneous coronary interventions. *Hum Mutat.* 2008;29(3):375-80.

Goran Rudež, Emile L.E. de Bruijne, Tamara N. Bongers, Diederik W.J. Dippel, Jaap W. Deckers, Don Poldermans, Huub H.D.M. van Vliet, Moniek P.M. de Maat, Frank W.G. Leebeek. Common variation in the platelet receptor *P2RY12* gene is associated with the risk of arterial thrombosis. (submitted)

Heleen J. Bouman, Jochem W. van Werkum, **Goran Rudež**, Frank W.G. Leebeek, Adrian Kruit, Christian M. Hackeng, Jurriën M. ten Berg, Moniek P. M. de Maat and Henk J.T Ruven. Genetic polymorphisms of the P2Y12 receptor influence in vitro inhibition of platelet aggregation with the potent P2Y12 receptor antagonist cangrelor. (submitted)

Goran Rudež, Niels F.M. Kok, Frank W.G. Leebeek, Kim van Gaalen, Carla C. Baan, Jan N.M. IJzermans, Moniek P.M. de Maat. C-reactive protein gene haplotypes determine the response of CRP serum concentration after surgery. (submitted)

AWARDS

Young Investigator Award of the International Society on Thrombosis and Haemostasis, Boston, USA, 2009.

Poster Prize of the British Society of Haematology, Edinburgh, 2005.

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Goran Rudež Erasmus MC Department: Hematology Research School: COEUR	PhD period: 2004 - 2009 Promotor(s): Prof.dr. F.W.G. Leebeek Supervisor: Dr. M.P.M. de Maat

1. PhD training		
	Year	**Workload (ECTS)**
General academic skills		
- Biomedical English Writing and Communication	2006	2
Research skills		
- Statistics (NIHES)	2005	5.7
In-depth courses (e.g. Research school, Medical Training)		
- PhD Courses at COEUR (8x) and MolMed (2x)	2004-2008	13.8
- PhD Courses of Dutch Society for Thrombosis and Haemostasis (3x)	2005-2007	3
- Other Basic Science PhD courses at Erasmus MC (2x)	2006-2008	0.6
Presentations		
- Oral (7x)	2005-2009	5.5
- Posters (10x)	2005-2008	5.9
International conferences (6x)	2005-2009	7.3
Seminars and workshops		
- COEUR Research seminars and lectures (10x)	2004-2008	3.7
- Symposia of the Dutch Society of Thrombosis and Haemostasis (4x)	2005-2009	2
- Other symposia and lectures (10x)	2004-2008	1.6
Didactic skills		
Other		
2. Teaching activities		
	Year	**Workload (ECTS)**
Lecturing		
Supervising practicals and excursions		
Supervising Master's theses		
Other		
- Student coaching (14 weeks)	2005 - 2008	0.5
Total		51.6 ECTS

ABBREVIATIONS

5-HT	5-hydroxytryptamine (Serotonin)
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
AMI	Acute myocardial infarction
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Activated protein C
aPTT	Activated partial thromboplastin time
AT	Antithrombin
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BMI	Body mass index
CABG	Coronary artery by-pass grafting
cAMP	Cyclic adenosine monophosphate
$CaCl_2$	Calcium chloride
CAD	Coronary artery disease
CAT	Calibrated automated thrombogram
CEPH	Centre d'Etude du Polymorphisme Humain
CHD	Coronary heart disease
CI	Confidence interval
CK-MB	Creatine kinase-MB
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
COX-1	Cyclo-oxygenase 1
CRP	C-reactive protein
CT	Closure-time; computer tomography
CV	Coefficient of variation
CVD	Cardiovascular disease
CYP2C19	Cytochrome P450 2C19
DEP	Diesel exhaust particles
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential
F1+2	Prothrombin activation peptide fragment 1 and 2
FV	Factor V
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FIX	Factor IX
FX	Factor X
FXa	Activated Factor X
FIX	Factor IX
FXII	Factor XII
FXIII	Factor XIII
GAM	Generalized additive models
GP	Glycoprotein
H_2O_2	Hydrogen peroxide
HR	Hazard ratio
Href	Reference haplotype
ht-SNP	Haplotype-tagging single nucleotide polymorphism
IL-6	Interleukin -6
IPA	Inhibition of platelet aggregation
IQR	Interquartile range
IS	Ischemic stroke
IVS	Intervening sequence
LAD	Left anterior descending artery branch of the left coronary artery
LD	Linkage disequilibrium
Ln[CRP]	Logarithmically transformed CRP
LPS	Lipopolysaccharide
LTA	Light-transmittance aggregometry
MI	Myocardial infarction
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NO	Nitrogen monoxide

NO_2	Nitrogen dioxide
O_3	Ozone
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
P200	Plasma with $200x10^9$ platelets/L
PAD	Peripheral arterial disease
PAF	Platelet activating factor
PAI-1	Plasminogen activator inhibitor 1
PARs	Protease activated receptors
PAR4-AP	Protease-activated receptor 4 activating peptide
PCI	Percutaneous coronary interventions
PDGF	Platelet-derived growth factor
PFA-100	Platelet function analyzer 100
PKA	Protein kinase A
PLC	Phospholipase C
PM	Particulate matter
PM_{10}	Particulate matter with aerodynamic diameter <10μm
$PM_{2.5}$	Particulate matter with aerodynamic diameter <2.5μm
PMN	Polymorphonuclear leukocyte
PPACK	D-phenylalanyl-prolyl-arginine chloromethyl ketone
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRU	P2Y12 reaction units
PT	Prothrombin time
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
TAT	Thrombin antithrombin
TGFβ	Transforming growth factor beta
TF	Tissue factor
TFBS	Transcription factor binding sites
TFPI	Tissue factor pathway inhibitor
TIA	Transient ischemic attack
TM	Thrombomodulin
TNF-α	Tumor necrosis factor alpha

TRAP	Thrombin receptor activated protein
TVR	Target vessel revascularization
TxA_2	Thromboxane A_2
UAP	Unstable angina pectoris
UFPs	Ultrafine particles with aerodynamic diameter <0.15 μm
UTR	Untranslated region
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells
VWF	von Willebrand factor