GENETIC AND PHARMACOGENETIC DETERMINANTS OF CARDIOVASCULAR DISEASE

Jeffrey J.W. Verschuren

Genetic and pharmacogenetic determinants of cardiovascular disease

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The studies described in this thesis were performed at the department of Cardiology of the Leiden University Medical Center, Leiden, The Netherlands

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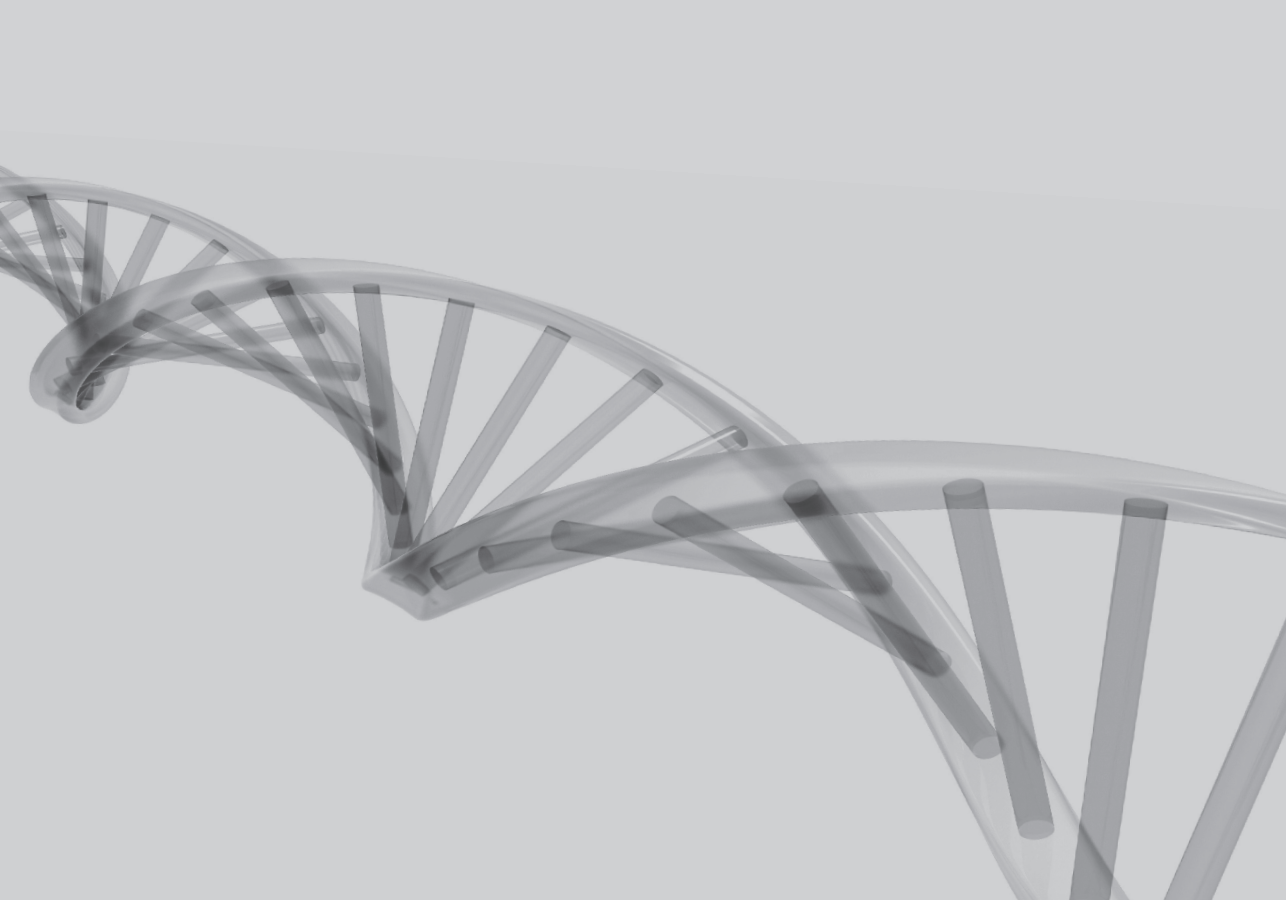
Chapter 1



General introduction

GENERAL INTRODUCTION

The last decades, tremendous progress has been made in improving treatment, and thereby prognosis, of patients with cardiovascular disease (CVD). Several breakthroughs in CVD management and prevention include the introduction of percutaneous coronary interventions in 1979[1] and the introduction of aspirin and cholesterol lowering with statins for treatment of CVD, both in the 1980s.[2,3] Despite this all, CVD remains a primary cause of morbidity and mortality in the western world. In the 2012 update of the heart disease and stroke statistics,[4] it was estimated that the prevalence of CVD in the United States in 2008 was 82.6 million individuals. Each year 785,000 Americans will have a new coronary event and approximately 470,000 will have a recurrent attack. In addition, 795,000 people will have a new or recurrent stroke each year. The 2008 overall rate of CVD death was 245 per 100,000 individuals. Although these numbers are somewhat better in the Netherlands,[5] also there the CVD burden is troublesome, indicating that we have a long road ahead of further improving the available treatment modalities and developing new and better treatment options and preventive measures.

Research on CVD has taught us a long time ago that disease development is an interplay of both genetic and environmental factors. In order to develop new treatment modalities, it is of paramount importance that we further increase our knowledge of the exact biological mechanisms and the genetic background of CVD. However, the multifactorial nature of CVD complicates the interpretation of genetic associations. Although many genetic factors have been associated with the different aspects of CVD, consistent replication has proven difficult.[6-9] The reported genetic factors have small effect sizes and, even taken together, only explain a minor part of the suspected genetic component of the disease.[10,11] This so called missing heritability has puzzled the scientific field and encouraged development of improved genotyping methods and data analysis possibilities.[12,13] First it was only possible to determine one single nucleotide polymorphism (SNP) at a time. Therefore, research focused on candidate genes that were hypothesized to be involved in the disease.[14] Examples of this approach include the angiotensin-converting enzyme gene,[15] the platelet glycoprotein IIIa gene[16] and the matrix metalloproteinase 3 gene.[17] This candidate gene approach was taken to a higher level when it became possible to genotype multiple SNPs at a time on so called multiplexes. After completion of the Human Genome Project[18] and the HapMap project[19] it became evident that SNPs were present in large quantities spread across the genome. In 2005 a new technique became available that enabled simultaneous genotyping of thousands of SNPs. This led to the development of genome-wide association studies, in which exploration of large amounts of common SNPs (> 100,000) across the whole genome became possible.[20] Since no prior biologic knowledge was needed for these types of analyses, new unforeseen risk loci could be identified, possibly leading to better

understanding of the disease in question and potentially resulting in novel treatment targets. However, this hypothesis-free approach soon proved to bring along new difficulties as well, since most identified loci were intergenic or even located in gene-deserts and therefore had unclear functions. This biological relevance item is currently the issue most studies try to resolve.

In the current thesis, several aspects of the above described development will be addressed. The first and second part of the thesis will focus on elucidating the genetic background of coronary restenosis and other aspects of CVD using candidate genes approaches, GWAS and post-GWAS analyses. The third and last part of this thesis will address the more clinical side of genetic research, pharmacogenetics. Pharmacogenetics, focusses on the genetic determinants of response to drug therapy with the ultimate goal of applying personalized medicine.

OUTLINE OF THIS THESIS

Part I – Genetics of coronary restenosis

Since its introduction, PCI has become widely accepted as an effective and safe treatment modality for single vessel and multi-vessel coronary atherosclerotic disease. However, an important drawback of PCI is the renarrowing of the treated vessel, resulting in renewed symptoms and the need for repeat intervention.[14] This phenomenon is called restenosis and it is an important cause of morbidity and possibly even mortality after PCI. [21] Restenosis is a complex disease for which the causative mechanisms have not yet been fully identified. In part 1, the genetic determinants of restenosis and the long-term consequences of this disease are explored.

In **chapter 2** and **chapter 3** an extensive overview of the current knowledge of restenosis is given. In **chapter 2** the different mechanisms involved in the development of restenosis are discussed together with the known clinical, biological and procedural risk factors contributing to the individual risk of restenosis. **Chapter 3** provides an overview of the latest innovations of optimizing outcomes of coronary stenting and discusses the available preventive and therapeutic measures of in-stent restenosis.

In **chapter 4** we describe the results of the very first GWAS published on restenosis that was performed in the GENetic DEterminants of Restenosis (GENDER) study population. Follow-up studies of this GWAS data resulted in the other 3 chapters in this part of the thesis. After obtaining the GWAS data, we used this data in **chapter 5** to clarify the inconsistent results, reported in literature, on the association of genetic variation in matrix metalloproteinases 2 and 3 and restenosis. Next, in **chapter 6** we examine all SNPs in the genomic region of all other previously described candidate genes of restenosis. The aim of this study is to explore whether the joint effect of all these SNPs together

indeed is associated with restenosis, despite all the inconsistent results from previous studies. **Chapter 7** describes the next step in unraveling the genetic background of restenosis. In this study we performed pathway analysis of a wide range of biological pathways, related to the key mechanisms involved in restenosis development, with the aim to identify new additional candidate genes for further research. Finally, in **chapter 8** the 10-year follow-up of the GENDER population is presented. In this chapter we examine whether the development of restenosis has an effect on long-term mortality rate. Moreover, we compare the GENDER study with the general Dutch population to see if patients with coronary artery disease still have a worse outcome, despite all therapeutic advances that were made during the last decade.

Part II – Genetics of (cardio)vascular diseases

In this second part of this thesis genetic determinants of two other (cardio)vascular conditions are studied. In **chapter 9** we investigate the possible role of DNA repair mechanisms in CVD development by performing a gene set analysis of DNA repair pathways for their association with the CVD events myocardial infarction and stroke. Besides the GENDER population, we include in this study the 5,244 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) study. In **chapter 10** we make a sidestep towards nephrology to study a condition with a likely mechanistic overlap with coronary restenosis, i. e. arteriovenous access failure in hemodialysis patients. We determine a wide range of SNPs in the Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) population and analyze them for their association with arteriovenous access failure.

Part III – Pharmacogenetics of cardiovascular diseases

The fact that not all patients have a similar adequate response to drug therapy poses a major problem in guideline-based clinical practice. Several underlying mechanisms for this aberrant response have been proposed and genetic variation is thought to play a major role. Pharmacogenetics is the field of research that examines genetic variation that influences responses to drug therapy in the individual patient. The ultimate goal of pharmacogenetics is the realization of personalized therapy in which determination of genetic polymorphisms can guide pharmacotherapy to choose agents with the greatest potential for efficacy and the least risk of toxicity. Pharmacogenetics also informs clinicians for dose adaptations to specific drugs in patients with aberrant metabolism.

In the first chapter of the final part of this thesis (**chapter 11**) a comprehensive overview is provided of the available pharmacogenetic evidence of the five major drug classes of cardiovascular diseases; statins, antiplatelet agents, anticoagulants, beta-blockers and angiotensin-converting enzyme inhibitors. In **chapter 12**, the eight best replicated SNPs, related to aspirin- and clopidogrel efficacy, are investigated in a

large population of ST-segment elevation myocardial infarction patients, to see whether their influence on recurrent thrombotic events could also be detected in this real-life unselected patient population.

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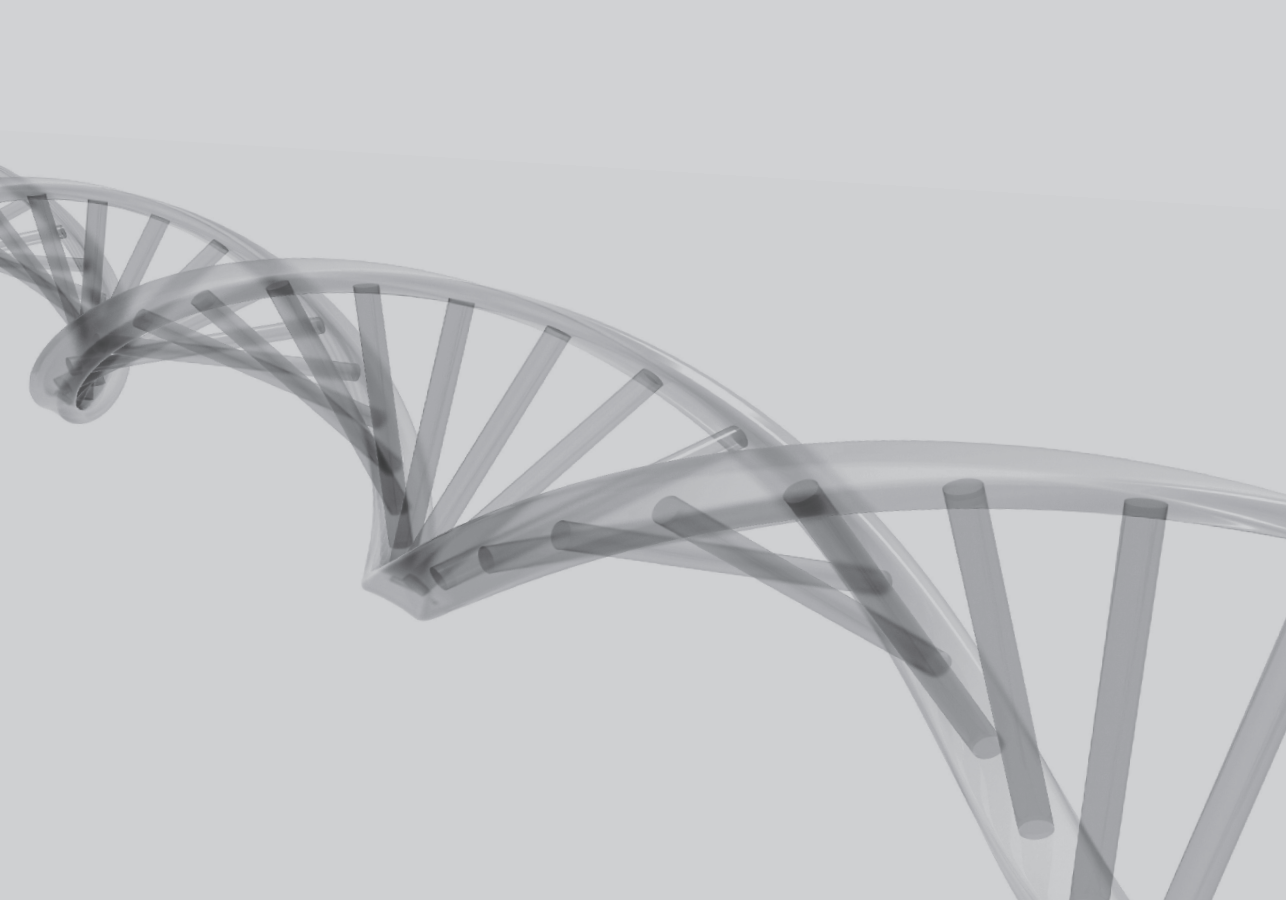
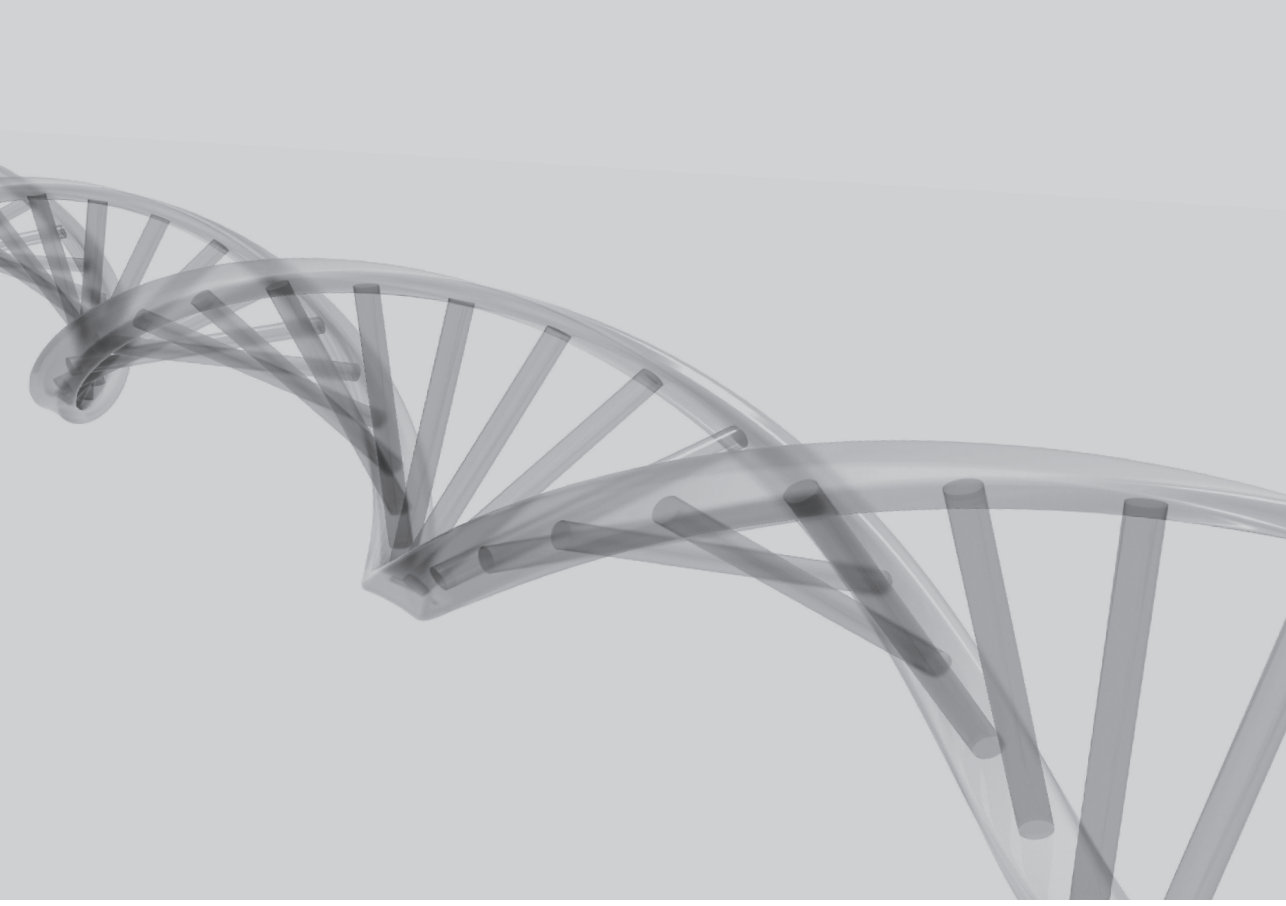
Part I



Genetics of restenosis

Chapter 2



Restenosis after PCI
Part 1:
pathophysiology and risk factors

Jeffrey J. W. Verschuren, J. Wouter Jukema, Tarek A. N. Ahmed and Paul H. A. Quax

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ABSTRACT

Restenosis is a complex disease for which the identified pathophysiological mechanisms have not yet been fully elucidated, but are thought to include inflammation, proliferation, and matrix remodeling. Over the years, many predictive clinical, biological, (epi)genetic, and procedural risk factors for restenosis have been identified. These factors are not only are useful in risk stratification of patients, they also contribute to our understanding of this condition. Furthermore, these factors provide evidence on which to base treatment tailored to the individual and aid in the development of novel therapeutic modalities. In this Review, we will evaluate the available evidence on the pathophysiological mechanisms of restenosis and provide an overview of the various risk factors, together with the possibility of clinical application of this knowledge.

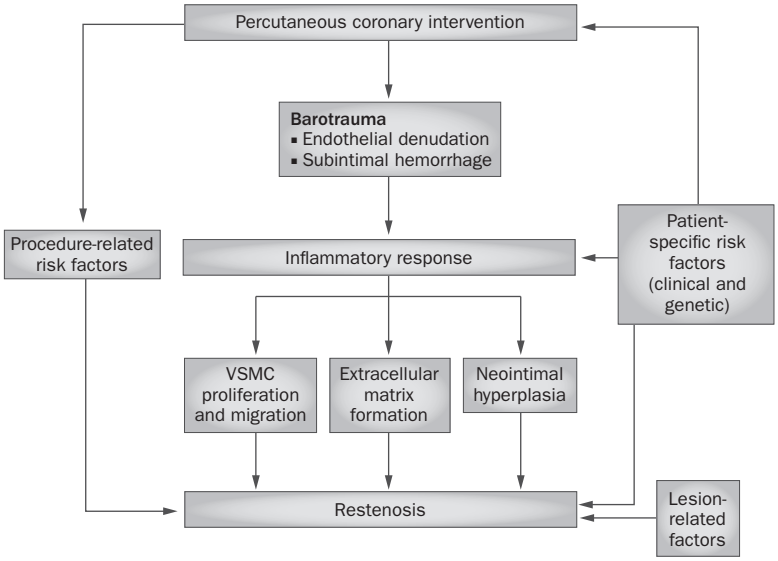
INTRODUCTION

Percutaneous coronary intervention (PCI) was introduced as an alternative means of coronary revascularization to CABG surgery in 1979.[1] Since then, PCI has become widely accepted as an effective and safe treatment modality for single vessel and multivessel coronary atherosclerotic disease. However, the major drawback of PCI is restenosis of the treated vessel, resulting in renewed symptoms and the need for repeat intervention.[2] Restenosis is a complex disease for which the causative mechanisms have not yet been fully identified. This condition can be defined by follow-up angiography or by recurrent ischemia-related symptoms in the patient. Binary angiographic restenosis is defined as the renarrowing of the vessel lumen to > 50% occlusion, usually within 3–6 months after PCI.[3] Clinical restenosis is characterized by recurrent angina pectoris requiring target vessel revascularization (TVR).[4] In many studies, follow-up coronary angiography has been used to define restenosis. Although the severity of angiographically determined restenosis correlates with TVR, up to 50% of patients with restenosis detected during angiography do not manifest clinical symptoms.[5,6] Results of a 2011 meta-analysis of 29 randomized controlled trials on the use of drug-eluting stents (DESs) demonstrated that two-thirds of patients with > 50% stenosis on angiography had ischemia-driven TVR.[7]

Before the introduction of the intracoronary bare-metal stent (BMS) in the late 1980s,[8] restenosis was a common complication that occurred in up to 50% of patients treated with balloon angioplasty.[9] Various pharmacological strategies were shown to be disappointing for the prevention of restenosis, probably owing to the failure to target the appropriate pathway and to the poor local effects and penetrance of the drug into the vessel wall. Only after the introduction of the BMSs did the incidence of restenosis begin to diminish (20-30%).[10] DESs have been proven to reduce restenosis even more effectively than BMSs (5-10%), although the problem was still not completely eradicated.[11] The effectiveness and safety of DESs were demonstrated by the results of large randomized controlled trials, leading to their current widespread use in daily clinical practice. However, implantation of DESs in unselected patients with complex lesions, and even off-label use, has resulted in a small resurge in the incidence of restenosis from 5% to 15%.[12-15] Furthermore, the new phenomena of 'in-stent restenosis' and, specifically for DESs, the 'late catch-up phenomenon' of restenosis beyond 1 year after implantation have been recognized.[16-18]

Restenosis is not a random phenomenon; certain patients appear to be at increased risk of developing this complication.[19] Owing to the increasing number of patients being treated with multiple PCI procedures, the increasing cost of interventional cardiology as a result of adjuvant medication and devices,[20] and the difficulty in treating in-stent restenosis, defining the subsets of patients at increased risk for restenosis would be useful. These patients could benefit from additional treatment modalities, such as

Figure 1 Factors involved in the development of restenosis.



Flowchart showing interaction between the main contributors to the development of restenosis.
Abbreviation: VSMC, vascular smooth muscle cells.

CABG surgery.[2] Until now, identification of at-risk subgroups of patients on the basis of clinical, lesion-related, procedural, and biological markers has been only partially successful.[2,19,21-24] Identification of the risk factors for the development for restenosis is not only helpful for patient stratification, but also provides valuable information on the underlying molecular and cellular mechanism of restenosis, with implications for new therapeutic modalities. An improved understanding of the processes of restenosis could lead to its prevention, saving patients from undergoing further procedures and reducing costs for society.

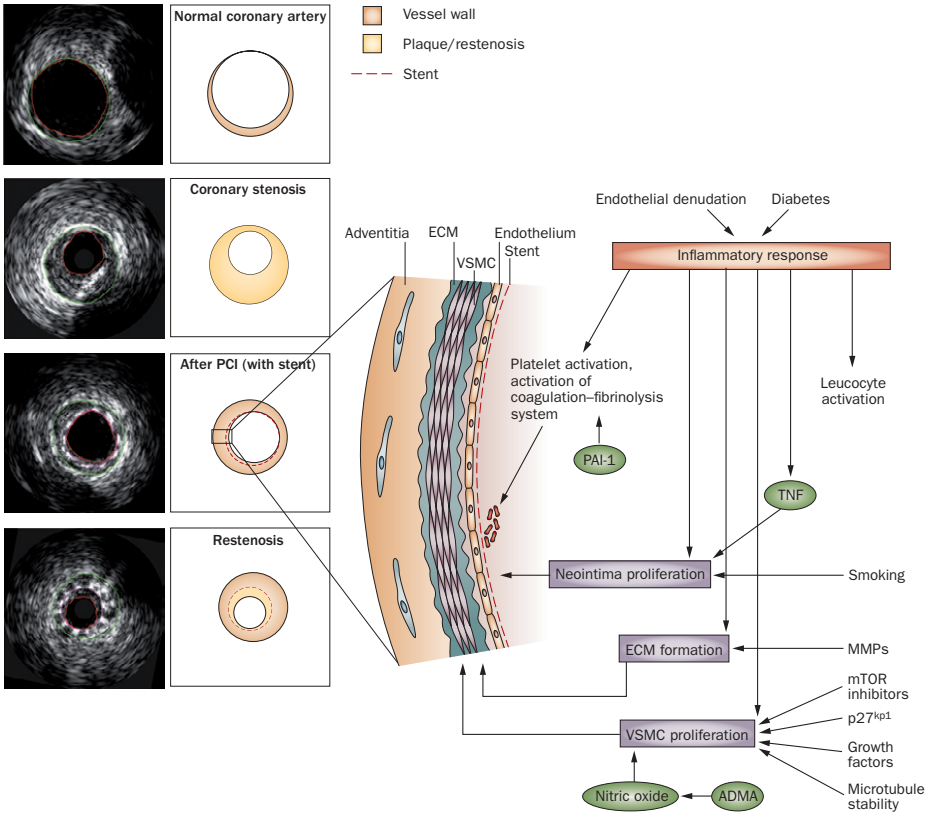
In this Review, we will evaluate the available evidence on the pathophysiological mechanisms of restenosis and explain that, although the exact mechanisms are not fully understood, an inflammatory response evoked by the procedure is probably is the main causative process (Figure 1).

We will also discuss many of the reported clinical, biological, (epi)genetic, lesion-related, and procedural risk factors for restenosis, the strength of the evidence for their association with this condition, and the possible clinical application of this knowledge.

PATHOPHYSIOLOGY

Although not completely elucidated, our current understanding of the restenotic process is that the stimulus triggering the cascade of events leading to restenosis comes from the vascular injury caused by balloon dilation (barotrauma) and stent placement during PCI.[19] This cascade is a very broad concept and many processes have been proposed to be involved (Figure 2). The inflammatory response to the endothelial denudation and subintimal hemorrhage caused by the balloon angioplasty has a key role, resulting in the onset of several proliferative processes, including vascular smooth muscle cell (VSMC)

Figure 2 Mechanisms of restenosis.



Intravascular ultrasound images of the various stages of coronary artery disease. Indicated are the outer border of the vessel (green), the lumen (red) and the coronary stent (pink). Schematic representations are shown to the right of these images. The processes occurring in the vessel immediately after PCI with stent placement are highlighted in detail. Abbreviations: ADMA, asymmetric dimethylarginine; ECM, extra cellular matrix; MMPs, matrix metalloproteinases; mTOR, mammalian target of rapamycin; PAI-1, plasminogen activator inhibitor-1; PCI, percutaneous coronary interventions; TNF, tumor necrosis factor; VSMC, vascular smooth muscle cells

proliferation and migration, extracellular matrix formation, and neointimal hyperplasia.[2,19] These events are set into motion by an elaborate interplay of systems—activation of platelets and related growth factors and proinflammatory cytokines, leukocytes, and the coagulation–fibrinolysis system, as well as events at the platelet surface.[2,25] Although this reaction is physiological, in some patients the proliferative response 'overshoots' resulting in renarrowing of the treated vessel. Why this hyper-response occurs in some individuals and not others is not well understood.

Although the mechanisms of restenosis are basically the same after balloon angioplasty and after PCI with stent placement, implantation of a stent does introduce some additional factors influencing the risk of restenose. Vessel recoil, a major contributor to restenosis after balloon angioplasty, is virtually eliminated by coronary stenting.[8] However, stent under-expansion during implantation is a procedure-related equivalent of vessel recoil.[26,27] In addition, the vascular reponse to the inflammatory response induced by the barotrauma of balloon angioplasty, as mentioned before, is partially avoided with stent placement.[26] Moses et al. reported that the margins of the region of balloon injury that were not covered by stents were the primary sites of restenosis, and thus recommended the use of longer single stents in order to ensure adequate stent coverage of the entire zone of balloon injury to decrease the influence of the barotrauma.[28]

Hypersensitivity to one or more components of the implanted stent is a rare, but nevertheless important, problem.[26] Nickel allergy is a proposed mechanism triggering the development of restenosis, although not confirmed by all studies.[29-31] Hypersensitivity reactions to DESs, especially to the drug-carrying polymers, have also been reported but are mainly associated with stent thrombosis and not restenosis,[32] as will be discussed in more detail in Part 2 of this Review.[33] Research into the molecular mechanisms involved in the interplay between the implanted stent and the vessel wall has contributed to our understanding of the role of stent components in restenosis development. Pallero et al. reported that stainless steel ions stimulated thrombospondin-1-dependent transforming growth factor β activation, which contributed to restenosis formation by increasing extracellular matrix and downregulating desmin in VSMC.[34]

Another factor related to the implanted stent itself is stent fracture. The process resulting in stent fracture is thought to involve mechanical fatigue caused by repetitive cardiac contraction exposing the stent to compression, torsion, bending, and shear stress.[35] The incidence of stent fracture is reasonably low (~4%),[35] but could be underreported because intravascular ultrasound (IVUS), which is the method of choice to detect stent fracture, is not routinely used in all studies. The mechanisms by which stent fracture leads to restenosis are not known; however it is conceivable that the integrity of the stent is lost with fracture, allowing vessel recoil to occur again, or that the fractured stent strut protrudes into the lumen thereby contributing to partial occlusion of the vessel, particularly in the presence of neointima formation. A meta-analysis by Chakravarty

et al. showed that lesions with fractured stents had a significantly higher rate of revascularization than those without stent fracture (17.0% versus 5.6%, $P < 0.01$). Conversely, the probability of stent fracture was higher in patients with restenosis than in those without this condition (12.8% versus 2.8%, $P < 0.01$).[35] Another study, not included in this meta-analysis, demonstrated that late restenosis was not observed at stent fracture sites without early restenosis during midterm follow-up, indicating that the late catch-up phenomenon reported with DES restenosis is not associated with stent fracture.[36] Further research in identification of patients and lesions at risk of stent fracture is needed.

RISK FACTORS FOR RESTENOSIS

Although most risk factors for restenosis are somehow associated with one or more of the pathophysiological processes discussed above, these relationships are not always clear or are as yet unknown.

Clinical risk factors

Since clinical parameters are collected in every trial, these baseline characteristics are relatively easy to analyze (Table 1). The most consistent clinical parameter that increases the risk of restenosis is diabetes mellitus.[37] Patients with insulin-dependent diabetes are at particularly high risk of adverse events following PCI.[38] The generally accepted hypothesis of this phenomenon is that hyperglycemia induces endothelial dysfunction and a proinflammatory state with increased growth factor and cytokine production.[25,39] These processes result in extensive neointima formation, thereby promoting the development of restenosis. The prothrombotic environment in patients with diabetes further increases the risk of events.[40] An increased risk of restenosis has not been detected in patients with metabolic syndrome for which the clinical characteristics are more varied than diabetes, thus resulting in a more heterogeneous patient group.[41]

Table 1 Clinical risk factors for restenosis

Risk factor	Risk of restenosis	Strength of evidence*
Male sex	Increased[42]	Poor
Older age	Increased[21]	Mediocre
Hypertension	Increased[43]	Mediocre
Diabetes mellitus	Increased[37]	Strong
History of restenosis	Increased[44]	Strong
Smoking	Decreased[45-48]	Strong

*Poor: one or two studies have shown this association with restenosis, but most studies did not find this relationship. Mediocre: around half of the studies report this association and the other half do not. Strong: commonly reported risk factor for restenosis.

Although some investigators have reported a higher risk of restenosis in male patients than in females,[42,49,50] the data are not consistent. The relationship between patient age and restenosis is also inconclusive; a trend towards increased risk of restenosis with increasing age[21] has not been not demonstrated in all studies.[24]

Hypertension has frequently been reported as a risk factor for restenosis,[43,45,51] although this association is probably not of a causal nature. One of the possible explanations for the observed relationship is the underlying endothelial dysfunction involved in both hypertension and restenosis.

A clinical factor applicable to the entire coronary system is a history of restenosis.[44] As will be discussed later in detail, some patients seem to have an inherent predisposition toward develop restenosis, which can be partly explained by their specific genetic background.

Paradoxically, although cigarette smoking is known to be a risk factor for coronary artery disease, a possible protective effect of smoking might exist for the development of restenosis after PCI. Although still controversial, the concept that cigarette smoking is associated with a lower rate of restenosis was already in existence over 10 years ago[46] and is supported by the results of several studies.[45,47,48] An interesting hypothesis of the mechanism was suggested by Binder in 2006.[52] He postulated that the contribution of bone-marrow-derived endothelial precursor cells to endothelial repair and inhibition of neointimal formation is hampered by the smoking-related inhibition of the release of these cells.[52]

Biological risk factors

Inflammation

Inflammation has probably the most important role of all the known biological risk factors in the development coronary restenosis[53] and many studies have explored this association. An extensive review on the subject was published by Niccoli et al. in 2010.[54] Elevation of plasma C-reactive protein (CRP) is commonly used as a marker of inflammation and is associated with the progression of cardiovascular disease.[55] Two meta-analyses on the relation between CRP and restenosis, which included different studies, both showed that increased serum CRP levels predicted the development of restenosis BMS implantation.[56,57] Moreover, elevated baseline CRP levels have also been associated with a diffuse pattern and, thus a more aggressive type, of restenosis.[58] However, no relationship between CRP and restenosis was demonstrated after implantation of DESs.[54] In a small study comparing CRP levels after implantation of various stents, Kim et al. concluded that patients receiving DESs had significantly lower CRP levels than those that received BMSs, which probably reflects the modulation of the inflammatory response by the eluted drug.[59] Whether the association between CRP and BMS-related restenosis

Table 2 Biological risk factors for restenosis

Risk factor	Risk of restenosis	Strength of evidence*
Genetic variation	Variable[19]	Variable
Elevated TNF level	Increased[60]	Mediocre
Elevated levels of certain complement components (C3a and C5a)	Increased[61]	Mediocre
Elevated levels of certain MMPs (MMP2, MMP3, MMP9)	Increased[62]	Mediocre
Elevated ADMA level	Increased[63]	Preliminary
Elevated CRP level	Increased[56]	Strong
Elevated PAI-1 level	Increased[64]	Strong
Elevated nitric oxide level	Decreased[65]	Strong

*Variable: dependent on the nature of the variation. Mediocre: around half of the studies report this association and the other half do not. Preliminary: one or two papers reporting the association, no extensive replication yet. Strong: commonly reported risk factor for restenosis. Abbreviations: ADMA, asymmetric dimethylarginine; CRP, C-reactive protein; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor 1; TNF, tumor necrosis factor

is a reflection of the inflammatory response evoked by angioplasty, or whether a possible direct response of CRP on the vascular wall also contributes to this phenomenon, remains to be elucidated (Table 2).[54]

Inflammatory responsiveness is highly genetically determined. Several studies have demonstrated associations between genetic polymorphisms in inflammatory factors and the risk of restenosis.[66-68] Comprehensive research on genetic inflammatory factors was performed by Monraats et al. in the Genetic Determinants of Restenosis (GENDER) study population, consisting of 3,104 patients treated with PCI.[69] Several significant genetic determinants were identified in an explorative study; the 16Gly variant of the β2-adrenergic receptor was associated with an increased risk of target vessel revascularization (TVR). By contrast, the rare alleles of the CD14 gene (-260T/T), colony-stimulating factor 2 (CSF2) gene (1944C/C; coding for 117Thr/Thr), and eotaxin (CCL11) gene (-1328A/A) were associated with decreased risk of TVR.[69] If validated in other cohorts, these factors might be valuable in stratification of patients with elevated risk for restenosis.

Tumor necrosis factor (TNF) is a key mediator in the inflammatory response. This cytokine acts locally at sites of tissue injury induced by vessel wall damage and has many biological functions.[66,70] In the GENDER study population, two polymorphisms (–238G>A and–1031T>C) in the TNF gene promoter were associated with a decreased risk of restenosis.[66] Both markers where hypothesized to lead to lower serum TNF levels. The concept was supported by experiments in a mouse model for reactive stenosis that showed upregulation of TNF gene expression upon vascular injury, and reduction of neointima formation in TNF-knockout mice as well as after administration of the anti-TNF compound thalidomide.[66] These results indicate that intervention in the development of

restenosis might be possible by modulating the TNF response. Other studies in rats[71] and in humans[60] confirmed these findings; however, by contrast, Koch et al. reported that TNF gene polymorphisms were not associated with an increased risk of restenosis in patients with coronary artery disease.[70]

Other interesting inflammatory markers for restenosis that have been identified, but have yet to be replicated in human studies, include complement components[61] and genetic variation in the genes for interleukin 10 (IL10),61 annexin A5 (ANXA5),68 and the vitamin D receptor (VDR).[67,72,73] Some of these associations were confirmed in animal models,[74,75] but not yet in clinical studies.

Several lines of evidence indicate that, besides its role in thrombus formation,the plasmin activation system is also involved in restenosis, by increasing neointima hyperplasia.[76-78] The role of plasminogen activator inhibitor 1 (PAI-1) has been investigated in multiple studies. Although conflicting results have been published,[79] most studies indicate that PAI-1 might be useful as a predictive marker.[64,80] In addition, therapeutic options utilizing inhibitors for plasminogen activators or PAI-1 have been explored in preclinical studies.[76,77,81-83] Moreover, variation in the PAI-1 gene (SERPINE1) has also been related to restenosis.[84]

Vascular remodeling and function

Matrix metalloproteinases (MMPs) are a group of enzymes with proteolytic activity against a variety of extracellular matrix components and they are involved in vascular remodeling and inflammation. Several studies have reported associations between increased serum levels of MMP2, MMP3, and MMP9 and an increased risk of restenosis.[62,85-87] However, no link between genetic variation in the MMP2 and MMP3 genes has been established.[88]

VSMCs have an important role in the development of restenosis. Cyclin-dependent kinase inhibitor 1B (p27kip1) has been implicated in the regulation of VSMC proliferation[89] and is, therefore, CDKN1B is a good candidate gene for involvement in the development of restenosis. Van Tiel et al. reported that the promoter polymorphism -838C>A (rs36228499) in CDKN1B was associated with a decreased risk of restenosis.[90] Moreover, in functional experiments, these investigators demonstrated a 20-fold increase in transcriptional gene activity in cases of the -838A allele.[90] Since this study was the first to implicate genetic variation in CDKN1B in the development of restenosis, more studies are needed to confirm these findings before the -838A allele can be established as a marker for stratification of patients at risk of restenosis.

Endogenous circulating bone-marrow-derived stem cells have been implicated in various aspects of vascular disease and function. With respect to restenosis the evidence is two-sided. On the one hand, circulating endothelial progenitor cells enhance re-endothelialization after vascular injury and thereby prevent excessive cell

proliferation.[91,92] This concept underlies the implementation of endothelial progenitor cell (EPC) capturing coronary stents, which will be discussed in more detail in Part 2 of this Review.[33] On the other hand, EPC homing has been suggested to contribute to the development of restenosis.[93] Pelliccia et al. demonstrated that patients who developed restenosis had increased concentrations of specific subpopulations of EPCs compared with controls.[93] A similar difference could not be demonstrated in patients with either stable or progressive coronary atherosclerosis, underlining the mechanistic differences between arteriosclerosis and restenosis. Variations in the time point at which stem cells were measured, and the differences between the specific subpopulations of EPCs and their functions, could provide an explanation for the conflicting results regarding the relationship between EPC and restenosis.[94] Therefore, although bone-marrow-derived stem cells have, without question, a role in the response to vascular injury, the exact mechanism involved is still unclear and needs to be further elucidated.

Nitric oxide is a vasodilatory molecule known to decrease proliferation and migration of VSMCs[95] and decreased nitric oxide synthase (NOS) expression has been related to restenosis.[65] However, conflicting reports on the influence of genetic variation in NOS have lead to the hypothesis that the effects of nitric oxide only become apparent under specific 'environmental' conditions such as endothelial dysfunction.[96] Pons et al. emphasized this hypothesis by reporting that several polymorphisms in the NOS3 gene were associated with restenosis in patients with, but not in those without, concurrent metabolic syndrome.[96] Owing to the advantageous effects of nitric oxide in the arterial lumen, a number of nitric oxide releasing systems, including drugs and polymers, have been explored for clinical application. Since the in vivo half-life of nitric oxide is very short (~2–5 s) and because it has potential cardiovascular side effects, precise and timely delivery is essential. Gene therapy is one method of achieving this goal, and preliminary studies in humans have shown the positive and safe results of this treatment.[97-99]

In 2010, another contributor to nitric oxide metabolism was associated with restenosis. Ari et al. reported that patients who developed restenosis had significantly higher asymmetric dimethylarginine (ADMA) serum levels than those who did not develop restenosis.[63] ADMA competitively inhibits nitric oxide synthesis by blocking NOS; therefore, an increase in ADMA level will lead to a decrease in nitric oxide activity and concentration. The serum level of ADMA can be reduced by inhibiting its synthesis or stimulating its excretion, meaning that ADMA is a potential treatment target as well as a predictive marker.[63]

Genetic factors

Much evidence exists to support a role for genetic factors in the risk of restenosis, independent of conventional clinical variables. In patients with multivessel disease, the incidence of restenosis of a second lesion was 2.5-times higher if the first lesion had

restenosis, even after adjustments for well-known patient-related risk factors, making a genetic predisposition to restenosis conceivable.[44] Identification of genetic markers for restenosis could prove to be useful in two ways. First, risk stratification according to the genetic make-up of the patient can enable treatment to be tailored to the individual. Second, identification of related genes might aid our understanding of the mechanisms involved in restenosis (Table 3), thereby leading to new prevention strategies. Many studies have focused on genetic variation influencing the risk of restenosis, together covering a wide array of candidate genes. Significant associations have been reported with polymorphisms in genes of the hemostatic system, for example, PIA1/A2 (rs5918) in the platelet glycoprotein IIIa gene (ITGB3),[100] the prothrombotic factor V Leiden polymorphism (rs6025) of the F5 gene,[84] and the platelet receptor P2Y12 gene haplotypes;[101] in the rennin–angiotensin system, for example, 1166A>C (rs5186) of the angiotensin II type 1 receptor gene (AGTR1);[102] and in glucose metabolism, for example 7,067,365C>A of the insulin receptor gene (INSR),[103] and 161C>T (rs3856806) of the peroxisome proliferator-activated receptor γ (PPARG) gene.[104]

Table 3 Genetic risk factors for restenosis

Risk factor (gene)	Alteration	Risk of restenosis	Strength of evidence*
β2-adrenergic receptor (*ADRB2*)	Arg16Gly	Increased[69]	Preliminary
Annexin A5 (*ANXA5*)	rs4833229 and rs6830321	Increased[72]	Preliminary
Vitamin D receptor (*VDR*)	Haplotypes	Increased[73]	Preliminary
$P2Y_{12}$ receptor (*P2Y12*)	Haplotypes	Increased[101]	Preliminary
Interleukin 10 (*IL10*)	–2849G>A, –1082G>A and +4259A>G	Increased[67]	Mediocre
Platelet glycoprotein IIIa (*ITGB3*)	PIA1/A2	Increased[100]	Mediocre
Nitric oxide synthase 3 (*NOS3*)	949A/G, -716C/T and Glu298Asp	Increased[96]	Strong
CD14	–260C/T	Decreased[69]	Preliminary
Colony-stimulating factor 2 (*CSF2*)	Ile117Thr	Decreased[69]	Preliminary
Eotaxin (*CCL11*)	–1328G/A	Decreased[69]	Preliminary
Factor V (*F5*)	Leiden mutation	Decreased[84]	Preliminary
cyclin-dependent kinase inhibitor p27kip1 (*CDKN1B*)	-838C>A	Decreased[90]	Preliminary
P300/CBP associated factor (*KAT2B*)	-2481G>C	Decreased[105]	Preliminary
Tumor necrosis factor (*TNF*)	–238G/A and –1031T/C	Decreased[66]	Mediocre
Matrix metalloproteinase 2 (*MMP2*) and matrix metalloproteinase 3 (*MMP3*)	Multiple single nucleotide polymorphisms	No association[88]	Strong
Angiotensin-converting enzyme (*ACE*)	I/D	No association[106]	Strong

*Preliminary: one or two papers reporting the association, no extensive replication yet. Mediocre: around half of the studies report this association and the other half do not. Strong: commonly reported risk factor for restenosis

Epigenetics is an upcoming field of increasing research interest. Epigenetic processes modulate gene expression patterns without modifying the actual DNA sequence. Part of the gene–environment interaction relevant for complex diseases is regulated by epigenetic mechanisms such as histone acetylation and DNA methylation.[107,108] Epigenetic processes have been related to restenosis by the association between the -2481G>C polymorphism (rs2948080) of the gene (KAT2B) encoding P300/CBP associated factor was associated with a reduced risk of both clinical and angiographic restenosis after PCI.[105] Since this study is the first on epigenetics and restenosis, the concept is preliminary; however, it is an interesting new development that is likely to shed new light on our understanding of the molecular mechanisms of restenosis.

The remaining issue with genetic studies is the problem of replication of the results in other cohorts. Many studies in which genetic determinants of restenosis have been investigated have fairly small sample sizes, increasing the risk of false positive, as well as false negative, findings. Furthermore, publication bias contributes to a distortion of the evidence. A striking example is the insertion/deletion polymorphism in the gene encoding angiotensin I converting enzyme (ACE I/D), a supposed risk marker of restenosis.[109,110] In a meta-analysis, Agema et al. demonstrated that the initial identification of this polymorphism as a risk factor for restenosis was made on the basis of results from a number of small positive studies, not specifically designed for genetic epidemiologic research, where positive studies were published and negative ones were not.[106] This meta-analysis clearly demonstrated that after correction for publication bias, the assumption that the ACE I/D polymorphism was associated with restenosis after PCI proved to be false.[106] Therefore, available evidence should be weighted and interpreted with caution. More, large studies specifically designed to investigate the genetics of restenosis are needed to confirm the currently available evidence and provide the opportunity to utilize our knowledge of genetic risk markers in clinical practice.

Risk factors related to lesion and procedure

Several lesion characteristics have been shown to increase the restenotic risk (Table 4). The lesion-related factor most consistently associated with restenosis is lesion length.[23,43,45,111,112] The longer the stented lesion the higher the risk of restenosis. Other factors include small vessel diameter and minimal lumen diameter after stenting.[43,113-115] In addition, more-complex lesions, such as ACC/AHA type C lesions, tortuous and calcified lesions, and chronic total occlusions, tend to be associated with an increased risk of restenosis, although not as consistently reported as the other factors (Table 4).[23,24,43,116] A history of restenosis increases the risk of a subsequent episode of restenosis;[44] however, PCI of a restenotic lesion carries an even higher risk.[23,24,45] Apparently the local tendency of renarrowing after dilatation, with or without stenting, seems to be considerable in these lesions.

Table 4 Lesion-related risk factors for restenosis

Risk factor	Risk of restenosis	Strength of evidence*
Chronic total occlusions	Increased[24]	Strong
Restenotic lesions	Increased[24]	Strong
Tortuous vessel	Increased[24]	Strong
Long lesion length	Increased[45]	Strong
ACC/AHA type C lesions	Increased[43]	Strong
Small vessel diameter	Increased[113]	Strong
Calcified lesions	Increased[116]	Strong

*Strong: commonly reported risk factor for restenosis.

Table 5 Procedural risk factors for restenosis

Risk factor	Risk of restenosis	Strength of evidence*
Multiple stents	Increased[23]	Mediocre
Stent fracture	Increased[35]	Mediocre
Minimal lumen diameter after PCI	Increased[113]	Strong
Balloon angioplasty‡	Increased[119]	Strong
Bare metal stent implantation§	Increased[119]	Strong
IVUS guidance	Decreased[120]	Strong

*Mediocre: around half of the studies report this association and the other half do not. Strong: commonly reported risk factor for restenosis. ‡Compared with stenting. §Compared with drug-eluting stent.
Abbreviations: PCI, percutaneous coronary intervention; IVUS, intravascular ultrasound.

Several characteristics related to the PCI procedure itself have been related to restenosis (Table 5). First, as mentioned previously, development of the technique has resulted in improved outcomes. Therefore, in studies where various revascularisation strategies are used - balloon angioplasty alone or with BMS of DES implantation - the treatment modality should always be taken into account.[117-119]

Second, implantation of multiple stents during PCI is related to an increased risk of restenosis.[22,23] This observation probably relates to the longer lesion length or the larger surface area covered by stent material, or both. A third factor, directly related to the procedure, is the use of IVUS to guide stent implantation. Several studies have shown that patients treated with IVUS guidance had lower rates of stent under-expansion[121] and a reduced risk of restenosis.[122] IVUS has been shown to be particularly beneficial for stenting of longer lesions (> 20 mm).[120] A meta-analysis on IVUS versus angiographic guidance of PCI with BMS implantation demonstrated that IVUS improved patient outcomes by reducing the incidence of angiographic restenosis, repeat revascularization, and major adverse cardiac events.[122] Routine use of IVUS during DES implantation has not yet been proven to be better than non-IVUS guided stenting.[123-125]

CONCLUSIONS

Restenosis is a complex disease for which the pathophysiological mechanisms are not yet fully understood, but are thought to include inflammation, proliferation, and matrix remodeling. Over the years many predictive clinical, biological, (epi)genetic, and procedural factors have been identified as being associated with the development of restenosis. These factors are not only useful in stratification of the patients at risk for restenosis, they also contribute to our understanding of this complex disease. Furthermore, they provide evidence for tailored therapy and subsequently aid in the development of novel treatment modalities.

Considering the multifactorial nature of the disease, development of a risk model including various factors, is likely to be the optimal method for implementation of the current evidence. Stolker et al. demonstrated that such a model, including six clinical and angiographic variables, could be used to identify individuals with a very low risk (< 2%) of developing restenosis and those with an increase risk (> 7%).[14] Although yet far from ideal, we believe that future studies exploring various risk models could optimize patient stratification enabling identification of individuals at risk so that appropriate treatment can be given.

Developments in genetic research, such as genome-wide association studies and sequencing, are likely to result in identification of new genetic loci involved in the pathophysiology of restenosis. Efforts are ongoing to replicate these findings and those from the small genetic trials in larger study populations. Validation of the genetic associations with functional experiments and animal models will greatly improve our knowledge about restenosis. Collaboration between different study groups might overcome the problem of the small sample size of the individual studies and will increase the power to detect associations. Increasing our understanding of the mechanisms involved in restenosis could lead to improved preventive measures and treatment modalities for this potentially devastating complication of PCI.

REVIEW CRITERIA

The articles on which this Review is based were identified by searching MEDLINE using the following keywords and Medical Subject Headings (MeSH) terms: "coronary restenosis", "risk factors", "inflammation", "genetics" and "epigenetics". We checked for papers published up to May 2011. Only papers in the English language were included. Although we realize that not all available evidence could be incorporated, the most relevant and influential articles were selected.

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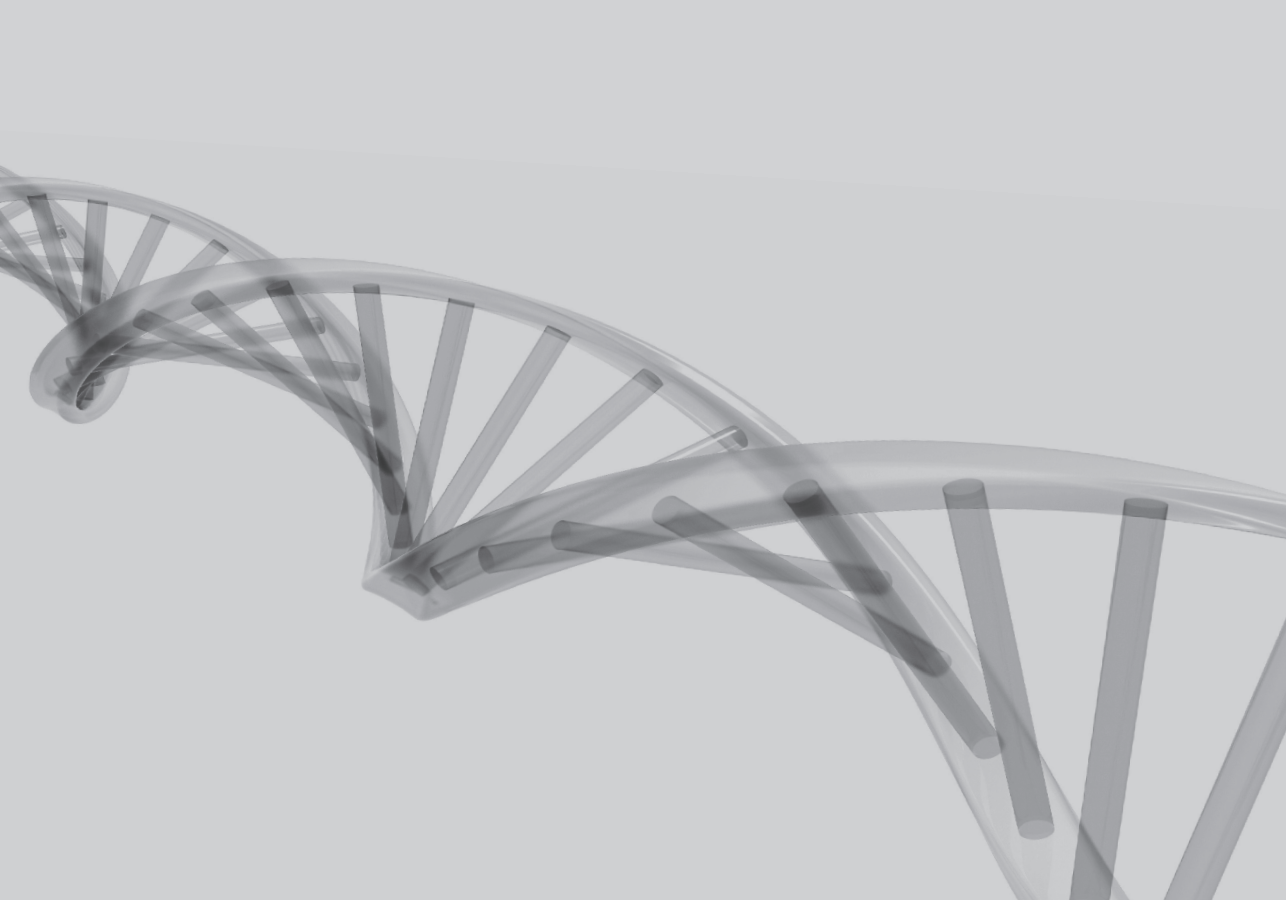
Chapter 3



Restenosis after PCI.
Part 2:
prevention and therapy

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ABSTRACT

The techniques and materials used during percutaneous coronary intervention have advanced considerably over the past three decades, yet restenosis remains one of the major drawbacks of this procedure. Many innovative technologies, including drug-eluting stents, with or without specific polymers, and fully biodegradable stents have been and continue to be developed in the search for a safe and effective antirestenosis therapy. Remarkable advances in stent design and nanoparticle delivery systems ('nano-vehicles') have already fueled revolutionary changes in the prevention and treatment of in-stent restenosis. In this Review we provide an overview of the latest innovations for optimizing outcomes of coronary stenting, and up-to-date information about prevention and treatment of in-stent restenosis.

INTRODUCTION

Since its introduction in the late 1970s,[1] percutaneous coronary intervention (PCI) has become the most important and widely used treatment for patients with obstructive coronary disease. Although the technique and the materials used during the procedure have advanced tremendously, restenosis—the renarrowing of the treated obstruction—remains one of the major complications of PCI.[2] Intravascular stents were developed as an adjunct to primary angioplasty for the management of early complications, including arterial dissection, and for the treatment for early elastic recoil. Despite the beneficial effects of stenting, however, rates of restenosis remained persistently high, giving rise to a new problem—in-stent restenosis (ISR). The introduction of drug-eluting stents (DESs) was seen as a solution to this problem and, initially, DESs reduced the incidence of ISR considerably.[3] However, these promising results led to increased use of DESs in a diverse range of complex coronary lesions, and for off-label indications, leading to a resurge in the rates of ISR.[4,5]

In light of the increasing number of PCI procedures being performed, the difficulty of treating ISR, and the increasing cost of adjuvant medication and devices, defining subsets of patients at increased risk for restenosis would be useful. These patients could benefit from additional treatment modalities. Until now, identification of subgroups has been only partially successful, as was discussed in detail in Part 1 of this Review.[6] The ongoing efforts to better understand the underlying pathophysiological mechanisms of restenosis and vascular biology continuously fuel research on the prevention and treatment of ISR. Here, in Part 2 of the Review, we will assess the most important innovations for optimizing the outcomes of coronary stenting, and data on the prevention and treatment of restenosis in general, and ISR in particular, published in the 5-year period up to August 2011.

Prevention of restenosis

In general, PCI with DESs is currently the best approach for the prevention of restenosis.[7] However, safety concerns about stent malapposition, late stent thrombosis, and delayed restenosis have arisen.[8,9] The main cause of these problems has, in addition to patient-related and lesion-related factors, been attributed to the stent polymer.[10] Additionally, the eluted antiproliferative agent and the stent platform (metal alloys and strut thickness) have been implicated in ISR. These concerns have fueled research in stent development, utilizing new antiproliferative agents, polymer technology, and metal stent platforms (Table 1).

Table 1 Developments in preventive measures for restenosis

Target	Development	Status
Antiproliferative drug	Sirolimus derivatives (biolimus A9[11-13] and novolimus[14])	Clinical*
Polymer	Biolinx® (® (Medtronic Vascular, Inc., Santa Rosa, CA, USA)[15,16]	Clinical*; preliminary‡
Polymer	Polyzene-F[17-19]	Clinical*; preliminary‡
Polymer	Biodegradable (polylactic acid, polylactic-co-glycolic acid,[20-22] SynBiosys® [InnoCore Technologies, Groningen, the Netherlands], Eureka® SOLO [Surmodics, Inc., Eden Prairie, MN, USA])[23]	Clinical*
Stent design	Polymer-free stent (Biofreedom®, Biosensors International Group, Hamilton, Bermuda)[24,25]	Clinical*; preliminary‡
Coating	Endothelial progenitor cell capturing stent (Genous®,[26-29] Combo Stent®[30,31] [both OrbusNeich Medical, Inc., Fort Lauderdale, FL, USA])	Clinical*
Coating	Titanium-nitride-oxide-coated stents (Titan2™ stent [Hexacath, Rueil-Malmaison, France])[29,32-37]	Clinical*
Stent platform	Bioabsorbable stent (magnesium stent [Biotronik, Berlin, Germany])[38-43]	Clinical*
Drug delivery	Nanomedicine[44,45]	Preclinical§
Stent platform/metal alloys	Platinum–chromium alloy stents (Promus Element® and Taxus Element® (Boston Scientific, Maple Grove, MN, USA)[46-49]	Clinical*
Drug delivery	Magnetic targeting stents[50]	Preclinical§
Miscellaneous	Gene-based therapy[51-59]	Preclinical§
Miscellaneous	Systemic treatment[60-69]	Clinical*

*Clinical: development is currently being tested in a clinical setting. ‡Preliminary: first clinical results in small studies await replication in larger trials. §Preclinical: development is currently being tested in animal models

Antiproliferative agents

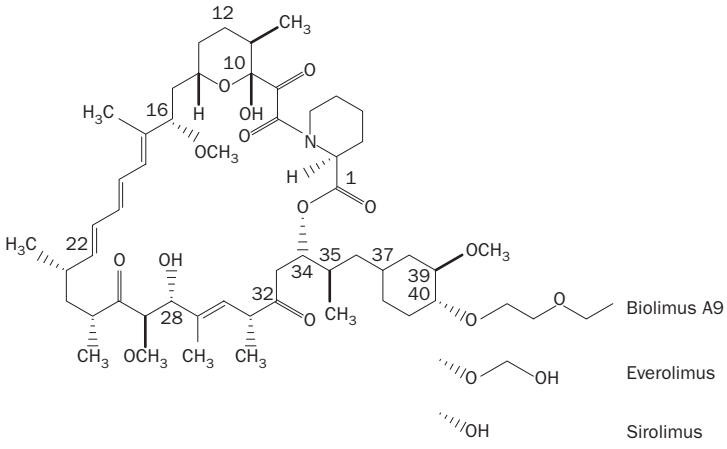
Sirolimus (rapamycin)-eluting stents (SESs; Cypher®, Cordis Corporation, Bridgewater, NJ, USA) and paclitaxel-eluting stents (PESs; Taxus®, Boston Scientific, Maple Grove, MN, USA) were the first two DESs to be approved by the FDA for use in humans (in 2003 and 2004, respectively). Although they both effectively reduce rates of restenosis compared with bare-metal stents (BMSs), their local adverse effects on the vasculature are fairly divergent. High concentrations of locally released paclitaxel have been shown to have detrimental effects on the vascular wall in a mouse model,[70] suggesting a narrower therapeutic range of this potent drug. On the other hand, sirolimus has a less-harmful effect than paclitaxel on the vascular wall.[70] Data from comparisons of first-generation DESs show that PESs are associated with a higher risk of ISR and stent thrombosis than SESs.[7,71] Two stents eluting sirolimus analogs—everolimus (Xience V®, Abbott Cardiovascular Systems, Inc., Santa Clara, CA, USA) and zotarolimus (Endeavor®, Medtronic Vascular, Inc., Santa Rosa, CA, USA)—have also been approved by the FDA.

Many studies have been dedicated to comparing the various available DESs. In 2011, the 2-year follow-up results of two large-scale, randomized, controlled trials showed a sustained benefit of everolimus-eluting stents (EESs) over PESs in terms of safety and efficacy.[72,73] On the other hand, zotarolimus-eluting stents (ZESs) were found to have a higher angiographic restenosis rate compared with PESs, although clinically driven repeat revascularization rates were similar for both stent types.[74]

In patients receiving routine clinical care, SESs have proven to be superior to ZESs.[75] The 2-year outcomes of the RESOLUTE All Comers trial[76] showed sustained similar safety and efficacy between ZESs and EESs. Unfortunately, few data are available on the direct EES versus SES comparison. Therefore, sirolimus and its analogs seem to be marginally superior to paclitaxel, whereas differences between the various limus-eluting stents remain to be elucidated.

In search for greater antirestenotic efficacy and improved long-term safety, new compounds specifically designed for use with DESs are being developed and studied. Biolimus A9 is a novel rapamycin derivative that, like sirolimus, inhibits smooth muscle cell proliferation via binding to the FK506-binding protein 1A and subsequent inhibition of the mammalian target of rapamycin (mTOR) and is specifically developed for local delivery to coronary arteries (Figure 1).[11] Besides its anti-inflammatory and antiproliferative potential and improved pharmacokinetic profile, the increased lipophilicity of biolimus A9 improves uptake by the coronary vessel wall, resulting in a more localized effect and

Figure 1 Chemical structure of biolimus A9.



This compound consists of a 31-membered triene macrolide lactone that preserves the core sirolimus ring structure with a 2-ethoxyethyl group addition to the hydroxyl group at position C (40) of the sirolimus molecule. One rationale for the inclusion of the ethoxyethyl group was to increase lipophilicity and, thus, to improve uptake by the coronary vessel wall. Permission obtained from John Wiley and Sons © Ostojic, M. et al. Catheter. Cardiovasc. Interv. 72, 901–908 (2008).

lower systemic drug exposure[12] than sirolimus eluted from the Cypher® stent.[77,78] Compared with first-generation DESs, biolimus A9-eluting stents (BESs) have been shown to be associated with better recovery of endothelial function in coronary arteries, which could be partly explained by the better drug release kinetics.[13]

Novolimus is a metabolite of sirolimus and represents another new antiproliferative mTOR inhibitor specifically developed for the stent. The newly developed DESyne® (Elixir Medical, Sunnyvale, CA, USA) novolimus-eluting stent has been tested in a clinical study, showing superiority over ZESs regarding angiographic in-stent late loss.[14]

Polymer technology

New-generation polymer coatings have been produced with the specific aim of mimicking the endothelial lining in order to prevent late thrombotic complications. Basic research has shown that some polymeric materials could potentially upregulate genes related to inflammation, proliferation, thrombosis, and vasoconstriction[79] —processes that are considered to be pivotal in the development of restenosis.[6]

One example of current progress is the Biolinx® (Medtronic Vascular, Inc.) polymer, currently used in the Endeavor Resolute® ZES. This blend of three different polymers—the hydrophobic C10 polymer to control drug release, the biocompatible and hydrophilic C19 polymer, and polyvinyl pyrrolidone—allows an early burst followed by controlled drug release[15] so that at least 85% of the zotarolimus is released within 60 days and the remainder within 180 days, avoiding long-term release of the drug. Such release patterns are designed to match the delayed healing times seen in complex lesions. The Resolute® ZES was shown to significantly lower target lesion revascularization (TLR) compared with an earlier Endeavor® ZES, which utilized a phosphorylcholine coating.[16]

Another polymer, polyzene-F is highly biocompatible and has anti-inflammatory, bacteria-resistant, and prohealing qualities. The coating ensures that the stent has a very low surface thrombogenicity, potentially reducing the risk of stent thrombosis. Evaluation of cobalt chromium stents nanocoated with polyzene-F in an animal model yielded favorable results.[17] Preliminary studies evaluating the Catania™ (CeloNova Biosciences, Newnan, GA, USA) stent in humans demonstrated a good safety profile and high-level efficacy.[18,19] Efforts to improve polymer stent coatings are ongoing.

Biodegradable polymers

Given the issues of polymer-induced inflammation, thrombosis, and restenosis, the development of biodegradable polymers has become a focus for research. The most-studied biodegradable polymers are polylactic acid and polylactic-co-glycolic acid, which degrade over time and could, therefore, eliminate the problems associated with lack of polymer biocompatibility and polymer-induced inflammation. To date, several biodegradable polymer stents eluting biolimus A9, sirolimus, or paclitaxel have been

clinically evaluated, which have so far proven to be effective and safe in the short term (≤ 30 days) and midterm (≤ 1 year).[20-22] In 2010, the 3-year follow-up data from the LEADERS trial[80] was presented, showing the sustained benefit of BESs with a biodegradable polymer over SESs with a durable polymer. Great expectations exist within the cardiology community that biodegradable-polymer DESs could become the stents of choice in years to come; the results of the ongoing ISAR-TEST-6 trial,[81] testing the safety and efficacy of the Nobori® (Terumo Corporation, Tokyo, Japan) biodegradable polymer BES and the Xience V® permanent polymer ZES will, therefore, be eagerly awaited.

New polymer technology presents some challenges, such as establishing the optimal degradation time, biocompatibility, composition, and formulation of the polymer. Several factors influence the velocity of degradation; therefore, the balance between drug-release kinetics and the rate of polymer degradation, as well as the effects of the degradation products all affect the efficacy of biodegradable polymer stent systems in the coronary vasculature.[82] Furthermore, studies in porcine coronary arteries have shown that even biodegradable polymers can cause inflammatory reactions, which could be attributable to the combination of the parent polymer compound and the biodegradation products.[83] Two new biodegradable polymers (SynBiosys® [InnoCore Technologies, Groningen, the Netherlands] and Eureka® SOLO [SurModics, Inc., Eden Prairie, MN, USA) have been tested in animal studies and yielded fewer acidic byproducts, and had a better degradation rate and biocompatibility profile than polylactic acid and polylactic-co-glycolic acid making them well-tolerated in vivo.[23] In a pig model, stainless-steel R Stents® (OrbusNeich Medical, Inc., Fort Lauderdale, FL, USA) with SynBiosys® coating and high-dose sirolimus (5 µg/mm) was associated with the lowest amount of neointima thickness after 28 days when compared with Xience V® and Cypher® stents.[30]

Polymer-free stents

In an attempt to overcome the problems encountered with polymers or their degradation products, 'polymerfree' DESs have been developed and have proven to be safe in clinical studies.[84,85] In an animal study, polymerfree biolimus A9 coated stents (Biofreedom®, Biosensors International Group, Hamilton, Bermuda) demonstrated more sustained intima inhibition, improved healing, and reduced inflammation compared with the polymercoated sirolimus eluting Cypher® stent at 180-day followup.[24] The ongoing first-in-man study of the Biofreedom® stent showed promising results compared with the Taxus® PES.[25]

Polymer-free, dual DESs have been tested over the past few years. No apparent benefit was observed by adding estradiol to a polymer-free SES in the ISAR-PEACE trial.[86] However, results from the ISAR-TEST-2 study[87] revealed that a novel, polymer-free sirolimus-eluting and probucol- eluting dual DES was noninferior to the Cypher® SES and the Endeavor® ZES. The antirestenotic efficacy of both the dual DES and the ZES

remained durable during the 2-year follow-up period.[87] The larger ISAR-TEST-5 study,[88] which was powered for clinical events, showed similarly durable results for the dual DES.

Novel prohealing stent coatings

Endothelial progenitor cell-capturing stent

An increased rate of endothelialization is thought to lead to reductions in restenosis and stent thrombosis.[89] This hypothesis underlies the development of the endothelial progenitor cell (EPC)-capturing stent. The bioengineered Genous® (OrbusNeich Medical, Inc.) EPC-capturing stent has a stainless-steel platform that is coated with an abluminal polysaccharide matrix and covalently coupled monoclonal murine antihuman CD34 antibodies. These antibodies bind bone-marrow-derived EPCs from the peripheral blood. These EPCs are hypothesized to differentiate into a functional endothelial layer after immobilization and populate the surface of the stent.[90] The safety and efficacy of the Genous® stent have been shown in preliminary human studies,[26,27] and further evaluation and comparison with other stents is currently ongoing.[28]

Despite its benefit in enhancing re-endothelialization, and thereby possibly preventing stent thrombosis, EPC capturing is not expected to potently inhibit neointimal proliferation. On the contrary, CD34 antibodies have even been shown to capture other progenitor cells, for example, smooth muscle cell progenitor cells, which could exaggerate restenosis.[91] Therefore, a major challenge in the development of an EPC-capturing DES is to maintain sustained inhibition of smooth muscle cell proliferation while promoting formation of a functional endothelial layer. This concept was tested in an animal study showing that immobilization of anti-CD34 antibody on SESs enhances endothelialization.[92] The REMEDEE study[30,31] investigators are currently testing the Combo Stent® (OrbusNeich Medical, Inc.), which incorporates low-dose abluminal sirolimus together with EPC-capturing technology and a biodegradable polymer. The combination of an EPC-capturing stent with a drug-eluting balloon is also an attractive alternative, as has been shown by the results of the PERFECT STENT study.[93]

Titanium-nitride-oxide-coated stent

The Titan2™ stent (Hexacath, Rueil-Malmaison, France) is a stainless-steel stent coated in titanium-nitride oxide that has been shown to inhibit platelet aggregation, minimize fibrin deposition, reduce inflammation, and promote healing.[32] This stent significantly reduced late lumen loss and TLR compared with a BMS at 6-months follow-up,[29] with preserved benefits up to 5-years.[33] Additionally, the Titan2™ stent demonstrated favorable results compared with the Taxus® PES in a randomized controlled trial of 425 patients with ST-segment elevation myocardial infarction,[34] as well as in routine clinical practice.[35] Despite the absence of an antiproliferative drug, use of the Titan2™ stent

resulted in less TLR than the Taxus® stent, although this reduction was not statistically significant.[34] The Titan2™ stent was noninferior to the Xience V® EES in the primary results of the large randomized controlled BASE-ACS trial[36] conducted in patients with acute coronary syndrome. However, the Titan2™ stent failed to prove noninferiority to the Endeavor® ZES in terms of angiographic in-stent late lumen loss at 6 months in the TIDE study,[37] although clinical outcomes at 1 year were comparable for both stent types.

Future prohealing stent designs

A step further to optimize the prohealing stent design is to create a bioactive stent that also elutes a drug (a bioeluting stent). Animal studies of a newly designed titanium-nitride-oxide stent eluting l-arginine, a precursor of nitric oxide with positive effects on endothelium function,[94] or a sulfated polysaccharide extracted from seaweed have shown up to 50% reduction in late lumen loss compared with the standard titanium-nitride-oxide stent.[95] A future clinical trial (the VINCI first-in-man study) is planned to test the efficacy and safety of this new generation of stents.[95]

Another approach to creating a prohealing stent would be to reduce the binding of platelets to an implanted stent, thereby reducing the inflammatory response and allowing surrounding endothelial cells to properly re-endothelialize the stent.[96] A stent created from a bioactive ligand, such as an integrin-binding motif, has been successfully used in noncardiac applications *in vivo* to promote device integration.[97] The ideal ligand should only interact with integrins uniquely present on endothelial cells and not on platelets, inflammatory cells, or smooth muscle cells.[96] Continued *in vitro* and *in vivo* studies with such biomaterials could lead to the creation of next-generation prohealing stent surfaces that promote the endothelialization of the stent while simultaneously inhibiting the adhesion and thrombus formation, and not stimulating smooth muscle cell proliferation.

Stent platforms

Metal alloys

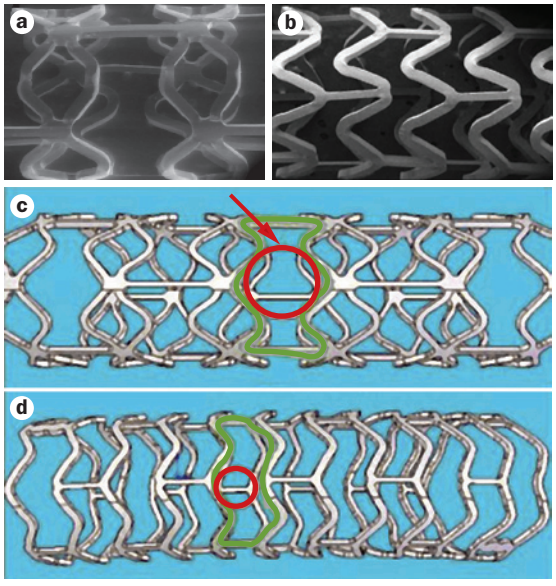
A platinum–chromium alloy developed in the early 2000s has been combined with everolimus on the Promus Element® (Boston Scientific) and with paclitaxel on the Taxus Element® (Boston Scientific) stents, which were granted 'CE' European safety marks in November 2009 and May 2010, respectively. Unlike stainless steel and cobalt–chromium alloys, platinum–chromium has the advantage of increased radial strength enabling the stent to have thinner struts, which have been proven to reduce clinical and angiographic restenosis.[46,47] In PLATINUM,[48] the Promus Element® stent was shown to have comparative efficacy and safety when compared with Xience V®. Similarly, the efficacy and safety of the Taxus Element® stent is comparable with that of the Taxus Express2® (Boston

Scientific) stent.[49] In a first-in-man study published in 2010, the Taxus® Petal™ (Boston Scientific) platinum–chromium bifurcation stent was successfully implanted in 25 of 28 patients (89.3%), with satisfactory clinical and angiographic outcomes at 1 year.[98]

Bioabsorbable platforms

The problems encountered with DES have encouraged research into innovative, temporary vascular scaffolds or bioabsorbable stent platforms, which gradually degrade until healing and re-endothelialization have occurred. Eventually, no foreign material is left exposed to the blood, thus mitigating the problem of late stent malapposition and stent thrombosis. These stents have the potential to preserve endothelial function, reactive vasomotion of the artery, and permit late lumen enlargement (expansive remodeling).[38] Initially, a high restenosis rate of 45% was observed in the PROGRESS-AMS trial[39] in which a non-drug-eluting bioabsorbable magnesium stent (Biotronik, Berlin, Germany) was evaluated. Yet more favorable results with stents with a poly-l-lactic acid backbone eluting everolimus (Abbott Vascular, Santa Clara, CA, USA) were reported in the ABSORB

Figure 2 The bioabsorbable stent.



a) the first-generation bioabsorbable stent (revision 1.0) and b) the second-generation bioabsorbable stent (revision 1.1). A clear change in the device design between the two generations is evident, with the out-of-phase zigzag pattern connected directly or by straight bridges in revision 1.0 being replaced by the in-phase hoops linked by straight bridges in revision 1.1. c) In addition, the maximum circular (red circles) unsupported cross-sectional areas (green contours) are larger in Revision 1.0 than in d) revision 1.1. Parts a) and b) reprinted from Journal of the American College of Cardiology, 56 (Suppl. 10), Garg, S. & Serruys, P. W., Coronary stents: looking forward. S43–S78, © 2010, with permission from Elsevier. Parts c) and d) Permission obtained from Wolters Kluwer Health © Serruys, P. W. et al. Circulation 122, 2301–2312 (2010).

trial[38,40] with sustained clinical benefit at 3-years follow-up. The first generation (revision 1.0) of bioabsorbable vascular scaffolds showed slight signs of shrinkage at 6 months contributing to late luminal loss.[41] However, the second generation (revision 1.1) showed substantial improvements with efficacy comparable to that of current DESs, and enhanced conformability to the angulations and curvatures of the vessel (Figure 2).[42,43]

Nanomedicine

In addition to its promising application in cancer chemotherapy, great interest has been generated in the application of nanotechnology in optimization of local drug delivery. Nanoparticles are liposomes consisting of lipids and polymers that can be loaded with a drug and used to nanotexture stents, in molding processes to make stents, and for drug delivery from stents.[99] Nanoparticle-mediated drug delivery systems are expected to revolutionize the development of innovative therapeutic devices, allowing local or targeted delivery of the drug with an excellent biocompatibility profile. This strategy controls the concentration and duration of drug release, thereby potentially reducing systemic toxicity.[100] Of the drugs investigated for restenosis prevention and treatment, only paclitaxel and sirolimus have been successfully administered through nanoparticle-based delivery systems and only in preclinical studies.79,80 Paclitaxel eluted from a cobalt–chromium stent coated with porous carbon–carbon nanoparticles showed promising results with respect to endothelialization and neointimal hyperplasia.[44] Sirolimus incorporated into nanoparticle delivery systems (poly-d,l-lactide) showed improved release kinetics.80 Furthermore, these sirolimus-loaded nanovehicles selectively inhibited cell viability and proliferation of cultured human coronary artery smooth muscle cells, while human coronary artery endothelial cells were inhibited to a lesser extent. Endothelial cells were, therefore, left viable to a degree that allowed re-endothelialization of the stented vessel, yet smooth muscle cell proliferation was still prevented.[45]

In another approach, Chorny *et al*. investigated the novel concept of 'magnetic targeting stents' by combining uniform field-induced magnetization and a biocompatible magnetic nanoparticle formulation in a rat model of carotid stenting.[50] Magnetic targeting allows a drug to be delivered on demand to an *in vivo* site with various dosing regimens. These investigators demonstrated that the magnetic nanoparticles loaded with paclitaxel adequately inhibited neointima formation after uniform-field-controlled targeting when compared with nonmagnetically treated animals.[50] Nanomedicine is, therefore, an innovative and promising perspective in stent design, but has yet to be demonstrated as safe and effective in clinical practice.

Gene-based therapy

Gene-based therapy has emerged over the past few years as a promising tool for the prevention of ISR. Numerous transgenes have been shown to be effective in reducing

ISR in animal models (Table 2) and various modes of local gene delivery have been developed. An effective method of gene delivery is by means of 'gene-eluting stents', which elute plasmid DNA or adenoviral vectors.[51,52] Pyrrole–imidazole polyamide is a powerful gene-regulating compound ('gene silencer') that inhibits the interaction between proteins, such as transcription factors, and DNA.[53] An *in vivo* animal study conducted by Yao *et al*. showed that synthetic pyrrole–imidazole polyamide can suppress neointimal hyperplasia by downregulation of transforming growth factor β1 and connective tissue growth factor,[54] as well as monocyte chemotactic protein 1, matrix metalloproteinase 9, and intercellular adhesion molecule 1,[55] making it a promising next-generation agent.

Nuclear 'orphan' receptors comprise a group of ligand-activated receptors for which specific ligands have not yet been identified, but which are known to directly bind and interact with the promoter of target genes.[56] Nuclear receptor related proteins 1 and 77 have been identified as having a role in ISR development; overexpression of these proteins inhibits intimal proliferation and ISR in animal models.[57,58] A small-molecule drug that enhances the activity of these receptors, namely 6-mercaptopurine, represents an attractive novel target for local intervention in restenosis.[58]

Table 2 Preclinical studies of gene therapy for restenosis

Study	Transgene (approved gene name)	Mode of delivery	Findings
Brito *et al*.[51]	eNOS (*NOS3*)	Plasmid-mediated gene delivery from lipopolyplex-embedded stents	Accelerated RE and reduced ISR
Takemoto *et al*.[52]	pE-NTPdase (*ENTPD*)	Plasmid-mediated gene transfer via cationic gelatin-coated stents	Accelerated RE, reduced ISR, and inhibition of subacute IST
Fishbein *et al*.[101]	iNOS (*NOS2*)	Adenoviral-mediated gene delivery from stents	Reduced ISR
Sharifi *et al*.[102]	eNOS (*NOS3*)	Adenoviral-mediated gene delivery from stents	Accelerated RE and inhibition of ISR
Johnson *et al*.[103]	TIMP3	Adenoviral-mediated gene delivery from stents	Reduced ISR
Egashira *et al*.[104]	Anti-MCP1 (anti- *CCL2*)	Plasmid-mediated gene delivery from stents	Reduced ISR
Walter *et al*.[105]	VEGF2 (*VEGFC*)	gene-eluting stent of naked plasmid DNA	Increased RE and reduced ISR
Brasen *et al*.[106]	EC-SOD (*SOD3*)	Catheter-mediated intramural delivery of adenovirus	Accelerated RE and reduced ISR

Abbreviations: Anti-MCP 1, antimonocyte chemoattractant protein 1; CCL2, chemokine (C-C motif) ligand 2; ENTPD, ectonucleoside triphosphate diphosphohydrolase; EC-SOD, extracellular superoxide dismutase; eNOS: endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; ISR, in-stent restenosis; IST, in-stent thrombosis; NOS2, nitric oxide synthase 2, inducible; NOS3, nitric oxide synthase 3 (endothelial cell); pE-NTPdase, placental ectonucleoside triphosphate diphosphohydrolase; RE, re-endothelialization; SOD3, superoxide dismutase 3, extracellular; TIMP3, tissue inhibitor of matrix metalloproteinase 3; VEGF2, vascular endothelial growth factor 2; VEGFC, vascular endothelial growth factor C.

Finally, reducing the proliferative capacity of vascular smooth muscle cells could be of benefit in reducing neointimal hyperplasia following PCI. The biology of microRNAs and their ability to modify smooth muscle biology has been reviewed by O'Sullivan and colleagues.[59] Two microRNAs, mir-143 and mir-145, were shown to have a key role in the regulation of vascular smooth muscle cells *in vivo* and might, therefore, have therapeutic potential.

Systemic treatment

Since local drug delivery does not eradicate ISR completely, systemic treatment has also been explored, despite the obvious risk of adverse effects. We will briefly discuss the evidence for the antirestenotic effects of several antiproliferative and anti-inflammatory drugs.

The major role of the inflammatory system in restenosis formed the rationale for using prednisone for the prevention of this condition. The IMPRESS trial[60] showed favorable results with oral prednisone in patients undergoing coronary BMS implantation both in angiographic and clinical outcomes. In addition, a subanalysis from the ongoing CEREA-DES trial,[107] reported by Pesarini *et al*. in 2010,[61] showed that high doses of oral prednisone reduced late lumen loss, probably via a reduction in the release of tumor necrosis factor.

Preclinical studies have demonstrated that systemically administered sirolimus (rapamycin) reduces neointimal proliferation after vascular injury.[108] Several clinical trials confirmed the benefit of oral sirolimus in reducing ISR after BMS implantation,[62,63] making it a possible effective and cost-saving alternative to DES implantation. However, the long-term results of the OSIRIS trial,[64] reported by Kufner and co-workers in 2009, showed an attenuated benefit of oral sirolimus after 4 years and, moreover, raised concerns regarding a related increase in newly diagnosed malignancies.

Cilostazol, a phosphodiesterase III inhibitor, has antiproliferative effects[109] and has been shown to reduce intimal hyperplasia and restenosis after both BMS and DES implantation.[65,66] In a meta-analysis published in 2011, Kamal *et al*. concluded that addition of cilostazol to standard dual antiplatelet therapy reduces angiographic restenosis without significantly affecting rates of major adverse cardiac events or bleeding.[67] However, cilostazol was associated with an increase in the incidence of minor adverse effects, such as headaches, gastrointestinal complaints, and palpitations.[110]

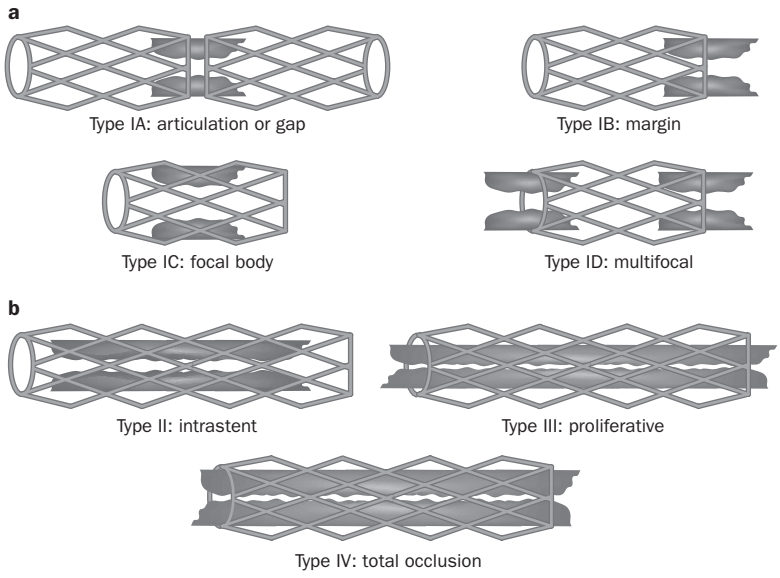
Pioglitazone, a thiazolidinedione peroxisome proliferator-activated receptor gamma agonist, is used for the treatment of diabetes. An additional antiatherogenic effect of the drug in vascular cells limiting lesion development in animal models of atherosclerosis has been described.[111] Several clinical studies have demonstrated a reduced incidence of ISR after stent deployment with thiazolidinedione (pioglitazone or rosiglitazone) administration.[68,69]

Although rates of ISR have been shown to be reduced with the systemic use of various drugs, the concentration of drug accumulated at the site of interest is limited by toxicity. Systemic treatment will, therefore, probably never be superior to local drug delivery with DESs. This strategy could, however, be useful as an adjunct to BMS implantation.

RESTENOSIS: PRESENTATION AND OUTCOMES

If measures to prevent the development of restenosis fail, the clinical presentation of this condition is not always benign and can have a spectrum of acuity.[112] Multiple mechanisms underlie myocardial infarction associated with ISR. An occlusive restenosis can be difficult to differentiate from a thrombotic event and a highly stenotic ISR lesion can promote local nonocclusive thrombosis and lead to a clinical presentation of non-ST-segment elevation myocardial infarction or troponin-positive unstable coronary

Figure 3 Patterns of in-stent restenosis.



Schematic image of four patterns of in-stent restenosis(ISR). a) Pattern I (focal ISR) contains four types (A–D). Restenotic lesions are ≤ 10 mm and are located at the articulation or gap between stents (type IA), at either the distal or proximal margin of the stent (type IB), within the body of a stent (type IC), or a combination of these distributions (type ID). b) Patterns II–IV (diffuse ISR) are defined according to the anatomical position of ISR in relation to the previously implanted stent. Type II lesions are > 10 mm and not extending beyond the margins of the stent; type III lesions are > 10 mm and extending beyond the stent margins; type IV lesions represent total occlusion and have a TIMI flow grade of 0. Permission obtained from Wolters Kluwer Health © Mehran, R. et al. Circulation 100, 1872–1878 (1999).

syndrome.[113] Whether a difference exists between the use of DESs and BMSs in this entity of 'thrombosis on top of restenosis' remains to be elucidated. A pathology study by Nakazawa *et al*. confirmed the occurrence of neoatherosclerosis in the neointimal growth after implantation of both BMSs and DESs, but unstable features of neoatherosclerosis were encountered more frequently and earlier with DESs.[114] Whether this finding translates into a difference in outcomes between stent types is still questionable.[115]

One factor that does influence outcomes associated with ISR is the angiographic pattern of restenosis (Figure 3), which can be broadly classified into focal (< 10 mm) and nonfocal (> 10 mm) lesions. Mehran *et al*. showed that the pattern of ISR independently predicts the long-term need for revascularization, with an increase in the rate of TLR with increasing ISR class.[116] The morphologic patterns of DES restenosis are different from those of BMS, favoring a more focal and easily treated pattern with expected improved clinical outcomes.[117] In DES-treated patients, the rates of TLR were significantly higher in diffuse ISR compared with focal ISR.[118]

TREATMENT OF RESTENOSIS

The optimal treatment for ISR remains debatable. The options include vascular brachytherapy; conventional balloon, cutting balloon, or drug-eluting balloon angioplasty; BMS or DES implantation; and CABG surgery. This diversity of available treatments and the variability in the underlying etiology of restenosis make selection of the most appropriate modality difficult. In other words, the treatment of ISR should be tailored and individualized according to the clinical situation and in view of the underlying etiological factors. Several of the treatment options for restenosis are discussed in detail below.

Vascular brachytherapy

Intracoronary brachytherapy was once recommended as an effective treatment for ISR on the basis of data from several randomized, controlled trials published in the early 2000s.[119,120] Antiproliferative δ (iridium-192) or β (phosphorus-32) irradiation is delivered locally to the target lesions via dedicated catheters. Currently, however, brachytherapy with either β or δ radiation is of very limited use. The difficulty in performing this procedure, particularly handling the radioactive substances, and the increasingly widespread use of DESs has gradually displaced brachytherapy from the armamentarium of the interventionist. Nevertheless, the 5-year follow-up data from the SISR study, presented at the 2011 ACC i2 summit, suggest that brachytherapy could be an equivalent treatment option to SES implantation for the treatment of ISR.[121]

Cutting-balloon angioplasty

The cutting balloon consists of a balloon catheter with three to four blades or 'atherotomes' designed to create discrete longitudinal incisions in the atherosclerotic lesion during balloon inflation. Such controlled dilatation theoretically reduces the force needed to dilate an obstructive lesion compared with standard balloon angioplasty and avoid slipping-induced vessel trauma during PCI, potentially decreasing the risk of ISR development.[122] Although this expected benefit was not demonstrated when the device first came into use in the early 1990s,[123] the later REDUCE III study did show that an IVUS-guided cutting balloon procedure followed by BMS implantation yielded restenosis rates similar to those achieved with DESs, thereby, providing an effective alternative.[124] However, use of the cutting balloon remains uncommon for the treatment of ISR, especially when used without stent placement. In 2010, Park *et al*. raised concerns that cutting-balloon angioplasty might be associated with a higher risk of myocardial infarction than conventional balloon angioplasty;[125] this technique is, therefore, unlikely to become an important ISR treatment modality.

Drug-eluting balloon angioplasty

Non-stent-based local delivery of an antiproliferative drug, particularly using drug-eluting balloons, theoretically represents a very attractive treatment for ISR that avoids the limitations associated with DES platforms. Drug-eluting balloons improve drug delivery by allowing homogenous drug transfer to the entire vessel wall rather than only to the areas covered by stent struts, as with DESs. All currently available drug-eluting balloons use paclitaxel in various coating formulations with a typical dose of 3 µg/mm2 of balloon surface. Drug-eluting balloon angioplasty has been shown to be more effective than conventional balloon angioplasty,[126] and as effective as PES implantation[127] for the treatment of ISR. However, drug-eluting balloons of course cannot prevent the almost immediate elastic recoil phenomenon.

Currently, research in this area is focused on comparisons of the various available drug-eluting balloons. For example, in a preclinical study in an advanced porcine model of coronary restenosis, Joner *et al*. found that the Pantera® Lux (Biotronik, Berlin, Germany; 3.0 µg/mm2 paclitaxel), the SeQuent® Please (B. Braun Melsungen AG, Berlin, Germany; 3.0 µg/mm2 paclitaxel), and the Elutax™ (drug-eluting balloons (Aachen Resonance, Aachen, Germany; 2.0 µg/mm2 paclitaxel) all resulted in delayed healing when compared with conventional balloon angioplasty.[128] However, the investigators also demonstrated significant heterogeneity in neointimal suppression between the balloons, with superiority of Pantera® Lux.[121] This difference was attributed to the 'excipient' used as an effective carrier for paclitaxel in the Pantera® Lux and SeQuent® Please balloons. The 6-month results of the PEPPER trial,[129] which were presented at the ACC i2 summit in April 2011, showed excellent results for the Pantera® Lux balloon for the

treatment of ISR both in BMSs and DESs. However, a subgroup analysis revealed a significantly lower in-stent late lumen loss in BMS-related ISR compared with DES-related ISR, suggesting that the drug effect of the balloon may be decreased in patients with DES-related ISR and may differ between DES drug types. A higher number of patients with diabetes in the DES-related ISR group may have confounded the results.[130]

In the 2010 guidelines for myocardial revascularization published by the European Society of Cardiology, drug-eluting balloons were considered a class IIa indication for the treatment of ISR.[131] Nevertheless, further large studies need to be implemented before these devices can be fully integrated into clinical practice. Patients are currently being recruited for an ongoing trial[132] to investigate the efficacy of a drug-eluting balloons for the treatment of ISR in patients with DESs.

Drug-eluting stents

DESs are known to have fairly low rates of ISR.[4,5] The proportion of restenotic lesions treated with DES in the studies providing this data is, however, low. Since restenotic lesions have a tendency towards recurrent restenosis, as discussed in Part 1 of this Review,[6] the outcomes associated with stent (DES) implantation in these lesions is likely to be different to those for DES placement in *de novo* lesions. In addition, ISR after BMS implantation differs from ISR associated with DES use and, therefore, a distinction between these two types of restenosis should also be made in terms of treatment.

Favorable outcomes of DES treatment for BMS-related ISR have been reported in several studies, even after long-term follow up.[124-127] Treatment with DES placement was found to be more effective and safer than conventional balloon angioplasty,[133] vascular brachytherapy,[134,135] or BMS implantation within the original stent.[136] DESs should, therefore, be the treatment of choice for the treatment of BMS-related ISR. By contrast, the same cannot be said for DES-related ISR, which continues to be a therapeutic challenge. To date, the treatment of this condition has been investigated in only one randomized controlled trial, which showed comparable efficacy for SES reimplantation or a switch to PES implantation in patients with SES-related ISR.[137] Other small nonrandomized trials have produced inconsistent results, limiting the possibility of drawing any definitive conclusions about the optimal treatment of DES-related ISR.[113]

An individual's resistance to a particular eluted drug can be a factor in restenosis development.[138,139] This hypothesis provides the rationale for switching to a different DES for the treatment of DES-related ISR. However, to date, no clinical study has demonstrated clear clinical benefit of implanting an alternative different DES.[137,140] Whether resistance to sirolimus also implies resistance to other limus derivatives remains questionable, as no reports have been published on the use of zotarolimus, everolimus, or biolimus A9-eluting stents for the treatment of SES-related ISR. Another uncertainty is whether the angiographic pattern of DES-related ISR provides a clue to the involvement of drug

resistance. Drug resistance is expected to cause a diffuse pattern of ISR, so perhaps a future study focused on patients with diffuse patterns of ISR would clarify the potential benefits of changing the agent eluted by the stent. In the ongoing prospective, randomized Italian GISE-CROSS trial,[141] treatment with a stent that elutes the same drug as the original restenosed stent (no CROSS group) is being compared with a crossover to an alternative DES in patients with ISR after either PES or SES implantation. The results of this study are eagerly awaited. Patients treated with DES for ISR are at high risk for recurrent ischemic events and should be maintained on dual antiplatelet therapy unless a complication emerges.[113] Therefore, individuals who have a contraindication for, or show noncompliance with, dual antiplatelet therapy should be considered for CABG surgery.

CABG surgery

CABG surgery is usually considered as the 'last resort' treatment for ISR in the clinic. However, this strategy is an appropriate first-line therapy for certain complex cases, such as multivessel ISR, diffuse ISR, multiple subsequent DES restenosis treated by repeat DES implantation, a strong genetic predisposition to ISR that precludes further interventional options, or in cases where dual antiplatelet therapy is not appropriate, as discussed above. To our knowledge, no studies of CABG surgery for the treatment of in-stent restenosis have been conducted.

CONCLUSIONS

Restenosis is a complex disease with a diversity of underlying mechanisms that are still not fully understood. Many innovative technologies, including DESs (with or without specific polymers) and fully biodegradable stents, have been and continue to be developed in the diligent search for an ideal antirestenosis therapy that is both effective and safe in the long term. Developments in the field of gene therapy might also impact future restenosis therapies. Advances in stent design and nanoparticle delivery systems ('nanovehicles') in the past 5 years have already fueled revolutionary changes in the concept of ISR prevention and treatment. In addition, several clinical algorithms for ISR treatment have been proposed on the basis of angiographic pattern of restenosis.[113,141,142] Treatment of ISR should be tailored to the individual, taking into consideration the available evidence and the best strategy for the patient, as well as the best method of treating the lesion. We believe that investing in the prevention of ISR is worth much more than investing in its treatment.

REVIEW CRITERIA

The articles on which this Review is based were identified by searching MEDLINE using the following keywords and Medical Subject Headings (MeSH) terms: "coronary restenosis", "drug-eluting stent", "biodegradable stent", "gene therapy of restenosis", and "nanomedicine". We checked for papers published up to August 2011. Only papers in the English language were included. Although we realize that not all available evidence could be incorporated, the most relevant and influential articles were selected for inclusion in this Review.

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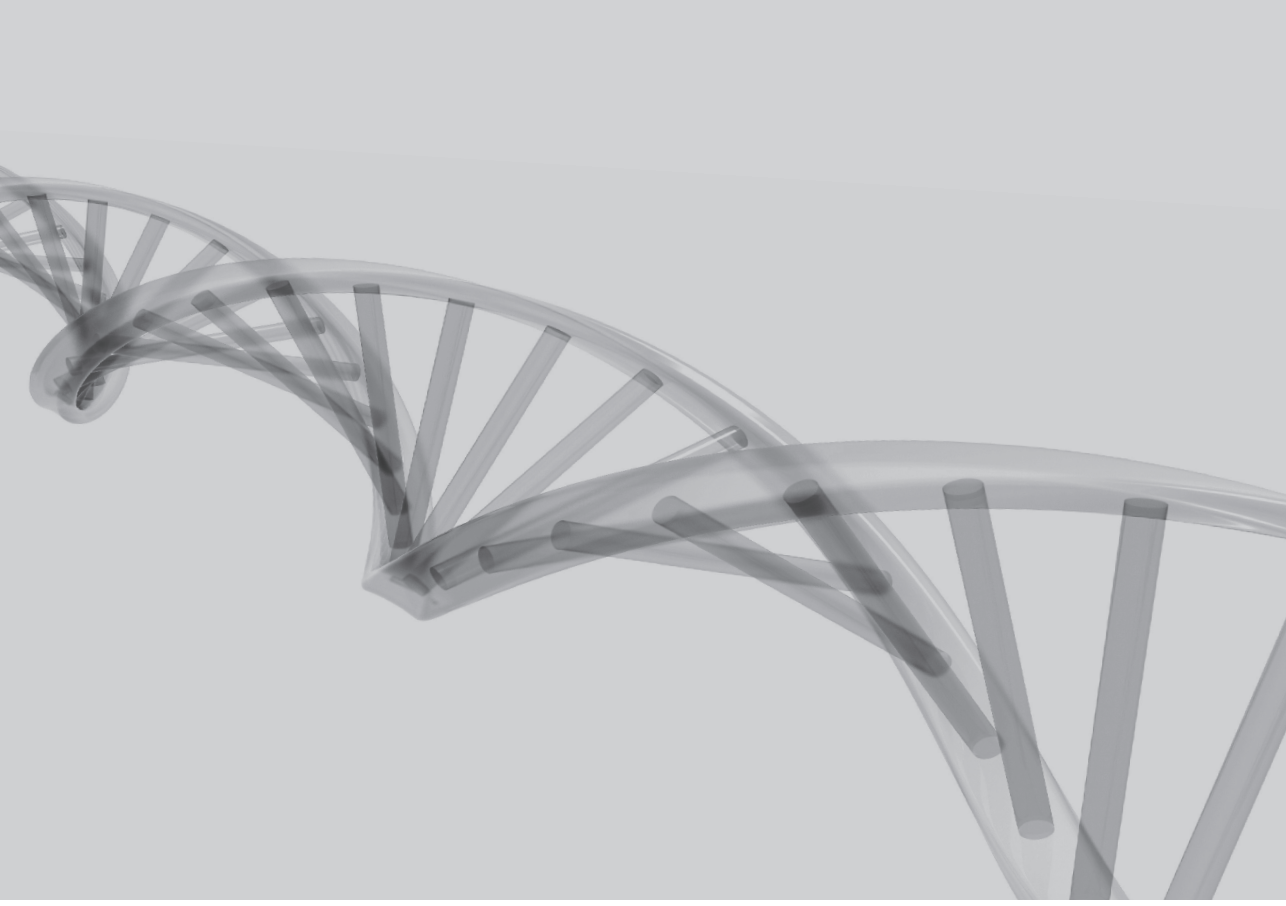
Chapter 4



A genome-wide association study identifies a region at chromosome 12 as a potential susceptibility locus for restenosis after percutaneous coronary intervention

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ABSTRACT

Percutaneous coronary intervention (PCI) has become an effective therapy to treat obstructive coronary artery diseases (CAD). However, one of the major drawbacks of PCI is the occurrence of restenosis in 5–25% of all initially treated patients. Restenosis is defined as the re-narrowing of the lumen of the blood vessel, resulting in renewed symptoms and the need for repeated intervention. To identify genetic variants that are associated with restenosis, a genome-wide association study (GWAS) was conducted in 295 patients who developed restenosis (cases) and 571 who did not (controls) from the GENetic Determinants of Restenosis (GENDER) study. Analysis of ~550 000 single nucleotide polymorphisms (SNPs) in GENDER was followed by a replication phase in three independent case–control populations (533 cases and 3067 controls). A potential susceptibility locus for restenosis at chromosome 12, including rs10861032 ($P_{combined} = 1.11 \times 10^{-7}$) and rs9804922 ($P_{combined} = 1.45 \times 10^{-6}$), was identified in the GWAS and replication phase. In addition, both SNPs were also associated with coronary events (rs10861032, $P_{additive} = 0.005$; rs9804922, $P_{additive} = 0.023$) in a trial based cohort set of elderly patients with (enhanced risk of) CAD (PROSPER) and all-cause mortality in PROSPER (rs10861032, $P_{additive} = 0.007$; rs9804922, $P_{additive} = 0.013$) and GENDER (rs10861032, $P_{additive} = 0.005$; rs9804922, $P_{additive} = 0.023$). Further analysis suggests that this locus could be involved in regulatory functions.

INTRODUCTION

Percutaneous coronary intervention (PCI) for unblocking a narrowed coronary artery is a widely used technique for treating patients with angina or an acute coronary event. Initially, PCI was performed only with balloon catheters, but technical advances made it possible to improve patient outcome by the placement of bare metal stents (BMS), or later, drug eluting stents (DES) at the site of blockage.[1-3] Patients undergoing PCI may suffer from a re-narrowing of the treated lesion, which is called restenosis, with renewed symptoms and the need for repeated intervention, typically within 3 to 6 months.[4] Restenosis occurs in ~5–25% of all treated patients depending on individual characteristics and the techniques used blockage[1-3,5,6], thereby still causing a significant clinical and economic burden for patients and society.

So far, the etiological basis of restenosis is only partly understood. The injury induced by PCI within the vascular wall causes segmental thrombus formation and subsequent invasion of macrophages and polymorphonuclear leukocytes in the blood vessel. This process is followed by release of numerous growth factors from blood cells and stretched smooth muscle cells that lead to the proliferation of smooth muscle cells in the treated lesion.[4,7,8]

In order to prevent restenosis, numerous systemic drugs have been studied for their inhibitory effect on smooth muscle cell proliferation but the results have been inconclusive.[9] A new solution has been the development of DES.[10,11] DES are made by applying a drug (such as sirolimus or paclitaxel) on a coronary stent. The drug is released directly into the (by PCI) injured area and is thereby preventing the inflammatory response and smooth muscle cell proliferation at the site of the coronary intervention. Although DES have decreased the incidence of restenosis, restenosis still occurs in all instances.[12,13] Moreover, although several clinical factors, lesion-related, procedural and biological markers have been shown to be associated with an elevated risk of restenosis[4,14-16], these associations have not been consistently replicated in all studies.

There is evidence that systemic factors can explain part of the risk of restenosis independently of conventional clinical factors or procedures. For instance, in patients with multilesion interventions, the risk that a lesion develops restenosis is 2.5 times higher when a companion lesion developed restenosis, independently of clinical factors.[17] The influence of genetic polymorphisms in the development of restenosis has been investigated by means of candidate gene approaches[18-21], with interesting, although sometimes controversial or inconsistent results. For instance, an insertion/deletion polymorphism in the angiotensin-converting enzyme was associated with restenosis within a French population[22], but not in a German or a Dutch population.[23,24]

We conducted the first genome-wide association study (GWAS) to identify new genetic risk factors for restenosis in a subpopulation of the GENetic DEterminants of

Restenosis (GENDER) study. This was followed by a replication phase in three independent restenosis case–control populations (replication I, replication II and replication III). In a secondary step, we tested if the most associated single nucleotide polymorphisms (SNPs) were also associated with other relevant clinical outcomes such as cardiovascular events and all-cause mortality in GENDER and in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) population. This additional validation effort may add worthwhile information with regard to the genetic factors involved in the pathophysiology of restenosis.

MATERIALS AND METHODS

We investigated the association between genetic variation and clinical restenosis in patients from four different cohort studies, a Dutch population (GENDER), an American population (replication I) and two populations from Germany, a BMS population (replication II) and a DES population (replication III). The GWAS was performed in the GENDER study (discovery set), whereas the replication was performed in replication I, replication II and replication III. In addition, the PROSPER and the GENDER studies were used to check whether the top associated SNPs found in the discovery set and validated in the replication cohorts are involved in other relevant clinical outcomes such as all-cause mortality and cardiovascular events.

All studies were approved by the medical ethical committees of the participating hospitals, had independent clinical event committees who adjudicated the endpoints in a blinded way. Blood samples were collected at the index procedure for DNA isolation after having obtained written informed consent from the patient and the trials were conducted in concordance with the Declaration of Helsinki.

Genome-wide association study

The main characteristics of the GENDER population have been described previously.[1] Briefly, 3104 consecutive symptomatic patients treated successfully by PCI for angina were included in four referral centers for interventional cardiology in the Netherlands. The follow-up protocol included a phone contact or a medical visit at the outpatient clinic at 30 days and around 9 months after stent placement. Clinical restenosis was defined as renewed symptoms requiring target vessel revascularization either by repeated PCI or coronary artery bypass graft surgery, by death from cardiac causes or myocardial infarction not attributable to another coronary event than the target vessel.[1] Within the 9-month follow-up period, 346 patients developed clinical restenosis. Clinical restenosis was not angiographically confirmed.

Table 1: Baseline clinical characteristics of GENDER (discovery population), Replication I, Replication II and Replication III.

Variables	GENDER[a], n = 866	P-value[b]	Replication I[a], n = 265	P-value[b]	Replication II[c], n = 1445	P-value[b]	Replication III[c], n = 1890	P-value[b]
Age (years)	62 ±11		64.5 ± 11.35		64 ± 11		66 ± 11	
Sex (male)	636 (73.44%)	0.50	182 (68.68%)	0.001	1110 (76.81%)	0.78	1478 (79.04%)	0.67
Stenting[d]	584 (67.43%)	0.95	265 (100%)	1	1445 (100%)	1	1890 (100%)	1
Diabetes	117 (20.43%)	0.75	97 (36.60%)	<0.001	301 (20.83%)	0.97	508 (26.88%)	0.58
Current smoker[e]	216 (24.94%)	0.40	147 (55.47%)	0.05	453 (31.34%)	0.41	276 (14.60%)	0.98
Hypercho-lesterolemia	520 (60.04%)	0.84	198 (74.72%)	0.93	624 (43.18%)	0.21	1341(70.95%)	0.51
Total occlu-sion	154 (17.78%)	0.45	14 (5.28%)	0.31	232 (16.05%)	0.18	144 (7.62%)	0.045
Residual stenosis (> 20%)	112 (12.93%)	0.07	11 (4.15%)	0.53	54 (3.37%)	0.92	48 (2.54%)	0.08

n, number of individuals in each cohort. [a]Endpoint: clinical restenosis. [b]P-values computed between cases and controls for each variable . [c]Endpoint: Target lesion revascularisation (TLR) within one year after PCI. [d]GENDER , Replication I and Replication II are BMS cohorts. Replication III is a DES cohort. [e]In replication 1, the data represented in the ever are ever smoke and not current smoker

The GWAS was performed in 325 cases of restenosis (all cases with enough quality DNA to perform the experiment) and 630 matched controls. Cases and controls were matched by gender, age and some possible confounding clinical variables for restenosis in the GENDER study, such as total occlusion, diabetes, current smoking and residual stenosis (Table 1).

All-cause mortality long-term follow-up data were collected in March 2011 and are defined as death from any cause and the information was collected from death certificates. Mean time follow-up was 9.5 years (SD = 3 years) and 239 (27.9%) patients died during the study. From the patients analysed in the GWAS, only nine subjects (1.0%) were not possible to long-term follow-up.

Genotyping and quality control

In the discovery stage, we conducted the genotyping using Illumina Human 610-Quad Beadchips and the infiniumII assay following manufacturer's instructions. These beadchips contain 620 901 SNPs and copy number variants covering 89% of the common genetic variation in the European population at $r^2 > 0.8$. Initially, 955 samples (325 cases and 630 controls) from the GENDER study were genotyped. After excluding bad performing samples (call rate < 0.98 and manually checked sample quality by means of B-allele

frequency and LogR ratio), genotype cluster definitions for each SNP were determined using the 'Cluster all SNPs' option in Illumina Bead-Studio Genotype module version 3.2. Genotype calls were made when a genotype yielded a quality metric (Gencall score) of 0.15 or higher. The final raw data set released from Beadstudio (eliminating intensity probes only) contained reliable called genotypes for 592 186 SNPs and 941 samples (321 cases and 620 controls), with 14 samples not released due to inadequate quality of genotypes. The remaining samples have a call rate ≥ 0.99. Additional quality-control measurements were then performed using PLINK.[25] Seven samples (four cases and three controls) were excluded due to sex discrepancies between the recorded sex and the inferred sex by the X-chromosome genotypes. We checked for the presence of population substructure in the GENDER study by means of multidimensional scaling (MDS). An identical by state distance matrix was calculated for each pair of individuals along with the 940 individuals from the human genome diversity project-centre d'Etude du polymorphisme humain (HGDP-CEPH) panel.[26] We observed that the vast majority of the individuals fell in the same cluster along with the European population, but 67 individuals were outside this cluster and were considered genetic outliers (see Supplementary Material, Fig. S1). One sample was eliminated because it showed a close genetic relationship with another sample from the GENDER study. Furthermore, we excluded SNPs for further analyses with a call rate lower than 95% (n = 1731), with a minor allele frequency lower than 1% (n = 34250) or with a significant deviation from Hardy–Weinberg equilibrium (HWE) in controls ($P < 0.00001$). The final data set consisted of 866 (295 cases, 571 controls) individuals and 556 099 SNPs that passed all quality-control criteria.

We applied the genomic-control method on the GWAS data and found that there was only a slight inflation of the genomic control parameter (l = 1.01581), which implies a high genetic homogeneity between the cases (n = 295) and the controls (n = 571). Given that the inflation factor was found to be minimal, all the statistics results are reported without genomic-control correction. At the tail end of the quantile–quantile plot (Q–Q plot), the P-values from the Cochrane–Armitage trend test deviate from the null distribution expected under the hypothesis of no association (Supplementary Material, Fig. S2), which indicates that several modest associations were present.

Replication stage

Replication I

The CardioGene Study was an IRB-approved, prospective cohort study of 358 patients enrolled at the time of BMS implantation to treat *de novo*, previously untreated native coronary artery lesions at William Beaumont Hospital (Royal Oak, Michigan, USA) and the Mayo Clinic (Rochester, Minnesota, USA). Patients were followed for 1 year to determine in-stent restenosis (ISR) outcomes. Enrolment began in February 2002 and was closed in

September 2003, prior to the approval and clinical use of DES in the USA. Additionally, 104 individuals were enrolled with historical in-stent restenosis in bare metallic stents, with two or more episodes of restenosis in native coronary arteries. The protocol was approved by the NHLBI IRB as well as the IRB at each of the clinical enrolment sites. Informed consent was provided by each patient. Standardized case report forms were used to collect baseline clinical data and outcome information in follow-up.[27]

For the clinical phenotype, consecutive patients presenting to the cardiac catheterization laboratories of the clinical enrolment sites were approached for participation in the study. Follow-up clinical evaluation was performed via patient interview and review of all available medical records at 6 months and 12 months post-stent. ISR was defined as clinical restenosis[27], which was defined by ischemic symptoms after stent implantation and evidence of flow limitation in the treated vessel by either invasive or non-invasive testing. Follow-up angiography was not specifically performed for the CardioGene Study. Any available angiographic data performed as part of each patient's clinical care was recorded.

Genotyping and quality control

Genotypes were assayed using the Affymetrix Genome-Wide Human SNP Array 6.0 platform, and genotypes were called using the Birdseed algorithm. For this analysis, the final sample with genotype data consisted of 265 samples, with 78 in-stent restenosis cases and 187 stented no-restenosis controls, from European ancestry participants among which call rates and deviations from HWE for all SNPs were calculated. Genotype call rates were 95% or greater for all samples. Of these, 35 samples were removed in data cleaning steps for sex mismatch, first degree relative of an included individual and genetic outlier based upon allele sharing and principal components analysis. Genetic analyses were conducted using an additive model, using logistic regression to evaluate the association between the allele dosage and the trait of interest. We adjusted the analysis for age and gender. Genomic control was not applied. Data management and statistical analysis used R, ProbABEL[28] and PLINK software.[25]

Replication II and replication III

Patients of replication II and replication III cohorts presented ischemic symptoms or evidence of myocardial ischemia in the presence of ≥ 50% de novo stenosis located in native coronary vessels. They were treated with PCI and stent implantation at Deutsches Herzzentrum München or 1. Medizinische Klinik rechts der Isar der Technischen Universität München. The main characteristics of the cohorts and the protocols of stent placement and post-stenting therapy have been described previously.[23,29] Briefly, replication II included 1445 patients treated with implantation of BMS and replication III consisted of 1890 patients treated with implantation of DES. TLR within 1 year after PCI was consid-

ered the primary endpoint for both cohorts. Re-hospitalization for repeat angiography was scheduled between 6 and 8 months or earlier if non-invasive evaluation or clinical presentation suggested the presence of ischemia. The secondary endpoint was defined as a diameter stenosis 50% at follow-up angiography at 6 months.

Genotyping and quality control

Initially, 3657 samples were genotyped by means of iPLEX assays. All SNPs showing a P-value $< 10^{-4}$ in the Cochran–Armitage trend test (additive model) in the discovery set (n = 91) were selected for replication. Four assays were designed using MassArray design software (Sequenom, San Diego, CA, USA). Genotyping was performed using iPLEX assays with the use of the MassARRAY methodology (Sequenom), with alleles discriminated by mass spectrometry, following manufacturer's instructions. Four SNP pairs were in complete LD ($r^2 = 1$) and thus we ascertained one tagSNP from each pair and the other was discarded.

Two SNPs did not fit the assay and four more SNPs failed in the experiment. Samples with a call rate < 75% per iPLEX or with a call rate < 90% when considering all iPLEXes were removed for further analysis. SNPs with call rate < 90% or out of HWE (P-value < 0.001 in controls) were also removed. Duplicate samples (~2.5%) showed identical genotypes. Blanks and positive controls were added in each experiment. Finally, 79 SNPs and 3335 samples (2880 controls and 455 cases) passed all quality criteria and were further analyzed.

In order to know if the most associated SNPs in the discovery set contain information to detect the presence of population substructure, we performed a MDS extracting the genotypes of these SNPs from the HGDP-CEPH panel[26], which contains samples belonging to 52 populations all over the world. The MDS did not show any cluster (data not shown) in the HGDP-CEPH panel, not even between populations from different continents, thus indicating that population substructure cannot be considered a confounding factor in the replication cohorts when considering these SNPs.

PROSPER study

The protocol of PROSPER (PROspective Study of Pravastatin in the Elderly at Risk) has been described elsewhere.[30] PROSPER is a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, subjects were screened and enrolled in Scotland (Glasgow), Ireland (Cork) and the Netherlands (Leiden). Men and women aged 70–82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension or diabetes. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. In this study, the predefined endpoints, coronary events, vascular events, vascular mortality and all-cause mortality were evaluated. In particular, coronary events

are a combination of fatal and non-fatal myocardial infarction. Information on all-cause mortality was received by post-mortem reports, death certificates, hospital records, general practitioners' records and/or interviews of family members or witnesses. All endpoints were adjudicated by the study endpoint committee.

Mean follow-up was 3.2 years (range 2.8–4.0) and 604 (10.4%) patients died during the study [31]. The SNPs were selected from the GWAS performed in 5244 subjects of the PROSPER study from whom genotype data were available.

Statistical analysis

Statistical analysis was undertaken using R (v2.8), PLINK v1.06[25], GenABEL and ProABEL softwares implemented in the R package.[28,32] Haploview software[33] was used to infer the LD in the targeted regions. LocusZoom[34] was used to draw regional plots for associated regions.

Each SNP was tested for association using a Cochran–Armitage test (additive model). Inflation in the test statistics was assessed using the genomic-control method and a Q–Q plot was computed.[35] The genotype counts of the discovery and replication stages were combined by means of the Fisher's trend combined P-value approach. All P-values are two-sided. Imputation of genotypes around the top associated SNPs were performed using MACH software.[36,37]

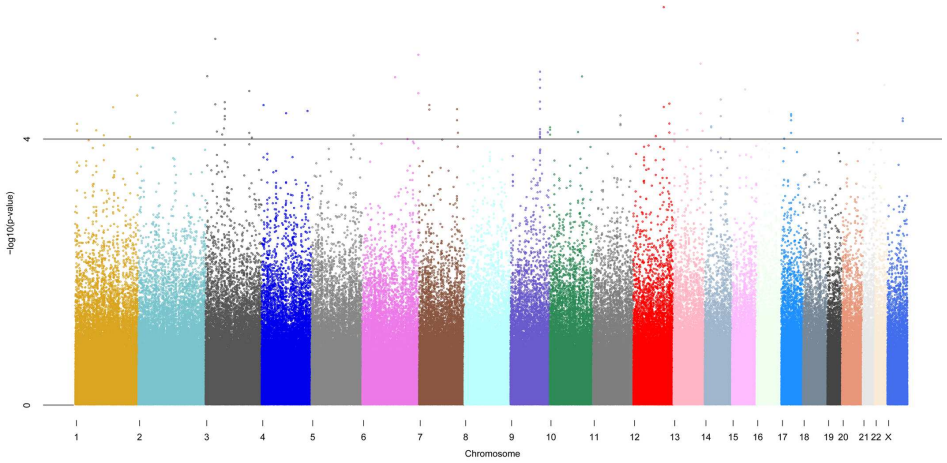
The promoter 2.0 Prediction Server[38] was used to predict promoter regions and is-rSNP for *in silico* regulatory detection.[39] The eQTL analysis was done following methodology described in reference [40].

RESULTS

The overall GWAS results are summarized in a Manhattan plot (Fig. 1). We found that 91 SNPs were associated with restenosis at P-value $< 10^{-4}$ assuming the additive model (Supplementary Material, Table S1).

In the replication stage, these 91 SNPs were genotyped in three restenosis populations, two BMS cohort (replication I and replication II) and a DES cohort (replication III) (Table 1). Associations found in the discovery set were replicated for an intergenic region on chromosome 12 (Table 2). This was specially found for replication I (Table 2). In replication I, rs10861032 showed an association with restenosis [$P = 3.99 \times 10^{-4}$; odds ratio (OR) = 3.06, 95% confidence interval (CI) (1.64–5.70)]; nominally significant at P-value < 0.05 even after Bonferroni correction (0.05/91 SNPs = 5.49×10^{-4}). Using the Fisher's trend combined P-value method, we observed that rs10861032 C allele was potentially associated with higher risk of restenosis ($P_{additive} = 1.11 \times 10^{-7}$) in all four populations combined. We computed I2 as a measurement of heterogeneity and we

Figure 1 Manhattan plot of the GWAS performed in the GENDER study.



P-values were obtained by Cochrane-Armitage trend test for 556099 SNPs and 866 individuals (295 cases and 571 controls) are plotted in –log10 scale according to their chromosomal position. A horizontal black line indicates an additive P-value of 10^{-4}.

Table 2 Association results for rs10861032 and rs9804922 in GENDER (discovery set), replication I, replication II and replication III.

Population	N(case/ control)	Chr	SNP	Position	Alleles[a]	MAF(case/ control)	$P_{additive}$ [b]	OR (CI 95%)[c]
GWAS	295/571	12	rs10861032	102436636	C/T	0.212/0.133	3.29E-05	1.75(1.35-2.27)
Replication I	78/187					0.192/0.099	3.98E-04	3.06 (1.64-5.70)
Replication II	275/1170					0.159/0.147	4.94E-01	1.09(0.85-1.41)
Replication III	180/1710					0.219/0.177	4.89E-02	1.31(1.00-1.7)
Combined							1.11E-07	
GWAS	295/571	12	rs9804922	102437572	T/C	0.114/0.049	1.03E-06	2.48(1.72-3.60)
Replication I	78/187					0.077/0.043	0.063	2.26(0.97-2.14)
Replication II	275/1170					0.077/0.074	8.83E-01	1.03(0.72-1.46)
Replication III	180/1710					0.119/0.092	1.03E-01	1.33(0.95-1.87)
Combined							1.45E-06	

Combined p-values are computed by means of Fisher's trend method. Positions are based on hg18 build. [a]The first allele is the minor allele. MAF, minor allele frequency. [b]Results of the Cochran-Armitage test. [c]OR of the minor allele from the two by two allele frequency table.

obtained a value of 77.04% for rs10861032 which can be interpreted as the percentage of total variation across studies due to heterogeneity.[41] A similar association for all four combined populations was observed for rs9804922 ($P_{additive} = 1.45 \times 10^{-6}$) for the risk allele (T).

Table 3: Association results with Coronary events and All-cause mortality for rs10861032 and rs9804922 in PROSPER.

	$P_{additive}$	HR(95%CI)
rs10861032 (MAF = 0.14)		
Coronary events	0.005	1.25(107-1.46)
All-cause mortality	0.007	1.25(1.06-1.47)
rs9804922 (MAF = 0.06)		
Coronary events	0.023	1.30(1.04-1.62)
All-cause mortality	0.013	1.33(1.06-1.67)

MAF, minor allele frequency; HR, Hazard Ratio. Adjusted for sex, age, country and pravastatin used.

Table 4: Association results for All-cause mortality for rs10861032 and rs9804922 in GENDER.

	$P_{additive}$	HR(95%CI)
rs10861032 (MAF = 0.16)		
All-cause mortality	0.005	1.39(1.10-1.74)
rs9804922 (MAF = 0.07)		
All-cause mortality	0.031	1.42(1.03-1.94)

MAF, minor allele frequency; HR, Hazard Ratio. Adjusted for sex and age

In addition, both SNPs were also associated with all-cause mortality in GENDER (rs10861032, $P_{additive} = 0.005$; rs9804922, $P_{additive} = 0.031$) and in the PROSPER study (rs10861032, $P_{additive} = 0.007$; rs9804922, $P_{additive} = 0.013$) (Tables 3 and 4), and also with coronary events (rs10861032, $P_{additive} = 0.005$; rs9804922, $P_{additive} = 0.023$) in PROSPER. Both rs10861032 and rs9804922 are located on an intergenic region. An open reading frame (*C12orf42*), a hypothetical protein of unknown function, is located 22.7 kb upstream of both SNPs, the gene STAB2 (Stabiline2) is located 68.5 kb downstream and the gene NT5DC3 253.5 kb downstream (Fig. 2). Non-genotyped SNPs in the 610-quad array were imputed from HapMap CEU reference panel (release 22) within a 500 kb window centered on rs1086103.

Analysis on 566 imputed and 140 genotyped SNPs within this region revealed three more imputed SNPs that were associated with restenosis, rs4147305 [$P = 1.72 \times 10^{-4}$, OR = 2.1 95% CI (3.14–1.41)], rs17034045 [$P = 1.72 \times 10^{-4}$ OR = 2.1 95% CI (3.14–1.41)] and rs10861033 ($P = 8.91 \times 10^{-4}$ OR = 1.55 95% CI [2.02–1.20)]. These SNPs are in LD with rs10861032 ($r^2 = 0.37$, $r^2 = 0.37$ and $r^2 = 0.85$, respectively).

Inspection of the UCSC genome browser (http://genome.ucsc.edu) database revealed that rs10861032 maps 110 bp downstream and rs9804922 457 bp upstream to a DNase I hypersensitivity site (chr12:102436746–102437115). In addition, the promoter 2.0 Prediction Server predicts with high scores that the surrounding region of the restenosis associated SNP could be a promoter region. Moreover, the is-rSNP algorithm[39] for *in silico* detection of genetic variation which affect the ability of a transcription factor

(TF) to bind to DNA predicts that both SNPs alter the binding affinity of TFs to the DNA (Supplementary Material, Table S2). Alleles of rs10861032 alter the binding affinity of *Htlf* (Helicase-like TF) ($P = 4.4 \times 10^{-3}$) and *Pax4* ($P = 5.7 \times 10^{-3}$). Members of *Htlf* family have helicase and ATPase activities and are thought to regulate transcription of certain genes by altering the chromatin structure around those genes.[42] Pax4 is a member of the paired box (*PAX*) family of TFs. These genes play critical roles in cancer growth.[43]

Alleles of rs9804922 alter the binding affinity of *TP53* (4.8×10^{-4}) and *HoxA* (3.3×10^{-4}). *TP53* is a tumor suppressor protein that regulates the cell cycle known to be very important in the process of restenosis (sirolimus and paclitaxel work by inhibiting the cell cycle).[44,45] *HoxA* belongs to the cluster A of the called homeobox genes which may regulate gene expression, morphogenesis and differentiation.[46]

We have also checked if both SNPs are expression quantitative loci (eQTLs) and regulate the expression level of genes at the locus (*cis*-regulation). This experiment was performed using *cis* expression-genotype data derived from 1469 human whole blood samples reflecting primary leukocyte gene expression.[40] However, in a window of 1 Mb surrounding rs10861032 and rs9804922, no significant eQTL effect was detected.

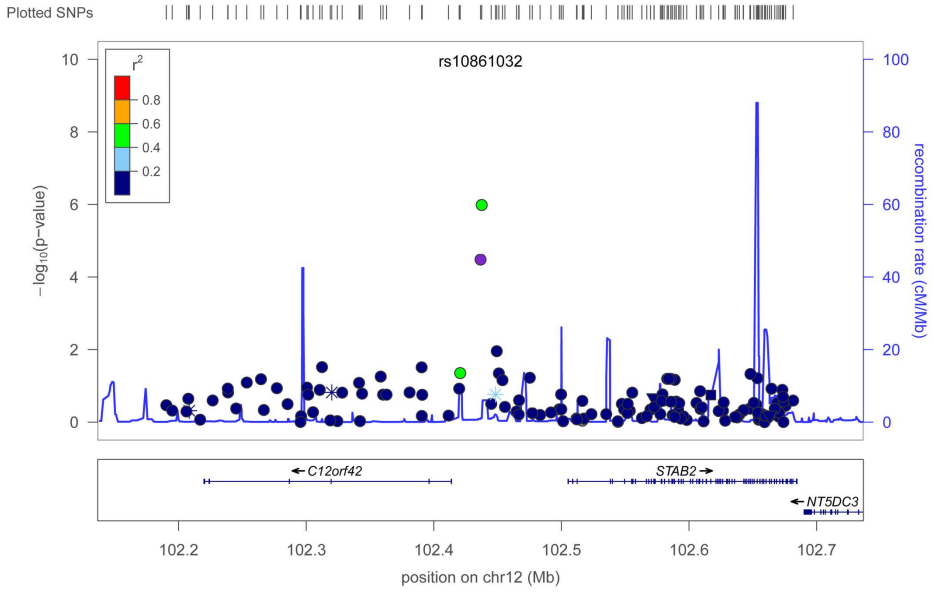
DISCUSSION

To our knowledge, this is the first GWAS that investigates the association between genetic markers and restenosis. Restenosis is a complex phenotype in which the individual contribution of genes to the development of the disease might probably be relatively small and therefore difficult to detect.[16] Using the GENDER population as a discovery set, we found several genomic regions that were associated with restenosis. However, this study presents some potential caveats. First, we combined patients that underwent PCI either by balloon angioplasty alone, BMS or DES as restenosis was and still is the major drawback of PCI in all these three cohort populations. The fact that we found similar results in the different cohorts independently of whether they used BMS or DES indicates that it is unlikely that the involvement of chromosome 12q23.3 region in the pathophysiology of restenosis is based only on the inhibitory effect on smooth muscle cell proliferation of the drugs coating the DES surface, which is only expected in subjects with DES. Secondly, it is also important to point out that in this study we have combined cohorts with two clinical endpoints [clinical restenosis (discovery set and replication I) and target lesion revascularization (TLR, replication II and replication III)]. Although both endpoints are highly comparable, it should be noted that clinical restenosis, although nowadays considered the most clinically relevant endpoint, is somewhat a broader endpoint for restenosis than TLR. The fact that the replication is mainly in replication I could indicate that the top SNPs are mainly associated with clinical restenosis and not with TLR. Finally,

it must be noticed that none of the detected SNPs shows traditional GWAS threshold significance. Nevertheless, the fact that similar association trends were observed in some of these regions when using other data sets suggests that these regions could be indeed potentially involved in the restenosis phenotype. From these SNPs, a region on chromosome 12q23.3 comprising rs10861032 and rs9804922 was replicated in three other restenosis populations, two BMS cohorts and a DES cohort ($P_{combined} = 1.11 \times 10^{-7}$ and $P_{combined} = 1.45 \times 10^{-6}$, respectively, when considering all four populations). Interestingly, both SNPs (rs10861032 and rs9804922) are also associated with all-cause mortality in GENDER and in PROSPER and also with coronary events in PROSPER (no data available for this endpoint in GENDER), which shows that probably this region on chromosome 12 may play a more general role in the development of coronary artery diseases. These findings require further research to disentangle whether the SNPs associated not only with restenosis but also with cardiovascular events and all-cause mortality are for instance the result of a pleiotropic effect of single alleles affecting multiple phenotypes.

The associated locus on 12q23.3 is an intergenic region flanked upstream by *C12orf42* and downstream by the gene STAB2 located 68.5 kb and the gene *NT5DC3* located 253.5 kb away from rs10861032 (Fig. 2). The *STAB2* gene encodes for a transmembrane recep-

Figure 2 Description of the loci on chromosome 12 associated with restenosis in the GWAS.



On the y axis, the $-\log_{10}$ (P-value) is depicted. The most significant SNP in the meta-analysis (rs10861032) is plotted in purple. LD is based on the HapMap CEU sample and is colour-code as red (r^2 from 0.8 to 1.0), orange (0.6-0.4), green (0.6-0.4), white blue (0.4 to 0.2) and dark blue (0.2 to 0). Recombination rate is depicted in light blue. Asterisks display TF site. The plot was generated using LocusZoom.[34]

tor protein which may function in angiogenesis, lymphocyte homing, cell adhesion and receptor scavenging.[47-49] All these processes are described to be of importance in the development of restenosis and coronary events[16], thereby making it a very plausible candidate. *NT5DC3* encodes for a protein involved in the progression of pancreatic cancer[50], therefore it is a gene involved in cell proliferation, an important biological process for the development of restenosis.

The top associated SNP in this study, rs10861032, is in low linkage disequilibrium (LD) ($r^2 < 0.2$) with SNPs in *STAB2*, *NT5DC3* and *C12orf42* (Fig. 2). Moreover, rs10861032 flanks upstream to a DNaseI hypersensitivity site (chr12:102436746–102437115), a universal feature of active *cis*-regulatory sequences, including promoters, insulators, enhancers, boundary elements and locus control regions.[51,52] Furthermore, the region flanked by rs10861032 and rs9804922 is likely to be a promoter region as predicted by the promoter 2.0 Prediction Server.[38] In addition, the is-rSNP algorithm[39] predicts that alleles of both SNPs alter the binding affinity of several TFs to the DNA, making them likely to be regulatory SNPs. Furthermore, it has recently been shown that 'gene desert' regions found by GWAS can be involved in *cis*-regulatory functions.[53-55] This could also be the case of rs10861032 and rs9804922 in the regulation of the expression of *STAB2* or *NT5DC3*, indicating that the region, associated with restenosis on chromosome 12, might be involved in the regulation of the expression of these two genes. However, all these bioinformatic predictions would require wet lab confirmation; in fact, analysis of gene expression and genetic variation on a 1 Mb region surrounding both rs10861032 and rs9804922 in 1469 whole blood samples[40] did not find any significant eQTL effect. Nevertheless, this result does not preclude *cis*-regulating effect in the case of restenosis, as this analysis was performed in whole blood and not yet in a restenosis population and eQTL effects are often cell-type specific.[56] It might be possible that the associated SNPs affect gene expression in a more specific tissue such as coronary or even carotid tissue. These tissues should be further investigated in order to disentangle the possible regulatory functions of rs10861032 and rs9804922 in the development of restenosis.

In conclusion, we have performed the first GWAS to look for genetic variants associated with the development of restenosis after PCI in the GENDER study followed by three independent replication steps and we have identified association for rs10861032 at the 12q23.3 region. The SNPs, rs10861032 and rs9804922, are also associated with all-cause mortality in GENDER and in PROSPER and also with coronary events in PROSPER which indicates that this region might play an important role in the broader range of coronary events. Further research will be needed to disentangle the biological implication of this region in restenosis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *Human Molecular Genetics* online.

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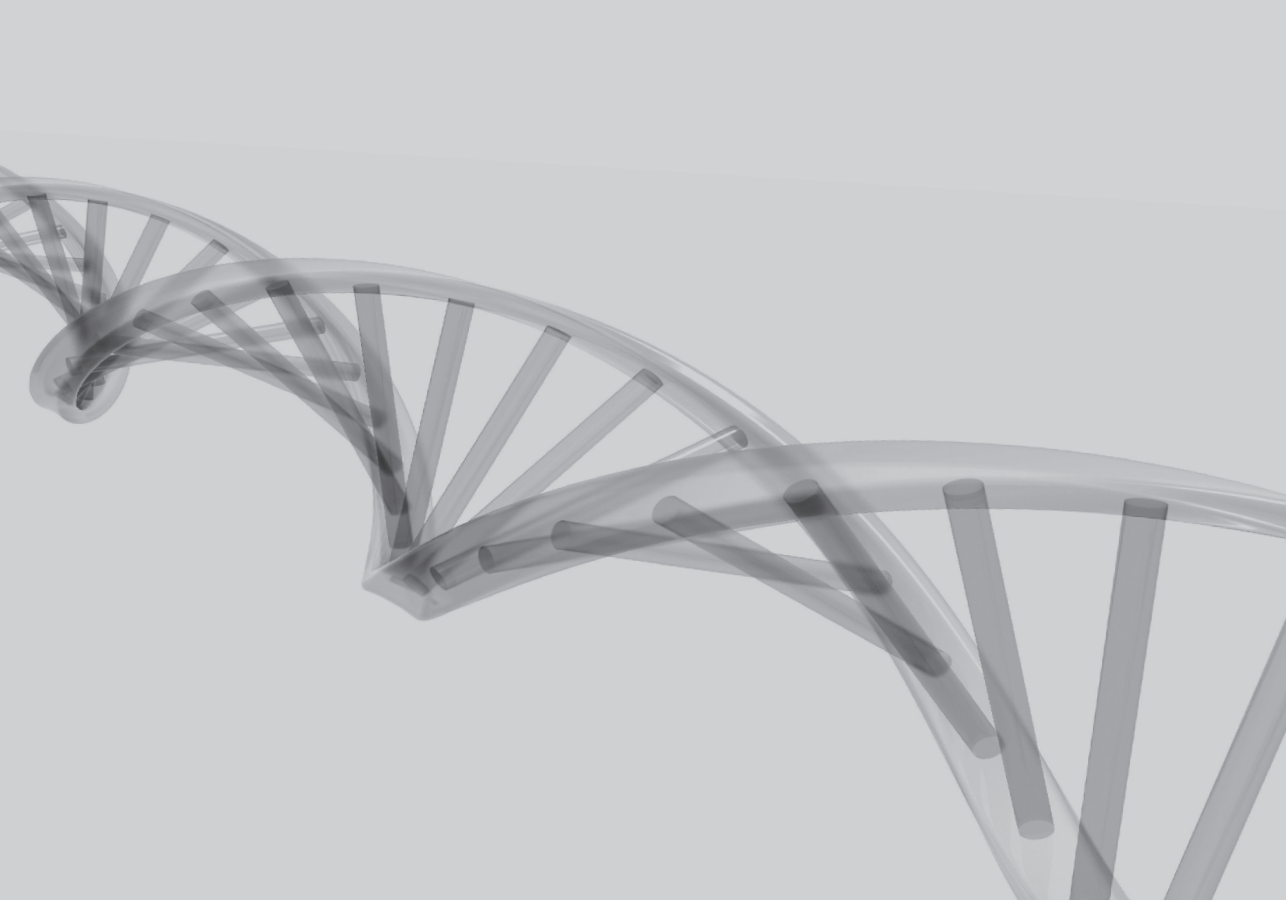
Chapter 5



Matrix metalloproteinases 2 and 3 gene polymorphisms and the risk of target vessel revascularization after percutaneous coronary intervention

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ABSTRACT

Objective: Mixed results have been reported of matrix metalloproteinases (MMP) and their association with restenosis after percutaneous coronary intervention (PCI). The current study examines whether multiple single nucleotide polymorphisms (SNPs), covering the full genomic region of MMP2 and MMP3, were associated with restenosis in the GENDER study population.

Methods and results: The GENetic DEterminants of Restenosis (GENDER) study enrolled 3104 consecutive patients after successful PCI. The primary endpoint was clinical restenosis, defined as target vessel revascularization (TVR), occurring in 9.8% of the patients. From the Hapmap database, 19 polymorphisms of MMP2 and 11 of MMP3 were selected. Furthermore, in a subpopulation, a genome-wide association analysis (GWA) was performed. No significant association was found with any of the investigated SNPs, including the previously reported 5A/6A polymorphism (rs3025058), with regard to TVR using single SNP analysis or haplotype analysis.

Conclusion: We found no significant association of MMP2 or MMP3 with TVR with this SNP-broad gene approach. Although we did not test all the known polymorphisms of these genes, using tagging analyses we examined those SNPs covering all known haplotypes of MMP2 and MMP3 to conclude that these genes do not correlate with a genetic risk of coronary restenosis after successful PCI.

INTRODUCTION

Matrix metalloproteinases (MMPs) form a family of over 20 zinc-dependent enzymes with proteolytic activity against a variety of extracellular matrix components including collagen, proteoglycans and elastin. All MMPs have distinct although overlapping substrate specificities.[1] Increased expression and activity of MMPs have been identified in various pathological processes, such as general inflammation, tumor metastasis, respiratory diseases, myocardial injury, vascular aneurysms, and vascular remodeling.[2] Vascular remodeling and inflammation are important features of restenosis after percutaneous coronary intervention (PCI) and several, mostly small, studies found associations of MMP plasma levels or genetic polymorphisms with restenosis.[3-5]

MMP2 (gelatinase A) is produced by vascular smooth muscle cells (VSMCs) to degrade basement membrane components and other matrix proteins during migration and proliferation of these cells. Different animal models for restenosis showed upregulation of MMP2 during intima formation.[6,7] Furthermore, MMP2 plasma levels were found to be increased after PCI and associated with restenosis.[4,8,9] Although genetic polymorphisms in the MMP2 gene have been associated with myocardial infarction (MI)[10,11] and heart failure[12,13], there are no studies published describing their association with coronary restenosis.

MMP3 (stromelysin-1) cleaves a wide variety of matrix proteins and is considered to reduce the matrix content of the vascular wall. Higher MMP3 expression is associated with the development of atherosclerotic plaques with less matrix protein and as a result smaller and more prone to rupture plaques (i.e. lipid rich plaques). On the other hand, low MMP3 expression genotypes are predisposed to develop relatively larger and more stable plaques (i.e. fibrotic plaques).[2] However, in a MMP3 knockout mouse model, Johnson et al. found that in absence of MMP3 the plaques where not only larger but that they also had a lower smooth muscle cell content, making them less stable.[14] Part of their explanation was that MMP3 has a broad substrate specific, including activation of other MMPs and that therefore many mechanisms could account for the effects of MMP3.[14] Functional studies have shown that the MMP3 -1612 5A/6A promoter polymorphism (rs3025058) is associated with decreased MMP3 expression levels.[15-17] In addition, several studies describe the association of this polymorphism with restenosis. For example, de Maat et al. found an association between the 6A6A MMP3 genotype and an increased risk of restenosis after balloon angioplasty.[3] The same conclusion was made by Humphries et al.[5]; however other studies could not identify this association.[18,19]

MMPs have been extensively studied on their association with different cardiovascular endpoints. The results with regard to restenosis have been mixed. To clarify these uncertainties we conducted this study in the large prospective GENetic DEterminants of Restenosis (GENDER) study population. With this study we systematically examined

whether multiple single nucleotide polymorphisms (SNPs), covering the full genomic region of MMP2 and MMP3, were associated with clinical restenosis in the GENDER study population.

METHODS

Study design

The present study population has been described previously.[20] In brief, the GENDER project was designed to study the association between various gene polymorphisms and clinical restenosis. All included patients were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in four out of the eight academic medical centers in the Netherlands. Patients treated for acute ST elevation myocardial infarction were excluded. Also excluded from analysis were patients suffering from events occurring within one month after the procedure, since these events were mostly attributable to sub-acute stent thrombosis or occluding dissections, and not to restenosis. During the study, no drug-eluting stents were used. Follow-up lasted for at least nine months, except when a coronary event occurred. Clinical restenosis, defined as renewed symptoms requiring target vessel revascularization (TVR), either by repeated PCI or CABG, was designated to be the primary endpoint. The study protocol met the criteria of the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating patients in advance of the PCI procedure. Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures.

Candidate gene approach

SNPs of MMP2 and MMP3 were selected from the HapMap database (http://www.hapmap.org). Using the data from the CEU population, tagging SNPs, with a minor allele frequency > 5%, were selected to cover each haplotype block within the genes (using Haploview program, version 4.1[21]). Five SNPs in MMP2 were analyzed (rs243866, rs857403, rs243849, rs2287076 and rs10775332) and 2 SNPs in MMP3 (rs679620 and rs646910). Furthermore, the 5A6A promoter polymorphism of MMP3 (rs3025058) was added to the tagging SNPs. All SNPs were genotyped by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), using the Sequenom MassARRAYtm methodology (Sequenom Inc, San Diego, CA, USA), following manufacturer's instructions. As quality controls, 368 (11%) of the samples were genotyped in duplo. No inconsistencies were observed. All the blanks (5%) were negative. Cluster plots were made of the signals from the low and the high mass allele. Independent researchers

carried out scoring. Disagreements or vaguely positioned dots (< 1%) produced by Typer 4.0 (Sequenom Inc.) were left out of the results.

Genome wide association study

A genome wide association study was performed in a selection of cases and controls from the GENDER study and has been described previously.[22] In brief, cases and controls were selected from the total GENDER study population and matched by age, gender and other clinical factors such as diabetes and current smoking that have been previously associated to restenosis. A genome-wide association analysis was conducted using the Illumina Human 610-Quad Beadchips following manufacturer's instructions. These beadchips contain 620,901 single nucleotide polymorphism (SNP) and copy number variants (CNV) probes, covering 89% of the common genetic variation in the European population at $r^2 > 0.8$. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol. Of the initially selected patients (321 cases and 620 controls), 295 cases and 571 controls fulfilled all criteria. The SNPs captured in the MMP2 region between positions 54060397 and 54102050 on chromosome 16 and the SNPs in the MMP3 region between positions 102206231 and 102225888 on chromosome 11 were selected for further analyses (see Table 2 and Table 3 for rs numbers).

Statistical analysis

Of the baseline characteristics the discrete variables are expressed as counts or percentages and were compared with chi-square tests. Continuous variables are expressed as mean (± standard deviation) and were compared by means with unpaired 2-sided t-test. Associations of the genotypes of the individual SNPs with TVR were calculated assuming an additive model using a Cox proportional hazards model. We controlled for age, sex and ethnicity in the Cox-regression by including them as covariates. Furthermore, based on the baseline characteristics, we also corrected for diabetes, stenting, total occlusion and type C lesions in the analysis of the candidate approach SNPs. These additional corrections were not necessary for the GWAS SNPs, since that subpopulation was matched for known risk factors as described above. Recessive and dominant models were also tested. After Bonferroni correction for multiple testing, we considered a statistically significant association (at $p < 0.05$) for any SNP with p-value < 0.0017.

The program Haploview was used to estimate allele frequencies, test the consistency of the genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium, and estimate and plot pairwise linkage disequilibrium (LD) measurements between SNPs.

Haplotypes and haplotype frequencies were calculated using PHASE software (http://www.stat.washington.edu/stephens/).[23-25] Haplotypes with a frequency of less than 5% were combined and included in all analyses, without reporting the results. The

haplotype analysis approach performed in this study assumes an additive effect of the haplotypes, and details of this approach have been described elsewhere.[26] Haplotype analyses were performed using STATA for haplotypes with a frequency > 5%. Their effects were calculated using a Cox proportional hazards model including sex, age, and ethnicity and weighted for the probability of having the haplotype.

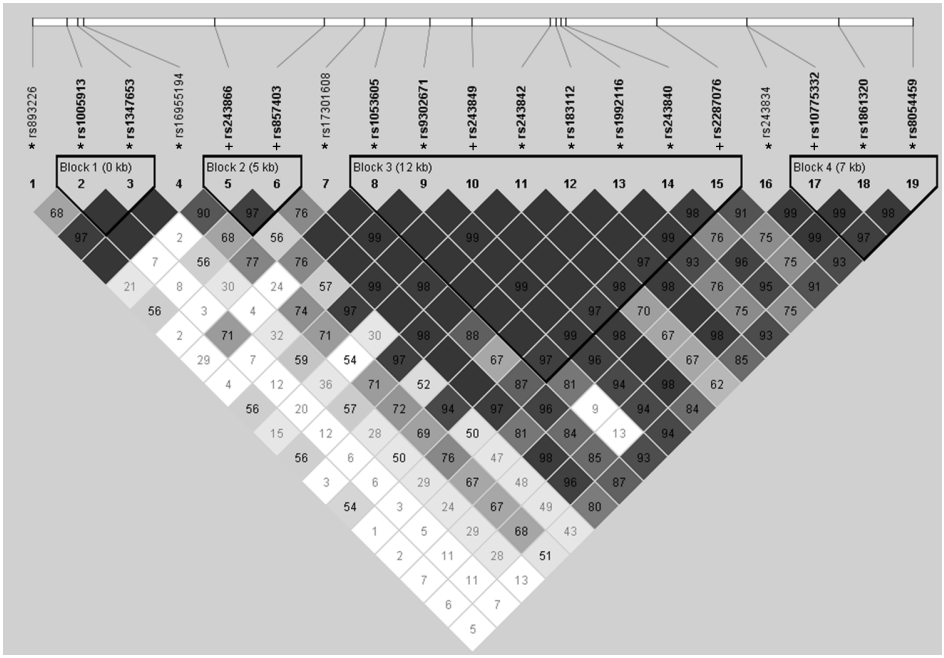
A p-value < 0.05 was considered statistically significant. Statistical analysis was carried out using SPSS 17.0 for Windows.

RESULTS

Baseline characteristics

A total of 3,146 patients had a complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9). Out of 3,146 patients 42 had an event in the first 30 days. These patients were excluded from further analysis, according to the protocol. Baseline characteristics of the population are shown in Table 1. Of the 3,104 patients, 304 (9.8%) patients underwent TVR during follow-up. 51 patients died (1.6%) and 22 (0.7%) suffered from MI. In the total GENDER population use of lipid lowering medication was equal in both cases and controls. Diabetes, total occlusion and C-type lesion were significantly

Figure 1 Linkage disequilibrium structure of 19 MMP2 SNPs on chromosome 16



Values shown in D', * SNP from GWAS, + SNP from candidate approach

Table 1 Demographic, clinical and lesion characteristics of the total GENDER population composed of 3,104 patients.

	Cases (n = 304)	**Controls** (n = 2,800)	**p-value**
Age (years)	61.7 ± 10.1	62.2 ± 10.8	0.46
BMI (kg.m^{-2})	26.9 ± 3.7	27.0 ± 3.9	0.70
Male sex	220 (72.4%)	1996 (71.3%)	0.69
Caucasian ancestry	295 (97.0%)	2714 (96.9%)	0.92
Diabetes	63 (20.7%)	390 (13.9%)	0.001
Hypercholesterolemia	188 (61.8%)	1702 (60.8%)	0.72
Hypertension	138 (45.4%)	1121 (40.0%)	0.07
Current smoker	62 (20.4%)	700 (25.0%)	0.08
Family history of MI	121 (39.8%)	977 (34.9%)	0.09
Previous MI	109 (35.9%)	1130 (40.4%)	0.13
Previous PCI	64 (21.1%)	493 (17.6%)	0.14
Previous CABG	36 (11.8%)	340 (12.1%)	0.88
Stable angina	198 (65.1%)	1881 (67.2%)	0.47
Acute coronary syndrome	106 (34.9%)	974 (31.2%)	0.19
Multivessel disease	148 (48.7%)	1284 (45.9%)	0.35
Peripheral vessel disease	12 (3.9%)	92 (3.3%)	0.54
Lipid lowering medication	171 (56.3%)	1516 (54.1%)	0.48
Restenotic lesions	27 (8.9%)	181 (6.5%)	0.11
Total occlusions	56 (18.4%)	372 (13.3%)	0.01
Type C lesion	94 (30.9%)	708 (25.3%)	0.03
Proximal LAD	70 (23.0%)	619 (22.1%)	0.71
RCX	75 (24.7%)	764 (27.3%)	0.33
Stenting	207 (68.1%)	2145 (76.6%)	0.001
Number of stents	0.98 ± 0.05	1.01 ± 0.01	0.58

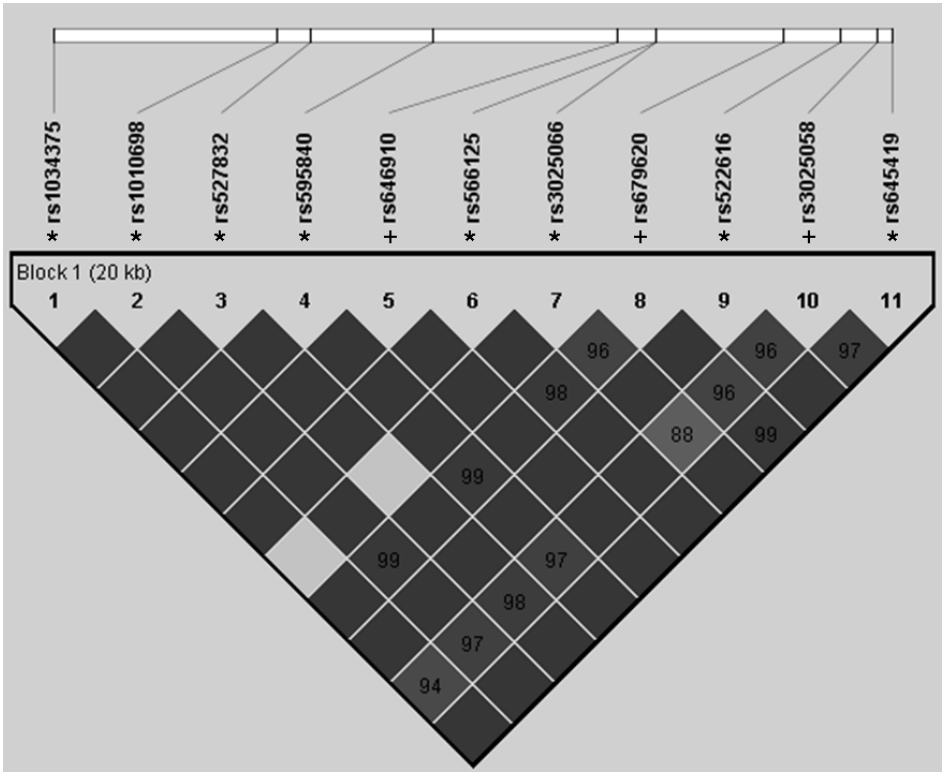
Values were given as n (%) or mean ± SD. BMI: body mass index, MI: myocardial infarction, LAD: left anterior descending branch of the left coronary artery, RCX: circumflex branch of the left coronary artery. p-value determined Pearsons Chi-Square (discrete variables) or unpaired 2-sided t-test (continuous variables).

more frequent in the cases (p-value = 0.001, 0.01 and 0.03 respectively), whereas the percentage of patients receiving a coronary stent was significantly higher in the control group (p-value = 0.001).

Genotypic analyses

The SNPs included in this study are listed in Table 2 and Table 3 for MMP2 and MMP3, respectively. The 19 polymorphisms of MMP2 and 11 polymorphisms of MMP3 provided coverage of all haplotype blocks (Figure 1 and Figure 2, respectively) and were all in Hardy-Weinberg equilibrium (p-value > 0.05). Associations with TVR as a marker of reste-

Figure 2 Linkage disequilibrium structure of 11 MMP3 SNPs on chromosome 11



Values shown in D', * SNP from GWAS, + SNP from candidate approach

nosis were calculated assuming an additive model. No significant associations were observed between any of the MMP2 and MMP3 polymorphisms, including the previously reported 5A/6A polymorphism (rs3025058), and TVR. Dominant and recessive models did not alter these effects (data not shown).

After analyzing the subpopulations of patients with (n = 2352) or without (n = 752) receiving coronary stents during PCI, similar results were obtained. None of the SNPs was associated with TVR in neither of the subpopulations.

Linkage disequilibrium structure is shown in Figure 1 and in Figure 2. MMP2 included 4 haplotype blocks, whereas MMP3 had only 1. The haplotypes of the different blocks of MMP2 were calculated as shown in Table 4. The most frequent haplotype was used as reference. None of the haplotypes was significantly associated with the outcome parameter TVR. In line with the single SNP analysis, haplotype analysis of MMP3 also did not show any significant association with TVR (Table 5).

Table 2. Single nucleotide polymorphisms of MMP2 and the association with target vessel revascularization

SNP	Major allele	Minor allele	MAF	Approach	Rel. position	Gene region	HR	95% CI	p-value
rs893226	G	T	0.40	GWAS	-10496		1,00	(0.840 1,20)	0,97
rs1005913	A	C	0.29	GWAS	-8871	Promoter	0.99	(0.82 - 1.20)	0.95
rs1347653	G	T	0.17	GWAS	-8352	Promoter	0.92	(0.72 - 1.17)	0.42
rs16955194	G	A	0.06	GWAS	-8064	Promoter	1.38	(0.90 - 2.11)	0.14
rs243866	G	A	0.24	Candid.	-1855	Promoter	0.90	(0.74 - 1.09)	0.28
rs857403	A	T	0.18	Candid.	3316	Intron	0.83	(0.67 - 1.04)	0.11
rs17301608	C	T	0.39	GWAS	5219	Intron	1.09	(0.92 - 1.29)	0.32
rs1053605	C	T	0.08	GWAS	6216	Coding exon	1.15	(0.84- 1.57)	0.40
rs9302671	G	T	0.37	GWAS	8334	Intron	1.09	(0.92- 1.30)	0.32
rs243849	C	T	0.17	Candid.	10313	Coding exon	0.82	(0.65- 1.04)	0.11
rs243842	T	C	0.34	GWAS	14031	Intron	0.96	(0.80 1.15)	0.63
rs183112	G	A	0.15	GWAS	14291	Intron	1.24	(0.97- 1.58)	0.09
rs1992116	C	T	0.46	GWAS	14500	Intron	1.16	(0.98- 1.37)	0.09
rs243840	A	G	0.20	GWAS	14768	Intron	0.83	(0.65 - 1.04)	0.10
rs2287076	T	C	0.47	Candid.	19066	Intron	1.17	(0.99 - 1.38)	0.08
rs243834	A	G	0.47	GWAS	23296	Intron	0.88	(0.74- 1.04)	0.14
rs10775332	C	T	0.46	Candid.	23335	Coding exon	1.10	(0.93 - 1.30)	0.22
rs1861320	G	T	0.47	GWAS	27649	Downstream	1.12	(0.94 - 1.33)	0.19
rs8054459	A	G	0.44	GWAS	31158	Downstream	0.84	(0.71- 1.01)	0.06

SNP, single nucleotide polymorphism; MAF, minor allele frequency; Rel., relative; HR, hazard ratio; CI, confidence interval; candid., candidate approach; GWAS, genome wide association scan.
Cox regression analysis with additive model with correction for sex, age and ethnicity in the GWAS SNPs and also correction for diabetes, total occlusion, stenting and type C lesions in the candidate SNPs.

DISCUSSION

Polymorphisms of MMPs have been associated with different cardiovascular events.[2,3,11,12,27,28] Studies of their association with restenosis after PCI are however scarce. Most of these studies look into the relation of the 5A6A promoter polymorphism of MMP3 and restenosis, however those results are mixed.[3,5,18,19] The main finding of the present study is that after testing several polymorphisms in MMP2 and MMP3 using a candidate gene approach we did not find any significant association with coronary restenosis. Since in the meantime a GWAS was carried out in a subpopulation of the GENDER study[22], we decided to include the genotyped polymorphisms in the genomic regions of both MMP2 and MMP3 in our analyses. None SNPs was associated with restenosis. Combining both approaches strengthened our findings of the lack of association between the two MMP genes and coronary restenosis after successful PCI.

Table 3 Single nucleotide polymorphisms of MMP3 and the association with target vessel revascularization

SNP	Major allele	Minor allele	MAF	Approach	Rel. position	Gene region	HR	95% CI	p-value
rs645419	A	G	0.48	GWAS	-2045	Promoter	1.04	(0.87 - 1.25)	0.66
rs3025058	5A	6A	0.50	Candid.	-1676	Promoter	1.09	(0.92 - 1.28)	0.33
rs522616	A	G	0.21	GWAS	-772	Promoter	1.03	(0.83 - 1.27)	0.80
rs679620	A	G	0.50	Candid.	657	Coding exon	1.07	(0.90 - 1.26)	0.46
rs3025066	A	G	0.06	GWAS	3794	Intron	1.09	(0.78 - 1.54)	0.61
rs566125	C	T	0.15	GWAS	3806	Intron	0.89	(0.69 - 1.15)	0.38
rs646910	T	A	0.13	Candid.	4755	Intron	1.06	(0.83 - 1.35)	0.67
rs595840	T	C	0.48	GWAS	9380	Downstream	1.03	(0.86 - 1.24)	0.72
rs527832	C	T	0.12	GWAS	12393	Downstream	0.94	(0.72 - 1.23)	0.67
rs1010698	A	C	0.48	GWAS	13256	Downstream	1.03	(0.86 - 1.24)	0.72
rs1034375	A	C	0.07	GWAS	18805	Downstream	1.23	(0.90 - 1.67)	0.19

SNP, single nucleotide polymorphism; MAF, minor allele frequency; Rel., relative; HR, hazard ratio; CI, confidence interval; candid., candidate approach; GWAS, genome wide association scan.
Cox regression analysis with additive model with correction for sex, age and ethnicity in the GWAS SNPs and also correction for diabetes, total occlusion, stenting and type C lesions in the candidate SNPs.

Table 4 Haplotype analysis of MMP2

	Haplotype	Frequency	HR	95% CI	p-value
Block 1	AG*	54.56%			
	CG	28.12%	1.00	(0.85 – 1.17)	0.99
	AT	17.28%	0.91	(0.75 – 1.11)	0.37
Block 2	AA	57.67%			
	GA*	23.89%	0.87	(0.72 – 1.05)	0.15
	AT	18.05%	0.82	(0.66 – 1.02)	0.08
Block 3	CTCTATAC	35.77%			
	CGCCACAT	35.42%	0.88	(0.73 – 1.06)	0.18
	CGTTGCGT	15.43%	0.90	(0.72 – 1.12)	0.35
	TGCTATAC	7.11%	1.08	(0.81 – 1.44)	0.61
Block 4	TTA	45.60%			
	CGG	44.40%	0.89	(0.76 – 1.05)	0.16
	CGA*	9.47%	1.10	(0.86 – 1.42)	0.44

* wild type; HR, hazard ratio; CI, confidence interval.

Previously, Hojo et al. reported a significant association of increased serum level of MMP2 four hours after PCI and the occurrence of restenosis.[4] Their study population was however very small and the patient characteristics of the 29 patients with follow-up were not well described. Recently, Katsaros et al. reported that in a population of 85 patients with stable angina pectoris receiving DES, increased MMP2 serum activity was

associated with dramatically increased restenosis rates.[8] Although these results look promising for possible identification of patients prone to develop restenosis, larger studies are needed to replicate these results. In our, much larger patient population, we could not confirm their results with genotypic data.

The increased risk of restenosis with the 6A6A MMP3 genotype was described by de Maat et.al. and Humphries et.al. in patients after balloon angioplasty without stenting.[3,5] The latter study also analyzed a subpopulation of patients who received an intracoronary stent and they could not identify an association with the 6A6A MMP3 genotype and restenosis. This difference probably indicates that different processes are involved in these two patient groups. However, since balloon angioplasty without stenting becomes more rare, the clinical relevance of this finding is decreasing. In the GENDER no significant association with TVR of any of the SNPs was detected in neither of the subpopulations. Also, the 5A6A polymorphism was also not related to restenosis, not in the total population nor in the one of the subgroups.

In the GENDER study all patients that were stented received one or more bare-metal stents (BMS). It could be argued that a possible role of investigated MMPs might be ascribed in restenosis in a patient group receiving drug-eluting stents (DES). This is however highly unlikely since DES are associated with a reduction in the development of restenosis, which would more likely diminish a possible genotype-dependent difference in neointima formation. Definite data on this topic is however lacking.

A limitation of our study is that the patients included in the GENDER study were mostly patients with a Caucasian ancestry (96.9%). Different results may be obtained in other ethnic groups. Furthermore, we did not test all known polymorphisms in the two genes of interest, but since SNPs were selected taking into account the LD measurements, all haplotype blocks were covered. Moreover, no significant associations were obtained by means of haplotype analysis. We therefore think it is valid to conclude that no major effects are to be expected from these genes. Also, our findings are based on one single study. However, since the GENDER study is a large prospective follow-up study and taken in account that the previous reports are heterogeneous and in smaller patients groups, the power of these results is large enough to draw these conclusions.

In conclusion, with this study we provide evidence that, with regard to restenosis, a solid association with genetic variation of MMP2 and MMP3 is absent. Although we did not test all the known polymorphisms of the MMP2 and MMP3 genes, using tagging analyses we examined those SNPs covering all known haplotypes of MMP2 and MMP3 to conclude that genetic variation of these genes does not correlate with a genetic risk of coronary restenosis after successful PCI. As the search for genetic factors involved in the process of restenosis continues, we merely exclude genetic variation in two previously proposed candidate genes for involvement in the increased risk for restenosis, so future research may focus on other targets.

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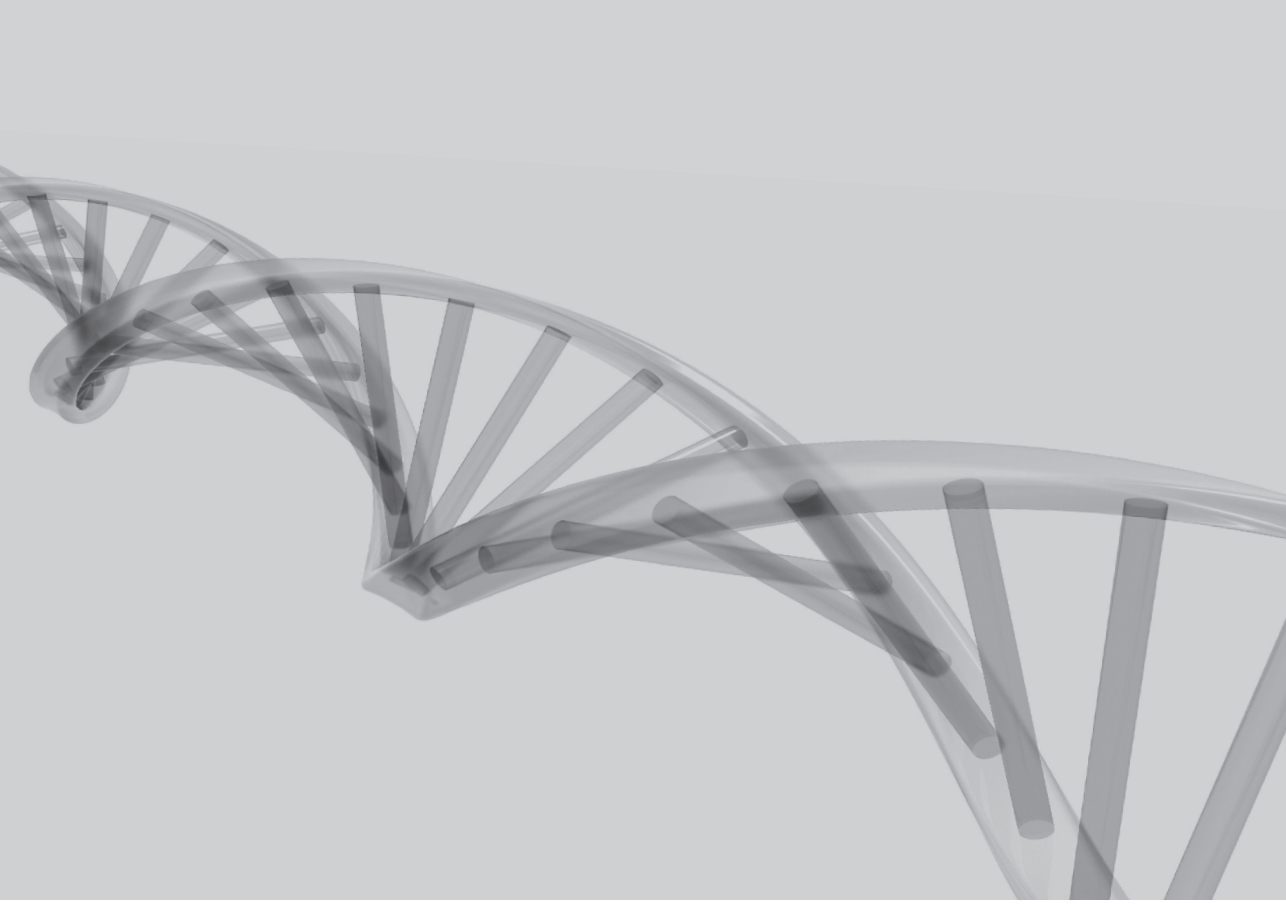
Chapter 6



Systematic testing of literature reported genetic variation associated with coronary restenosis
Results of the GENDER study

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ABSTRACT

Background: Coronary restenosis after percutaneous coronary intervention still remains a significant problem, despite all medical advances. Unraveling the mechanisms leading to restenosis development remains challenging. Many studies have identified genetic markers associated with restenosis, but consistent replication of the reported markers is scarce. The aim of the current study was to analyze the joined effect of previously in literature reported candidate genes for restenosis in the GENetic DEterminants of Restenosis (GENDER) databank.

Methods and results: Candidate genes were selected using a MEDLINE search including the terms 'genetic polymorphism' and 'coronary restenosis'. The final set included 36 genes. Subsequently, all single nucleotide polymorphisms (SNPs) in the genomic region of these genes were analyzed in GENDER using set-based analysis in PLINK. The GENDER databank contains genotypic data of 2,571,586 SNPs of 295 cases with restenosis and 571 matched controls. The set, including all 36 literature reported genes, was indeed significantly associated with restenosis, $p = 0.024$ in the GENDER study. Subsequent analyses of the individual genes demonstrated that the observed association of the complete set was determined by 6 of the 36 genes.

Conclusion: Despite overt inconsistencies in literature, with regard to individual candidate gene studies, this is the first study demonstrating that the joint effect of all these genes together, indeed is associated with restenosis.

INTRODUCTION

Restenosis is a complex disease for which the causative mechanisms have not yet been fully identified. Despite medical advances, restenosis still remains a significant complication after percutaneous coronary intervention (PCI).[1] Identification of risk factors and underlying mechanisms could not only be useful in risk stratification of patients, they also contribute to our understanding of this condition. In addition, these factors could provide evidence on which to base individually tailored treatment and aid in the development of novel therapeutic modalities.[2] Unraveling the mechanisms leading to restenosis development remains challenging. Genetic susceptibility is known to play a role in the individuals risk of developing this complication.[1] Many studies have focused on identification of genetic markers associated with restenosis. Over the last decades genetic research has developed from candidate gene approaches[3-5] to multiplex arrays[6] and finally to genome wide association studies (GWAS).[7] Genetic variation in large array of plausible candidate genes have been associated with restenosis, however, consistent replication of the reported markers is scarce.[1] Possible explanations for this lack of consistency are the small sample size of many (especially relative more dated) studies, phenotype heterogeneity and lack of proper replication cohorts.

Currently more and more GWAS are being performed, investigating many diseases, including cardiovascular diseases.[8,9] An advantage of GWAS is the hypothesis-free approach of this method, enabling identification of new genetic loci associated with the disease of interest. With respect to restenosis, a disadvantage of the GWAS approach is that due to the complexity of the disease the effect size of individual genetic markers is likely to be small and therefore hard to detect. Moreover, the availability of (large) replication cohorts is very limited. In 2011, the first GWAS on restenosis in the GENetic DEterminants of Restenosis (GENDER) study identified a new susceptibility locus on chromosome 12.[7] The fact that this GWAS only identified this previously unknown locus does not mean that genetic variation in the previously proposed candidate genes does not affect restenosis development. It merely indicates that the influence of other individual markers is probably too small to detect in the GWAS setting. Especially for the complex traits, a more appropriate approach to interpret GWAS data is to analyze the combined effect of a single nucleotide polymorphism (SNP) set, grouped per pathway or gene region.[10] To date, investigation into a possible joined effect of multiple genetic markers for restenosis has not been performed.

The goal of the current study is to investigate whether the last decade of research on genetics of restenosis has led to a set of genes that is associated with restenosis in a set-based analysis using the available genotypic data of the GENDER databank.

METHODS

Gene Selection

Candidate genes previously associated with restenosis were selected after a search of literature of papers published up to November 2011. Genes were identified searching MEDLINE using keywords as 'genetic polymorphism', 'candidate gene', 'restenosis' and 'percutaneous coronary intervention'. Selection criteria included a sample size of > 250 patients and the observation of a significant association of a SNP with restenosis. The final set included 36 genes (Table 2). All available SNPs from the GENDER GWAS databank within a 10-Kb window around these genes were analyzed.

Study Population

The design of GENDER and the genome-wide association study (GWAS), which has been performed in a subset of this study population, have both been described previously.[7,11] In brief, GENDER included 3,104 consecutive unrelated symptomatic patients treated successfully by PCI for angina. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure. During a follow-up period of 9 months, the endpoint clinical restenosis, defined as renewed symptoms requiring target vessel revascularization (TVR) either by repeated PCI or CABG, by death from cardiac causes or myocardial infarction not attributable to another coronary event than the target vessel, was recorded. During follow-up, 346 patients developed clinical restenosis. Blood samples were collected at the index procedure for DNA isolation. The GWAS was performed in 325 cases of restenosis and 630 controls matched by gender, age, and some possible confounding clinical variables for restenosis in the GENDER study such as total occlusion, diabetes, current smoking and residual stenosis. Genotyping was performed using the Illumina Human 610-Quad Beadchips following the manufacturer's instructions. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol. The final dataset consisted of 866 individuals (295 cases, 571 controls) and 556,099 SNPs that passed all quality control criteria, together covering 89% of the common genetic variation in the European population.[7,12] Imputation was performed with MACH software based on the HapMap II release 22 CEU build 36 using the default settings.[13] This program infers missing genotypes based on the known genotypic data of the samples together with haplotypes from a reference population provided by HapMap taken into account the degree of linkage disequilibrium (LD). After subsequent quality control, we excluded SNPs for further analyses with a call rate lower than 95% (n = 3335) or with a significant deviation from Hardy–Weinberg equilibrium (HWE) in controls ($P < 0.00001$) (n = 79). The final GENDER Biobank dataset consisted of 866 (295 cases, 571 controls) individuals and 2,571,586 SNPs.

Statistical Analysis

The statistical analyses were performed using the set-based test of PLINK v1.07.[14] During this test, first a single SNP analysis of all SNPs within the set is performed. Subsequently a mean SNP statistic is calculated from the single SNP statistics of a maximum amount of independent SNPs below a certain p-value threshold. If SNPs are not independent and the LD (expressed in R^2) is above a certain threshold, the SNP with the lowest p-value in the single SNP analysis is selected. This analysis is repeated in a certain amount of permutations of the phenotype. An empirical p-value for the SNP set is computed by calculating the number of times the test statistic of the simulated SNP sets exceeds that of the original SNP set. For the analysis of this study, the parameters were set to p-value threshold < 0.05, R^2 threshold < 0.1, maximum number of SNPs = 5 and 10,000 permutations.

Initially, the set including all 36 genes is tested as a whole for the association with restenosis. Subsequent analysis of the individual genes will be justified only when the complete set is significantly associated with the endpoint.

RESULTS

Patient characteristics are presented in Table 1. No significant differences were found between cases and controls regarding the known risk factors for restenosis (age, diabetes, smoking, stenting and previous restenosis). Hypertension and multivessel disease were more common in the cases compared to the controls.

In Figure 1 the QQ-plot of the GENDER GWAS after imputation is shown, demonstrating that no genomic inflation has occurred in this analysis (lambda = 1.027). The complete set of 36 genes, previously associated with restenosis in literature, contained 2,581 SNPs. A detailed description of the individual studies and candidate genes can be found in Table 2. The largest gene was chemokine (C-X3-C motif) receptor 1 (CX3CR1) of 316.54kb, contributing 384 SNPs (14.8%), and glutathione peroxidase 1 (GPX1) was with 1.18kb the smallest gene, only contributing 8 SNPs (0.3%). Analysis of the complete set using the set-based test demonstrated a significant association with clinical restenosis, with an empirical p-value of 0.024.

To determine which genes are mainly responsible for this association we subsequently investigated the association of the individual gene based sets. Six of the 36 genes were demonstrated to have an empirical p-value below 0.05 (Table 3). In order of descending p-values the associated genes are; angiotensin II receptor type 1 (AGTR, p = 0.028), glutathione peroxidase 1 (GPX1, p = 0.025), K(lysine) acetyltransferase 2B (KAT2B, also known as PCAF, p = 0.023), matrix metallopeptidase 12 (MMP12, p = 0.019), fibrinogen beta chain (FGB, p = 0.013) and vitamin D receptor (VDR, p = 0.012). Detailed

Table 1, Demographic, clinical and lesion characteristics of the study population.

	Cases (n = 295)	**Controls** (n = 571)	**p-value**
Age (years)	62.8 ± 10.6	62.4 ± 10.9	0.59
BMI (kg.m^{-2})	26.7 ± 3.6	27.1 ± 3.7	0.20
Male sex	213 (72)	421 (74)	0.63
Diabetes	58 (20)	119 (21)	0.68
Hypercholesterolemia	179 (61)	341 (60)	0.79
Hypertension	138 (47)	211 (37)	0.005
Current smoker	68 (23)	148 (26)	0.36
Family history of MI	117 (40)	210 (37)	0.41
Previous MI	119 (40)	246 (43)	0.44
Stable angina	188 (64)	400 (68)	0.06
Multivessel disease	155 (53)	248 (43)	0.01
Restenotic lesion	23 (8)	48 (8)	0.76
Total occlusion	57 (19)	97 (17)	0.40
Type C lesion	95 (38)	154 (27)	0.11
Stenting	199 (68)	385 (67)	0.99

Values were given as n (%) or mean ± SD. Patients using anti-diabetic medication or insulin at study entry were considered to be diabetics. Hypertension was defined as a blood pressure of either above 160 mmHg systolic or 90 mmHg diastolic. Hypercholesterolaemia was defined as total cholesterol concentrations of above 5 mmol/L. BMI: body mass index, MI: myocardial infarction. P-values are determined by Pearsons Chi-Square (discrete variables) or unpaired 2-sided t-test (continuous variables)

Table 2 Candidate genes and the studies that reported their association with restenosis.

Candidate gene			**Literature based study characteristics and results**					
Gene	**Entrez nr**	**Location**	**Study size**	**% of cases**	**FU (mo)**	**Top SNP**	**Effect size (95% CI)[a]**	**Ref**
adrenergic beta-2-receptor (ADRB2)	154	5q31-q32	3104	9.8	9	rs1042713	HR 1.33 (1.06-1.68)	[6]
advanced glycosylation end product-specific receptor (AGER)	177	6p21.3	267	UK	6-9	rs1800624	↓	[15]
			297	25.9	6	rs2070600	NS	[16]
angiotensin II receptor, type 1 (AGTR1)	184	3q24	272	29.8	6	rs5186	NS	[17]
			3104	9.8	9	rs5186	OR 1.85 (1.28-2.66)	[18]
Butyrylcholinesterase (BCHE)	590	3q26.1-q26.2	461	23.2	6	rs1803274	OR 5.5 (1.6-21.4)	[19]
chemokine (C-C motif) ligand 11 (CCL11)	6356	17q21.1-q21.2	3104	9.8	9	rs4795895	HR 0.73 (0.58-0.93)	[6]
CD14	929	5q31.1	129	24	6	rs2569190	RR 3.8 (1.2-11.6)	[20]
			3104	9.8	9	rs2569190	HR 0.74 (0.55-0.99)	[6]

Table 2 Candidate genes and the studies that reported their association with restenosis. (*continued*)

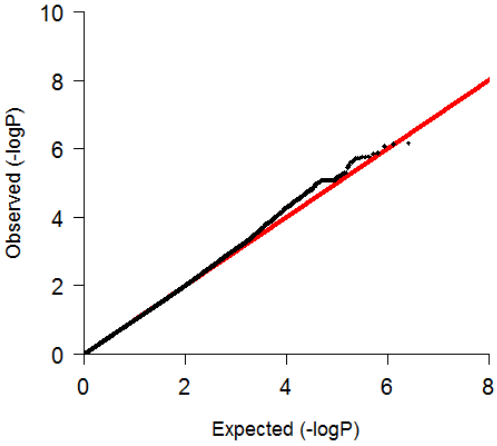
Candidate gene			Literature based study characteristics and results					
Gene	**Entrez nr**	**Location**	**Study size**	**% of cases**	**FU (mo)**	**Top SNP**	**Effect size (95% CI)[a]**	**Ref**
cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDKN1B)	1027	12p13.1-p12	433	11.3	12	rs34330	NS	[21]
			2309	8.8	9	rs36448499	HR 0.61 (0.40-0.93)	[22]
collagen, type III, alpha 1 (Col3A1)	1281	2q31	527	9.1	6	rs1800255	OR 4.2 (1.4-11.2)	[23]
colony stimulating factor 2 (CSF2)	1437	5q31.1	3104	9.8	9	rs25882	HR 0.76 (0.61-0.94)	[6]
chemokine (C-X3-C motif) receptor 1 (CX3CR1)	1524	3p21.3	365	25.5	6	rs3732379	OR 2.4 (1.3-4.2)	[24]
cytochrome b-245, alpha polypeptide (CYBA)	1535	16q24	730	35.8	6	rs4673	OR 0.5 (0.3-0.8)	[25]
cytochrome P450, family 2, subfamily C, polypeptide 19 (CYP2C19)	1557	10q24	928	19.1	12	rs12248560	↓	[26]
fibrinogen beta chain (FGB)	2244	4q28	527	9.1	6	rs1800790	OR 2.7 (1.2-6.2)	[23]
			2257	8.8	9	rs1800790	NS	[27]
coagulation factor V (F5)	2153	1q23	3104	9.8	9	rs6025	HR 0.40 (0.19-0.85)	[28]
glutathione peroxidase 1 (GPX1)	2876	3p21.3	461	23.2	6	rs1050450	OR 2.1 (1.2-3.8)	[19]
interleukin 10 (IL10)	3586	1q31-q32	162	39.5	UK	rs1800871	HR 0.39 (0.16-0.94)	[29]
			1850	17.6	12		NS	[30]
			3104	9.8	9	rs3024498	HR 2.0 (1.4-2.8)	[31]
interleukin 1 receptor antagonist (IL1RN)	3557	2q14.2	183	46.4	12	VNTR	HR 5.24 (1.63-16.81)	[32]
			779	43.9	6	VNTR	NS	[33]
			1850	20.3	12	rs419598	OR 0.73 (0.58-0.92)	[3]
insulin receptor (INSR)	3643	19p13.3-p13.2	461	23.2	6	7,067,365C>A	OR 1.9 (1.2-3.1)	[19]
integrin, beta 2 (ITGB2)	3689	21q22.3	1207	21.2	12	rs235326	OR 0.71 (0.55-0.92)	[4]
lipoprotein lipase (LPL)	4023	8p22	3104	9.8	9	rs328	OR 0.62 (0.44-0.86)	[34]
matrix metallopeptidase 12 (MMP12)	4321	11q22.3	527	9.1	6	rs2276109	OR 3.9 (1.0-12.4)	[23]
matrix metallopeptidase 9 (MMP9)	4318	20q11.2-q13.1	461	23.2	6	rs2664538	OR 2.0 (1.0-3.9)	[19]

Table 2 Candidate genes and the studies that reported their association with restenosis. (*continued*)

Candidate gene			Literature based study characteristics and results					
Gene	**Entrez nr**	**Location**	**Study size**	**% of cases**	**FU (mo)**	**Top SNP**	**Effect size (95% CI)[a]**	**Ref**
methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR)	4524	1p36.3	260	36.9	6	rs1801133	OR 3.58 (1.51-8.46)	[35]
			800	18.9	12	rs1801133	NS	[36]
nitric oxide synthase 3 (NOS3)	4846	7q36	205	29.3	6	rs2070744	OR 2.06 (1.08-3.94)	[37]
			901	10.2	9	rs1799983	HR 1.67 (1.09–2.54)	[38]
			1556	20.8	12	rs1799983	NS	[39]
purinergic receptor P2Y, G-protein coupled, 12 (P2RY12)	64805	3q24-q25	2062	8.4	9	Haplotype of 5 SNPs	HR 1.6 (1.2-2.0)	[40]
serpin peptidase inhibitor, clade E, member 1 (SERPINE1)	5054	7q21.3-q22	1850	20.3	12	rs1799899	NS	[41]
			3104	9.8	9	rs1799899	HR 1.26 (1.07-1.49)	[28]
K(lysine) acetyltransferase 2B (KAT2B, PCAF)	8850	3p24	3104	9.8	9	rs2948080	HR 0.80 (0.67-0.97)	[42]
peroxisome proliferator-activated receptor gamma (PPARG)	5468	3p25	565	28.7	6	rs3856806	↓	[43]
			935	18.3	12	rs3856806	NS	[44]
c-ros oncogene 1 , receptor tyrosine kinase (ROS1)	6098	6q22	461	23.2	6	rs529038	HR 1.8 (1.1-2.8)	[19]
thrombomodulin (THBD)	7056	20p11.2	730	35.8	6	rs1042579	OR 2.1 (1.3-3.53)	[25]
thrombospondin 4 (THBS4)	7060	5q13	628	UK	6-10	rs1866389	OR 2.67 (1.04-6.80)	[45]
thrombopoietin (THPO)	7066	3q27	527	9.1	6	rs6141	OR 2.4 (1.1-5.3)	[23]
tumor necrosis factor (TNF)	7124	6p21.3	1850	17.6	12	rs1800629	NS	[30]
			3104	9.8	9	rs361525	HR 0.60 (0.37-0.98)	[5]
tumor protein p53 (TP53)	7157	17p13.1	132	0	UK	rs1042522	↑	[46]
			433	11.3	12	rs1042522	NS	[21]
			779	43.9	6	Haplotype of 3 SNPs	OR 0.58 (0.40-0.83)	[47]
uncoupling protein 3 (UCP3)	7352	11q13.4	527	9.1	6	rs1800849	OR 5.2 (1.9-13.0)	[23]
vitamin D receptor (VDR)	7421	12q13.11	3104	9.8	9	rs11568820/ rs4516035 haplotype	HR 0.72 (0.57-0.93)	[48]

[a]The direction of the association between genetic variation and the risk of restenosis, when effect size is not available; ↓ protective effect, ↑ deleterious effect. Entrez nr; unique gene ID number used in NCBI database. Abbreviations: FU, follow-up; UK, unknown; NS, not significant; OR, odds ratio; HR, hazard ratio; RR, relative risk; Ref, reference.

Figure 1 Q-Q plot for the GWAS after imputation on clinical restenosis in the GENDER study population.



Lambda = 1.027

information on the individual SNPs in these genes is depicted in Table 4. The SNP with the lowest individual p-value was rs11574027 in the VDR gene, $p = 1.4 \times 10^{-4}$. In the complete GWAS analysis, which has been published in 2011,[7] this SNP ranked 116th. The strongest association in that analysis was found with a SNP in an intergenic region on chromosome 12, $p = 1.0 \times 10^{-6}$.

Logistic regression models with and without the 11 SNPs described in Table 4 demonstrated that together these SNPs explained 9.0% (R Square improved from 0.008 to 0.098) of the occurrence of clinical restenosis in this cohort.

As a final analysis we removed the 6 significantly associated genes from the complete set. Subsequent analysis of the subset of the other 30 genes did not demonstrate a remaining joined effect, p = 0.65 after 10,000 permutations.

DISCUSSION

With this study we aimed at clarifying the ambiguities regarding genetic predisposition for developing restenosis after PCI. We show that the joined effect of the complete spectrum of candidate genes, so far proposed to be involved in the restenotic process, results in a significant association with restenosis. This association is determined by six individual genes. Analyzing a subset containing the 30 genes not associated with the endpoint on an individual basis, did not show a remaining joined effect, making the involvement of genetic variation in these genes on restenosis development more unlikely.

The six associated genes span a wide range of different functions underlining the complexity of the disease. When examining the biological pathways with involvement

Table 3 Results of individual gene set-based analysis of genes previously associated with restenosis.

Gene	Chr	Start (bp)	End (bp)	Size (kb)	SNPs	Sign. SNPs	Indep. SNPs	P-value
ADRB2	5	148 186 349	148 188 381	2.03	32	8	2	0.088
AGER	6	32 256 724	32 260 001	3.28	37	1	1	0.228
AGTR1	3	149 898 348	149 943 480	45.13	100	5	1	**0.028**
BCHE	3	166 973 387	167 037 944	64.56	101	8	2	0.314
CCL11	17	29 636 800	29 639 312	2.51	18	0	0	1.000
CD14	5	139 991 501	139 993 439	1.94	22	4	2	1.000
CDKN1B	12	12 761 576	12 766 569	4.99	13	0	0	1.000
Col3A1	2	189 547 344	189 585 717	38.37	97	2	2	0.649
CSF2	5	131 437 384	131 439 757	2.37	28	0	0	0.965
CX3CR1	3	39 279 990	39 596 531	316.54	384	3	1	0.358
CYBA	16	87 237 199	87 244 958	7.76	14	1	1	0.182
CYP2C19	10	96 512 453	96 602 660	90.21	43	1	1	1.000
FGB	4	155 703 596	155 711 686	8.09	25	2	1	**0.013**
F5	1	167 747 816	167 822 393	74.58	200	1	1	1.000
GPX1	3	49 369 615	49 370 795	1.18	8	1	1	**0.024**
IL10	1	205 007 571	205 012 462	4.89	30	5	1	**0.053**
IL1RN	2	113 601 609	113 608 063	6.45	62	0	0	0.991
INSR	19	7 063 266	7 245 011	181.75	172	20	5	0.263
ITGB2	21	45 130 299	45 165 303	35.00	57	6	4	0.663
LPL	8	19 841 058	19 869 049	27.99	75	14	5	1.000
MMP12	11	102 238 675	102 250 922	12.25	36	3	3	**0.019**
MMP9	20	44 070 954	44 078 606	7.65	23	10	3	0.067
MTHFR	1	11 768 374	11 788 702	20.33	61	1	1	1.000
NOS3	7	150 319 080	150 342 608	23.53	20	0	0	0.987
P2RY12	3	152 538 066	152 585 234	47.17	121	0	0	1.000
SERPINE1	7	100 556 303	100 558 421	2.12	27	0	0	0.863
KAT2B	3	20 056 528	20 170 898	114.37	144	19	4	**0.023**
PPARG	3	12 304 349	12 450 854	146.51	144	14	5	1.000
ROS1	6	117 716 223	117 853 711	137.49	206	1	1	0.631
THBD	20	22 974 271	22 978 301	4.03	22	0	0	1.000
THBS4	5	79 366 747	79 414 861	48.11	61	3	2	0.292
THPO	3	185 572 467	185 578 626	6.16	16	1	1	0.165
TNF	6	31 651 329	31 654 089	2.76	41	2	2	0.370
TP53	17	7 512 445	7 531 642	19.20	17	1	1	0.120
UCP3	11	73 388 958	73 397 778	8.82	34	1	1	0.183
VDR	12	46 521 589	46 585 081	63.49	93	2	2	**0.012**

Chromosome and genomic region based on HapMap Rel 28 Phase II+III. P-value based on permutation (10,000). Abbreviations: SNPs, number of SNPs in genomic region including 10kb window; Sign.SNPs, number of SNPs with $p < 0.05$; Indep.SNPs, number of significant and independent SNPs, considering threshold of $R^2 < 0.1$.

Table 4 Significantly associated SNPs of the 6 top genes.

Gene	SNP	Chr	bp	Function	Alleles	MAF		OR	p-value	Origin	Imputation quality
						case	control				
AGTR1	rs5182	3	149942085	Exon, synonymous	T/C	0.43	0.50	0.75	0.0040	Genotyped	-
FGB	rs1044291	4	155712802	3'UTR	T/C	0.38	0.30	1.40	0.0028	Imputed	0.970
GPX1	rs8179164	3	49372288	Promoter	A/T	0.02	0.04	0.42	0.0077	Imputed	0.993
MMP12	rs12808148	11	102238373	Downstream	C/T	0.16	0.09	1.82	0.00021	Imputed	0.953
	rs17099726	11	102257062	Promoter	G/T	0.03	0.06	0.54	0.032	Imputed	0.957
KAT2B	rs6776870	3	20126544	Intron	G/C	0.14	0.21	0.62	0.00064	Imputed	0.999
	rs2929404	3	20069570	Intron	T/C	0.21	0.15	1.49	0.0026	Imputed	0.981
	rs17796904	3	20096353	Intron	T/C	0.16	0.12	1.43	0.012	Genotyped	-
	rs4858767	3	20141941	Intron	G/C	0.29	0.34	0.79	0.037	Imputed	0.994
VDR	rs11574027	12	46573640	Intron	T/G	0.03	0.007	4.19	0.00014	Genotyped	-
	rs11574077	12	46539194	Intron	G/A	0.07	0.04	1.60	0.029	Genotyped	-

SNP, single nucleotide polymorphism; Chr, chromosome; bp, base pair; MAF, minor allele frequency in control group; OR, odds ratio. The imputation quality indicates the average posterior probability for the most likely genotype generated by MACH, ranging from 0-1.

of these genes, only the VDR and KAT2B genes share a common pathway. The genes are both involved in the Vitamin D receptor pathway described by BioCarta.[15] This pathway mainly involves the transcriptional regulating capacities of this receptor and is involved in controlling cellular growth, differentiation and apoptosis. Since these processes are all thought to be important contributors to the restenotic process, this indeed is a plausible pathway to be involved in restenosis development.[1]

The rationale of set-based analysis is to overcome the marginally weak effect of single SNPs by analyzing a set of SNPs, since these SNPs could jointly have strong genetic effects. Most studies utilizing the candidate gene approach analyzed only one or at most a few SNPs within the gene of interest. The likelihood that exactly those SNPs are the causal or associated SNPs is of course small. A broader approach, like this set-based analysis, is therefore more likely to detect an associated gene by combining multiple SNPs with a possible marginal individual effect.[16,17] For the current study we used the PLINK software[14], although multiple statistical programs are available for this type of analysis. Gui et al. compared 7 tests analyzing the WTCCC Crohn's Disease dataset.[18] One of their overall conclusions was that the set-based test in PLINK was the most powerful algorithm. Another study, applying PLINK set-based test, Global test, GRASS and SNP ratio test, for the analysis of three pathways regarding human longevity observed similar results with the different tests.[19]

For the current study we analyzed the data using a threshold of linkage disequilibrium defined by $R^2 \geq 0.1$. The standard setting in PLINK is a R^2 of 0.5. In our opinion this threshold is too high for the intended analysis for this study. A higher threshold will include more SNPs in higher LD, which would be unfavorable, since we were interested in independent loci contributing to the risk of restenosis. By decreasing this threshold, only SNPs were selected that had a R^2 below 0.1, and thus independent of each other.

Although hypertension and multivessel disease were more frequent in cases compared to controls we decided not to correct for these variables. In the complete GENDER population these variables were not independent predictors for restenosis development[11], so the differences in the current subpopulation likely resulted by chance during the selection process. Also, other studies provide no convincing data that hypertension is related to restenosis.[1] It is therefore unlikely that previous associations of some of the current candidates genes (VDR, FGB, AGTR1 and GPX1) with hypertension[20-23], have influenced our results, although this cannot be completely excluded.

A limitation of the current study could be that we analyzed imputed genotypic data, which introduces some amount of uncertainty. However, since we were interested in the combined effect of SNPs, an extensive genomic coverage was paramount for this analysis. Only analyzing the genotyped GWAS data would have resulted in the coverage of some of the smaller genes by only 1 or 2 SNPs. Therefore we decided that the more extensive genomic coverage of the imputed dataset outweighed the small introduction of possible error. A second limitation is that the analyses were only performed in the GENDER population. Availability of other populations with thorough genetic data on restenosis is however very limited. To our knowledge, the GWAS on restenosis in the GENDER population is the first, and only, examining this endpoint on a genome wide scale. Finally, the conclusions of this study are only based on genetic analyses. Functional studies should be performed to elucidate the biological consequences of these findings.

In conclusion, with these results we demonstrate that the efforts in unraveling the genetic factors influencing the risk of restenosis of the last years has resulted in a set of genes that joint together is indeed likely to be associated with restenosis, despite the overt inconsistencies of the individual studies. Confirmation of the association of these genes with the occurrence of restenosis after PCI helps our understanding of the genetic etiology of the disease. Future additional research strategies, like biological pathway analysis of GWAS data or even (exome) sequencing, might help us find the missing heritability of restenosis after PCI and increase our knowledge of the biological mechanistic background of restenosis development. This knowledge could subsequently result in identification of new treatment targets or development of novel preventive measure or risk stratification models.

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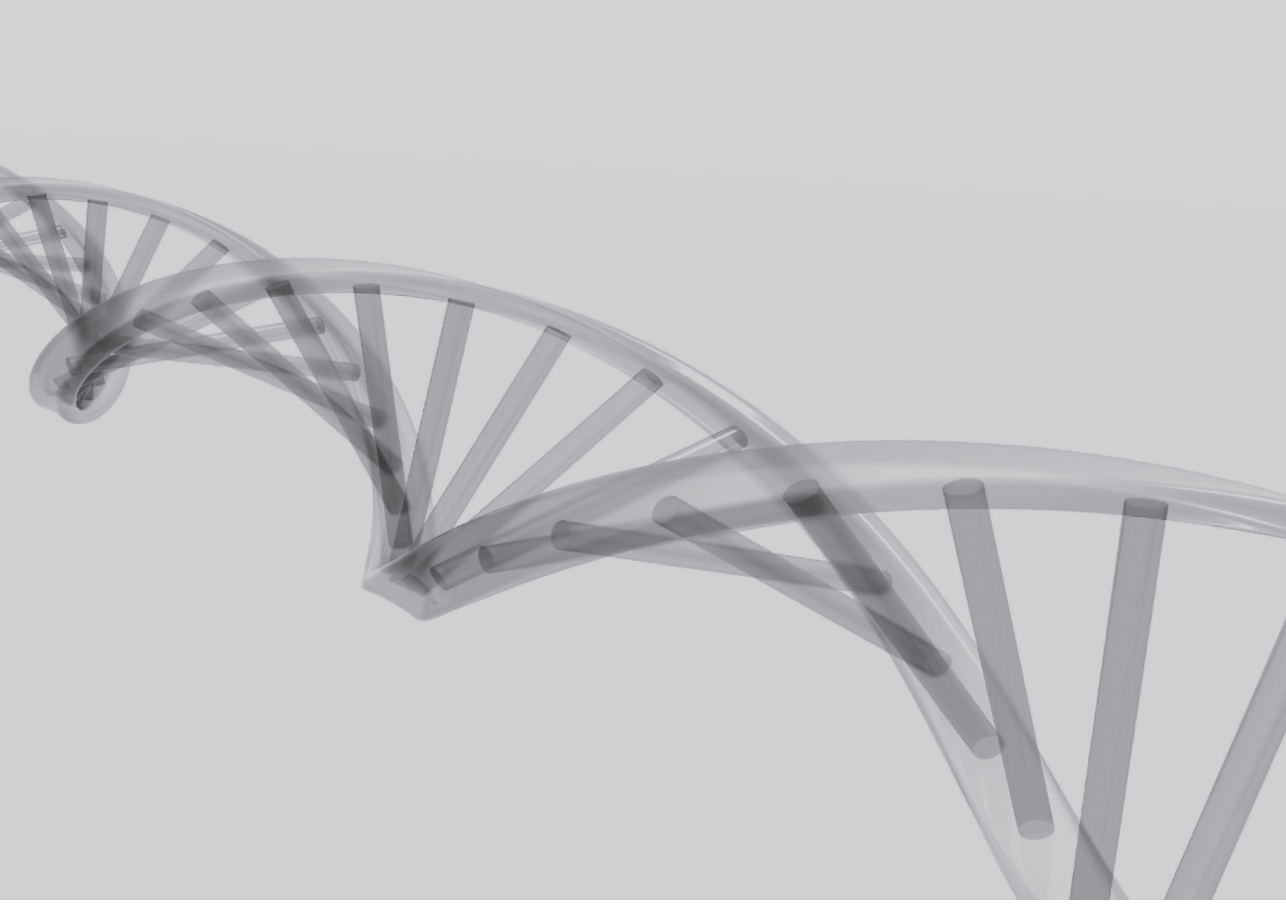
Chapter 7



Pathway analysis using genome-wide association study data for coronary restenosis
A potential role for the PARVB gene

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ABSTRACT

Background: Coronary restenosis after percutaneous coronary intervention (PCI) still remains a significant limitation of the procedure. The causative mechanisms of restenosis have not yet been fully identified. The goal of the current study was to perform gene-set analysis of biological pathways related to inflammation, proliferation, vascular function and transcriptional regulation on coronary restenosis to identify novel genes and pathways related to this condition.

Methods: The GENetic DEterminants of Restenosis (GENDER) databank contains genotypic data of 556,009 SNPs of 295 cases with restenosis and 571 matched controls. Fifty-four pathways, related to known restenosis-related processes, were selected. Gene-set analysis was performed using PLINK, GRASS and ALIGATOR software. Pathways with a $p<0.01$ were fine-mapped and significantly associated SNPs were analyzed in an independent replication cohort.

Results: Six pathways (cell-extracellular matrix (ECM) interactions pathway, IL2 signaling pathway, IL6 signaling pathway, platelet derived growth factor pathway, vitamin D receptor pathway and the mitochondria pathway) were significantly associated in one or two of the software packages. Two SNPs in the cell-ECM interactions pathway were replicated in an independent restenosis cohort. No replication was obtained for the other pathways.

Conclusion: With these results we demonstrate a potential role of the cell-ECM interactions pathway in the development of coronary restenosis. These findings contribute to the increasing knowledge of the genetic etiology of restenosis formation and could serve as a hypothesis-generating effort for further functional studies.

INTRODUCTION

Although interventional cardiology has advanced tremendously in recent years, restenosis after percutaneous coronary intervention (PCI) still remains a significant limitation of the procedure.[1] Restenosis is a complex disease for which the causative mechanisms have not yet been fully identified. Genetic susceptibility is known to play a role in the development of this complication.[1] Many studies have focused to identify these genetic markers associated with restenosis. Candidate gene approaches investigating restenosis have resulted in identification of genetic variation in genes involved in the inflammatory response[2,3] and genes related to vascular remodeling[4,5] and proliferation[6]. In 2012, our group analyzed the joint effect of the genetic variation of 36 previously described candidate genes of restenosis, demonstrating that the joint effect of all the single nucleotide polymorphisms (SNPs) in these genes together was indeed significantly associated with restenosis development.[7] This association was mainly driven by SNPs in 6 individual genes, together spanning a wide range of different functions.

The first genome-wide association study (GWAS) on restenosis only identified one intergenic region on chromosome 12.[8] Although this finding, after further functional confirmation, potentially helps our understanding of the molecular background of restenosis, it also complicates things since none of the previously described markers were identified at a GWAS significant level. The main explanation for this is likely the multifactorial nature of the disease and thus the small individual effect size of the SNPs. It has been described that instead of examining individual SNPs, analyzing the joint effect of multiple SNPs is a better method to find genetic variation in complex diseases.[9-11] Incorporating biological knowledge of the interplay of several genes within functional pathways may improve the power to detect associated genes not previously identified for their involvement. Thus far, the reported genetic variants only explain a small part of the expected genetic component of restenosis development.[7] This so-called "missing heritability" is not uncommon in complex diseases, and pathway approaches are expected to fill at least part of this gap.[12,13] During the last years, several studies have been reported that successfully applied this approach, for instance, identifying biological pathways related to human longevity[14], pancreatic cancer[15], crohn's disease[16] and psychiatric disorders.[17,18]

The goal of the current study was to examine the joint effect of genetic variation in biological pathways related to inflammation, proliferation, vascular function and transcriptional regulation on coronary restenosis in the GENetic DEterminants of Restenosis (GENDER) study population to identify novel genes and pathways related to this condition.

METHODS

GENDER study population

The main results of the GENDER study[19] and the design and results of the genome-wide association study (GWAS)[8,20], have been described previously. In brief, GENDER included 3,104 consecutive unrelated symptomatic patients treated successfully by PCI for angina. During a follow-up period of 9 months, the endpoint clinical restenosis, defined as renewed symptoms requiring target vessel revascularization (TVR) either by repeated PCI or CABG, by death from cardiac causes or myocardial infarction not attributable to another coronary event than the target vessel, was recorded. During follow-up, 346 patients developed clinical restenosis. Blood samples were collected at the index procedure for DNA isolation. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of each participating institution (the ethics committee of the Leiden University Medical Center, the ethics committee of the Academic Medical Center Amsterdam, the ethics committee of the University Medical Center Groningen and the ethics committee of the Maastricht University Medical Center). Written informed consent was obtained from each participant before the PCI procedure.

The GWAS was performed in a subpopulation of GENDER of 325 cases of restenosis and 630 controls matched by gender, age, and several other known risk factors for restenosis like total occlusion, diabetes, current smoking and residual stenosis. Genotyping was performed using the Illumina Human 610-Quad Beadchips following the manufacturer's instructions. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol.[20] The population substructure was analyzed by means of multidimensional scaling. The vast majority of the individuals fell in the same cluster along with the European population but 67 individuals were outside this cluster and were considered as genetic outliers and therefore excluded from further analyses. The final dataset consisted of 866 individuals (295 cases, 571 controls) and 556,099 SNPs that passed all quality control criteria, together covering 89% of the common genetic variation in the European population.[20]

Pathway selection

Pathways related to known restenosis-related processes (inflammation, vascular function, proliferation and transcription) were selected from the MSigDB database[21], which included curated gene sets from KEGG[22], BioCarta[23] and Reactome[24,25]. A total of 54 pathways were selected (Supplementary Table S1).

Replication cohort

The replication cohort consisted of a German restenosis cohort, i.e. patients presenting with ischemic symptoms or evidence of myocardial ischemia in the presence of ≥ 50% de

novo stenosis located in native coronary vessels. All patients were treated with PCI and stent implantation at Deutsches Herzzentrum München or 1. Medizinische Klinik rechts der Isar der Technischen Universität München. The study protocol conformed to the Declaration of Helsinki and was approved by the institutional ethics committee responsible for both participating centers, Deutsches Herzzentrum München and 1. Medizinische Klinik, Klinikum rechts der Isar, Technische Universität München. All subjects gave their written, informed consent for participation in the study. The main characteristics of this cohort and the protocols of stent placement and post-stenting therapy have been described previously[26,27]. Briefly, this population included 1537 patients treated with implantation of bare-metal stents (BMS) and 1920 patients treated with implantation of drug-eluting stents (DES). Target lesion revascularization (TLR) within 1 year after PCI was considered as the primary endpoint. Re-hospitalization for repeat angiography was scheduled between 6 and 8 months or earlier if non-invasive evaluation or clinical presentation suggested the presence of ischemia. For the current study we only analyzed the patients receiving BMS, since this was the only type of stent implanted during the GENDER study.

The samples during the replication stage were genotyped by means of iPLEX assays[28]. The SNPs were selected for replication after step-down fine-mapping of the significant associated pathways. Finally only SNPs within these pathways showing a p-value <0.10 and linkage disequilibrium (LD) <0.5 in the GENDER study were selected. Two assays were designed using MassArray design software (Sequenom, San Diego, CA). Genotyping was performed using iPLEX assays with use of the MassARRAY methodology (Sequenom), with alleles discriminated by mass spectrometry, following manufacturer's instructions. One SNP, rs5764560, did not fit the assay. All the genotyped SNPs had a call rate >95%. One SNP, rs7292425, was out of Hardy Weinberg equilibrium (p-value<0.001), and was excluded for further analysis. Duplicate samples (~2.5%) showed identical genotypes. Finally, 39 SNPs passed all quality criteria and were further analyzed.

Statistical Analysis

Analyses were performed using PLINK[29],GRASS[30] and ALIGATOR[31] software. We will briefly describe all three methods. During the set-based test of PLINK the joint effect of all genetic variation, fulfilling the test constraints, within the set of genes of pathway of interest is evaluated. First a single SNP analysis of all SNPs within the pathway set is performed. Subsequently, a mean SNP statistic is calculated from the single SNP statistics of a maximum amount of independent SNPs with a p-value < 0.2. SNPs are considered independent when the LD expressed in R^2 is < 0.5. Of the SNPs that are in LD with R^2 > 0.5, the SNP with the lowest p-value in the single SNP analysis is selected. This analysis is repeated 10,000 times in simulated datasets with permutation of the phenotype. An empirical p-value for the SNP set is computed by calculating the number of times the test statistic of the simulated SNP sets exceeds that of the original SNP set.

GRASS calculates "eigenSNPs" for each gene in the pathway set by summarizing the variation of a gene using principal component analysis. Subsequently, one or more of these "eigenSNPs" per gene are selected using regularized logistic regression to calculate a test statistic for each pathway set. This analysis is repeated 10,000 times in simulated datasets with permutation of the phenotype. The p-value per pathway SNP set is calculated by comparing the test statistic of the original pathway SNP set with that of the combined simulated pathway SNP sets.

ALIGATOR (Association LIst Go AnnoTatOR) analyses gene sets for genes enriched with significant SNPs. Enrichment is defined as a gene set containing a larger number of significant genes than expected by chance. Replicate gene lists of the same length as the original are generated by randomly sampling SNPs (thus correcting for variable gene size). The lists are used to obtain p-values for enrichment for each gene set (by comparing the number of significant genes observed on the actual gene list to that observed on each replicate list), to correct these for testing multiple non-independent categories, and to test whether the number of significantly enriched categories is higher than expected. ALIGATOR uses data from all the SNPs tested in a gene and corrects for the variable numbers of SNPs per gene. Each gene is counted once regardless of how many significant SNPs it contains, thus eliminating the influence of LD between SNPs within genes. We used p-value cutoff < 0.005 for SNPs, 5000 replicate gene lists and 1000 permutations as parameters to run ALIGATOR. Pathways were included when 2 or more individual genes contained significantly associated SNPs.[31,32]

Considering the exploratory nature of the analysis and the considerate overlap between the pathways, associations of pathways with restenosis were considered worthwhile exploring with P<0.01. Since it has been suggested that PLINK, GRASS and ALIGATOR provide complementary information[13,33], we proceeded with the secondary analysis when a pathway was associated with restenosis with p<0.01 in at least one of the analyses. The pathways meeting this criterion were explored in more detail by fine-mapping of firstly the genes within those pathways and secondly the individual SNPs. For these secondary analyses, as well as during the replications stage p<0.05 was considered significant. When applying a strict Bonferroni correction, correcting for the 54 tested pathways and three different tests, the threshold for statistical significance was set at p<0.0003 (=0.05/(54*3).

RESULTS

The 295 patients with restenosis were well matched with the 571 patients without restenosis, for the known risk factors for restenosis, like age, diabetes, current smoking, stenting of the culprit lesion and previous restenosis (Table 1). Hypertension and multivessel disease were more common in the cases compared to the controls.

Table 1, Baseline characteristics of the GENDER study population.

	Cases (n = 295)	**Controls** (n = 571)	**p-value**
Age (years)	62.8 ± 10.6	62.4 ± 10.9	0.59
BMI ($kg.m^{-2}$)	26.7 ± 3.6	27.1 ± 3.7	0.20
Male sex	213 (72)	421 (74)	0.63
Diabetes	58 (20)	119 (21)	0.68
Hypercholesterolemia	179 (61)	341 (60)	0.79
Hypertension	138 (47)	211 (37)	0.01
Current smoker	68 (23)	148 (26)	0.36
Family history of MI	117 (40)	210 (37)	0.41
Previous MI	119 (40)	246 (43)	0.44
Stable angina	188 (64)	400 (68)	0.06
Multivessel disease	155 (53)	248 (43)	0.01
Restenotic lesion	23 (8)	48 (8)	0.76
Total occlusion	57 (19)	97 (17)	0.40
Type C lesion	95 (38)	154 (27)	0.11
Stenting	199 (68)	385 (67)	0.99

Values were given as n (%) or mean ± SD. Diabetes was defined by treatment with anti-diabetic medication or insulin at study entry. Hypertension was defined as a blood pressure of either > 160 mmHg systolic or > 90 mmHg diastolic. Hypercholesterolaemia was defined as total cholesterol concentrations > 5 mmol/L.
BMI: body mass index, MI: myocardial infarction. P-values are determined by Pearsons Chi-Square (discrete variables) or unpaired 2-sided t-test (continuous variables)

A total of 54 pathways were analyzed in the current study. Half of these pathways (n=27) were related to inflammation, 13 pathways were related to proliferation, six pathways were related to vascular function and finally, eight pathways were related to transcriptional regulation. The pathways associated with restenosis with a p-value below 0.01 in either PLINK, GRASS or ALIGATOR are shown in Table 2. An overview of the results of all the pathways can be found in Supplementary Table S1. When applying Bonferroni correction none of the pathways were significantly associated.

In the set-based analysis using PLINK, the IL6 signaling pathway described by BioCarta was associated most significantly with clinical restenosis with a p-value of 0.0008. Using the GRASS software, this pathway had a p-value of 0.08 and the ALIGATOR analysis resulted in p=0.01. The strongest associated pathway using GRASS was the BioCarta mitochondria pathway with a p-value of 0.006. However, this pathway was not associated with restenosis using PLINK (p=0.80) or ALIGATOR. The insulin signaling pathway, described by BioCarta, was the strongest associated pathway using ALIGATOR (p=0.001). Using PLINK this pathway had a p-value of 0.02, whereas GRASS resulted in p=0.14. Other pathways that were associated in one of the analyses, considering the p<0.01 cut-off, include the inflammatory IL2 signaling pathway (PLINK p=0.006, GRASS p=0.012

Table 2 Pathways associated with restenosis with PLINK or GRASS

Pathway	Database	PLINK	GRASS
Inflammation			
IL2 signaling pathway	BioCarta	**0.006**	0.012
IL6 signaling pathway	BioCarta	**<0.001**	0.08
Proliferation			
Role of Mitochondria in Apoptotic Signaling	BioCarta	0.80	**0.006**
PDGF Signaling Pathway	BioCarta	**0.008**	0.03
Vascular function			
Cell-extracellular matrix interactions	Reactome	**0.009**	0.17
Transcription			
Control of Gene Expression by Vitamin D Receptor	BioCarta	**0.008**	0.07

A complete overview of all pathways can be found in Supplementary Table S1.

and ALIGATOR p=0.08) and the IL4 signaling pathway (PLINK p=0.02, GRASS p=0.04 and ALIGATOR p=0.008), the proliferation platelet derived growth factor (PDGF) pathway (PLINK p=0.008, GRASS p=0.03 and ALIGATOR p=0.02) and a transcriptional regulatory pathway, the vitamin D receptor (VDR) pathway (PLINK p=0.008, GRASS p=0.07 and ALIGATOR p=0.003). Finally, the cell-extracellular (ECM) matrix interactions pathway, as described by Reactome, was demonstrated to be associated in PLINK (p=0.009), but not in GRASS (p=0.17) or ALIGATOR (p=0.14).

Subsequently, fine mapping analysis was performed of six pathways with a p-value <0.01 in one of the three analyses. The individual genes of each pathway were analyzed using set-based analysis of PLINK (Supplementary Table S2). Five of the 22 genes of the IL6 signaling pathway were significantly associated, as shown in Table 3. These genes were the RAF1 gene with a p-value of 0.001, the JAK2 gene with p=0.006, the STAT3 gene with p=0.006, the IL6 gene with p=0.04, and the GRB2 gene with a p-value of 0.04 (Table 3). The IL2 signaling pathway and the PDGF signaling pathway showed considerable overlap with the IL6 pathway. Thirteen of the 22 genes (59%) of the IL2 pathway and 14 of the 32 genes (44%) of the PDGF pathway are also involved in the IL6 pathway. In addition to RAF1, JAK2 and GRB2, mentioned above, STAT5A, STAT5B and PIK3R1 were also significantly associated, p=0.04, p=0.01 and p=0.04 respectively. The association of the VDR pathway was driven by three genes, NCOR1 (p=0.003), VDR (p=0.01) and BAZ1B (p=0.02). The association of the cell-ECM interactions pathway was caused by two genes. The PARVB gene was associated with restenosis with a p-value of 0.001 and the LIMS2 gene with a p-value of 0.01. Finally, DIABLO was the only associated gene of the mitochondria pathway with a p-value of 0.03.

In Table 3 the results of the single SNP analysis of the significantly associated genes is reported showing which SNPs are responsible for the significant association of these genes. Of the in total 54 individual SNPs, 34 were associated with restenosis with a p-

Table 3, Fine mapping of the associated pathways and replication of the top SNP

Pathway	Gene	SNPs (total)	SNPs (final)	P (gene)	GENDER (N = 866)					Replication cohort (N = 1537)			
					SNP	MAF		OR	P (SNP)	MAF		OR	P (SNP)
						case	control			case	control		
IL6 signaling pathway													
	RAF1	19	2	0.001	rs1532534	23%	16%	1.58	<0.001	19%	18%	1.07	0.59
					rs1532533	46%	41%	1.26	0.02	37%	39%	0.91	0.34
	JAK1	30	8	0.006	rs2256298	20%	28%	0.64	<0.001	**23%**	**27%**	**0.81**	**0.06**
					rs310219	22%	30%	0.66	<0.001	25%	27%	0.88	0.23
					rs2780900	8%	12%	0.61	0.005	11%	12%	0.94	0.69
					rs17127169	7%	11%	0.62	0.01	7%	8%	0.90	0.56
					rs310247	41%	47%	0.79	0.02	**40%**	**44%**	**0.84**	**0.07**
					rs974019	11%	14%	0.76	0.09	[c]			
					rs3790541	15%	12%	1.28	0.09	11%	10%	1.05	0.76
					rs12745769	22%	20%	1.19	0.16	[d]			
	STAT3	7	1	0.006	rs6503695	42%	34%	1.35	0.004	35%	36%	0.95	0.61
	IL6	10	1	0.04	rs11766273	8%	5%	1.58	0.02	7%	8%	0.87	0.43
	GRB2	11	3	0.04	rs12950752	29%	22%	1.41	0.003	25%	26%	0.93	0.53
					rs16967789	17%	13%	1.35	0.03	15%	14%	1.01	0.94
					rs930297	13%	11%	1.28	0.11				
IL2 signaling pathway													
	RAF1	19	2	<0.001	[a]								
	JAK1	30	8	0.006	[a]								
	STAT5B	5	1	0.01	rs8064638	38%	31%	1.33	0.007	31%	31%	0.97	0.77
	GRB2	11	3	0.04	[a]								
	STAT5A	3	1	0.04	rs8072785	13%	9%	1.44	0.02	11%	10%	1.16	0.32

Table 3, Fine mapping of the associated pathways and replication of the top SNP (*continued*)

Pathway	Gene	SNPs (total)	SNPs (final)	P (gene)	GENDER (N = 866)					Replication cohort (N = 1537)			
					SNP	MAF		OR	P (SNP)	MAF		OR	P (SNP)
						case	control			case	control		
PDGF Signaling pathway													
	RAF1	19	2	0.001	[a]								
	STAT3	7	1	0.006	[a]								
	JAK1	30	8	0.006	[a]								
	STAT5B	5	1	0.01	[b]								
	PIK3R1	31	5	0.04	rs17318918	16%	11%	1.58	0.002	10%	9%	1.20	0.25
					rs10515074	27%	22%	1.31	0.02	[e]			
					rs1043526	17%	13%	1.36	0.03	13%	13%	1.00	0.99
					rs4122269	32%	37%	0.82	0.06	35%	36%	0.98	0.80
					rs40419	40%	43%	0.87	0.19	[d]			
	STAT5A	3	1	0.04	[b]								
	GRB2	11	3	0.04	[a]								
VDR pathway													
	NCOR1	14	1	0.003	rs3744328	4%	1%	2.73	0.002	3%	3%	0.99	0.96
	VDR	33	4	0.01	rs11574027	4%	1%	4.19	<0.001	2%	1%	1.08	0.83
					rs11574077	7%	4%	1.60	0.03	4%	5%	0.85	0.47
					rs11168287	42%	47%	0.83	0.07	48%	48%	0.96	0.70
					rs9729	46%	50%	0.88	0.19	[d]			
	BAZ1B(WSTF)	6	1	0.02	rs17400042	3%	6%	0.49	0.009	2%	3%	0.71	0.30
Cell-extracellular matrix interactions pathway													
	PARVB	93	24	0.001	rs139107	16%	9%	1.91	<0.001	12%	13%	0.97	0.81
					rs2092351	31%	40%	0.67	<0.001	37%	37%	1.01	0.89

Table 3, Fine mapping of the associated pathways and replication of the top SNP (*continued*)

Pathway	Gene	SNPs (total)	SNPs (final)	P (gene)	GENDER (N = 866)					Replication cohort (N = 1537)				
					SNP	MAF		OR	P (SNP)	MAF		OR	P (SNP)	
						case	control			case	control			
					rs6006636	18%	12%	1.53	0.002	15%	16%	0.90	0.42	
					rs2179559	18%	24%	0.70	0.01	21%	19%	1.13	0.29	
					rs2267617	52%	45%	1.33	0.01	49%	49%	1.03	0.72	
					rs139069	23%	17%	1.41	0.01	20%	18%	1.14	0.24	
					rs2072949	8%	5%	1.72	0.01	7%	6%	1.12	0.54	
					rs2267604	28%	23%	1.34	0.01	[f]				
					rs139096	31%	25%	1.33	0.01	27%	29%	0.88	0.21	
					rs5764455	39%	44%	0.80	0.03	**36%**	**42%**	**0.77**	**0.007**	
					rs9614207	33%	38%	0.80	0.04	39%	37%	1.10	0.31	
					rs6006611	50%	45%	1.23	0.04	48%	46%	1.12	0.23	
					rs5764066	22%	18%	1.27	0.05	**26%**	**22%**	**1.23**	**0.05**	
					rs2092437	14%	11%	1.33	0.07	15%	13%	1.20	0.18	
					rs7292425	26%	22%	1.24	0.07	[g]				
					rs133911	26%	30%	0.81	0.07	30%	29%	1.05	0.65	
					rs2073080	17%	20%	0.78	0.07	16%	17%	0.91	0.45	
					rs5764560	41%	36%	1.20	0.08	[h]				
					rs8140244	13%	16%	0.81	0.14	[d]				
					rs5764495	47%	51%	0.87	0.16	[d]				
					rs2896022	46%	50%	0.87	0.17	[d]				
					rs2281292	38%	41%	0.87	0.19	[d]				
					rs6006615	19%	16%	1.19	0.19	[d]				
					rs6006486	28%	25%	1.16	0.19	[d]				

Table 3, Fine mapping of the associated pathways and replication of the top SNP (*continued*)

Pathway	Gene	SNPs (total)	SNPs (final)	P (gene)	GENDER (N = 866)					Replication cohort (N = 1537)			
					SNP	MAF		OR	P (SNP)	MAF		OR	P (SNP)
						case	control			case	control		
	LIMS2	14	2	0.01	rs2288657	5%	2%	2.52	<0.001	4%	5%	0.90	0.65
					rs2243934	6%	4%	1.38	0.17	[d]			
Mitochondria pathway													
	DIABLO	2	1	0.03	rs12870	48%	42%	1.27	0.02	44%	43%	1.03	0.72

In the first five columns the analysis of the separate genes is depicted. In the second five columns the single SNP analysis is shown and in the final four columns the replication stage is shown. SNPs (total) indicates the total number of available SNPs in the gene. SNPs (final) indicates the number of SNPs with $P < 0.2$ and $LD < 0.5$ that were included in the set-based analysis. MAF, minor allele frequency. [a] single SNP analysis shown in IL6 pathway [b] single SNP analysis shown in IL2 pathway [c] SNP in LD ($R^2 = 0.52$) with rs310219, not selected for replication [d] p-value > 0.1 of SNP in GENDER, not selected for replication [e] SNP in LD ($R^2 = 0.57$) with rs17318918, not selected for replication [f] SNP in LD ($R^2 = 0.54$) with rs6006636, not selected for replication [g] SNP out of HWE ($p = 0.0001$) during replication. [h] SNP did not fit the iPlex designs.

value below 0.05. The strongest associated SNP was located in the PARVB gene of the cell-ECM interactions pathway (rs139107, p<0.001)

Baseline characteristics of the replication cohort can be found in Supplementary Table S3. In the replication stage, two SNPs in the PARVB gene, which was part of the cell-ECM interactions pathway, were the only SNPs found to be significantly associated with restenosis (Table 3). The minor allele of rs5764455 was significantly more prevalent in the control group than in the cases, 42% and 36% respectively, p=0.007. In GENDER the minor allele frequency (MAF) of this SNP was 44% and 39% in controls and cases respectively, p=0.03. For rs5764066 the MAF was 22% in controls and 26% in cases, p=0.05, compared to 18% versus 22% in GENDER. In addition, two SNPs in the JAK1 gene, which was part of the IL6 pathway, the IL2 pathway and the PDGF pathway, showed a borderline significant association with target lesion revascularization in the replication cohort. No wet-lab experiments were performed to elucidate the function of the two PARVB SNPs, rs5764455 and rs5764066, but online databases were explored for available data. Both SNPs were located in introns and the UCSC genome browsers database (http://genome.ucsc.edu) did not indicate that they were located in a DNAse I hypersensitive site or in other important regulatory regions. Moreover, neither of two SNPs were reported in two publically available eQTL databases (VarySysDB[34] and the eQTL database of the Pritchard lab[35]).

DISCUSSION

We explored the joint effect of genetic variation in biological pathways on coronary restenosis development. When using a strict Bonferroni correction, none of the pathways reached the threshold of significance. However, we show a potential role for the cell-ECM interactions pathway as described by Reactome. This pathway, related to vascular remodeling, was associated with coronary restenosis in the GENDER study, in particular the PARVB gene and several SNPs in this gene were replicated in an independent study cohort. The SNPs of the five other pathways, associated in the GENDER study, did not replicate.

ECM remodeling and vascular smooth muscle cell migration and proliferation are thought to be the key processes in restenosis development.[1] Cell-ECM interactions, as described by the cell-ECM interactions pathway, play a critical role in regulating a variety of cellular processes in multicellular organisms including motility, shape change, survival, proliferation and differentiation. The PARVB (beta-parvin, also known as affixin, Gene ID: 29780) in particular, encodes a member of the parvin family of actin-binding proteins, which play a role in cytoskeleton organization and cell adhesion. Unlike other family members like alpha-parvin and gamma-parvin, beta-parvin has been shown to be expressed the highest in heart and skeletal muscle tissue.[36] PARVB is known to

interact with integrin-linked kinase (ILK) and this complex is involved in the early stage of cell-substrate interaction through integrins.[37] Moreover, it has been suggested that it is a crucial modulator of cell survival[38] and that it has an important role in the angiogenic process[39], making it a likely candidate for restenosis development.

Since the introduction of GWAS many new insights on a wide array of disease have been reported.[40] However, extending these findings to biological mechanisms that explain disease development is a major ongoing challenge. The most likely explanation for this problem is that GWAS only focuses on single SNP associations. The reported loci often are located in intergenic regions or gene deserts and often are of unknown function. Furthermore, these loci have exhibited only modest effect sizes. Since biological pathways are the natural causative mechanisms resulting in disease and it is conceivable that genes functioning within these pathways interact within biological networks, it is only logical that the next step in the post-GWAS era is the exploration of these pathways. The rationale of set-based analysis is to overcome the marginally weak effect of single SNPs by analyzing a set of SNPs, since these SNPs could jointly have strong genetic effects. Examination of genetic variation of complete biological pathways, like in this set-based analysis, is therefore more likely to detect an associated mechanism by combining multiple SNPs with a possible marginal individual effect.[41,42] In this study, the SNP with the strongest association with restenosis in the GENDER population was located in the PARVB gene of the cell-ECM interactions pathway (rs139107, $p<0.001$). As a reference, none of the SNPs currently examined, were located in the top 100 strongest associated SNPs of the complete GWAS analysis.[8]

For the current study we performed the analyses with PLINK, GRASS and ALIGATOR software. To date, many different statistical programs have been described to perform this type of analysis with. All methods have advantages and disadvantages.[9] In the case of PLINK and GRASS, both methods use the raw genotypes as input data. An advantage of this method is that they can take into account the LD structure within genes during their computations, which is not the case with methods performing their analyses using p-values as input data. Furthermore they both are self-contained tests that deal with the complexity of the SNP sets by permutation of the case-control status. However, differences between PLINK and GRASS include that PLINK analyzes SNP sets as a whole, and does not take the SNP distribution over the genes within the pathway into account, whereas GRASS first calculates "eigenSNPs" for each gene in the pathway set by summarizing the variation of a gene using principal component analysis. Compared to PLINK and GRASS, ALIGATOR is not a self-contained test but a competitive test, using SNP p-values instead of raw-genotypes, aiming to test whether genes in one pathway are enriched with a greater number of associated SNPs. Several comparative analyses have been performed, not resulting in a consensus of the best method, but rather suggesting to use two or more algorithms resulting in complementary findings.[14,33]

For the current study we analyzed the data using a p-value threshold for including individual SNPs in the PLINK analyses of $p<0.2$ for their association with restenosis in the primary single SNP analysis. A standard setting in PLINK is often $p<0.05$, on relatively arbitrary grounds. Since we aimed at analyzing the combined effect of SNPs with small individual effect sizes we decided to increase this threshold to $p<0.2$. By increasing this threshold, more SNPs will be selected for further analyses ensuring that also the SNPs that were not associated with restenosis on their own, but that potentially did contribute to the overall effect of a pathway were taken into account. Before the start of this study we decided to apply a threshold of $p<0.01$ for the association of a pathway with restenosis, to identify pathways of interest. Although this threshold could be considered arbitrary, we decided that is was appropriate considering the considerate overlap between the pathways and the exploratory nature of this study. When using a strict Bonferroni correction for the 54 analyzed pathways and 3 applied tests ($0.05/(54*3)=p<0.0003$), none of the pathways would have qualified for further investigation. This could indicate that either the population is too small to detect the subtle genetic influence on this complex phenotype or that no solid genetic association is present. However, since to our knowledge, other (larger) restenosis study populations with GWAS data do not exist, we are limited in exploring this hypothesis regarding the sample size issue. A more strict multiple testing correction than currently used increases the risk of false negative results. Future functional studies are needed to further elucidate the biological background of restenosis development. The first step of the functional analyses would likely be to see whether the SNPs affect expression or function of the gene they are located in. If this is not the case, they could influence on other (nearby) genes, which could for instance be explored by mRNA expression analyses or allelic expression imbalance.[43] Furthermore, sequencing of the genes of interest could result in identifying functional variants in LD with the SNPs identified in the current study.

Limitations

Proper replication of these analyses requires other datasets with GWAS data. To our knowledge, the GWAS performed in the GENDER study population is the first and unfortunately is currently the only GWAS on coronary restenosis. The desired replication cohorts are therefore unavailable. Since we did want to analyze our findings in an independent study cohort we decided to genotype only the top SNPs of the associated pathways instead. Considering the minimal replication of the analyzed SNPs, the robustness of the reported associations is limited. However, the presented data do provide evidence that ECM interactions and inflammation contribute to coronary restenosis development. And the results of the current study can serve as a hypothesis-generating effort for further functional studies.

All patients in the GENDER study and in the replication cohort were either treated with balloon-angioplasty (33% in GENDER) or BMS implantation. It is unknown whether these findings are generalizable to patients treated with DES or endothelial-progenitor-cell covered stents.

Conclusion

In conclusion, although these results do not comply to the required strict multiple testing correction, we do demonstrate a potential role of the cell-ECM interactions pathway in the development of coronary restenosis. After performing additional studies, these findings could contribute to the increasing knowledge of the genetic etiology of restenosis formation. Unraveling the mechanisms leading to restenosis development remains challenging. There is a need for larger study populations specifically designed for genetic association analyses. In the select population that will still develop coronary restenosis despite advances in therapy, it will remain an imported cause of morbidity and therefore further understanding of the condition will be essential if we want to achieve personalized treatment and eradicate this costly and clinically very relevant limitation of PCI.

ACKNOWLEDGEMENTS

We want to thank Q. Helmer for her technical assistance in performing the statistical analyses.

SUPPLEMENTARY MATERIAL

Supplementary Material is available upon request.

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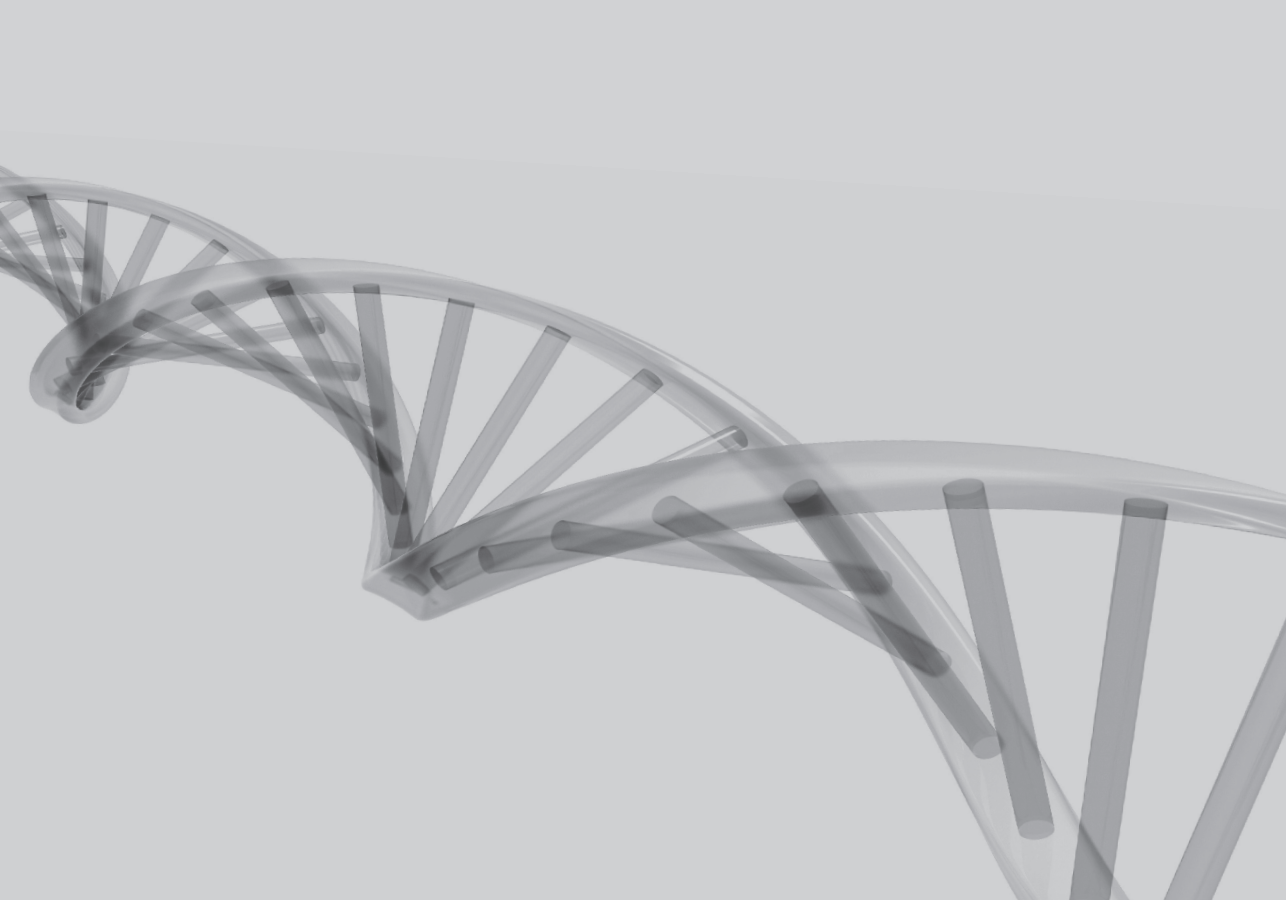
Chapter 8



10-year mortality risk of patients undergoing elective PCI: Long-term follow-up of the GENetic Determinants of Restenosis (GENDER) study

No increased mortality risk by restenosis, only by coronary artery disease itself

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ABSTRACT

Background: Data on long-term follow-up of patients developing coronary restenosis after percutaneous coronary intervention (PCI) are scarce. To explore the relation of restenosis with long-term mortality, we analysed the 10-year survival data of the GENetic DEterminants of Restenosis (GENDER) study population.

Methods: GENDER was designed to study the genetic background of restenosis development after successful PCI. This multicentre prospective follow-up study had clinical restenosis within nine months as its primary endpoint. After this follow-up period, patients returned to usual clinical care. In total 3104 patients were included between March 1999 and June 2001 of which 304 developed restenosis. Long-term mortality data were obtained from the Dutch Central Bureau of Statistics. As a control population, a random sample of 15,000 individuals, matched for age and gender, was extracted from the Dutch general population.

Results: Of the patients with restenosis, 86 (28.6%) died, which was similar to the 770 (27.7%) of the patients without restenosis, $p = 0.28$. Also no difference was observed for cardiovascular death (12.3% versus 11.4%, respectively, $p = 0.64$). When comparing the total GENDER population with the general population, a higher mortality rate was observed in the GENDER population, 27.8% versus 21.6%, $P < 0.001$.

Conclusion: The current study demonstrates that, in the long-term, patients suffering from restenosis do not have an increased mortality risk. However, this study population does have an increased mortality risk compared with the general population, indicating that it seems that there is still room to improve current treatment strategies for cardiovascular disease.

INTRODUCTION

Although the death rates from cardiovascular diseases (CVD) have declined over the last decades, the burden of disease remains high and CVD remains one of the leading causes of morbidity and mortality in Western developed countries[1,2] including the Netherlands.[3-6] Tremendous efforts worldwide are put into research and development of novel treatment modalities for CVD to further halt the disease burden, and frequent updates of primary and secondary prevention guidelines should result in the most optimal treatment of each patient with CVD. Besides major improvements in pharmacological treatment with for instance statins, beta-blockers, angiotensin-converting-enzyme inhibitors and antiplatelet agents, interventional treatment modalities such as percutaneous coronary intervention (PCI) have markedly improved the outcome of patients with coronary artery disease.

An important complication of percutaneous coronary intervention (PCI) that is still present is coronary restenosis.[7] Coronary restenosis - the renarrowing of the treated obstruction - results in high morbidity [8] and is even reported to be associated with an increased risk of mortality.[9,10] Data on long-term follow-up of patients with restenosis are, however, scarce. To explore the relation of restenosis development with long-term mortality, we analysed the 10-year survival of the patients included in the GENetic DEterminants of Restenosis (GENDER) study.[11] The second objective of this report was to investigate whether the treatment strategies after PCI, applied in daily clinical practice over the past 10 years, resulted in a shift in mortality rates and causes of death of these confirmed coronary artery disease (CAD) patients compared with the general population.

METHODS

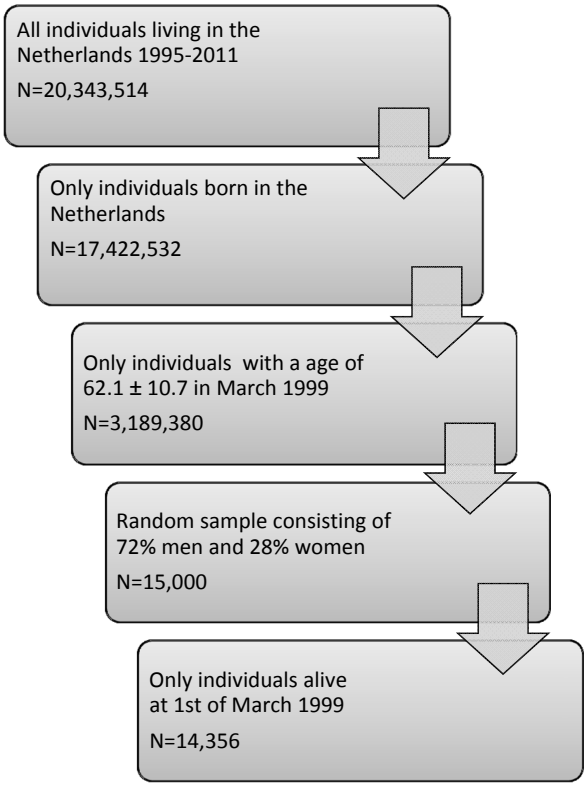
The GENDER project was designed to study the genetic background of coronary restenosis development after successful PCI. It is a multicentre prospective follow-up study with clinical restenosis within nine months as its primary endpoint. The first patient was included in March 1999 and inclusion lasted up to June 2001. In total 3104 consecutive patients were treated successfully by PCI for stable angina, non-ST-elevation acute coronary syndromes or silent ischaemia in four referral centres for interventional cardiology in the Netherlands: Academic Medical Center in Amsterdam, University Medical Center Groningen, Leiden University Medical Center and University Hospital Maastricht. Only patients treated for acute ST-elevation myocardial infarction (MI) were excluded. The study was primarily supported by the Interuniversity Cardiology Institute of the Netherlands.

The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of each participating institution. After having obtained written

informed consent, blood was sampled for DNA isolation and future analysis. Clinical and procedural data were gathered prospectively. Clinical restenosis was defined as the composite of death, myocardial infarction and target vessel revascularisation. An independent endpoint committee evaluated all potential endpoints. During the in-study follow-up period, 346 of the patients developed clinical restenosis. After the follow-up period patients returned to usual guideline-based clinical care, either with the general practitioner or if deemed necessary with the cardiologist. Currently, approximately 10 years after completion of the study, we have obtained long-term mortality data, including specific causes of death, for the complete GENDER population from the Dutch Central Bureau of Statistics (CBS). Data were available up to 31 December 2011 and the primary cause of death was described by ICD-10 codes.

As a control population for the GENDER study we extracted a random sample of 15,000 individuals, matched for age and gender, from the general population of individuals born in the Netherlands and living in the Netherlands in the timeframe 1995-2011. Individuals who died before March 1999 were excluded from further analyses (N = 644) (Fig. 1).

Figure 1 Random sample of general Dutch population, matched for age and sex.



RESULTS AND DISCUSSION

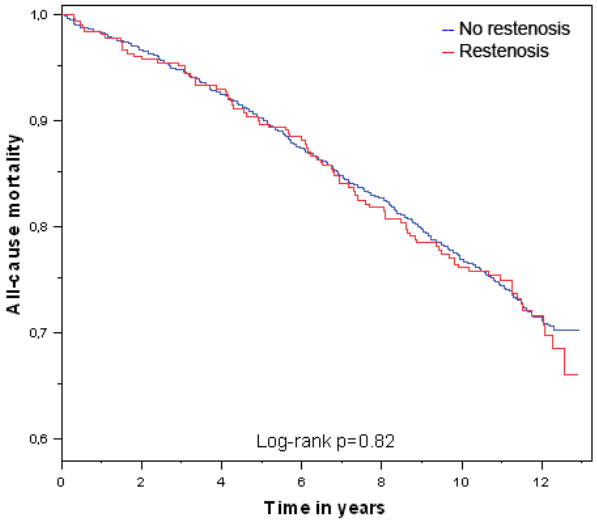
Restenosis and mortality

Baseline characteristics of the GENDER study population are shown in Table 1. Twenty patients (0.6%) of the total GENDER population could not be identified in the CBS database and were considered lost to follow-up. A total of 865 patients died between inclusion in the GENDER study and 1 January 2012. When comparing the patients suffering from coronary restenosis after PCI with those who did not develop restenosis, no differences in mortality rates were observed (Fig. 2). Of the 300 patients with restenosis, 86 (28.7%) died,

Table 1 Baseline characteristics of the GENDER study population

Parameter	GENDER study population (N = 3,104)
Age	62.1 ± 10.7
Sex (male)	2,219 (71.5%)
Diabetes	453 (14.6%)
Hypertension	1,259 (40.6%)
Hypercholesterolaemia	1,890 (60.9%)
Current smoker	762 (24.5%)
Multi-vessel disease	1,462 (46.1%)
Total occlusion	428 (13.8%)
Stenting	2,309 (74.4%)
Residual stenosis > 20%	350 (11.3%)

Figure 2 Kaplan-Meijer curve comparing mortality of patients with and without coronary restenosis of the GENDER study population

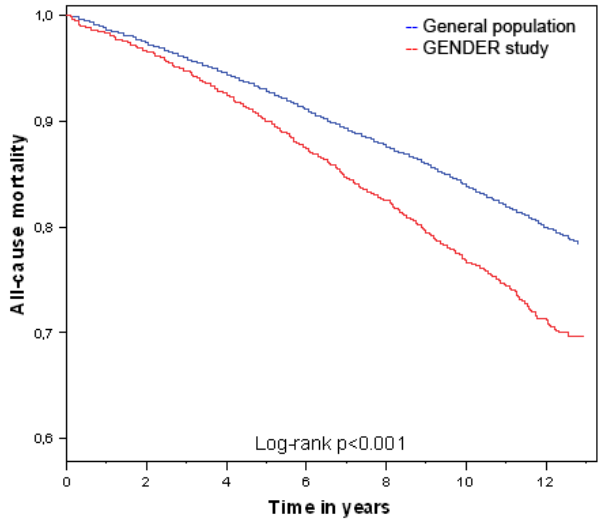


which was similar to the 770 (27.7%) of the patients without restenosis (N = 2,784), hazard ratio (HR) 1.13, 95% confidence interval (CI) 0.90-1.41, p = 0.28, with adjustment for age and sex. Also no difference was observed for death due specifically to CVD (12.3% versus 11.4% in the group with restenosis and the group without respectively, p = 0.64).

All-cause mortality in GENDER compared with the general population

When comparing the total GENDER population with a random sample from the general population, we observed a higher mortality rate in the GENDER study population than in the control cohort, 27.8% versus 21.6%, P < 0.001. This difference is visualised in Fig. 3. However, when adjusting for age and sex, this increased 10-year mortality risk was no longer statistically significant, HR of 1.05, 95% CI 0.97-1.14, p = 0.24. This was mainly caused by the influence of sex. The male subjects of GENDER did not have an increased all-cause mortality risk compared with the general population (HR 0.97, 95% CI 0.87-1.05). In contrast, female patients with known coronary artery disease had an 1.39-fold (95% CI 1.18-1.63, p < 0.001) increased risk to die during long-term follow-up, compared with the female individuals in the general population.

Figure 3 Kaplan Meijer curves comparing mortality of the GENDER study population (n = 3,084) with a random sample from the Dutch general population (n = 14,356)



Primary cause of death

In Table 2, the primary causes of death are shown. The majority of the patients died of a cardiovascular cause (N = 355, 11.5% of the total population and 41.5% of all-cause mortality). Compared with the random sample from the general Dutch population, cardiovascular causes of death were more frequent in the GENDER study, 11.5% (N = 355)

versus 6.7% (N = 957), p < 0.001. Patients in the GENDER cohort had a 1.29-fold increased risk (95% CI 1.13-1.48, p < 0.001) for CVD death compared with the control cohort, adjusted for age and sex. Most of these CVD deaths were caused by acute myocardial infarction in both cohorts, 91 (3.0% of the total population) in GENDER and 244 (1.6% of the total population) in the control group respectively. Death due to stroke accounted for a similar percentage of all deaths in both populations, N = 47 (1.5%) in GENDER versus N = 199 (1.3%) in the control cohort.

The second major cause of death in GENDER was cancer-related. A total of 2,045 patients (7.9% of the population and 28.6% of all-cause mortality) died of cancer, which was slightly less frequent than in the general population (8.6% of the population and 41.3% of all-cause mortality). However, this difference was not statistically significant (p = 0.08). The patients included in GENDER did have a higher risk of dying from a metabolic disease, mostly diabetes, a well-known risk factor for CVD. Moreover, also death caused by urological disorders or diseases of the reproductive organs was higher in the GENDER population than in the general population. No differences were observed between other causes of death. Approximately 4% of deaths were of infectious nature, 2% due psychiatric disorders, 3% due pulmonary disease and 2% due to non-natural causes.

Table 2 Causes of death of the GENDER study population and of the Dutch general population from March 1999 up to December 2011

Cause	ICD-10 code	GENDER (N = 3,084)		General population (N = 14,356)		P-value
		N (%)	%*	N (%)	%*	
Cardiovascular disease	I000-I999	355 (11.5%)	41.5%	957 (6.7%)	30.8%	< 0.001
Cancer	C000-D489	245 (7.9%)	28.6%	1,283 (8.9%)	41.3%	0.08
Infectious	A000-B999, J120-J180	34 (1.1%)	4.0%	140 (1.0%)	4.5%	0.39
Metabolic disease	E000-E909	36 (1.2%)	4.2%	80 (0.6%)	2.6%	< 0.001
Psychic disorders	F000-F999	17 (0.6%)	2.0%	56 (0.4%)	1.8%	0.21
Respiratory system**	J000-J999 (excl. J120-J180)	41 (1.3%)	4.8%	192 (1.3%)	6.2%	0.97
Gastro-intestinal system	K000-K939)	29 (0.9%)	3.4%	101 (0.7%)	3.2%	0.17
Urinary tract and reproductive organs	N000-N999	23 (0.7%)	2.7%	43 (0.3%)	1.4%	< 0.001
Non-natural cause of death	V010-Y899	20 (0.6%)	2.3%	68 (0.5%)	2.2%	0.21
Others		21 (0.7%)	2.5%	79 (0.6%)	2.5%	0.41
Unknown	R000-R999	35 (1.1%)	4.1%	109 (0.8%)	3.5%	0.04
Total		856 (27.8%)	100.0%	3,108 (21.6%)	100%	< 0.001

* Percentage of all-cause mortality. ** Excluding pneumonia

Cardiovascular death in GENDER

When looking more specifically into the cardiovascular causes of death in the GENDER population, ischaemic heart disease was the most frequent cause of death, responsible for almost half (47%) of all CVD deaths (Table 3). Of these 167 patients dying from ischaemic heart disease, 91 died as the consequence of an acute myocardial infarction. Heart failure was the next most frequent occurring CVD death (15.4%), followed by cerebrovascular disease (13.1%) and valvular disease (7.9%). Rhythm and conductions disorders and aneurysmal disease each accounted for approximately 4% of the CVD related deaths.

Table 3, Specific cardiovascular causes of death of the GENDER population

Cause of death	ICD-10 code	GENDER	
		N	%*
All cardiovascular disease	I000-I999	355	100%
Ischaemic heart disease	I200-I259	167	47.0%
Acute myocardial infarction	I210-I219	91	25.6%
Valve disease	I340-I389	28	7.9%
Rhythm and conduction disorders	I440-I499	16	4.5%
Heart failure and cardiomyopathies	I420-I439, I500-I509	54	15.2%
Cerebrovascular disease	I600-I699	47	13.2%
Aneurysmal disease	I710-I729	15	4.2%
Other cardiovascular disease		28	7.8%

* Percentage of all cardiovascular death. Other cardiovascular disease includes hypertensive heart disease, rheumatic heart disease, pulmonary heart disease, peripheral vascular disease and unspecified heart disease

Limitations

One study limitation should be mentioned. For this study we analysed the primary cause of death recorded by the Dutch CBS. Although this is the most specific mortality data available a small margin of error should be taken into account. For instance, the coronary artery disease patients in GENDER who are now classified with diabetes as their primary cause of death, likely died from a cardiovascular cause of death instead. Autopsy would result in the most specific cause of death, but this is not performed in most individuals.

CONCLUSIONS

The data presented above allow two conclusions. First, although previous studies have shown that patients developing coronary restenosis after PCI have a higher (CVD) morbidity and mortality[9,10], the current study demonstrates that, in the long-term, no differ-

ences in mortality rates are present between patients who develop restenosis and those who do not develop restenosis. This indicates that the current treatment of coronary restenosis, by repeated intervention, is good enough to prevent an increased mortality risk in the long-term. However, this patient population with confirmed coronary artery disease, despite guideline-based treatment, does have an increased mortality risk compared with the general population, especially pronounced in female patients. Since this increased risk is still attributable to CVD mortality, and not to other causes of death, there still seems room to improve current treatment strategies for CVD. Although speculative, likely the most room for improvement is in primary prevention and not secondary prevention as in the current study population. Future studies in both primary and secondary prevention cohorts will show us whether we are still heading in the right direction for optimal treatment of our patients, ultimately shifting their mortality risk all the way back to that of the general population.

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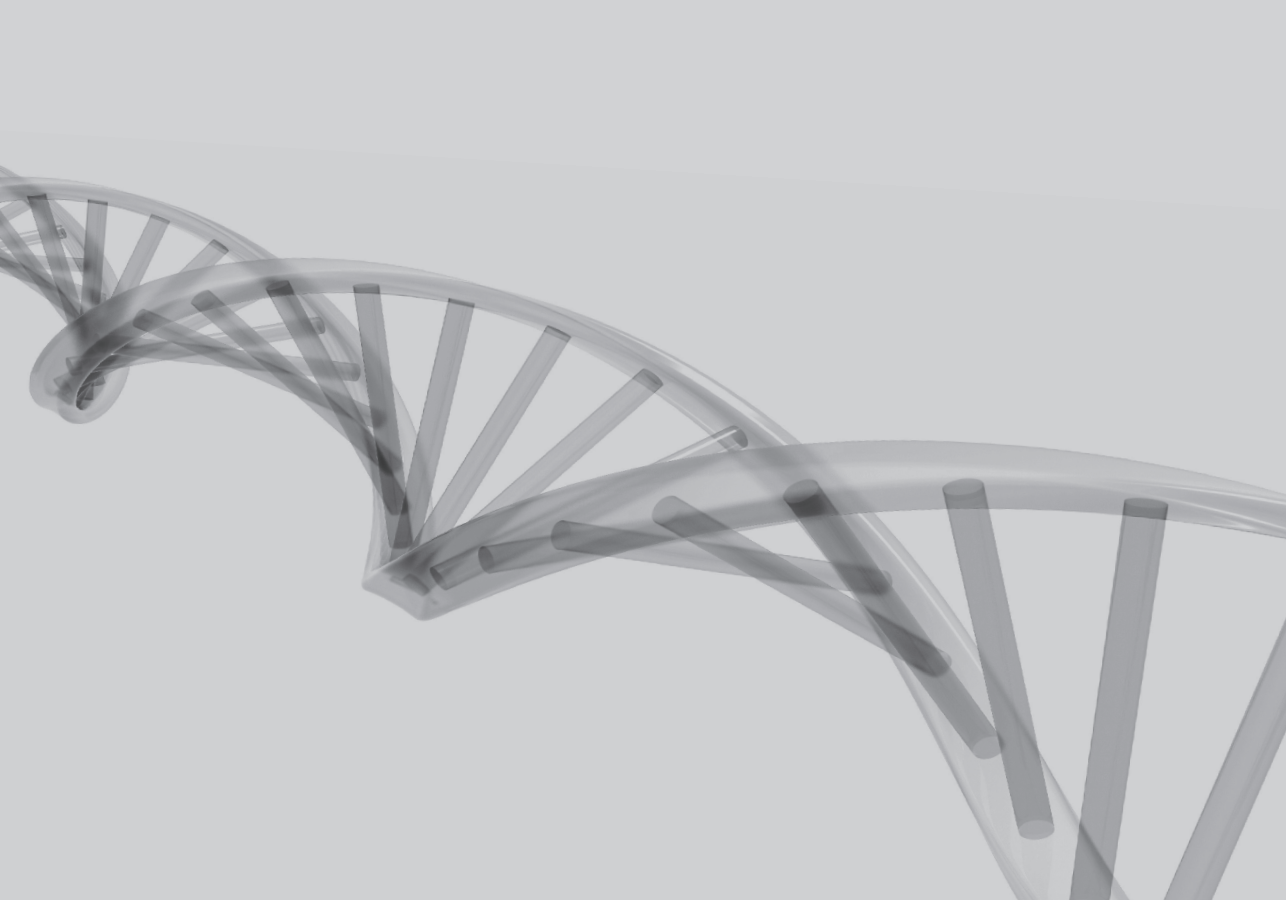
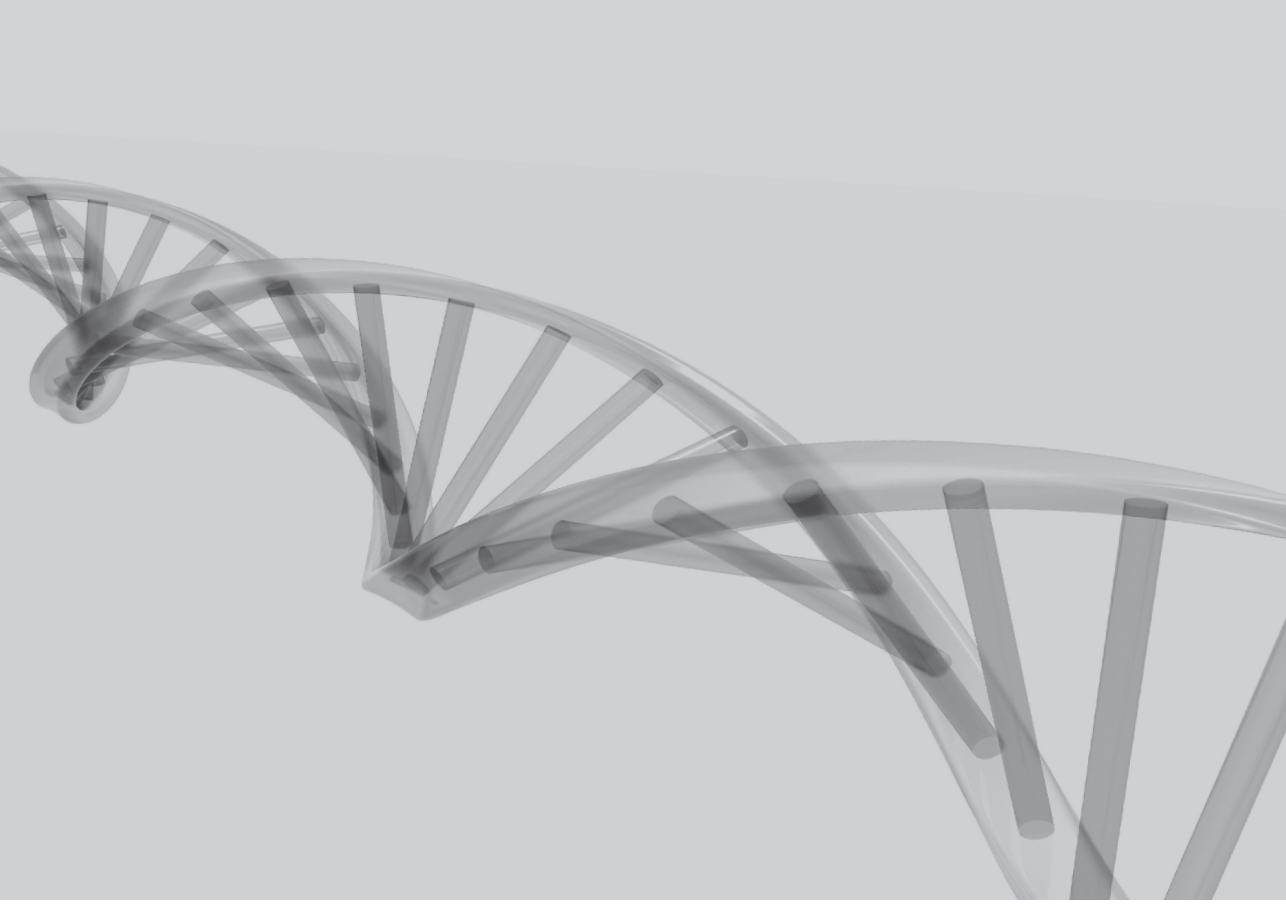
Part II



Genetics of (cardio) vascular diseases

Chapter 9



Non-homologous end-joining pathway associated with occurrence of myocardial infarction

Gene set analysis of genome-wide association study data

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ABSTRACT

Purpose: DNA repair deficiencies have been postulated to play a role in the development and progression of cardiovascular disease (CVD). The hypothesis is that DNA damage accumulating with age may induce cell death, which promotes formation of unstable plaques. Defects in DNA repair mechanisms may therefore increase the risk of CVD events. We examined whether the joint effect of common genetic variants in 5 DNA repair pathways may influence the risk of CVD events.

Methods: The PLINK set-based test was used to examine the association to myocardial infarction (MI) of the DNA repair pathway in GWAS data of 866 subjects of the GENetic DEterminants of Restenosis (GENDER) study and 5,244 subjects of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) study. We included the main DNA repair pathways (base excision repair, nucleotide excision repair, mismatch repair, homologous recombination and non-homologous end-joining (NHEJ)) in the analysis.

Results: The NHEJ pathway was associated with the occurrence of MI in both GENDER ($P = 0.0083$) and PROSPER ($P = 0.014$). This association was mainly driven by genetic variation in the MRE11A gene ($P_{GENDER} = 0.0001$ and $P_{PROSPER} = 0.002$). The homologous recombination pathway was associated with MI in GENDER only ($P = 0.011$), for the other pathways no associations were observed.

Conclusion: This is the first study analyzing the joint effect of common genetic variation in DNA repair pathways and the risk of CVD events, demonstrating an association between the NHEJ pathway and MI in 2 different cohorts.

INTRODUCTION

Cardiovascular disease (CVD) is caused by interplay of environmental factors and multiple predisposing genes. DNA damage, caused by for instance oxidative stress and cigarette smoking, has been recognized as a significant contributor to the pathogenesis of CVD.[1,2] Mechanistically, in cells where the DNA damage is beyond repair apoptosis is induced.[3] The effect of this damage induced cell death is dependent on the cell type. Death of endothelial cells is implicated in plaque erosion and subsequent vessel thrombosis.[4] Vascular smooth muscle cell (VSMC) death has been associated with thinning of the fibrous cap and increasing the risk of plaque rupture.[5,6] To further complicate matters, apoptosis is not the only response of cells to DNA damage, also cellular senescence has been described.[7,8] Cellular senescence is a state in which cells remain in cell cycle arrest and in which they have lost their optimal function. With respect to the pathogenesis of atherosclerosis, senescence of for instance vascular endothelial cells can result in a provasoconstrictor and a proinflammatory phenotype.[7] So besides cell death, DNA damage could also increases the risk of CVD by inducing cell senescence.

Adequate DNA repair is crucial for survival of an organism, as the DNA is continuously exposed to various types of external factors, like mutagenic chemicals and radiation, and endogenously generated triggers like reactive oxygen species (ROS) and DNA replication errors, all capable of inducing DNA damage. Human cells possess several innate DNA repair processes to protect against the harmful consequences of DNA damage.[9] Single-strand DNA damage can be repaired by excision repair and mismatch repair pathways that use the undamaged strand as template during the repair process. For the repair of double-strand breaks other repair mechanisms like non-homologous end-joining (NHEJ) or homologous recombination are required.[10]

Evidence of the relation between genomic integrity and cardiovascular disease in the ageing population has been growing over the last years. Up to now, this evidence consists in various forms, ranging from cellular biology studies in vascular endothelial cells[11,12], vascular smooth muscle cells[13,14] and macropahges[15], histological examination of human atherosclerotic plaques[14,16] and animal studies in telomerase deficient mice[17] and DNA repair defective mice.[8,18,19] In contrast, only limited studies have focused on single nucleotide polymorphisms (SNPs) in genes related to DNA repair processes and CVD events, although some associations have been reported. The only SNP with some consistent results is the Arg399Gln (rs25487) SNP in the XRCC1 base excision repair (BER) gene which was reported to be associated with stroke[20] and coronary atherosclerosis[21]. Other genes from the excision repair pathway such as OGG1, XRCC3, ERCC2 (XPD)and ERCC5, were found to moderately associated as a combined score to the risk of large artery atherosclerotic stroke in the smoking subset of a Chinese population [22]. The

authors suggested that the individual vulnerability to smoking-induced oxidative stress was influenced by carriers of these SNPS.

In genome-wide association studies (GWAS) investigating the genetic background of CVD, no association was found with SNPs in genes related to DNA repair processes.[23,24] However, considering the multifactorial nature of the condition, it is possible that by a joint effect, genetic variants with small individual effect sizes, could contribute to disease risk and are undetected in a GWAS.[25,26] The goal of the current study was to examine whether common genetic variants in DNA repair genes are related to the risk of CVD events by using a gene set analysis of the 5 main DNA repair pathways in two large representative CVD populations.

METHODS

GENDER study population

The design of the GENetic DEterminants of Restenosis (GENDER) study has been described previously.[27] In brief, GENDER included 3,104 consecutive unrelated symptomatic patients treated successfully by PCI for angina. The study protocol conforms to the Declaration of Helsinki and was approved by the ethics committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure. Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. During the study, no drug-eluting stents were used. Blood samples were collected at the index procedure for DNA isolation. During a follow-up period of 9 months, the endpoint clinical restenosis, defined as renewed symptoms requiring target vessel revascularization (TVR) either by repeated PCI or CABG, by death from cardiac causes or myocardial infarction (MI) not attributable to another coronary event than the target vessel, was recorded. Furthermore, of each patient the occurrence of MI or stroke prior to inclusion into the study, as well as during the follow up period, was recorded. For this study the combination of prevalent and incident MI or stroke was analyzed.

PROSPER study population

The design and population of the PROSPective study for the Elderly at Risk (PROSPER) has been described previously.[28] PROSPER is a prospective multicenter randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in elderly individuals. Between December 1997 and May 1999, subjects were screened and enrolled in Scotland (Glasgow), Ireland (Cork), and The Netherlands (Leiden). Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5,804 subjects were randomly assigned to

pravastatin or placebo. In this study several cardiovascular endpoints were evaluated during a mean follow-up of 3.2 years; the primary endpoint consisted of a composite of fatal/non-fatal MI or fatal/non-fatal stroke. Secondary and tertiary endpoints included stroke and MI separately, all-cause mortality and death due to a vascular cause.[29] The institutional ethics review boards of all centers approved the protocol, and all participants gave written informed consent. The protocol was consistent with the Declaration of Helsinki. For the current study we combined the incident events ,myocardial infarction and stroke, that occurred during the follow-up period with the prevalent events that occurred before inclusion into PROSPER, to obtain a lifetime risk for these events.

Genotyping

In GENDER, a GWAS was performed in 325 cases of restenosis and 630 controls matched by gender, age, and some confounding clinical variables for restenosis in the GENDER study such as total occlusion, diabetes, current smoking and residual stenosis.[30] Genotyping was performed using the Illumina Human 610-Quad Beadchips following manufacturer's instructions. . After stringent quality control, bad performing samples (call rate < 99%) and assays (call rate < 95%, minor allele frequency < 1% and deviation from Hardy-Weinberg equilibrium) were excluded from further analysis. The final dataset consisted of 866 individuals (295 cases, 571 controls) and 556,099 SNPs.

In PROSPER, a GWAS was performed using Illumina Human 660-Quad Beadchips following manufacturer's instructions. After stringent quality control, bad performing samples and assays were excluded from further analysis. Genotypic data was available in 5,244 subjects and a total of 557,192 SNPs[31].

Both datasets were imputed using MaCH software[32] up to ~2.5 million SNPs based on the HapMap Phase I + II CEU release 22 (hg18/build36) reference.

Gene set analysis

We analyzed SNPs within a 10-kb window around the genes encoding proteins belonging to the 5 DNA repair pathways described in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database[33,34]; the BER pathway, the nucleotide excision repair (NER) pathway, the mismatch repair (MMR) pathway, the homologous recombination pathway and the NHEJ pathway. Gene set analyses were performed with the PLINK set-based test v1.07 in a case-control setting.[35] In the first step of this test, a single SNP analysis of all SNPs within the set is performed. Subsequently, a mean SNP statistic is calculated from the single SNP statistics of a maximum amount of SNPs below a certain P-value threshold. For the current study this threshold was set on 0.20 to ensure that all SNPs with minor effect will be analyzed. If SNPs are not independent, i.e. the LD (expressed in R2) is above a certain threshold, the SNP with the lowest P-value in the single SNP analysis is selected. This analysis is repeated with 10,000 simulated SNP sets,

in which the phenotype status of the individuals is permuted. An empirical P-value for the SNP set is computed by calculating the number of times the test statistic of the simulated SNP sets exceeds that of the original SNP set. For the set-based analysis of this study, the parameters were set to P-value threshold < 0.20, R2 threshold < 0.5, and maximum number of SNPs = 99999. The associations were considered significant, after correction for the 5 analyzed pathways, if the P-value < 0.01 (0.05/5). For the pathway analysis we only used the genotyped GWAS sets of both studies, since imputed SNPs were obtained based on their LD pattern with the genotyped SNPs and the LD threshold of the set-based analysis corrects for this, making their added value minimal.

RESULTS

Participant characteristics of the two study populations are presented in table 1. The main differences in the baseline characteristics between the two study populations

Table 1, Baseline characteristics and endpoints of the GENDER and PROSPER studies

	GENDER N = 866	PROSPER N = 5,244
Baseline characteristics		
Age (years)	62.5 ± 10.8	75.3 ± 3.4
Male gender (N)	634 (73)	2,524 (48)
Current smoking (N)	216 (25)	1,392 (27)
History of diabetes (N)	177 (20)	544 (10)
History of hypertension (N)	349 (40)	3,257 (62)
History of angina (N)	288 (68)	1,424 (27)
History of myocardial infarction (N)	365 (42)	708 (14)
Total cholesterol (mmol/L)	4.9 ± 1.0a	5.7 ± 0.9
Body mass index (kg/m2)	27.0 ± 3.7	26.8 ± 4.2
Statin treatment	465 (54)	2,605 (50)
Endpoints		
Myocardial infarction b	389 (45)	1145 (22)
Stroke b	49 (6)	731 (14)
Myocardial infarction or stroke b	416 (48)	1714 (33)
Clinical restenosis c	295 (34)	NA
All cause mortality	237 (27)d	548 (11)c
Vascular mortality c	NA	266 (5)

Data are presented as mean ± SD or number (%). a Cholesterol levels available in only 177 patients. b Before inclusion or during follow-up period. c During follow-up period. d During follow up of 10 years after inclusion in GENDER.

are the mean age of the participants (GENDER; 62.5 years, PROSPER; 75.3 years) and the proportion of women (GENDER; 27%, PROSPER; 52%) Moreover, diabetes and complaints of stable angina pectoris were more frequent in GENDER, whereas a history of hypertension was more frequent in PROSPER. The incidence of MI in GENDER was 45% and in PROSPER 22%.

Of the 5 selected DNA repair pathways, as described by the KEGG pathway database, the NER pathway was the largest one (44 genes), (Table S1), while the NHEJ pathway was the smallest (13 genes). The BER pathway consisted of 35 genes, the MMR pathway of 23 genes and the homologous recombination pathway of 28 genes.

The set-based analysis of the 5 pathway sets in the GENDER population resulted in a significant association of the homologous recombination pathway with MI (P = 0.011) and the combined endpoint of MI or stroke (P = 0.0039) (Table 2). A significant association with the same endpoints was also found for the NHEJ pathway (P = 0.0083 for MI and P = 0.0089 for MI or stroke). No significant association of any of the DNA repair pathways was found with stroke alone.

Analysis in the PROSPER dataset resulted in an association of the NHEJ pathway with MI (P = 0.014). A borderline significant association of the BER pathway with MI was also observed (P = 0.049). The other pathways did not show significant associations in PROSPER (Table 2).

To determine by which genes the association of the NHEJ pathway with MI was driven, we examined the SNP set from each gene of this pathway separately. We found that

Table 2, Set-based analysis of DNA repair pathways in the GENDER and PROSPER study populations

Pathway			MIa		Strokea		MI or Strokea	
	Genes	SNPs	SNPs	P	SNPs	P	SNPs	P
GENDERb			N = 389/477		N = 49/817		N = 416/450	
Base excision	35	315	37	0.48	36	0.47	37	0.59
Nucleotide excision	44	401	57	0.38	49	0.34	60	0.38
Mismatch repair	23	285	39	0.34	40	0.42	47	0.35
Homologous recombination	28	418	64	0.011	51	0.71	60	0.0039
Non-homologous end joining	13	137	19	0.0084	14	0.43	19	0.0089
PROSPERb			N = 1,145/4,099		N = 731/4,513		N = 1,714/3,530	
Base excision	35	298	43	0.049	42	0.39	39	0.43
Nucleotide excision	44	392	62	0.34	57	0.47	49	0.49
Mismatch repair	23	285	39	0.43	33	0.46	31	0.91
Homologous recombination	28	426	61	0.14	42	0.49	51	0.24
Non-homologous end joining	13	144	19	0.014	30	0.35	22	0.11

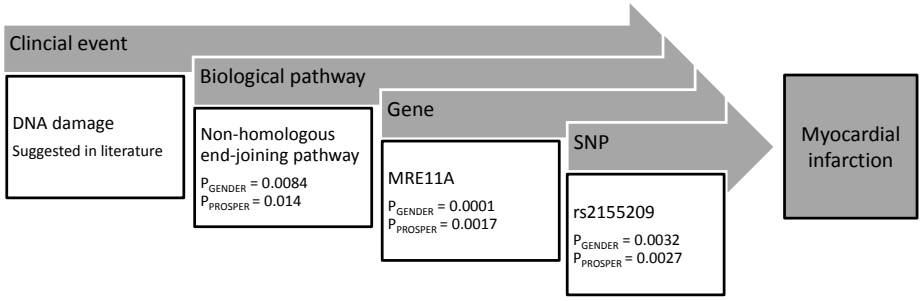
The SNPs per endpoint indicate the number of independent SNPs that passed the test constrains (P < 0.2 and R2 < 0.5) and were thus jointly analyzed in 10,000 permutations. a Before inclusion or during follow-up period. MI, myocardial infarction. b Cases/controls

Table 3, Results of the gene set analysis of the non-homologous end joining pathway with myocardial infarction in GENDER and PROSPER

Gene	GENDER							PROSPER						
	SNPs	Sig. SNPs	P (gene)	Top SNP	MAF	OR	P (SNP)	SNPs	Sig. SNPs	P (gene)	Top SNP	MAF	OR	P (SNP)
XRCC5	19	3	0.23	rs3821107	0.24	0.79	0.04	22	1	0.89	rs828704	0.20	0.93	0.19
NHEJ1	15	1	0.55	rs7588654	0.03	0.65	0.14	15	0	1.00				
XRCC4	29	4	0.055	rs13178127	0.05	2.02	0.001	30	4	0.38	rs35271	0.14	1.15	0.039
RAD50	8	0	1.00	-				10	1	0.52	rs2237060	0.44	1.07	0.13
POLM	4	2	0.12	rs11769882	0.25	0.79	0.040	4	1	0.26	rs11769882	0.22	0.93	0.14
PRKDC	14	1	0.69	rs7003908	0.34	1.16	0.14	14	4	0.026	rs10109984	0.38	1.15	0.005
POLL	3	0	1.00	-				3	1	0.28	rs3730477	0.21	1.09	0.14
DCLRE1C	8	1	0.22	rs12572872	0.23	1.24	0.06	8	0	1.00				
DNTT	9	1	0.35	rs1923703	0.12	0.80	0.14	9	0	1.00				
MRE11A	14	3	0.0001	rs535801	0.31	1.52	0.00006	13	2	0.0017	rs2155209	0.35	0.86	0.002
FEN1	5	0	1.00	-				5	1	0.27	rs695867	0.35	0.80	0.10
LIG4	7	3	0.31	rs9520823	0.30	1.21	0.07	7	3	0.030	rs1151403	0.42	0.87	0.003
XRCC6	2	0	1.00	-				4	1	0.45	rs17002523	0.01	1.34	0.16

SNPs, total number of SNPs per gene; Sig.SNPs indicate the number of SNPs that passed the test constrains ($P < 0.2$ and $R2 < 0.5$) and were thus jointly analyzed in 10,000 permutations; OR odds ratio; P, p-value; MAF, minor allele frequency

Figure 1 Fine mapping from DNA damage through the identification of an associated DNA repair pathway, the responsible gene in this pathway, to the single nucleotide polymorphism (SNP)



the association was driven by several genes from this pathway. In GENDER the XRCC4 gene demonstrated a borderline significant association (P = 0.055) and in PROSPER the genes PRKDC (P = 0.029) and LIG4 (P = 0.030) were individually associated with MI. The strongest association was found with the MRE11A gene in both GENDER (P = 0.0001) and PROSPER (P = 0.0017), driven by 3 and 2 SNPs, respectively, which differ between the studies (Table 3).

By using imputed data we performed in silico fine mapping of the individual SNPs in the MRE11A genetic region on chromosome 11 (Figure 1). Within the range of 10Kb around the MRE11A gene genotypic data of 104 SNPs were available. We identified 8 sets of SNPs that were in high LD (R2 > 0.8) but only one set (10 SNPs) associated with MI in both GENDER and PROSPER (P = 0.0033 and P = 0.0023 respectively). This set contained the top SNP in MRE11A in PROSPER (rs2155209), that was identified in the individual gene analysis (Table 4). This SNP is located in a DNase I hypersensitivity site (UCSC genome browsers database [36]). The promotor 2.0 Prediction Server[37] reported

Table 4, Genomic region of MRE11A divided in LD blocks for the association with myocardial infarction

Set	SNPs	R2	Tagging SNP	MAF*	GENDER		PROSPER	
					OR (95% CI)	P	OR (95% CI)	P
1	10	0.91	rs2155209	0.36	0.74 (0.61-0.91)	0.0033	0.86 (0.78-0.96)	0.0023
2	10	0,90	rs535801	0.31	1.52 (1.24-1.87)	0.000059	1.00 (0.90-1.11)	0.94
3	17	0.88	rs1270146	0.43	1.32 (1.09-1.60)	0.0049	1.02 (0.93-1.13)	0.63
4	13	0.91	rs529126	0.26	1.44 (1.16-1.80)	0.00090	1.02 (0.91-1.12)	0.71
5	5	0.85	rs499952	0.35	1.32 (1.08-1.61)	0.0059	1.03 (0.92-1.12)	0.54
6	4	0.91	rs13447720	0.23	0.94 (0.75-1.18)	0.57	1.18 (1.05-1.30)	0.0027
7	18	0.91	rs10765682	0.09	0.91 (0.65-1.27)	0.58	1.01 (0.87-1.20)	0.95
8	18	1.00	rs12788248	0.01	1.22 (0.43-3.49)	0.71	1.02 (0.58-1.72)	0.95
other**	9		-					

R2 indicates lowest LD between SNPs within the set. Tagging SNP is a genotyped SNP with the lowest P-value per set. * MAF, minor allele frequency in GENDER. ** SNPs not in LD with other SNPs within the gene.

that the region surrounding rs2155209 is not a promotor region. In addition, the is-rSNP algorithm[38] reported that the DNA binding affinity of three transcription factors is significantly affected by rs2155209 (LM221 P = 0.014, estrogen receptor 2 (ESR2) P = 0.028 and LM168 P = 0.037). Unfortunately, rs2155209 is not reported in three publically available eQTL databases (mRNA by SNP browser[39,40], VarySysDB[41] and the eQTL database of the Pritchard lab[42]).

To explore whether the found associations were caused by specific subgroups we analyzed the NHEJ pathway in male and female patients, smokers and non-smokers and in patients with and without diabetes separately. Moreover, in the PROSPER study we also performed the analyses in the pravastatin and placebo group. No clear associations were detected in these subgroups (Table S2).

DISCUSSION

The current study is the first to use a DNA repair pathway approach for the identification of new candidate genes related to cardiovascular outcomes. We show that genetic variation in several of these genes are indeed associated with cardiovascular related endpoints and that the joint analyses of these genetic markers demonstrates a significant association of the NHEJ pathway with prevalent and incident MI in two study populations. Variation in the gene encoding meiotic recombination 11 homolog A (MRE11A) drives the association.

This is the first study that demonstrates a relation between the NHEJ pathway and MI or even with CVD. This particular DNA repair pathway is mainly involved in the repair of double-strand breaks (DSBs), which are considered to have the highest risk of evoking deleterious events, such as chromosomal translocations, cancer and cell death. The main driver of this association, MRE11A, is a highly conserved protein, existing in vivo as a dimer, forms together with RAD50 and NBS1 the MRN complex.[43] The MRN complex has a critical role in the recognition of DNA damage lesions or the chromatin alterations that follow DNA damage[3] and a key role in the cellular response to DSBs.[44] Moreover, the MRN complex has been implicated in telomere maintenance, meiosis, DNA replication and checkpoint activation.[45-47] Genetic variation in MRE11A has previously been associated with several types of cancer [48-50] and antaxia-telangiectasis-like disease.[51] To our knowledge, no association of this gene with CVD events has yet been described, also not in the previous GWAS on CVD.[23,52,53] The SNP rs2155209, significantly associated with MI in both of our study populations, has been associated with an 1.5-fold increased risk of bladder cancer, although the authors of that study suggest that it might has been a false-positive finding. [49] This MRE11A SNP is located in the 3'UTR of the gene, and the possible functional effect has not yet been studied.

When examining the LD structure of MRE11A, we found that the structure is not conform the expected LD block formation, meaning that nearby SNPs are organized into regions of high LD separated by short segments of very low LD. This discontinuous LD structure is not uncommon, and has been described before for this gene.[54] Allen-Brady et al.[54] describe 4 tagging SNPs together accounting for 99% of the genetic variance within the gene region. In the current study the genetic coverage of the gene was more thorough (104 SNPs compared to 11 in the former study), resulting in 8 tagging SNPs. Interestingly, one of the 4 described tagging SNPs, rs556477, is in very high LD (R2 = 0.92) with our top SNP rs2155209. It is unknown whether rs2155209 has any direct functional effects. That this SNP is located in a DNase I hypersensitivity site, which often associated with cis-regulatory sequences, including promoters, insulators, enhancers and locus control regions, increases the likelihood that rs2155209 influences one of these features and thereby exerting its clinical effects, although this remains to be proven. Another method of predicting the possible regulatory abilities of non-coding SNPs is the in silico rSNP algorithm[38]. This approach indicated that rs2155209 affects binding of ERS2. The estrogen receptor 2 belongs to a family of nuclear receptor transcription factors, activating transcription upon binding to specific DNA sequences. Moreover, the SNP was associated with LM221 and LM168, two conserved motifs in the human genome described to be involved in gene regulation, likely serving as insulators.[55] Wet lab confirmation of these bioinformatic predictions will be necessary before definite conclusions can be drawn from these findings.

Cigarette smoking is considered to be an important risk factor in atherosclerotic vascular disease and it is a well-known external factor associated with DNA damage, like single- and double strand breaks and the formation of oxidative DNA-adducts.[56,57] The association of the NHEJ pathway with MI, demonstrated in the current study, could indicate that this pathway is the underlying mechanism of the strong relation between smoking and CVDs. However, this hypothesis could not be confirmed in subgroup analyses. Whether this absence of association is caused by lack of power of the subgroup analysis, or because the underlying mechanism causing MI is not through smoking, is uncertain. Moreover, the hypothesis that patients with diabetes mellitus have increased oxidative stress which could lead to DNA damage[58,59], could also not be confirmed in our population. However, considering that the incidence of diabetes was 20% in GENDER and only 10% in PROSPER, there was not enough power to detect a small effect. Furthermore, we cannot exclude that another DNA repair pathway than NHEJ might be responsible for the DNA repair in diabetic patients, but considering the small subgroup size and the fact that the other DNA repair pathways were not significantly associated in the complete populations, we did not perform further analyses for these pathways. The subgroup analysis did demonstrate that the association of the NHEJ pathway with MI was possibly driven by the male subjects, since in PROSPER no association was found

in female subjects. Although in GENDER a similar trend was observed, these results were not significant.

The strength of gene set analysis, opposed to GWAS analysis, is that it tests the joint effect of multiple individual SNPs within a larger set. Considering the a priori small effect size of the individual SNPs on complex disease endpoints, like MI, analysis of the joint effect of multiple markers, in this study comprising complete DNA repair pathways, will increase the likelihood of finding biological plausible associations.

Several possible limitations to our study have to be mentioned. For the current study we performed the pathway analysis using the PLINK software.[35] Other software packages have been described, although to date none has been proven to be clearly superior to the others. Gui and colleagues compared 7 tests analyzing the WTCCC Crohn's Disease dataset.[60] One of their overall conclusions was that the set-based test in PLINK was the most powerful algorithm. Another study, applying PLINK set-based test, Global test, GRASS and SNP ratio test, for the analysis of three pathways regarding human longevity observed similar results with the different tests.[61] Although other software packages could lead to different results, the fact that our fine mapping strategy led to the identification of a single LD block associated in two independent populations, increased the likelihood of a true positive association.

The five DNA repair pathways analyzed were derived from the publically available KEGG database[34]. The KEGG database is however not the only database providing biological pathways and there is no consensus on the best database. In our opinion these particular pathways were more elaborately described by KEGG than in other databases (for instance Reactome or BioCarta). Moreover, only the KEGG database provided a description of all 5 DNA repair pathways. Since the overlap of certain pathways of different databases is substantial, we decided only to test the DNA repair pathways described by KEGG.[33] It is important to realize that probably none of these databases provide a perfect representation of the actual biological mechanism, simple because our current knowledge is not that far evolved yet. Likely not all genes incorporated within the current pathways directly influence the actual DNA repair process of interest. These unrelated gene product could therefore interfere with the actual associations, however to what extent this is the case in the current study remains unknown.

As stated above, DNA damage and DNA damage repair are associated with cancer. Since we are interested in the effects of DNA damage repair on clinical events other than cancer, and because the two included study populations are of rather old age, especially PROSPER, we cannot exclude that the competing risk of cancer related mortality and CVD events have led to a selection bias of the patients. Therefore, it could be possible that the role of DNA repair pathways is being underestimated. However, since we cannot correct for this potential selection bias, this remains speculative. Another potential confounder is age. The GENDER study is considerably younger than the PROSPER cohort, possibly

explaining part of the different results of both studies. However, since in the set-based analysis of PLINK correction for confounders is not possible, the actual magnitude of the influence of age on the current results remains therefore uncertain.

In conclusion, with this study we demonstrate that genetic variation in the NHEJ pathway of the human DNA repair machinery, and specifically genetic variation in the MRE11A gene, is associated with the occurrence of MI. Results of this study need to be validated by functional studies to further elucidate the precise mechanistic role of NHEJ in atherosclerotic lesion formation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *PLoS One* online.

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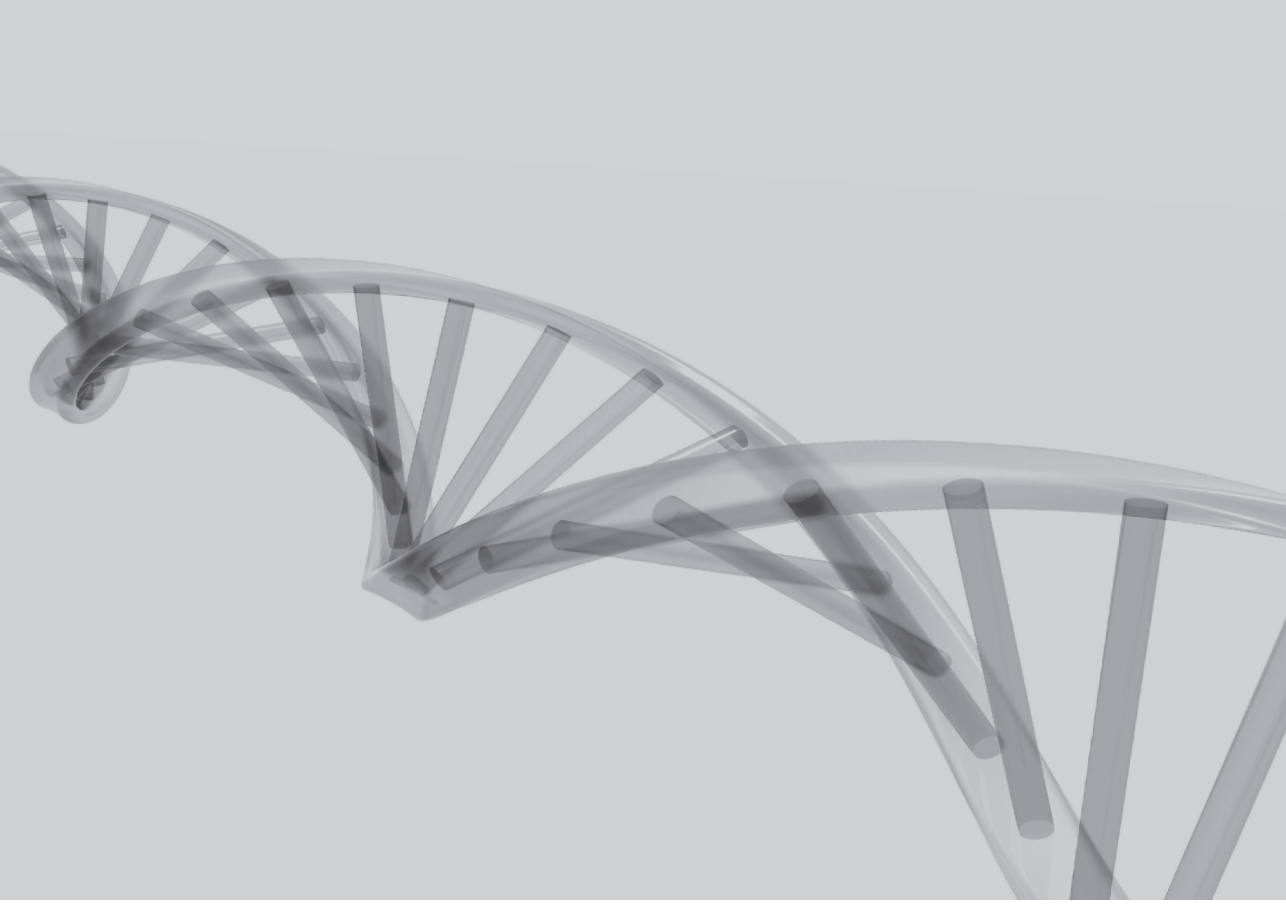
Chapter 10



Candidate gene analysis of arteriovenous fistula failure in hemodialysis patients

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ABSTRACT

Background: Arteriovenous fistula (AVF) failure remains an important cause of morbidity in hemodialysis patients. The exact underlying mechanisms responsible of AVF failure are unknown but processes like proliferation, inflammation, vascular remodeling and thrombosis are thought to be involved. The current objective was to investigate the association between AVF failure and single nucleotide polymorphisms (SNPs) in genes related to these pathophysiological processes in a large population of incident hemodialysis patients.

Methods: A total of 479 incident hemodialysis patients were included between January 1997 and April 2004. Follow-up lasted two years or until AVF failure, defined as surgery, percutaneous endovascular intervention, or abandonment of the vascular access. Forty-three SNPs in 26 genes, related to proliferation, inflammation, endothelial function, vascular remodeling, coagulation and calcium/phosphate metabolism, were genotyped. Relations were analyzed using Cox regression analysis.

Results: In total, 207 (43.2%) patients developed AVF failure. After adjustment, two SNPs were significantly associated with an increased risk of AVF failure. The hazard ratio of LRP1 rs1466535 was 1.75 (95% CI 1.15-2.66) and patients with factor V Leiden had a hazard ratio of 2.54 (95% CI 1.41-4.56) to develop AVF failure. The other SNPs were not associated with AVF failure.

Conclusion: In this large cohort of hemodialysis patient, only two of the 43 candidate SNPs were associated with an increased risk of AVF failure. Whether other factors, like local hemodynamic circumstances, are more important or that other SNPs play a role in AVF failure remains to be elucidated.

INTRODUCTION

A durable vascular access to the bloodstream is of vital importance for patients undergoing chronic hemodialysis. However, vascular access dysfunction is currently the Achilles' heel of hemodialysis therapy, accounting for 20% of all hospitalizations in hemodialysis patients leading to unacceptable high morbidity and economic burden.[1,2] For chronic hemodialysis, arteriovenous fistulas (AVFs) are the preferred modality in view of the superior patency rates as compared to arteriovenous synthetic grafts. Nonetheless, the durability of AVFs is far from optimal with one-year primary patency rates ranging from 60-65%.[3,4]

The vast majority of arteriovenous (AV) access failure is caused by thrombosis, secondary to disproportionate intimal hyperplasia (IH) and impaired outward remodeling of the venous outflow tract.[5-8] The stimuli responsible for the localized intimal hyperplastic response in the venous outflow tract are multifactorial and include hemodynamic factors such as turbulent flow, the prothrombotic environment that results from endothelial damage as well as vascular inflammation.[9] The stenotic vascular lesions that arise from this intimal hyperplastic response mainly consist of vascular smooth muscle cells (VSMCs), myofibroblasts and extracellular matrix proteins.[10] Excessive accumulation of extracellular matrix is mediated by several growth factors.[5,9,11] Morphologically, these stenotic lesions closely resemble restenotic lesions after percutaneous coronary intervention (PCI).[12,13] Additional specific pathophysiological stimuli for IH in vascular access stenosis include the abnormal calcium/phosphate metabolism in patients with chronic kidney disease that result in arterial as well as venous calcification of the tunica media.[14] Therefore, processes related to vascular function and remodeling, growth factors for extracellular matrix formation, inflammation, coagulation and calcium/phosphate metabolism probably play an important role in AVF failure. Currently, it is unknown why AVF failure occurs in some individuals but not in others. It has been suggested that genetic factors could play a role in the development of AVF failure.[15] However, limited studies have investigated the effects of genetic risk factors that play a role in these processes on AVF failure.

The aim of the current study was to investigate the association between AVF failure and single nucleotide polymorphism (SNPs) involved in processes related to endothelial function and vascular remodeling, growth factors, inflammation, coagulation, and calcium/phosphate metabolism in a large population of incident hemodialysis patients.

METHODS

Patients

The Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) is a prospective multicenter cohort study in which incident end-stage renal disease patients from 38 dialysis centers in the Netherlands were included. The study was in accordance with the Declaration of Helsinki and was approved by all local medical ethics committees. All patients gave informed consent. We followed patients until AVF failure (defined as surgery, percutaneous endovascular intervention, or abandonment of the vascular access in the first two years on dialysis), death or censoring, i.e. transfer to a nonparticipating dialysis center, withdrawal from the study, switch to peritoneal dialysis, transplantation, or end of the follow-up period (April 2006).

Eligibility included age older than 18 years, no previous renal replacement therapy, and survival of the initial three months of dialysis. For the current analyses, we used data from patients included between January 1997 and April 2004 with a functional fistula within three months after the first dialysis session from whom DNA was available. Patients that were dialyzed using a central venous catheter and patients on peritoneal dialysis were excluded.

Demographic and clinical data

Data on age, sex, and primary kidney disease were collected at the start of dialysis treatment. Primary kidney disease was classified according to the codes of the Euro-

Table 1 Baseline characteristics

	Arteriovenous fistula N = 479
Age (years) (IQR)	65.3 (54.3-73.7)
Sex, female (n, %)	174 (36.3%)
Race, white (n, %)	440 (91.9%)
Primary kidney disease (n, %)	
Diabetes mellitus	73 (15.2%)
Glomerulonephritis	54 (11.3%)
Renal vascular disease	99 (20.7%)
Others	253 (52.8%)
interstitial nephropathy	48 (10.0%)
cystic kidney disease	63 (13.2%)
congenital and hereditary kidney disease	5 (1.0%)
Multisystem disease	28 (5.8%)
other	18 (3.8%)
unknown	91 (19.0%)

pean Renal Association-European Dialysis and Transplant Association (ERA-EDTA).[16] We grouped patients into four classes of primary kidney disease: glomerulonephritis, diabetes mellitus, renal vascular disease, and other kidney diseases. Other kidney diseases consisted of patients with interstitial nephritis, polycystic kidney diseases, other multisystem diseases, and unknown diseases.

SNP selection and genotyping

Single nucleotide polymorphisms, previously associated with AV access failure, were selected after a systematic search of literature. Furthermore, we also selected SNPs that were associated with coronary restenosis[13,17,18] and vascular aneurysm formation,[19,20] since underlying mechanisms of these diseases are thought to overlap. Searching MEDLINE using keywords including 'hemodialysis', 'arteriovenous access failure', and 'single nucleotide polymorphism' identified 6 genes previously associated with AV access failure (tumor necrosis factor alpha (TNF),[21] klotho,[22] fibrinogen beta (FGB),[23] factor V,[24] and matrix metallopeptidase 1 (MMP1).[25] To broaden the search to coronary restenosis and vascular aneurysm formation, the keywords 'coronary restenosis', 'percutaneous coronary intervention' and 'aortic aneurysm' were added, identifying another 20 candidate genes. Only SNPs with a minor allele frequency higher than 1% were included. Two multiplex assays were designed using Assay designer software. The final set included 43 SNPs in 26 genes, involved in processes related to endothelial function and vascular remodeling, growth factors, inflammation, coagulation and calcium/phosphate metabolism (Supplementary Table S1). All SNPs were genotyped by MALDI-TOF mass spectrometry, using the MassARRAY^{tm} methodology (Sequenom Inc., San Diego, CA, USA), following manufacturer's instructions. As quality control, 5% of the samples were genotyped in duplicate. No inconsistencies were observed. All the negative controls (2%) were negative. All SNPs were in Hardy-Weinberg equilibrium ($p \geq 0.01$) and had a call rate above 90% (Supplementary Table S2).

Statistical analysis

Continuous variables are presented as median and interquartile range (IQR). Categorical variables are presented as numbers with percentages. We calculated hazard ratios (HRs) with 95% confidence intervals (95% CIs) using Cox regression analysis for heterozygote and mutant genotypes as compared with wild-type genotypes for AVF failure (first event) within two-years of follow-up for the 43 SNPs. For the purposes of epidemiological comparison, we used false discovery rate (FDR) to adjust for multiple-testing.[26] Although no universal FDR significance threshold has been defined, a cut-point of 0.20 has been suggested for candidate gene association studies, meaning that one should expect at most 20% of declared discoveries to be false.[27] We therefore used a cut-point of 0.20, which resulted in a corrected level of significance of $p = 0.009$ instead of $p = 0.05$.

We also investigated the association between clinical factors and AVF failure and the interaction between these clinical factors and SNPs associated with AVF failure using the relative excess risk due to interaction (RERI) method. All analyses have been performed using SPSS statistical software version 20.0 (SPSS, Chicago, Ill, USA).

RESULTS

A total of 479 incident hemodialysis patients with an AVF from the NECOSAD cohort were genotyped. Of the 479 patients, 207 (43.2%) reached the endpoint of AVF failure during follow up. The absolute incidence of AVF failure was 340 per 1000 person-years. Baseline characteristics of the patients are shown in Table 1. The median age was 65.3 years, 36.3% were female, and 15.2% had diabetes mellitus as their primary kidney disease.

Genes implicated in endothelial function and vascular remodeling

The results of the analyses of this set of SNPs are summarized in Table 2. Carriers of the AA genotype of the low-density lipoprotein receptor-related protein 1 (LRP1) rs1466535 had a significant 1.75-fold (95% CI 1.15-2.66) higher risk of AVF failure with a p-value of 0.009 and a FDR of 0.009. The Annexin A5 SNPs, MMP1 rs11292517, nitric oxide synthesis 3 (NOS3) rs1799983, Elastin rs2071307 and Quaking rs3763197 were not associated with AVF failure. The two intergenic SNPs, identified in a genome-wide association study (GWAS) for coronary restenosis, were also not associated with AVF failure, although they both demonstrated a non-significant trend towards a higher risk in the variant allele carriers.

Growth factors and related genes

We genotyped 12 SNPs in 8 growth factor related genes (Table 3). None of the SNPs showed a significant association with AVF failure.

Genes implicated in inflammation

In total, 8 SNPs were genotyped in several genes implicated inflammatory pathways (Table 4). We did not find an association between AVF failure and the SNPs in interleukin 6 (IL6), interleukin 10 (IL10), lymphotoxin alpha, CD180 and toll-like receptor 4. The TNF rs1800629 AA genotype was associated with a 1.97-fold higher AVF failure risk as compared with the GG genotype with a 95% CI of 1.00 to 3.87, a p-value of 0.05 and a FDR of 0.01, which was just not significant considering the threshold of FDR < 0.009. Both IL10 rs1800896 and rs3024498 GG genotypes as compared with AA genotypes were associated with a non-significant lower risk of AVF failure (HR 0.73, 95% CI 0.49-1.10, p = 0.14 and HR 0.73, 95% CI 0.42-1.25, p = 0.25).

Table 2 Polymorphisms related to endothelial function and vascular remodeling and association with AVF failure

Gene	Name	SNP	Genotype	N	HR with 95% CI	P
MMP1	Matrix metallopeptidase 1	rs11292517	-/-	132	1 Reference	
			-/C	213	0.92 (0.67-1.28)	0.64
			CC	103	0.86 (0.58-1.28)	0.47
NOS3	Nitric oxide synthesis 3	rs1799983	GG	224	1 Reference	
			GA	188	1.26 (0.94-1.69)	0.12
			AA	44	0.88 (0.52-1.50)	0.64
ELN	Elastin	rs2071307	GG	185	1 Reference	
			GA	207	1.30 (0.96-1.75)	0.09
			AA	62	1.01 (0.64-1.60)	0.97
ANXA5	Annexin A5	rs4833229	CC	146	1 Reference	
			TC	218	0.85 (0.62-1.16)	0.30
			TT	91	0.85 (0.57-1.26)	0.42
ANXA5	Annexin A5	rs6830321	GG	132	1 Reference	
			GA	216	0.79 (0.57-1.09)	0.15
			AA	106	0.88 (0.61-1.29)	0.52
LRP1	low density lipoprotein receptor-related protein 1	rs1466535	GG	192	1 Reference	
			GA	207	1.31 (0.97-1.78)	0.08
			AA	56	1.75 (1.15-2.66)	0.009
QKI	Quaking	rs3857504	CC	308	1 Reference	
			TC	126	1.16 (0.85-1.58)	0.35
			TT	12	1.67 (0.78-3.57)	0.19
QKI	Quaking	rs3763197	TT	323	1 Reference	
			TC	122	1.10 (0.81-1.50)	0.54
			CC	9	1.10 (0.41-2.96)	0.86
QKI	Quaking	rs2759393	CC	268	1 Reference	
			CA	157	0.96 (0.72-1.30)	0.80
			AA	24	0.56 (0.26-1.20)	0.13
-	12q23.2	rs10861032		316	1 Reference	
				122	0.99 (0.72-1.37)	0.97
				17	1.48 (0.76-2.92)	0.25
-	12q23.2	rs9804922		385	1 Reference	
				68	0.81 (0.53-1.24)	0.33
				2	1.32 (0.18-9.40)	0.78

SNP, single nucleotide polymorphism; N, number of individuals; HR, hazard ratio; CI confidence interval.

Table 3 Polymorphisms in growth factor related genes and association with AVF failure

Gene	Name	SNP	Genotype	N	HR with 95% CI		P
CDKN1B	Cyclin-dependent kinase inhibitor 1B	rs36228499	CC	147	1	Reference	
(p27kip1)			CA	234	1.01	(0.74-1.39)	0.93
			AA	73	1.16	(0.76-1.76)	0.49
CTGF	Connective tissue growth factor	rs6918698	CC	128	1	Reference	
			GC	216	0.87	(0.63-1.20)	0.40
			GG	108	0.87	(0.59-1.28)	0.48
FGFR4	Fibroblast growth factor receptor 4	rs351855	GG	210	1	Reference	
			GA	170	1.10	(0.82-1.49)	0.53
			AA	52	0.69	(0.41-1.16)	0.16
KLF5	Kruppel-like factor 5	rs3812852	AA	393	1	Reference	
			GA	62	0.97	(0.65-1.44)	0.86
			GG	1	1.95	(0.27-13.95)	0.51
PDGFD	Platelet derived growth factor D	rs974819	CC	225	1	Reference	
			TC	183	0.93	(0.69-1.25)	0.62
			TT	46	1.18	(0.75-1.86)	0.48
PDGFD	Platelet derived growth factor D	rs496339	AA	366	1	Reference	
			GA	84	0.99	(0.69-1.42)	0.95
			GG	4	0.49	(0.07-3.51)	0.48
TGFBR1	Transforming growth factor, beta receptor1	rs1626340	AA	282	1	Reference	
			GA	136	1.06	(0.77-1.44)	0.74
			AA	19	1.01	(0.50-2.07)	0.97
TGFBR2	Transforming growth factor, beta receptor2	rs1036095	GG	266	1	Reference	
			GC	165	0.86	(0.64-1.17)	0.34
			CC	24	0.64	(0.32-1.32)	0.23
TGFBR2	Transforming growth factor, beta receptor2	rs4522809	AA	126	1	Reference	
			GA	237	1.34	(0.94-1.89)	0.10
			GG	90	1.42	(0.93-2.16)	0.10
VEGF	Vascular endothelial growth factor	rs2010963	GG	200	1	Reference	
			GC	196	1.17	(0.87-1.58)	0.30
			CC	59	1.01	(0.65-1.58)	0.96
VEGF	Vascular endothelial growth factor	rs3025039	CC	344	1	Reference	
			TC	102	0.95	(0.68-1.33)	0.77
			TT	10	1.20	(0.49-2.92)	0.69
VEGF	Vascular endothelial growth factor	rs699947	CC	124	1	Reference	
			CA	226	1.00	(0.72-1.40)	0.99
			AA	100	1.01	(0.68-1.50)	0.98

SNP, single nucleotide polymorphism; N, number of individuals; HR, hazard ratio; CI confidence interval.

Table 4 Polymorphisms related to inflammatory genes and association with AVF failure

Gene	Name	SNP	Genotype	N	HR with 95% CI		P
IL6	Interleukin 6	rs1800795	GG	175	1	Reference	
			GC	217	1.06	(0.78-1.43)	0.73
			CC	63	1.11	(0.72-1.71)	0.65
IL10	Interleukin 10	rs1800896	AA	130	1	Reference	
			GA	222	1.01	(0.73-1.39)	0.96
			GG	102	0.73	(0.49-1.10)	0.14
IL10	Interleukin 10	rs3024498	AA	238	1	Reference	
			GA	176	1.03	(0.76-1.38)	0.87
			GG	42	0.73	(0.42-1.25)	0.25
LTA	lymphotoxin alpha (TNF	rs1799964	TT	264	1	Reference	
	superfamily, member 1)		TC	168	0.99	(0.74-1.33)	0.95
			CC	24	0.71	(0.35-1.45)	0.35
RP105	CD180	rs5744478	TT	386	1	Reference	
			TC	67	0.97	(0.66-1.44)	0.88
			CC	3	2.36	(0.75-7.38)	0.14
TNF	Tumor necrosis factor alpha	rs1800629	GG	311	1	Reference	
			GA	129	1.05	(0.77-1.44)	0.75
			AA	16	1.97	(1.00-3.87)	0.05
TNF	Tumor necrosis factor alpha	rs361525	GG	420	1	Reference	
			GA	34	0.74	(0.41-1.33)	0.31
			AA	1	NE		
TLR4	Toll-like receptor 4	rs4986790	AA	393	1	Reference	
			GA	61	0.79	(0.51-1.23)	0.29
			GG	2	2.91	(0.72-11.74)	0.13

SNP, single nucleotide polymorphism; N, number of individuals; HR, hazard ratio; CI confidence interval; NE, not estimable

Genes implicated in calcium/phosphate metabolism

Eight SNPs were genotyped in the klotho, vitamin D receptor (VDR), and fetuin-A gene (Table 5). We did not find an association between AVF failure and the SNPs in the klotho gene (rs9527025, rs564481, rs397703 and rs577912), the VDR (rs11574027, rs2238135, and rs4516035) or the fetuin-A gene (rs4918).

Genes implicated in coagulation

Factor V rs6025, also known as factor V Leiden, was associated with a 2.54-fold (95% CI 1.41-4.56, p = 0.002, FDR 0.005) higher risk of AVF failure. Rs1044291 and rs1800787 in the FGB gene were not associated with AVF failure (Table 6).

Table 5 Polymorphisms related to calcium/phosphate metabolism and association with AVF failure

Gene	Name	SNP	Genotype	N	HR with 95% CI		P
Klotho		rs9527025	GG	358	1	Reference	
			GC	89	0.94	(0.66-1.34)	0.72
			CC	6	0.69	(0.17-2.80)	0.61
Klotho		rs564481	CC	161	1	Reference	
			TC	204	1.25	(0.90-1.72)	0.18
			TT	82	1.28	(0.86-1.92)	0.23
Klotho		rs397703 *	TT	296	1	Reference	
			TC	133	1.16	(0.85-1.57)	0.35
			CC	26	0.70	(0.34-1.43)	0.33
Klotho		rs577912	GG	328	1	Reference	
			TG	114	0.88	(0.63-1.22)	0.43
			TT	11	0.72	(0.27-1.95)	0.52
VDR	Vitamin D Receptor	rs11574027	GG	443	1	Reference	
			TG	12	0.80	(0.33-1.93)	0.61
			TT	0	NE		
VDR	Vitamin D Receptor	rs2238135	GG	258	1	Reference	
			GC	163	1.15	(0.86-1.54)	0.36
			CC	30	0.60	(0.30-1.19)	0.14
VDR	Vitamin D Receptor	rs4516035	AA	140	1	Reference	
			GA	209	1.04	(0.75-1.43)	0.82
			GG	98	0.90	(0.60-1.35)	0.61
AHSG	alpha-2-HS-glycoprotein (Fetuin-A)	rs4918	CC	212	1	Reference	
			GC	193	1.06	(0.79-1.43)	0.70
			GG	44	1.31	(0.82-2.09)	0.26

* rs397703 is a proxy for rs1207568 ($R^2 = 0.70$). SNP, single nucleotide polymorphism; N, number of individuals; HR, hazard ratio; CI confidence interval; NE, not estimable

Our results did not change when we calculated hazard ratios in another model by combining heterozygote genotypes and mutant genotypes or heterozygote genotypes and wild-type genotypes. The 41 SNPs that were not associated with AVF failure remained unassociated in other models.

Interactions and combined effects

Female sex was associated with a 1.48-fold (95% CI 1.12-1.95) higher risk of AVF failure, $p = 0.005$. Furthermore, diabetes mellitus as primary kidney disease, as compared with other causes, was associated with a 2.01-fold (95% CI 1.42-2.85) higher risk of AVF failure, $p < 0.001$. The combination of factor V Leiden with diabetes mellitus or female sex and the combination of LRP1 rs1466535 AA genotype and female sex did not result

Table 6 Polymorphisms related to coagulation and association with AVF failure

Gene	Name	SNP	Genotype	N	HR with 95% CI	P
FBG	Fibrinogen-beta	rs1044291	CC	204	1 Reference	
			TC	193	1.20 (0.89-1.62)	0.23
			TT	56	1.12 (0.71-1.77)	0.63
FBG	Fibrinogen-beta	rs1800787 *	CC	292	1 Reference	
			TC	137	0.90 (0.65-1.23)	0.50
			TT	21	0.68 (0.32-1.46)	0.33
F5	Factor 5	rs6025	GG	435	1 Reference	
			GA	17	2.54 (1.41-4.56)	0.002
			AA	1	NE	
ITGB3	integrin, beta 3	rs17218711 †	GG	326	1 Reference	
	(platelet glycoprotein IIIa)		GC	115	1.05 (0.76-1.45)	0.78
			CC	15	1.12 (0.53-2.40)	0.76

* rs1800787 was a proxy for rs1800790 ($R^2 = 1.0$). † rs1718711 was a proxy for rs5918 ($R^2 = 0.93$). SNP, single nucleotide polymorphism; N, number of individuals; HR, hazard ratio; CI, confidence interval; NE, not estimable

in an higher risk on an additive scale using the RERI method (Supplementary Table S3). Patients with LRP1 rs1466535 AA genotype with diabetes mellitus had a 2.97-fold higher risk (95% CI 1.10-8.05, $p = 0.03$) of AVF failure as compared with patients without diabetes mellitus and without LRP1 rs1466535 AA genotype (reference), while patients without LRP1 rs1466535 AA genotype with diabetes mellitus had a 2.11-fold (95% CI 1.48-3.01, $p < 0.001$) higher risk of AVF failure and patients with LRP1 rs1466535 AA genotype without diabetes mellitus had a 1.63-fold (95% CI 1.07-2.47, $p = 0.02$) higher risk of AVF failure (Supplementary Table S3).

When analyzing the two significantly associated SNPs (LRP1 rs1466535 and factor V Leiden) together, 269 patients were carrier of at least 1 risk allele of these two SNPs. Of these carriers, 48.3% developed AVF failure. In comparison, 36.7% of the patients with the wild type genotype of these SNPs ($n = 210$) developed AVF failure ($p = 0.01$).

DISCUSSION

We investigated the association between AVF failure and 43 SNPs in 26 genes involved in processes related to endothelial function and vascular remodeling, growth factors, inflammation, coagulation, and calcium/phosphate metabolism. To our knowledge, this study is the largest study that investigated genetic risk factors of AVF failure. We showed that, after adjustment for multiple comparisons by using FDR, LRP1 rs1466535 and factor V rs6025 (factor V Leiden) were significantly associated with a higher risk of

AVF failure. TNF rs1800629 was not significantly associated with AVF after adjustment. No significant associations of AVF failure were observed with the other SNPs.

The rs1466535 SNP in the LRP1 gene was the only SNP in the 'endothelial function and vascular remodeling' group that was significantly associated with AVF failure. In 2011, this SNP was identified in a GWAS on abdominal aortic aneurysms and that this SNP has a possible functional role in LRP1 expression.[20] The mechanism, by which LRP1 could influence vascular remodeling leading to aneurysm formation, was suggested to involve regulation of MMP9 expression.[20,28] Moreover, LRP1 was shown to be essential for the maintenance of vascular wall integrity mediated through PDGF receptor beta and Smad signaling.[29] Interestingly, in our study patients carrying the variant A allele had a higher risk of developing AVF failure, whereas in the above mentioned study,[20] the wild type allele was the risk allele. Whether this opposite effect indicates that AVF failure is mediated through a lack of vascular expansion, which is necessary for AVF maturation, of that another function LRP1 is involved in AVF failure is unclear. Nevertheless, the observed association of the LRP1 SNP with AVF failure suggests a potential role for LRP1 in vascular remodeling and AVF maturation.

We also showed that factor V Leiden was associated with a 2.54-fold (95% CI 1.41-2.56, $p = 0.002$) higher risk of AVF failure. Factor V Leiden is one of the most common inherited pro-coagulatory defects and is a well-known risk factor for venous thrombosis.[30] A previous study also showed that factor V Leiden was associated with vascular access failure.[31] In agreement with these observations, another recent study showed that another SNP in the factor V gene was associated with AV access failure in hemodialysis patients.[24] Furthermore, thrombophilia (including factor V Leiden) was associated with an increased risk of vascular access dysfunction in a yet another study.[32] Together, these studies provide growing evidence of a role of factor V Leiden mutation in AV access failure.

Although the -308G>A (rs1800629) SNP in the TNF gene did not remain significantly associated with AVF failure after multiple testing correction, we think it is worthwhile discussing. Since the vascular inflammatory response is likely an important contributor to AVF failure, genetic variation in inflammatory related genes could be associated with AVF failure. In our study, individuals with the AA genotype of rs1800629 had a non-significant higher risk of AVF failure. This SNP has been the focus of several previous studies, focusing on a wide variety of endpoints, including cardiovascular events in rheumatoid arthritis patients,[33] irritable bowel syndrome,[34] life-expectancy,[35] tuberculosis susceptibly,[36] mortality after acute renal failure[37] and comorbidity and functional status scores in hemodialysis patients.[38] The other analyzed SNP in the TNF gene (rs361525) was also not associated with AVF failure in our study, although it was previously associated with coronary restenosis.[39] The two SNPs in the anti-inflammatory cytokine IL10 demonstrated a non-significant lower risk of AVF failure development, possibly due to lack of power.

Remarkably, these two SNPs were shown to increase the risk of coronary restenosis[18] and cardiovascular events during hemodialysis.[40] Whether this discrepancy is caused by the differences in the mechanisms of AVF failure and coronary restenosis remains to be elucidated.

For this study, besides previously reported candidate genes for AV access failure, we also selected SNPs in genes related to coronary restenosis and aortic aneurysm formation. Processes involved in AVF patency, in particular VSMC proliferation and inflammation, play also a key role in the development of coronary restenosis after PCI.[13] In contrast with the scarcely available data on genetic determinants of AV access failure, the genetic background of coronary restenosis has been more established.[12,13] Although our SNP selection covered a wide range of candidate genes in all involved mechanisms, the obtained results do not indicate an important role for our selected SNPs in the development of AVF failure. One explanation for these observations could be that other SNPs are involved in AVF failure or that genetic susceptibility does not play an important role in the development of AVF failure. Based on other studies that also did not find an association between most candidate SNPs and AV access failure,[23-25,41] it might be that local factors, such as hemodynamics and vascular damage, have a more important role in the failure of the vascular access required for hemodialysis than the genetic background.

Diabetes mellitus is a well-known risk factor for both renal disease and cardiovascular complications as well as for AVF failure in dialysis patients.[42-44] The prevalence of diabetes among dialysis patients differs considerably worldwide. According to the European Renal Association-European Dialysis and Transplant Association (ERA-EDTA) Registry, the prevalence of diabetes mellitus among dialysis patients in Europe is 22%,[45] in line with the relative low prevalence in our study population. Although the interaction analysis does show some influence of diabetes on the genetic associations, elaborate subgroup analyses are not appropriate considering the low proportion of diabetic patientsin our population.

Our study has several potential limitations. We had no information about AVF failure before the first successful dialysis session. Whether inclusion of non-maturating fistula would have affected our results is questionable. It is conceivable that the maturating process of an AVF is mechanistically different compared to AVF failure of a well-maturated fistula. Excluding the non-maturating AVF likely resulted in a more homogeneous study endpoint. However, this hypothesis remains speculative since we do not have data to explore this. Also we had no information about the protocols that were used in the various centers for access surveillance to detect stenosis prior to failure. Furthermore, we had no information about the cause of fistula failure (i.e. thrombosis, stenosis or other). A previous suggested that hospital specific aspects contribute to AVF failure.[46] Unfortunately, we do not have information about differences of fistula creation in the involved centers, about the experience of surgeons in fistula creation or about the surgical tech-

niques used to create a fistula. Another limitation is that, despite of our large number of patients, we had limited power to find an association between AVF failure and several SNPs. In addition, we investigated the association between multiple SNPs and fistula failure, thereby increasing the chance of false positive findings. However, since we selected only candidate genes based on previous studies, and because we adjusted for multiple comparisons by using false discovery rates (with the threshold set at 20%, previously suggested to be an appropriate threshold for candidate gene association studies[27]), we have tried to minimize the chance of false positive findings. The general strength of this study was the large and well-defined Dutch cohort of incident hemodialysis patients with available DNA for investigation of genetic risk factors. Another potential strength of our study was that we only included incident hemodialysis patient, thereby limiting survivor bias.

Current guidelines for prevention of vascular access failure recommend uniform surveillance of all patients.[47] Identification of genetic risk factors might lead to a more directed approach for surveillance techniques. Risk factors for primary patency loss could be used to focus on specific patient groups for more intensive surveillance. Furthermore, increasing your understanding to the molecular mechanisms of AVF failure, could aid the development of better preventive measures or new treatment modalities.

In conclusion, we found that LRP1 rs1466535 and factor V Leiden were associated with an increased risk of AVF failure. Other SNPs in vascular function and remodeling related genes, growth factors genes, inflammation genes, coagulation genes, and calcium metabolism genes were not significantly associated with AVF failure. Further studies on the genetics of AVF failure are needed to unravel the underlying mechanisms of this deleterious condition and thereby help us improve prevention and treatment of AVF failure in this diseased population.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *PLoS One* online.

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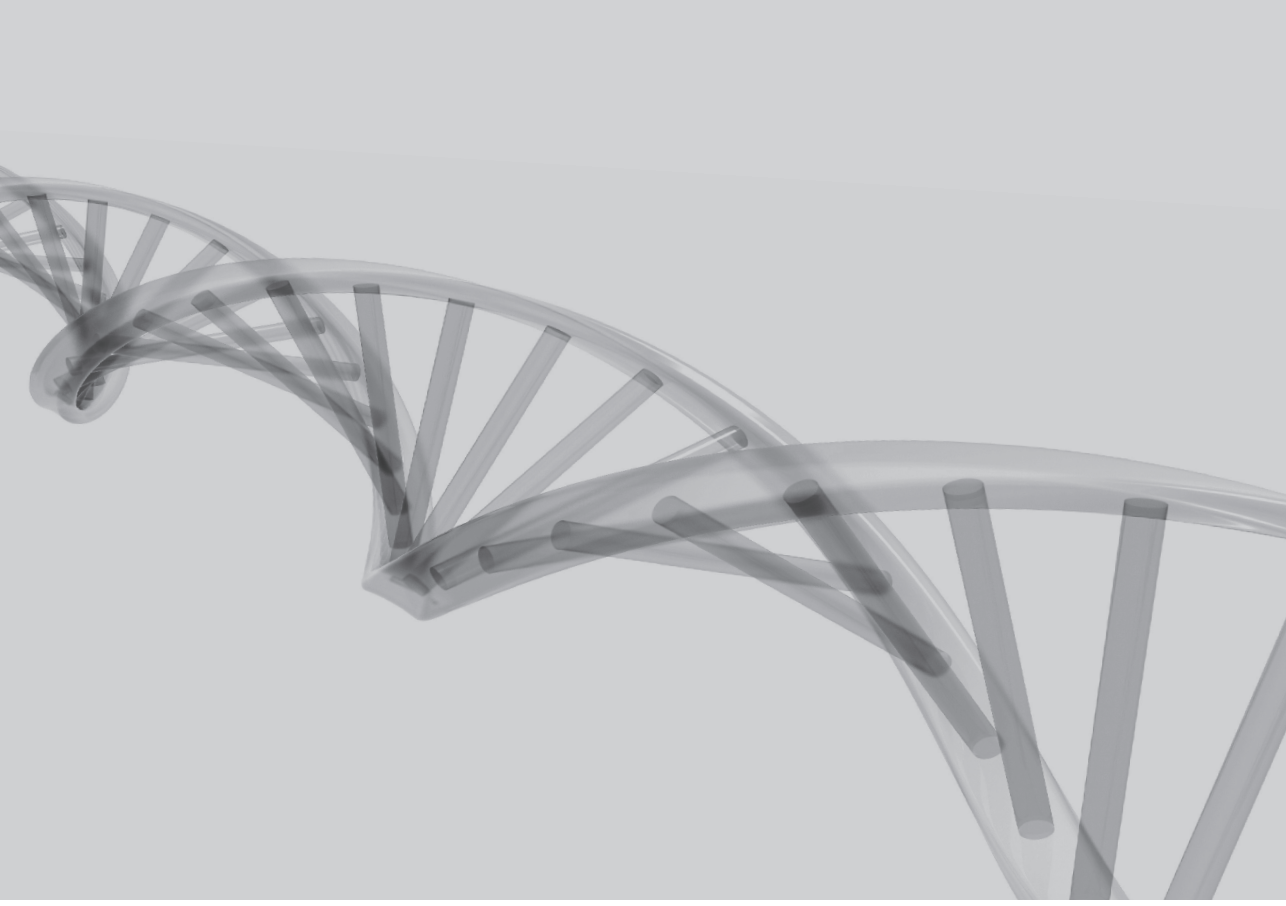
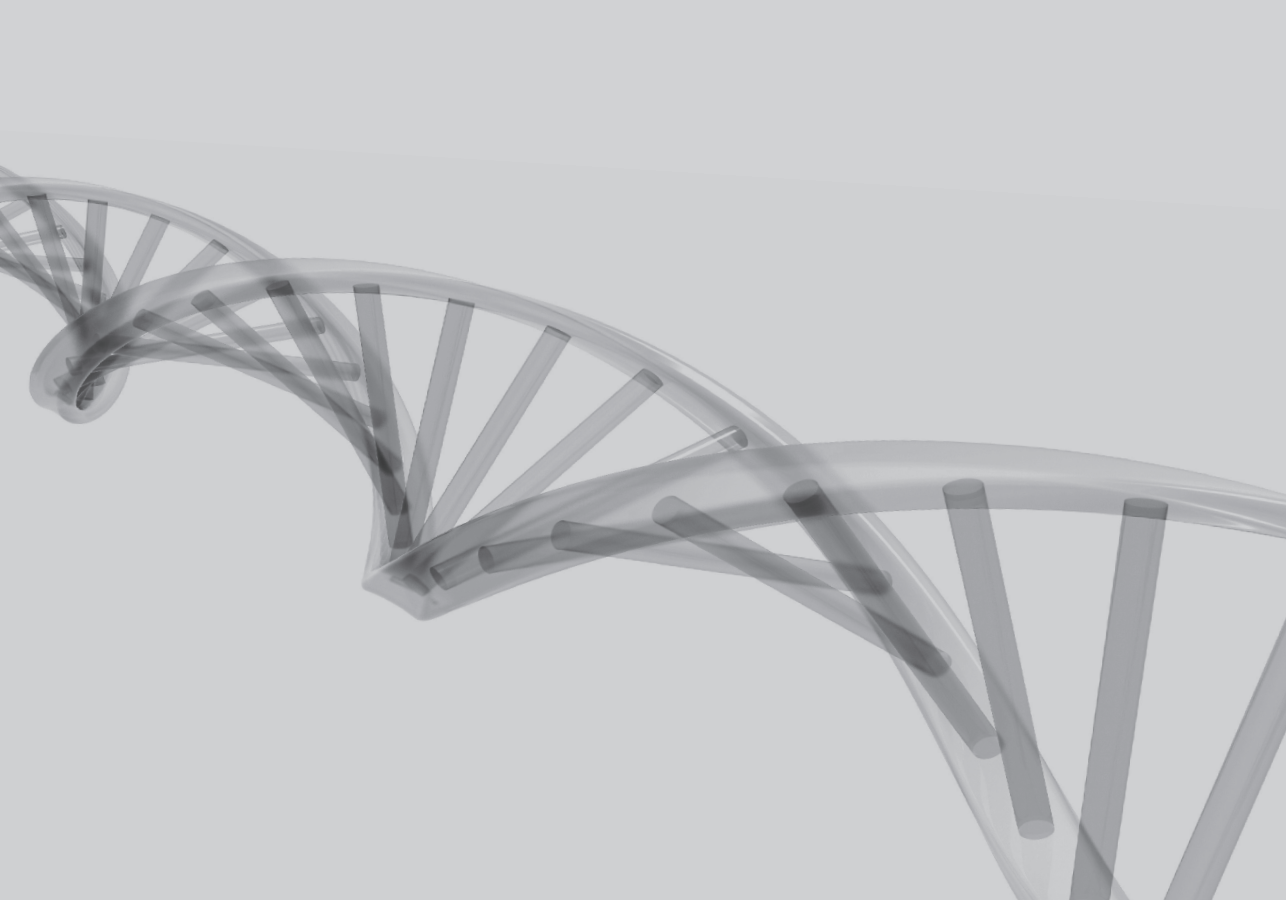
Part III



Pharmacogenetics in cardiovascular diseases

Chapter 11



A systematic review on pharmacogenetics in cardiovascular disease

Is it ready for clinical application?

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ABSTRACT

Pharmacogenetics is the search for heritable genetic polymorphisms that influence responses to drug therapy. The most important application of pharmacogenetics is to guide choosing agents with the greatest potential of efficacy and smallest risk of adverse drug reactions. Many studies focussing on drug-gene interactions have been published in recent years, some of which led to adaptation of FDA recommendations, indicating that we are on the verge of the clinical application of genetic information in drug therapy. This systematic review provides a comprehensive overview of the current knowledge on pharmacogenetics of all major drug classes currently used in the treatment of cardiovascular diseases.

INTRODUCTION

Pharmacogenetic studies search for heritable genetic polymorphisms that influence responses to drug therapy. Pharmacogenetics has many possible applications in cardiovascular pharmacotherapy including screening for polymorphisms to choose agents with the greatest potential for efficacy and least risk of toxicity. Pharmacogenetics also informs dose adaptations for specific drugs in patients with aberrant metabolism.

Each year an estimated 785 000 Americans suffer a myocardial infarction (MI) and 610 000 people experience a new stroke. Despite advances in management, 470 000 recurrent MIs and 185 000 recurrent strokes occur annually.[1] The numbers from Europe are not much better, indicating the importance of optimizing treatment strategies.[2]

Although several reviews on pharmacogenetics in cardiology have been published, most focused on specific drug subgroups. This systematic review offers a comprehensive summary of the current knowledge of pharmacogenetics in cardiology, elaborates on progress towards individualized drug therapy, and incorporates conclusions made during the Colloquium "Pharmacogenetics of cardiovascular drugs: implications for a safer and more efficient drug therapy" held by the Royal Netherlands Academy of Arts and Sciences in October 2010. In particular, the review considers the most relevant and well studied polygenic markers for some pressing clinical issues: (i) statins: focusing on variability in efficacy and risk of myopathy; (ii) resistance to antiplatelet medication; (iii) dosing issues with oral anticoagulants; (iv) suboptimal responses to β-blockers; and (v) the variable response in blood pressure and clinical events in patients taking angiotensin converting enzyme (ACE) inhibitors (Table 1).

Table 1 Major issues in cardiovascular drug pharmacogenetics and the strength of the genetic evidence

Drug class	Problem	Evidence
Statins	Variable lipid lowering	Mediocre
	Risk of myopathy	Strong
Antiplatelet drugs	Resistance	Strong
Anticoagulants	Dosing problems	Strong
β-blockers	Variable response	Poor
ACE-inhibitors	Variable blood pressure reduction	Mediocre
	Variable benefit to decrease risk of events	Mediocre

METHODS

Relevant articles were identified by searching MEDLINE using the following keywords and Medical Subject Headings (MeSH) terms: pharmacogenetics, genetic heterogeneity, genetic polymorphism, single nucleotide polymorphism (SNP), haplotypes, treatment outcome, adverse effects, drug therapy, cardiovascular diseases (CVDs), and/or coronary disease. We screened the title and abstract of possibly relevant citations and retrieved, if potentially interesting, full reports or abstracts. We checked the references of retrieved publications for additional relevant papers and included all available literature until May 2011. We excluded reviews, editorials and articles in languages other than English.

STATINS: VARIABILITY IN EFFICACY AND RISK OF MYOPATHY

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely prescribed to lower plasma cholesterol levels, thereby reducing cardiovascular risk. Despite the clinical effectiveness of statins, proven in large randomized controlled trials,[3,4] inter-individual variation in response means that many patients fail to achieve adequate reduction of cholesterol levels even after multiple dose adjustments.[5] Genetic factors are thought to be partly responsible for this inter-individual variation Indeed, more than 40 candidate genes have been described with respect to the differential effect of statins in decreasing the risk of clinical endpoints including cardiovascular death, MI and lipid lowering abilities (see Supplementary material online, *Table S1*).

Variability in lipid lowering

Several pharmacogenetic studies examined the gene encoding the cholesterol ester transfer protein (CETP) involved in cholesterol metabolism and, in particular, the TaqIB variant (rs708272). Initial studies associated the B2B2 genotype with lower CETP levels,[6] higher high density lipoprotein cholesterol (HDL-C) levels and lower risk of progression of coronary artery disease (CAD), compared with the B1B1 genotype.[7-11] However, on statin treatment B1B1 patients showed lower progression of CAD than B2B2 carriers. A meta-analysis confirmed the firm association between TaqIB and HDL-C levels and CAD risk, while no significant interaction between this polymorphism and pravastatin treatment could be demonstrated.[12] This meta-analysis included studies that enrolled patients with and without a history of CAD. In contrast, the original study included only patients with significant CAD.[7] Furthermore, long-term results from the Regression Growth Evaluation Statin Study (REGRESS), the first study to report the possible pharmacogenetic interactions between the *CETP* polymorphism and statin treatment,[13] also contrast with the meta-analysis. REGRESS demonstrated significantly higher 10-

year mortality in statin-treated male patients carrying the B2 allele, compared with the B1B1 genotype.[14] Therefore, although untreated B2B2 patients have a lower risk of CAD progression, statin treatment is more beneficial in patients with the B1B1 genotype, negating the initial advantage of the B2 allele in CAD.

The extensive studies of apolipoprotein E (APOE), and in particular the ε2/ε3/ε4 variants defined by combinations of two polymorphisms (Cys112Arg, rs429358 and Arg158Cys, rs7412) are equivocal.[15] Several studies report an association between ε2 carriers and an increased lipid lowering response to statins or reduced cardiovascular outcomes, such as nonfatal MI and cardiovascular-related mortality, whereas other studies did not find significant associations.[16] Although the first genome-wide association study (GWAS) on statin effects showed a significant association between the three *APOE* polymorphisms and the low-density lipoprotein cholesterol (LDL-C) response,[17] a recent meta-analysis did not confirm this association.[18] Therefore, it seems unlikely that a true association exists between APOE polymorphisms and the lipid lowering response to statin treatment.

Since most studies discussed so far had insufficient power to detect the small effect produced by individual genetic loci, Barber *et al.*[19] combined three statin GWASs encompassing 3936 patients. Carriers of a polymorphism (rs8014194) in the calmin (*CLMN*) gene on chromosome 14 had a significantly greater reduction of 3% in total cholesterol compared to non-carriers. The exact function of calmin is unknown, but it contains a calponin-domain expected to have actin-binding activity[20] and is strongly expressed by liver and adipose tissue.[21] Nevertheless, this polymorphism explained only 1% of the variability in statin response.[19] A SNP near *APOE*, in the *APOC1* gene (rs4420638), showed moderate evidence for being associated with statin-induced LDL-C changes. Most other putative associations appeared to be independent of statin treatment and were in loci previously associated with lipid or lipoprotein traits.[19]

Kinesin-like protein 6 and variability in clinical outcome

Next, carriers of the arginine-coding allele (rs20455) of kinesin-like protein 6 (*KIF6*) showed a substantially higher benefit with pravastatin treatment compared with non-carriers in three large clinical trials, which appeared to be independent from lipid and C-reactive protein-lowering effects.[22-24] Kinesin-like protein 6 is a member of the molecular motor super family involved in the intracellular transport of several important molecules, including mRNA.[25] A recent meta-analysis of 19 studies (in total 17 000 CAD cases and 39 369 controls) concluded that the SNP in question was not associated with the risk of CAD.[26] This study was, however, unable to explore the effect of statins, since this information was not available of all included studies, but the probability of a strong influence on the effect of statin decreases with this publication. Nevertheless, further (functional) studies are needed to explore the role of KIF6.

Risk of myopathy

Genetic variations are also associated with an altered risk of adverse drug reactions (ADRs), especially statin-induced myopathy. The incidences of myopathy and rhabdomyolysis are estimated at 11.0 and 3.4 per 100 000 person-years respectively. The case mortality rate with rhabdomyolysis is 10%.[27]

The polypeptide organic anion transporter P1B1 (OATP1B1) encoded by the *SLCO1B1* gene is involved in the hepatic uptake of statins.[28,29] Two common polymorphisms (521T>C, rs4149056 and 388A>G, rs2306283) influence the transporter function of OATP1B1,[30] markedly altering statin pharmacokinetics.[31] A GWAS showed a strong association between a non-coding polymorphism (rs4363657) and statin-induced myopathy in patients treated with high-dose simvastatin.[32] This polymorphism is in almost complete linkage disequilibrium ($R^2 = 0.97$) with the 521T>C polymorphism and was expressed by more than 60% of patients who developed myopathy with an odds ratio of 4.7 per copy of the C allele.[32] A subsequent study confirmed this pharmacogenetic effect in simvastatin-induced myopathy,[33] but not with either atorvastatin or pravastatin,[33] probably indicating a simvastatin specific effect.[34]

In conclusion, the reports on genetic variability and response to statin treatment are difficult to interpret. Variability in study designs and endpoint definitions probably contribute to these mixed results. Since statins' full pharmacodynamic spectrum, besides their lipid-lowering properties, remains largely unknown, it is not surprising that clear cut pharmacogenetic results are lacking. It is likely that future research will further elucidate statins' exact mechanisms of action and help explain the variability in response. Newly identified loci influencing lipid concentration identified in recent GWAS, might also prove to have pharmacogenetic associations with statin treatment.[35,36] Pharmacogenetic data on ADRs of statin is limited and more research dedicated to this problem is needed before concrete advises can be given on this notion.

RESISTANCE TO ANTIPLATELET MEDICATION

Blood platelets maintain appropriate hemostasis upon vascular injury, but also contribute to the pathophysiology of thrombosis. Thrombosis of atherosclerotic plaques is the most important underlying mechanism of CAD and embolic stroke, and consequently antiplatelet therapy is the cornerstone of secondary prevention in modern CVD treatment.[37-40] Inter-patient variations in platelet reactivity and responses to antithrombotic treatment result in marked differences in the benefits derived from antiplatelet treatment. Indeed, up to 25% of patients continue to experience new thrombotic events despite guideline therapy.[41,42] The term 'resistance' is widely used in such cases, although a clear-cut definition is lacking.[43] The current clinical definition regards resistance as

recurrent cardiovascular events despite supposed adequate therapy. Biochemical definitions which are based on residual platelet activity during treatment measured ex-vivo by several laboratory tests, are however also clinically relevant. Trenk *et al.*[44] for example, found a three-fold increase (95% confidence interval (CI) 1.4-6.8, $P = 0.004$) in the 1-year incidence of death and MI among patients with high residual platelet activity at discharge after percutaneous coronary intervention (PCI) despite clopidogrel. However, although it is likely that biochemically non-responsive patients are also clinically non-responsive, biochemical 'resistance' is neither constant in an individual nor over time.[42] Furthermore, there is an evident lack of concordance between laboratory tests used to express resistance.[45-47]

Against this background, recent research suggests that genetic variation makes an important contribution to variation in responses to the three main groups of antiplatelet therapy: aspirin (see Supplementary material online, *Table S2*); thienopyridine derivates [clopidogrel (see Supplementary material online, *Table S3*), prasugrel (see Supplementary material online, *Table S4*), and the P_2Y_{12} receptor antagonist ticagrelor]; and GP IIb/IIIa receptor inhibitors (orbofiban and abciximab) (see Supplementary material online, *Table S5*).

Aspirin

The antiplatelet effects of aspirin (acetylsalicylic acid) arise from irreversible acetylation of cyclooxygenase (COX)-1, which catalyzes the formation of thromboxane A2, prostaglandin and related metabolites from arachidonic acid.[48,49] Blocking COX-1 decreases the formation of thromboxane A2, thereby reducing platelet aggregation.[50] Aspirin resistance is poorly defined: a patient can be a non-responder in one test and a normal responder in another. Because of this lack of uniformity, the reported prevalence of aspirin resistance differs widely between studies. A recent systematic review reported a mean prevalence of aspirin resistance of 24% with a range between 0% and 57%.[51]

Conflicting results on aspirin efficacy

Of the many pharmacogenetic studies on aspirin, Hanlushka *et al.*[49] were the first to describe an effect of two polymorphisms in the *COX-1* gene, -842A>G (rs10306114) and 50C>T (rs3842787), that are in complete linkage disequilibrium. Healthy individuals heterozygous for these polymorphisms showed greater inhibition of platelet aggregation compared to homozygotes of the wild-type allele. Two later studies confirmed this relationship in CAD patients.[52,53] However, many other studies failed to find significant associations between variation of the *COX-1* gene and residual platelet activity[54,55] or clinical events, including non-fatal MI or cardiac death.[56]

Platelet glycoprotein (GP) IIb/IIIa receptors bind fibrinogen, thereby cross-linking platelets and von Willebrand factor (vWF).[57] Studies that focused on the 1565T>C

polymorphism (rs5918; PlA1/A2) of the *GP IIIa* (integrin β3) gene produced highly variable conclusions (see Supplementary material online, *Table S2*). A meta-analysis that included most of these studies concluded that the PlA1/A2 variant was significantly associated with aspirin resistance, but only in healthy individuals.[45] Co-medications used by CVD patients potentially obscured aspirin resistance, while differences in laboratory techniques may have also influenced outcome.

The meta-analysis also revealed that variations of four other genes [*GP Ia* (807C>T, rs1126643), *COX-1* (-842A>G/50C>T), P_2Y_{12} (H1/H2, constituted by rs10935838, rs2046934, rs5853517 and rs6809699) and P_2Y_1 (1622A>G, rs701265)] were not associated with aspirin resistance.[45] However, a later relatively large study, including 200 high-risk atherosclerosis patients, found a strong association between aspirin resistance and the T allele of the 807C>T polymorphism of the *GP Ia* gene.[58] A further study ruled out any influence of the -842A>G/50C>T polymorphism of *COX-1* and PlA1/A2 of *GP IIIa* on platelet response in a healthy Chinese population, in which these sites were non-polymorphic. However, the study showed attenuated antiplatelet effect during aspirin treatment in the presence of the P_2Y_1 893CC genotype (rs1065776).[55] This last association contrasts with results from 469 Caucasian patients with a history of MI. Here, carriers of the 893T allele had an almost three-fold increased risk of aspirin resistance compared to the CC genotype.[59]

Complications of aspirin therapy

A small number of studies focused on genes involved in aspirin-related ADRs. Park *et al.*[60,61] reported that the promoter polymorphisms -863C>A (rs1800630) and -1031T>C (rs1799964) of *TNF-α* and -509C>T (rs1800469) of *TGF-β1* may contribute to aspirin-induced urticaria. Piazuelo *et al.*[62] related the carriage of the A allele of the 27 bp variable number tandem repeats of *eNOS* with a decreased risk of upper GI bleeding. Further pharmacogenetic reports on ADRs of aspirin are necessary given the widespread prescription of aspirin.

In conclusion, the precise role of genetic variation in the variability of response to aspirin treatment has yet to be defined. The only consistent association is that of the PlA2 variant of the *GP IIIa* gene and aspirin resistance in healthy individuals. Standardization of techniques to measure and define response is a prerequisite for future studies, and to establish genetic variation's contribution to aspirin resistance.

Thienopyridine derivates/P_2Y_{12} receptor inhibitors

Clopidogrel resistance is common: the incidence was 21% (95% CI 17-25%) in a meta-analysis of patients after PCI.[42] Estimates of the incidence vary widely between studies, mainly due to differences in the time of measurement and dose of clopidogrel. However,

the technique used to assess clopidogrel responses did not result in a different prevalence of clopidogrel resistance between studies.[42]

Clopidogrel is an orally administered prodrug. Intestinal absorption is limited by P-GP, an efflux pump encoded by the *ABCB1* gene. Most of the bioavailable fraction undergoes hydrolysis to an inactive metabolite. Approximately 15% is oxidized by the hepatic cytochrome (CYP) P450 system in two sequential steps to generate an active metabolite,[63] which binds to, and irreversibly inhibits, the platelet ADP receptor P_2Y_{12}. By blocking this receptor, clopidogrel prevents platelet degranulation and inhibits conversion of the GP IIb/IIIa receptor to the form that binds fibrogen and links platelets, thereby inhibiting platelet aggregation.[64-67]

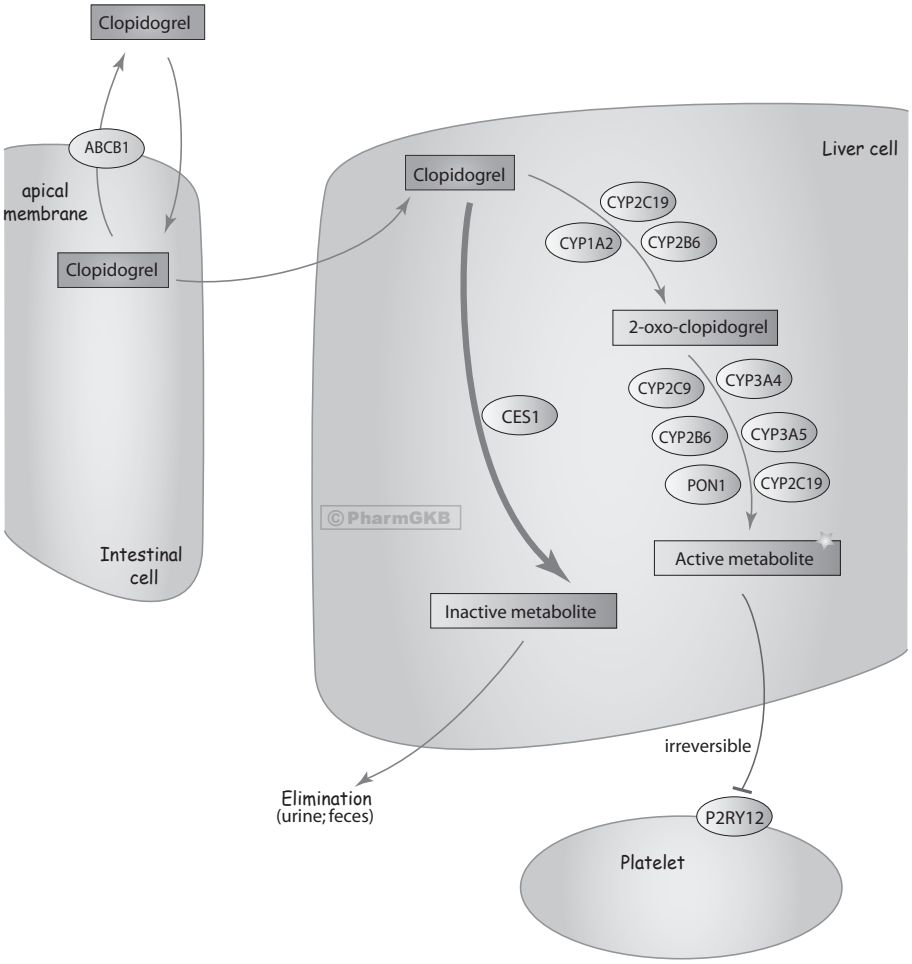
Metabolism and resistance

Several studies examined the influence of genetic variation in one or more enzymes of the CYP450 system on clopidogrel resistance. The most consistent finding is that the *2 allele of the *CYP2C19* gene (rs4244285) (involved in both hepatic oxidative steps) leads to the formation of an inactive enzyme and, therefore, decreased platelet responsiveness to clopidogrel[68] (*Figure 1*). After the first report,[68] which enrolled healthy male volunteers, studies replicated the reduced platelet responsiveness *in vitro* and established an increased risk of cardiovascular events and death during clopidogrel treatment in different patient populations (see Supplementary material online, *Table S3*). Other loss-of-functions alleles (*3, rs4986893; *4, rs28399504 and *5, rs56337013) have been associated with clopidogrel resistance although not as strongly and as consistently as the *2 allele, probably reflecting their lower allele frequencies.[69-72] Furthermore, recent studies associated a gain-of-function allele (*17, rs1248560) and a higher platelet inhibition by clopidogrel and, as a consequence, increased bleeding risk.[73,74]

The CYP2C9 loss-of-function allele *3 (rs1057910) has been associated with increased residual platelet activity and poor responder status after a 300 mg clopidogrel loading dosage in healthy subjects[69] and CVD patients.[76] Surprisingly, the latter study could not detect a similar effect of the risk allele during clopidogrel maintenance therapy. The authors hypothesized that since this enzyme is involved in the second metabolization step, the reduced enzyme capacity of the *3 allele becomes critical only with higher clopidogrel plasma concentrations.[63]

Studies on genetic variation in other involved CYP450 enzymes, especially CYP3A4 and CYP3A5, did not show a significant influence on platelet responsiveness after clopidogrel treatment (see Supplementary material online, *Table S3*). From a clinical point of view, an important finding came from Gladding *et al.*,[77] who demonstrated that increasing the clopidogrel dose in patients with non-responder CYP2C19 polymorphisms can increase the antiplatelet response, indicating that personalizing doses using pharmacogenetics could potentially optimize treatment.

Figure 1 Clopidogrel metabolism.



From Sangkuhl et al.[75] Used with permission of PharmGKB and Stanford University

Drug transport and receptors

Although *CYP2C19* polymorphisms probably account for most of the variability in clopidogrel response, variations in the function of intestinal efflux transporters might also influence clopidogrel bioavailability. For instance, the 3435C>T SNP of *ABCB1* (rs1045642) was shown to influence P-glycoprotein transporter expression, resulting in two- to four-fold lower peak concentrations of clopidogrel and its active metabolite in TT carriers after oral administration.[78] Two recent studies confirmed these findings[70,79]. Mega *et al.*[79] also reported that the increased risk of events of 3435TT homozygotes increased further in combination with a *CYP2C19* loss-of-function allele. However, not all published papers show consistent results[76] and a recent GWAS could not reproduce the

association between this polymorphism and clopidogrel pharmacodynamics in healthy volunteers.[80]

Another candidate gene encodes the P_2Y_{12} receptor, which is essential for platelet inhibition exerted by clopidogrel, prasugrel and ticagrelor.[81-83] Ziegler *et al.*[84] reported a four-fold increased risk for cerebrovascular events in patients that carried the 34C>T (rs6809699) variant of the P_2Y_{12} receptor receiving clopidogrel for peripheral artery disease. Moreover, Staritz *et al.*[85] associated another polymorphism (52G>T, rs6785930) to clopidogrel resistance in patients receiving dual antiplatelet therapy (clopidogrel and aspirin) after PCI. However, other studies could not confirm these findings with respect to platelet response or cardiovascular events.[70,86,87]

Finally, T allele carriers of the *GP Ia* gene 807C>T (rs1126643) polymorphism show increased platelet aggregation during clopidogrel treatment than non-carriers, increasing their thrombotic risk.[88] The same group confirmed these results in patients receiving dual antiplatelet therapy.[89] However, another study could not confirm these results in 600 acute coronary syndrome (ACS) patients on dual antiplatelet therapy.[90] The PlA polymorphism of the platelet *GP IIIa* gene (rs5918) probably does not influence the effect of thienopyridine derivates, although small studies report positive as well as negative effects.[91,92] Simon *et al.*[70] did not find a significant association of the PlA polymorphism in a population consisting of 2208 patients with acute MI treated with clopidogrel and the rate of cardiovascular events (225 deaths and 94 non-fatal MIs or strokes).

In conclusion, resistance to clopidogrel is a major problem, and prediction of the problem could be of great value. The *CYP2C19* loss-of-function allele *2 shows the most promise in predicting clopidogrel resistance and subsequent treatment failure, although this was not demonstrated by all studies.[93] Many other polymorphisms in theoretically promising target genes do not produce consistent results. Furthermore, proton pump inhibitors, especially omeprazol, show high affinity for CYP2C19 and might inhibit clopidogrel metabolism, raising concerns about concomitant use.[94] Clinical evidence of this adverse drug-drug interaction, however, derives mainly from observational studies. A recent review concluded that although definite evidence is lacking and further studies are needed, clinicians should keep in mind this possibly clinically relevant interaction when considering prescribing this combination.[94]

Prasugrel

The pharmacogenetics of prasugrel, a newer thienopyridine, has not been as comprehensively investigated as clopidogrel. Nevertheless, several studies indicate that the main contributor to clopidogrel resistance, CYP2C19*2, does not affect prasugrel's efficacy.[69,95] In a large study, no significant attenuation of the prasugrel response was found in carriers of at least one of the known loss-of-function alleles of the tested CYP 450 genes. Furthermore, in ACS patients none of the genotypes was associated with clinical

cardiovascular events[95]. Prasugrel's metabolic pathway explains this lack of association; CYP2C19 makes only a minor contribution to prasugrel's metabolism. CYP3A4 and CYP2B6 are largely responsible for converting prasugrel to its active form.[95,96] *CYP3A4* appears not to be very polymorphic and no clear association emerged between *CYP2B6* variants and prasugrel function.[95] Prasugrel's strong antiplatelet effect leads to a highly reduced residual platelet activity, making any effect of genetic variation difficult to determine. Prasugrel inhibits platelets sufficiently to reduce clinical endpoints despite decreased efficacy arising from the polymorphisms.

Ticagrelor

The newest P_2Y_{12} receptor antagonist, ticagrelor, appears to be superior to clopidogrel[81,83] and has similar potency as prasugrel.[82] A genetic sub study of the largest ticagrelor trial to date, the PLATelet inhibition and patient Outcomes (PLATO) trial, genotyped 10,285 patients randomized to ticagrelor or clopidogrel treatment. No interaction between ticagrelor and any loss-of-function *CYP2C19* allele (*2 till *8), gain-of-function *CYP2C19**17 allele or *ABCB1* 3435C>T genotype emerged with regard to the either primary efficacy endpoint (composite of cardiovascular death, MI or stroke) or any type of major bleeding after up to 12 months treatment.[74]

Glycoprotein IIb/IIIa antagonists

Glycoprotein IIb/IIIa antagonists are mechanistically different from aspirin, thienopyridine derivates and other P_2Y_{12} inhibitors. Glycoprotein IIb/IIIa antagonists act primarily outside the platelet by competitively inhibiting the receptor essential for platelet bridging and aggregation[97]. Several studies have focused on polymorphisms in the GP IIIa gene and antiplatelet effect, but the results appear inconclusive. One study on orbofiban[98] and two on abciximab[99,100] found a relationship between the PlA2 allele with reduced platelet inhibition and increased cardiovascular event rate. However, other studies did not replicate this association.[101-103] In addition, Shields *et al.* developed a model to predict recurrent events during orbofiban treatment that included 10 candidate genes: platelet receptors *GP IIIa*, PlA1/A2 (rs5918) , *GP Ia* 807C>T (rs1126643), *GP Iba*, -5C>T (rs2243093); platelet ligands β-fibrinogen, -455G>A (rs1800790) and *vWF*, -1051G>A; interleukins *IL-1RN**2, and *IL-6*, -174G>C (rs1800795); adhesion proteins E-selectin, 128A>C (rs5361) and P-selectin, 715A>C; and metalloproteinase *MMP-9*, -1562C>T. In 924 ACS patients an association emerged between a combination of genetic variants and both recurrent MIs and bleeding outcomes during orbofiban treatment.[104] Since the influence of individual genetic polymorphisms is usually small, combining multiple variants in a model might be useful. However, interpreting the results may be more difficult.

DOSING ISSUES WITH ORAL ANTICOAGULANTS

Coumarin anticoagulants (vitamin K antagonists), are widely used to treat and prevent venous and arterial thromboembolisms. However, anticoagulants' narrow therapeutic range necessitates frequent monitoring of the INR (International Normalized Ratio) coagulation status, which is expensive for health care systems and inconvenient for patients. The large individual variability in drug response with all three of the most frequently prescribed anticoagulants (warfarin, acenocoumarol and phenprocoumon) complicates therapy and makes defining a standard fixed dose unachievable. Besides several patient-related (amongst others age, weight, body surface area and diet) and clinical factors (hepatic or renal disease, anemia, co-medication), pharmacogenetics explains a large part of the variability in dose requirements[105-107] (see Supplementary material online, *Table S6*).

Increased metabolism

Furuya *et al.*[108] first reported the influence of polymorphisms in CYP2C9, the primary enzyme involved in warfarin metabolism, on dose requirement in vivo. Carriers of the *2 allele (rs1799853) required a 20% lower warfarin dose to maintain a target INR between 2 and 4. Later studies associated the *3 (rs1057910) allele with an increased sensitivity to warfarin.[109] In vitro, the *2 and *3 genotypes decrease CYP2C9 activity by 30% and 80% respectively.[110,111] A large meta-analysis of 39 studies encompassing 7907 patients[112] concluded that, compared to the wild type *CYP2C9**1/*1 genotype, all variant genotypes (*1/*2, *1/*3, *2/*2, *2/*3 and *3/*3) required lower maintenance doses of warfarin. For instance, maintenance doses in the *2 and *3 homozygotes were 36% and 78.1% lower respectively.

However, using this pharmacogenetic knowledge in clinical practice is difficult. A meta-analysis investigating the safety and efficacy of genotype-guided dosing of warfarin in reducing the occurrence of major bleeding events and over-anticoagulation found a non-significant trend towards more rapid achievement of a stable dose in the genotype-guided dosing arm.[113] Several ongoing randomized controlled trials, which are expected to encompass more than 2500 patients will help elucidate the role of genetic testing in warfarin management.[113]

A second contributor

Another extensively studied gene is that encoding VKORC1 (vitamin K epoxide reductase complex subunit 1), which converts the oxidized, inactive form of vitamin K to the reduced, active form. Coumarins inhibit VKORC1, thereby inhibiting activation of the vitamin K dependent clotting factors II, VII, IX and X.[114] A recent meta-analysis analyzed the relationship between mean maintenance dose of warfarin and three *VKORC1*

polymorphisms (1173T>C, rs9934438; 3730G>A, rs7294 and -1639A>G, rs9923231).[115] Carriers of 1173T>C and -1639A>G needed higher maintenance warfarin doses.

Genetic variation in several other genes—including *APOE*, glutamyl carboxylase (*GGCX*), calumenin (*CALU*), *CYP4F2*, epoxide hydrolase 1 (*EPHX1*) and factor VII (*F7*)—may also influence warfarin dosage requirements. However, although some studies found significant associations, results were inconsistent.[106,116-120] Finally, recent GWASs identified *CYP2C9* and *VKORC1* only as major hotspots, indicating that polymorphisms in other genes probably only have minor influence on warfarin dosing[117,121].

Although warfarin is usually the anticoagulant of choice, acenocoumarol and phenprocoumon are used in most European countries.[122] All three drugs are metabolized by CYP2C9. However, phenprocoumon appears to be less influenced by *CYP2C9* polymorphisms[123] due to significant metabolism by the non-polymorphic *CYP3A4*.[122] The effect of the *VKORC1* polymorphism is similar for all three drugs.[122,124,125]

Dosing advice with genetic guidance

In conclusion, the association between warfarin dose and polymorphisms of the *CYP2C9* and *VKORC1* genes seems unquestionable, as demonstrated by numerous studies and several meta-analyses. Polymorphisms in *CYP2C9* and *VKORC1* together with additional clinical factors including co-medication, account for 50-60% of the inter-individual warfarin variability.[106,126-128] Based on this evidence, a free web site is available to help clinicians estimate the therapeutic dose in patients new to warfarin (http://www.warfarindosing.org) (*Figure 2*). Furthermore, the U.S. Food and Drug Administration (FDA) now recommends considering lower initiation doses of warfarin for patients with certain polymorphisms in *CYP2C9* and *VKORC1*.[129]

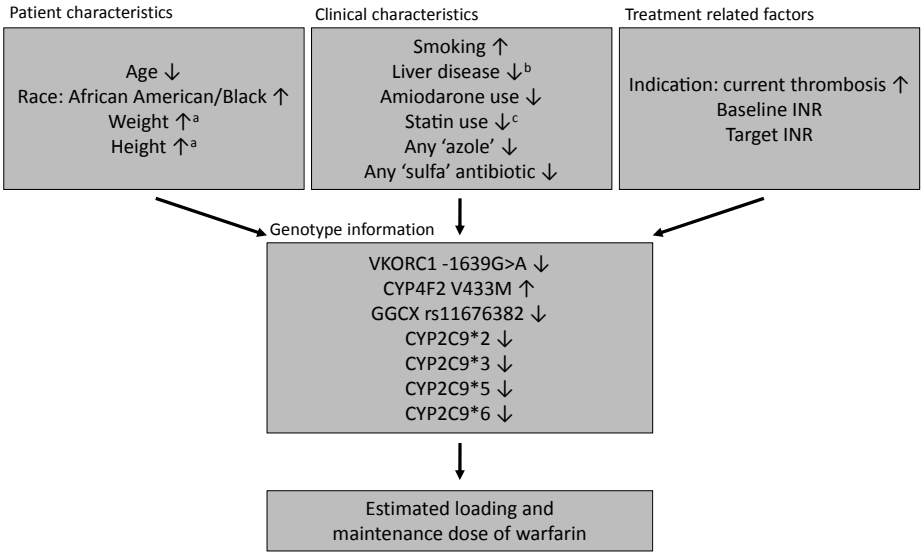
SUBOPTIMAL RESPONSES TO B-BLOCKERS

β-blockers competitively antagonize β-adrenergic receptors (β-AR). Chronic β1-AR activation contributes to the pathogenesis of heart failure and β-AR blockade improves survival, remodeling and left ventricular ejection fraction (LVEF).[130-132] Other major indications for β-blocker therapy are hypertension, angina pectoris and MI. As with most other drugs, not all patients benefit from β-blockers to the same extent, possibly due to genetic variation (see Supplementary material online , *Table S7*).

Receptors and treatment efficacy

The primary focus of studies examining pharmacogenetic determinants of β-blocker response has been the β-AR genes and two polymorphisms - Ser49Gly (rs1801252) and Arg389Gly (rs1801253) - in the beta-1 adrenergic receptor (*ADRB1*) gene in particular.

Figure 2 Dosing algorithm warfarin.



The flow chart illustrates the publically available warfarin dosing algorithm (http://www.warfarindosing.org). This algorithm incorporates patient factors, clinical parameters and genetic information to provide an estimation of the loading dose as well as the maintenance dose of warfarin in patient new to this drug. Clinical testing of the algorithm is currently ongoing (GIFT trial, NCT01006733). ↓, lower warfarin dose; ↑, higher warfarin dose. [a] Higher dose with higher body surface area. [b] Severe liver disease may predispose to an elevated INR. [c] Concomitant statin use (except pravastatin) decreases warfarin maintenance dose.

Ser49Gly and Arg389Gly are in linkage disequilibrium and 65% of the population carry the Ser49/Arg389 haplotype.[133] Not all studies reported consistent results, but a meta-analysis combining 3 studies (encompassing 504 heart failure patients), concluded that Arg389 homozygote individuals showed significantly greater improvement of LVEF when treated with β-blockers, compared to Gly389 carriers.[133] Other more recent studies drew the same conclusion.[134,135] However, two large studies reported inconsistent results on mortality and heart failure related hospitalization. In the BEST study, the combination of Arg389 homozygote genotype and bucindolol treatment was associated with decreased mortality and hospitalization.[136] However, no association emerged in MERIT-HT.[137] Furthermore, several,[138-140] but not all,[141-143] studies associated Arg389 homozygotes with an increased effect of β-blockers in decreasing systolic and diastolic blood pressure or heart rate. Furthermore, the Gly allele of Ser49Gly was more commonly associated with a favorable outcome or improved response to β-blockers.[144-146] However, many studies did not find any association.[143,147,148]

Most studies assessing two common polymorphisms in *ARDB2* (Gly16Arg, rs104213 and Gln27Glu, rs1042714) did not observe any association.[147,148] However, two small studies associated the Glu27 allele with increased LVEF after β-blocker treatment compared

to the Gln27 allele.[149,150] A third polymorphism in *ARDB2*, Thr164Ile (rs1800888), occurs in only 1% of Caucasians.[135,147] Although the Ile164 variant did not have a major impact on outcome in a population of 443 heart failure patients, multivariate analysis suggested a possible negative impact of β-blocker therapy on survival in heterozygous subjects.[151]

Dosing problems of β-blockers

Many β-blockers are metabolized predominately by hepatic CYP2D6,[152] which is highly polymorphic with many alleles having a decreased or absent function.[153] Several studies report that *CYP2D6* genotype profoundly affects β-blocker concentrations and the effect seems especially pronounced with metoprolol.[154-157] Poor metabolizing genotypes increase metoprolol plasma concentrations, which results in greater reductions of heart rate and blood pressure,[158-160] and an increased risk of ADRs.[160] Although the evidence is derived from a few studies, dose adjustments should be considered in patients vulnerable to complications, such as heart failure patients.[161]

The use of different β-blockers between, and even within, studies complicates the evaluation of the evidence on the pharmacogenetics of β-blocker therapy. Differences in receptor specificity of the individual β-blockers could explain this heterogeneity. For instance, metoprolol and bisoprolol are β1-AR selective, while carvedilol and bucindolol are non-selective.[162] In summary, the most convincing evidence suggests that the variable benefit from β-blocker treatment is influenced by the Arg389Gly polymorphism of the *ADRB1* gene. Furthermore, indisputable evidence shows that polymorphisms of the *CYP2D6* gene influence β-blocker metabolism and several reports indicate that poor metabolizers have increased efficacy and a greater risk of side effects. The data on other possible related polymorphisms is too thin to draw definite conclusions. Combining different risk alleles could provide more answers.[163]

THE VARIABLE RESPONSE IN PATIENTS TAKING ANGIOTENSIN-CONVERTING ENZYME-INHIBITORS

The angiotensin-converting enzyme gene insertion/deletion polymorphism

Most studies examining genetic factors influencing responses to ACE-inhibitor treatment evaluated the *ACE* gene insertion/deletion (I/D) polymorphism (rs4646994) (see Supplementary material online, *Table S8*), which correlates strongly with ACE plasma concentrations.[164,165] Nevertheless, initial results were inconsistent, partly due to limited sample size. Recently, however, the Genetics of Hypertension-Associated Treatment (GenHAT) study did not find an association between the I/D *ACE* polymorphism and the 6-year non-fatal cardiac events.[166] Similarly, the Perindopril Protection Against Recurrent Stroke Study (PROGRESS) did not found find an association between this polymorphism

and 4-year risk of cardiac events, mortality, neurologic events or decline or with blood pressure reduction during perindopril treatment.[167] In contrast, in the population-based Rotterdam Study, hypertensive patients on ACE-inhibitors had an increased 10-year mortality risk when carrying the DD genotype compared to the II genotype, with the ID genotype in an intermediate position.[168] However, the Rotterdam Study's observational nature represents a limitation.

In conclusion, although the *ACE* gene I/D polymorphism was a prime candidate, an association with therapeutic response seems unlikely. Indeed, since Cambien *et al.*[169] classified this polymorphism as a potent risk factor for coronary heart disease, many predominantly small positive trials reported similar observations. But a meta-analysis, examining the ACE I/D polymorphism with regard to restenosis, showed that small positive studies were much more likely to be published than negative ones. This publication bias distorted the actual association.[170]

Other targets in the renin–angiotensin–aldosteronesystem

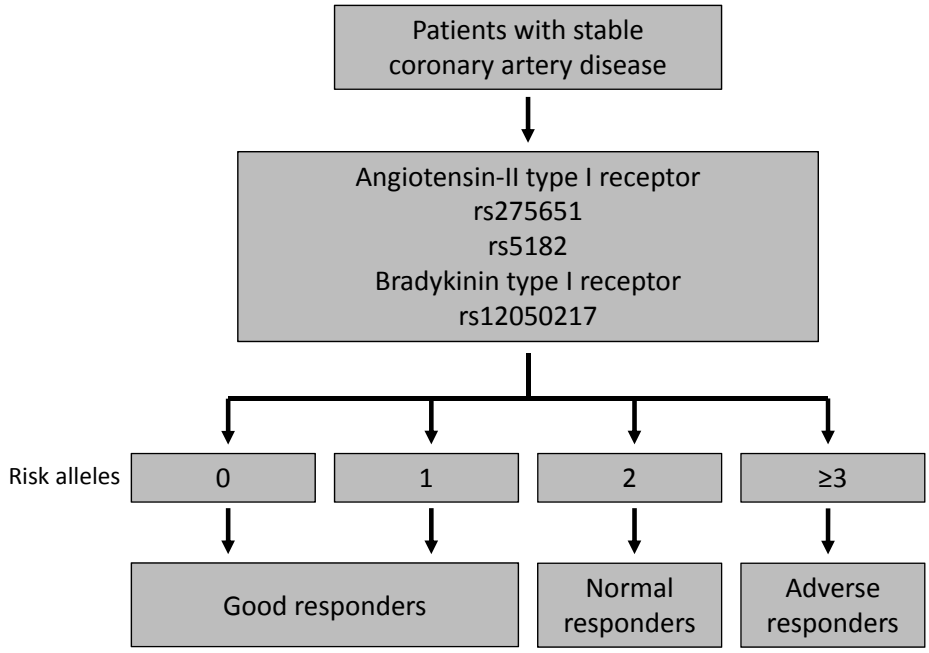
Angiotensinogen (*AGT*) is another candidate gene in pharmacogenetic research on ACE-inhibitors. In another cohort of the Rotterdam study, 4097 subjects were analyzed for the Met235Thr (rs699) polymorphism of *AGT*.[171] The Thr allele increased the risk of MI and stroke in ACE-inhibitor users. No significant association was found with β-blockers. The same authors published a paper on a different cohort of 2216 subjects from the Rotterdam study investigating whether the Met235Thr AGT polymorphism modified the risk of atherosclerosis associated with β-blocker or ACE-inhibitor therapy.[172] They also examined the possible relation with the I/D ACE polymorphism and the angiotensin II receptor type 1 (*AGTR1*) 573C>T (rs5182) polymorphism. None of the candidate polymorphisms strongly modified the risk of atherosclerosis, measured by arterial disease, carotid atherosclerosis and aortic atherosclerosis in hypertensives taking a β-blocker or ACE-inhibitor.

Furthermore, Su *et al.* genotyped a Chinese population of 1447 hypertensive patients from a benazepril trial for several polymorphisms in AGT, AGTR1 and AGTR2.[173] No association with blood pressure response emerged for the Met235Thr AGT polymorphism. However, subjects with the AA genotype of the rs7079 polymorphism of the AGT gene showed an enhanced antihypertensive effect. Although mortality risk seems to be higher among 235Thr allele carriers of the AGT gene receiving an ACE-inhibitor based regimen,[171] the risk of atherosclerosis[172] or blood pressure reduction[173] does not seem to be increased. Further studies examining the influence of this AGT polymorphism might better characterize this relationship. Moreover, common haplotypes composed of 7 polymorphisms in the AGTR1 gene were also associated with a greater antihypertensive effect on ACE-inhibitor therapy

Prediction of benefit

Finally, the first results of the Perindopril Genetic Association study (PERGENE) were recently published.[174] PERGENE included 8907 subjects and was designed to assess the feasibility of pharmacogenetic profiling of treatment benefit of ACE-inhibitors in patients with stable CAD. PERGENE investigated 52 haplotype-tagging polymorphisms in 12 candidate genes within the pharmacodynamic pathway of ACE-inhibitors and their relation with cardiovascular mortality, non-fatal MI, and resuscitated cardiac arrest during 4.2 years of follow-up. Two polymorphisms located in the AGTR1 gene (rs275651 and rs5182) and one in the bradykinin type I receptor (rs12050217) gene were significantly associated with perindopril's treatment benefit. Combining these 3 polymorphisms in a pharmacogenetic score identified patients who did not benefit from perindopril (26.5%) (*Figure 3*). The event rate increased significantly in patients allocated to perindopril (6.3 to 10.4%, $P_{interaction} < 0.0001$). The authors replicated this pharmacogenetic score in a subgroup of the PROGRESS trial[174]. Five polymorphisms of the *ACE* gene (one in complete

Figure 3 Pharmacogenetic score proposed by the PERGENE study for prediction outcome of treatment with angiotensin-converting enzyme-inhibitors.



Results of the PERGENE study indicated that carriage of 3 or more minor alleles (risk alleles) of rs275651 A>T, rs5182 C>T and rs12050217 A>G corresponded with an increased risk of cardiovascular mortality, myocardial infarction or resuscitated cardiac arrest during treatment with perindopril compared to placebo. Clinical consequences of these findings, whether indeed patients with this specific risk profile should not receive angiotensin-converting enzyme -inhibitor treatment, remain to be prospectively studied. Modified from Brugts *et al.*[174]

linkage disequilibrium with the I/D polymorphism) were shown not to be associated with the endpoints, again stressing that the ACE I/D gene variation does not seems to carry clinical value. The PERGENE trial takes an important step closer to realizing individualized therapy for ACE-inhibitors, but requires replication.

Angiotensin receptor blockers (ARB) inhibit the renin-angiotensin-aldosteron system through a different mechanism than ACE-inhibitors. Although some small studies investigated pharmacogenetics of ARBs, replication in larger populations is necessary before drawing conclusions (see Supplementary material online, *Table S8*).

FUTURE PERSPECTIVES AND CONCLUSION

During the Colloquium, mentioned in the introduction, all above discussed subjects were covered and the following conclusions were made. Elucidating genetic variation in patients with CVD may enable tailored therapy, thereby optimizing efficacy while minimizing costs and adverse effects. Nevertheless, despite the vast amount of available publications, pharmacogenetics of CVD is not yet established in clinical practice. Indeed, studies of most polymorphisms produced inconsistent results due to the complex systems underlying CVD, which means variation in a single gene usually produces only a small effect on clinical phenotypes. Very large patient cohorts are needed to convincingly detect these subtle differences. Large studies designed to detect pharmacogenetic association, (e.g. large GWAS) are ongoing and will probably be of great value in unraveling the current inconsistencies as well as elucidating associations of genetic variation in other target genes. Moreover, new approaches like sequencing might result in new targets for subsequent research.

The relevance and usefulness of GWAS have recently been reviewed by Daly[175] (Table 2) and commercially available arrays, like the Drug Metabolizing Enzymes and Transporters (DMET) platform, offer systematic exploration of potential relevant pathways.[176] However, pharmacogenetics needs evidence other than GWAS to convince cardiologists of its clinical utility. In particular, large prospective studies should investigate the effect of genetic testing on clinical outcomes and stratify patients to determine groups for which genetic testing is relevant. For example, the JUPITER trial showed that statin treatment is more beneficial in subjects with high C-reactive protein.[177] Development of risk models combining clinical characteristics (age, gender, and history), patient characteristics (for instance blood pressure, weight, and echocardiographic parameters), biomarkers and genetics will be most useful and informative.

Besides the genetic variation depicted in Table 3, the evidence for most other pharmacogenetic associations is not strong enough for clinical application. Nevertheless, currently several guidelines on drug prescription already contain pharmacogenetic infor-

Table 2 GWAS on pharmacogenetics of cardiovascular drugs

Study name	Drug	Outcome measure	Population size (disc./repl.)	Lowest P-value	Associated gene(s)	Ref
TNT	Atorvastatin	Lipid lowering response	1984/3761	5.5E-30	*APOE, PCSK9, HMGCR*	(17)
TNT, CAP and PRINCE	Statin	Lipid lowering response	3936[a]	1.9E-08	*CLMN, APOC1*	(19)
SEARCH and HPS	Simvastatin	Myopathy	85|90[b]/21|16643[b]	4.00E-09	*SLCO1B1*	(32)
PAPI	Clopidogrel	Platelet aggregation	429/227	1.5E-13	*CYP2C19*	(79)
-	Warfarin	Maintenance dose	181/374	2.6E-13	*VKORC1, CYP2C9*	(120)
WARG	Warfarin	Maintenance dose	1053/588	5.4E-78	*VKORC1, CYP2C9, CYP4F2*	(116)
Rotterdam Study	Acenocoumarol	Maintenance dose	1451/287	2.0E-123	*STX4A (VKORC1), CYP2C9*	(124)

[a] Combined number of patients included in the meta-analysis. [b] Case|control. TNT, Treating to New Targets study; CAP, Cholesterol And Pharmacogenetics study ; PRINCE, PRavastatin/Inflammation CRP Evaluation study; SEARCH, Study of the Effectiveness of Additional Reductions in Cholesterol and Homocysteine; HPS, Heart Protection Study; PAPI, the Amish Pharmacogenomics of Anti-Platelet Intervention study; WARG, WARfarin Genetics study; disc., discovery cohort; repl., replication cohort

mation. Very recently Swen *et al.*[178] published an update of the current Dutch guidelines implementing pharmacogenetics in several therapeutic recommendations. Also, over 60 FDA-approved drug labels contain pharmacogenetic information.[179] For instance, in 2007, the FDA recommended initiation warfarin doses for carriers of certain *CYP2C9* and *VKORC1* polymorphisms. Moreover, in 2009 the FDA added a warning to the clopidogrel prescribing information to highlight the impact of the poor metabolizer genotypes of *CYP2C19*. Finally, the pharmacogenetic score developed in the PERGENE trial seems to be able to identify patients that will not benefit from ACE-inhibitor treatment and the clinical consequences of this knowledge, whether indeed patients with this specific risk profile should not receive ACE-inhibitor treatment, should be prospectively tested.

Ongoing large clinical trials, primarily designed as pharmacogenetic studies, and GWAS with large study populations promise to provide more convincing evidence.

Table 3 Best explored single nucleotide polymorphisms in field of cardiovascular pharmacogenetics

Gene	SNP	Drug	Effect of variant
CYP2C19	*2 (rs4244285)	Clopidogrel	Decreased effect
CYP2C9	*2 (rs1799853) *3 (rs1057910)	Warfarin	Increased effect
VKORC1	-1639A>G (rs9923231) 1173T>C (rs9934438)	Warfarin	Decreased effect
ACE	I/D (rs4646994)	ACE-inhibitors	No effect

Overall, there are enough reasons to remain optimistic that, even though we are taking small steps at a time, we are heading to an era where we can finally use pharmacogenetics in clinical practice to optimize treatment for the individual patient.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *European Heart Journal* online.

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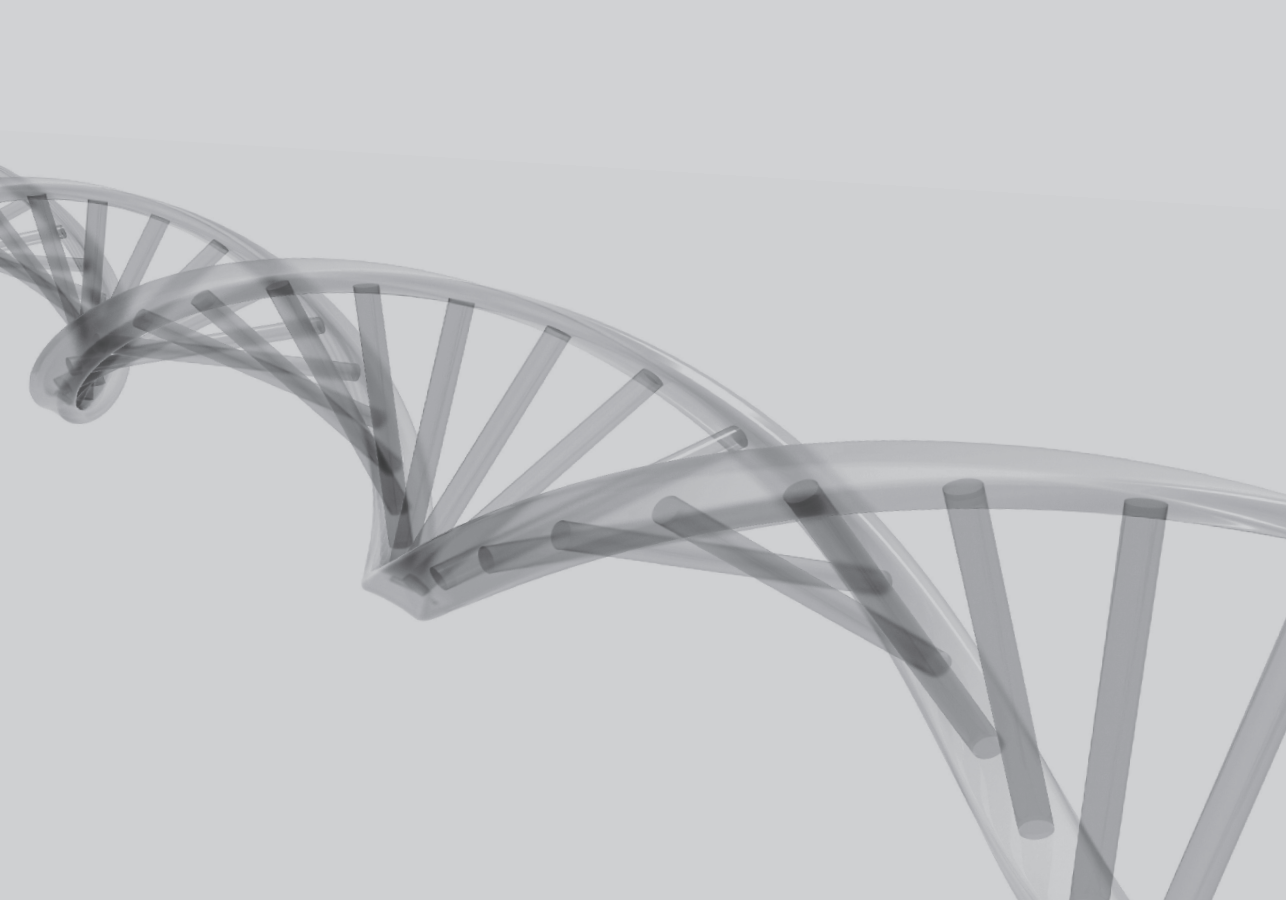
Chapter 12



Value of platelet pharmacogenetics in common clinical practice of patients with ST-segment elevation myocardial infarction

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ABSTRACT

Background: Antiplatelet drug resistance is a well-known problem, causing recurrent cardiovascular events. Multiple genetic polymorphisms have been related to antiplatelet resistance by several large trials, however data from common clinical practice is limited. We examined the influence of previously described polymorphisms, related to aspirin and clopidogrel resistance, on treatment outcome in a real life unselected population of patients presenting with ST-segment elevation myocardial infarction (STEMI) treated with percutaneous coronary intervention.

Methods and Results: This cohort study consisted of 1327 patients with STEMI. Patients were treated according to a standardized guideline-based protocol. Nine polymorphisms, COX1 (-842A>G), $P2Y_1$ (893C>T), GPIa (807C>T), GPIIIa (PlA1/A2), CYP2C19 (*2, *3 and *17), ABCB1 (3435T>C) and PON1 (576A>G), were genotyped. During 1 year of follow-up the primary endpoint, a composite of cardiac death or recurrent myocardial infarction, was reached in 86 patients. The COX1 and CYP2C19*2 polymorphisms were associated with the primary endpoint, HR 2.55 (95% CI 1.48-4.40), $p = 0.001$ and HR 2.03 (1.34-3.09) $P = 0.001$, respectively. The combined analysis demonstrated a 2.5-fold increased risk for individuals with ≥ 2 risk alleles, $p = 6.9 \times 10^{-9}$. The association of COX1 was driven by mortality related events whereas that of CYP2C19*2 was mainly attributed to myocardial infarction and stent thrombosis.

Conclusion: In this unselected, real life population of STEMI patient on dual-antiplatelet therapy, the polymorphisms COX1 -842A>G and CYP2C19*2 were determinants of thrombotic complications during follow-up. We show that in a clinical setting, testing for these polymorphisms could be of value in the identification of STEMI patients at risk for recurrent cardiovascular events.

INTRODUCTION

Insufficient platelet inhibition during adequate guideline antiplatelet therapy is a well-known problem in the secondary prevention of coronary artery disease, causing a considerable amount of patients to suffer from recurrent thrombotic events.[1] Individual differences in the intrinsic rate of platelet reactivity and variability in the response to antiplatelet therapy are the main underlying mechanisms responsible for this so-called antiplatelet therapy resistance. For both aspirin and clopidogrel this problem has been recognized. Several factors play a role in this inadequate response to antiplatelet agents. Clinical factors like increased body mass index (BMI), diabetes mellitus and drug-drug interactions have been implicated.[1] Moreover, genetic polymorphisms causing individual variability in drug absorption, metabolism and availability of biological targets, like platelet receptors, influence the platelet inhibition during therapy in each individual.[1]

Pharmacogenetics is the upcoming field of research exploring the influence of genetic variation on response on drug therapy, to pursue achievement of individualized therapy. There is growing evidence that aspirin- and especially clopidogrel resistance is partially determined by carrying risk alleles of several single nucleotide polymorphisms (SNPs) in genes resulting in altered drug efficacy.[1] In comparison with the largely inconsistent pharmacogenetic evidence on aspirin resistance due to the high variability in laboratory tests and the small sample size of most studies,[2] clopidogrel pharmacogenetics has become a well-recognized risk factor for resistance to treatment. Over the last years, multiple studies consistently reported an association of the reduced-function alleles of CYP2C19 and clopidogrel resistance and occurrence of thrombotic events.[3-5] However, the most recent meta-analyses did not demonstrate this association.[6,7] The studies included in these analyses comprise several patient groups, including patients with stable and unstable angina and the spectrum acute coronary syndromes. Specific data on patients with ST-segment elevation myocardial infarction (STEMI), a subpopulation specifically at high risk for thrombotic events, is limited. Furthermore, data on the generalizability of this evidence to the patients seen in the daily clinical practices is largely lacking.

The only large population based study on this subject is that of Simon *et al*. in the French Registry of Acute ST-Elevation and Non-ST-Elevation Myocardial Infarction (FAST-MI) population (n = 2208).[8] Although this well designed study investigated probably a good representative population, they still applied several exclusion criteria such as pre-specified levels of biomarkers and duration of complaints. Moreover, of the total included population, only 53% were STEMI patients and only 70% of the patients were treated with percutaneous coronary intervention (PCI), making it a quite heterogeneous population. Also the genotypic analysis was limited to 4 candidate genes. All other cohort studies were smaller and had even less patients with STEMI.[9-11]

To obtain answers on the role of pharmacogenetics in the outcome of patients suffering from STEMI, our study investigated the effect of the best described polymorphisms implicated in antiplatelet therapy efficacy on thrombotic complications in a prospectively gathered real life unselected population of patients presenting with STEMI. The goal was to evaluate whether the described effects of these polymorphisms were detectable in this high risk population and could therefore possibly be of value for application in the common clinical practice.

METHODS

Study population

The population of the current study consists of patients who were admitted with the diagnosis STEMI to the Leiden University Medical Center (LUMC), Leiden, The Netherlands, between February 2004 and January 2010. All patients underwent primary PCI and were treated according to the previously described standardized guideline-based MISSION! AMI care program[12]. In brief, the protocol includes a pre-hospital triage system based on 12-lead electrocardiography and when eligible pre-hospital administration of aspirin (300mg), clopidogrel (600mg) and abciximab (25µg/kg bolus, followed by 10µg/kg/min for 12 h) was performed to pursue early reperfusion. Patients were directly transferred to the catheterization laboratory for primary PCI. Beta-blockers and angiotensin-converting enzyme (ACE) inhibitors were titrated to achieve an optimal heart rate and blood pressure control. Moreover statin treatment was started in all patients. Patients without complications were discharged at day 3 after education on lifestyle changes and drug compliance. Aspirin (100mg/day) was prescribed indefinitely, and clopidogrel (75mg/day) for 12 months, irrespective of implanted stent type. All patients were offered an outpatient rehabilitation program.

Follow-up and study endpoints

During the first year, 4 outpatient clinic visits were scheduled. During this 1 year of follow-up all thrombotic cardiovascular events were recorded. In this study, the primary endpoint was the composite of cardiac death and recurrent myocardial infarction (MI). Secondary endpoints included cardiac death and MI separately, as well as definite stent thrombosis, repeat revascularization and all-cause mortality. All deaths were defined as cardiac, unless clearly proven non-cardiac. Myocardial infarction was defined as a troponin T level above the upper limit in the presence of ischemic complaints or PCI or a re-rise of > 25% after recent MI in the presence of symptoms or PCI. Stent thrombosis was defined as angiographic or pathological confirmation of a partial or total thrombotic occlusion within the stent or 5 mm proximally or distally to the stent.[13] Finally, data on

revascularization of any coronary artery, irrespective of the treatment modality (PCI or CABG) was collected. Target vessel revascularizations were all clinically driven.

Genotyping

EDTA blood was prospectively collected on admission and DNA was extracted following standard procedures. DNA was available from 1370 of the 1674 consecutive patients, reasons for missing DNA were death before the collection of blood or failure of the DNA extraction.

The most well replicated genetic polymorphisms related to aspirin and clopidogrel resistance were selected after a systematic search of literature, as described previously.[1] In brief, relevant articles were identified by searching MEDLINE using keywords and Medical Subject Headings (MeSH) terms including the following: pharmacogenetics, single nucleotide polymorphism, treatment outcome, adverse effects, drug therapy and cardiovascular disease (CVD). All available literature until May 2011 was included and reviews, editorials, and articles in languages other than English were excluded. A multiplex assay was designed using Assay designer software. When a SNP did not fit the multiplex, a proxy of that SNP was selected with the highest R^2 value. The final set included cyclooxygenase 1 (*COX1*) -842A>G (rs10306114), P2Y purinoceptor 1 ($P2Y_1$) 893C>T (rs1065776), $P2Y_{12}$ 52G>T (rs6809699), glycoprotein (GP) Ia 807C>T (rs1126643), *GPIIIa* rs8069732 (in complete linkage disequilibrium (LD) ($R^2 = 1.0$) with PlA1/A2 [rs5918]), cytochrome P450 (CYP) 2C19*2 (rs4244285), CYP2C19*3 (rs4986893) and rs11188072 (in complete LD ($R^2 = 1.0$) with CYP2C19*17 [rs12248560]), ATP-binding cassette sub-family B member 1 (*ABCB1*) rs2235048 (as a proxy for 3435T>C [rs1045642], in complete LD with $R^2 = 1.0$) and paraoxonase-1 (*PON1*) 576A>G (rs662) (Supplementary table 1).

These SNPs, except the SNP in *PON1*, were genotyped by MALDI-TOF mass spectrometry, using the MassARRAY™ methodology (Sequenom Inc., San Diego, CA, USA), following the manufacturer's instructions. The *PON1* 576A>G (rs662) SNP was genotyped using a TaqMan Drug Metabolism Genotyping Assay (Assay ID C___2548962_20; Applied Biosystems, Foster City, CA, USA). As quality control, 5% of the samples were genotyped in duplo. No inconsistencies were observed. All the negative controls (2%) were negative. Call rate of all SNPs was above 98%. Two SNPs deviated significantly from Hardy-Weinberg (HW) equilibrium. $P2Y_{12}$ 52G>T (rs6809699) (HW ChiSq 362.7 , $P = 7.4 \times 10^{-81}$) was excluded from further analysis. The CYP2C19*3 polymorphism (HW ChiSq 64.0, $P = 1.3 \times 10^{-15}$) was included, since the deviation was caused by 1 homozygote of the minor allele of this low frequency SNP (0.3% in the current population). This minor allele frequency was lower than the reported frequency in HapMap CEU reference panel (1.7%) (http://www.hapmap.org). All other SNPs matched with the reported frequencies. Finally, individuals with 50% or more failed SNPs were excluded (3%). The final analysis included 1327 patients.

Table 1 Baseline and procedural patient characteristics.

Characteristics	Without primary event (N = 1241)	Primary event (N = 86)	P-value
Baseline			
Sex – male (%)	949 (77)	61 (71)	0.24
Age – mean (SD), yr.	60.4 (11.8)	66.9 (13.3)	< 0.001
BMI – median (IQR), kg/m^2	26.0 (4.4)	26.5 (6.2)	0.28 [a]
Hypertension – no. (%)	416 (34)	38 (45)	0.03
Hypercholesterolemia – no. (%)	250 (20)	21 (25)	0.29
Diabetes mellitus – no. (%)	122 (10)	22 (26)	< 0.001
Family history of CAD – no. (%)	528 (43)	23 (28)	0.008
Previous or current smoker – no. (%)	724 (58)	48 (57)	0.82
Previous MI – no. (%)	101 (8)	13 (15)	0.03
Previous PCI – no. (%)	75 (6)	6 (7)	0.73
Previous CABG – no. (%)	23 (2)	1 (1)	1.00
Procedural			
Abciximab during intervention	1189 (96)	81 (94)	0.40
Killip class ≥2– no. (%)	42 (4)	13 (18)	< 0.001
Culprit lesion – no. (%)			
LAD	558 (45)	46 (54)	0.13
RCA	485 (40)	20 (23)	0.003
RCX	183 (15)	12 (14)	0.84
Left main	8 (1)	7 (8)	< 0.001
Bypass	7 (1)	1 (1)	0.42
Multi vessel disease	662 (53)	65 (77)	< 0.001
Stenting of culprit lesion – no. (%)	1213 (98)	82 (95)	0.15
Number of coronary stents - no (%)			
1	752 (61)	47 (55)	0.28
> 1	461 (37)	35 (41)	0.51
Type of stent – no. (%)			
Bare metal stent	377 (31)	38 (44)	0.008
Drug-eluting stent	802 (65)	41 (48)	0.001
EPC stent	11 (1)	3 (4)	0.06
Different types	18 (2)	0 (0)	0.63
Cardiac shock during PCI[b] – no. (%)	24 (2)	18 (21)	< 0.001
TIMI flow < 3 after PCI – no. (%)	73 (6)	21 (25)	< 0.001
Medication at discharge – no. (%)			
Aspirin	1195 (96)	56 (90)	0.02
Clopidogrel	1238 (> 99)	61 (98)	0.18

Table 1 Baseline and procedural patient characteristics. (*continued*)

Characteristics	Without primary event (N = 1241)	Primary event (N = 86)	P-value
Statin	1230 (99)	60 (97)	0.12
Beta-blocker	1184 (95)	54 (87)	0.003
ACE inhibitor or ARB	1210 (98)	60 (97)	0.67

[a] P-value calculated with Mann-Whitney test, [b] cardiac shock requiring treatment with inotropic agents, an intra-aortic balloon pump or other medical devices. Abbreviations: SD, standard deviation; BMI, body mass index; IQR, interquartile range; CAD, coronary artery disease; MI, myocardial infarction; PCI, percutaneous cononary intervention; CABG, coronary artery bypass grafting; EPC, endothelial progenitor cell capturing stents; ACE, angiotensin-converting enzyme; ARB angiotensin-receptor blocker.

Statistics

To compare the group with and the group without a primary outcome event, categorical parameters are compared with Pearson chi-square or Fisher's exact test when appropriate. Continuous data were compared with unpaired 2-sided Student's t-test. In the case of non-Gaussian distribution, variables were compared with the Mann-Whitney test. All SNPs were tested for deviation from Hardy-Weinberg equilibrium using chi-square analysis.

Associations of SNPs with outcome events were calculated using multivariable, stepwise, forward Cox analyses assuming an additive genetic model. This multivariable model included the clinical variables that were significantly different between the primary event group and the group without. For the polymorphisms associated with $p < 0.05$, recessive and dominant models were tested. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.[14]

RESULTS

Baseline characteristics

All patients were treated with a primary PCI and received dual antiplatelet therapy with aspirin and clopidogrel. In-hospital mortality of the final study population was 1.8% (24 patients). At discharge from the hospital admission following the primary intervention, aspirin and clopidogrel were prescribed to 96.0% and 99.7% of the patients respectively. However, all patients received the loading of clopidogrel.

During follow-up 86 patients (6.5%) reached the primary composite endpoint consisting of cardiac death or recurrent myocardial infarction. A total of 57 patients (4.3%) died, of which 48 of a cardiac cause. Forty-four patients (3.3%) suffered from a recur-

rent myocardial infarction and 237 patients (17.9%) required repeat revascularization, of which 72 (30.3% of all revascularizations) were treated for an acute indication like unstable complaints or MI. Ninety-two procedures (6.9% of all patients) were revascularizations of the target vessel. Stent thrombosis was recorded in 15 patients (1.1%). Several baseline and procedural characteristics were significantly different between the primary endpoint group and the patients without the primary endpoint (Table 1). The event group was older, more likely to have hypertension, diabetes mellitus and a previous myocardial infarction in their medical history, but less likely to have a positive family history for cardiovascular disease. The location of the culprit lesion in the right coronary artery (RCA) was less often found in the primary event group, whereas it was more frequent in the left main (LM) coronary artery. The patients with a primary event were more often found to have multivessel disease and had a higher Killip class. The type of the implanted stent was also associated with clinical outcome events. Patients receiving drug-eluting stents (DES) had a lower risk of an event. Moreover, during the primary intervention, in the event group more patients suffered from cardiac shock requiring medical intervention and the classification of the final TIMI flow after the intervention was lower compared to the group without an event. Aspirin and beta-blockers were less frequently prescribed at discharge to the patients that suffered from a primary event during follow up.

Genotypic analysis

Of the 9 SNPs that were analyzed, the COX1 -842A>G polymorphism and the CYP2C19*2 reduced-function polymorphism were significantly associated with the primary endpoint (Table 2). For both SNPs there was an increase in the hazard ratio for an outcome event with each additional minor allele, HR 2.55 (95% CI 1.48-4.40), p = 0.001 for COX1 -842A>G (Figure 1) and HR 2.03 (95% CI 1.34-3.09), p = 0.001 for CYP2C19*2 (Figure 2). The other SNPs were not significantly associated with the primary endpoint.

The additional testing of dominant and recessive models indicated that the association of the CYP2C19*2 with primary endpoints was best described with a the recessive ge-

Table 2 Cox analysis of pharmacogenetic SNPs in the MISSION! population.

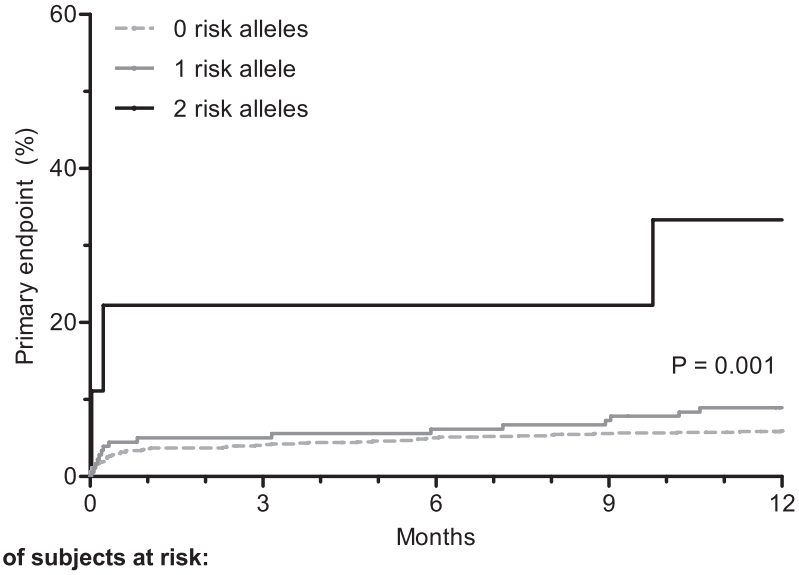
Gene, nucleotide change (dbSNP)		Without primary event (N = 1241)	Primary event (N = 86)	HR (95% CI)	P
Genotype	MAF				
COX1, -842A>G (rs10306114)				2.55	0.001
AA – no. (%)	7.5%	1063 (86)	66 (78)	(1.48-4.40)	
AG – no. (%)		163 (13)	16 (19)		
GG – no. (%)		7 (1)	3 (4)		

Table 2 Cox analysis of pharmacogenetic SNPs in the MISSION! population. (*continued*)

Gene, nucleotide change (dbSNP)		Without primary event (N=1241)	Primary event (N=86)	HR (95% CI)	P
Genotype	MAF				
$P2Y_1$, 893C>T (rs1065776)				NS	0.71
CC – no. (%)	3.5%	1145 (93)	80 (95)		
CT – no. (%)		85 (7)	4 (5)		
TT – no. (%)		2 (<1)	0 (0)		
GPIa, 807C>T (rs1126643)				NS	0.38
CC – no. (%)	39.3%	426 (35)	39 (45)		
CT – no. (%)		635 (52)	32 (37)		
TT – no. (%)		169 (14)	15 (17)		
GPIIIa, T>C (rs8069732)[a]				NS	0.51
TT – no. (%)	15.2%	889 (72)	69 (81)		
TC – no. (%)		317 (26)	11 (13)		
CC – no. (%)		32 (3)	5 (6)		
CYP2C19, 681G>A, *2 (rs4244385)				2.03	0.001
GG – no. (%)	16.7%	861 (70)	55 (65)	(1.33-3.09)	
GA – no. (%)		339 (28)	22 (26)		
AA – no. (%)		31 (3)	8 (9)		
CYP2C19, 636G>A, *3 (rs4986893)				NS	0.50
GG – no. (%)	0.3%	1233 (99)	86 (100)		
GA – no. (%)		7 (1)	0 (0)		
AA – no. (%)		1 (<1)	0 (0)		
CYP2C19, C>T (rs11188072)[b]				NS	0.93
CC – no. (%)	20.6%	773 (63)	55 (66)		
CT – no. (%)		401 (33)	23 (27)		
TT – no. (%)		51 (4)	6 (7)		
ABCB1, 3435C>T (rs1045642)				NS	0.91
CC – no. (%)	46.0%	354 (29)	26 (31)		
CT – no. (%)		618 (50)	40 (48)		
TT – no. (%)		256 (21)	18 (21)		
PON1, (rs662)				NS	0.23
TT – no. (%)	29.7%	592 (48)	45 (53)		
TC – no. (%)		532 (44)	34 (40)		
CC – no. (%)		100 (8)	6 (7)		

[a] genotyped SNP is in complete LD with PlA1/A2 (rs5918) polymorphism. [b] genotyped SNP is in complete LD with CYP2C19*17 (rs12248560) polymorphism. P-values were calculated with a multivariable, stepwise, forward Cox analyses assuming an additive model and including adjustment for age, hypertension, diabetes, family history, previous myocardial infarction, Killip score, culprit lesion, multi vessel disease, stent type, cardiac shock during PCI, final TIMI flow < 3, aspirin at discharge and betablocker at discharge. Genotyping was not successful for all SNPs in each individual subject, therefore genotype counting per SNP might not compute to the total number of subjects in each group.

Figure 1 Rates of primary outcome events (cardiac death and recurrent myocardial infarction) according to -842A>G COX1 genotype.



Number of subjects at risk:

0 risk alleles	1133	1085	1072	1065	1037
1 risk allele	179	170	168	166	160
2 risk alleles	9	7	7	7	6

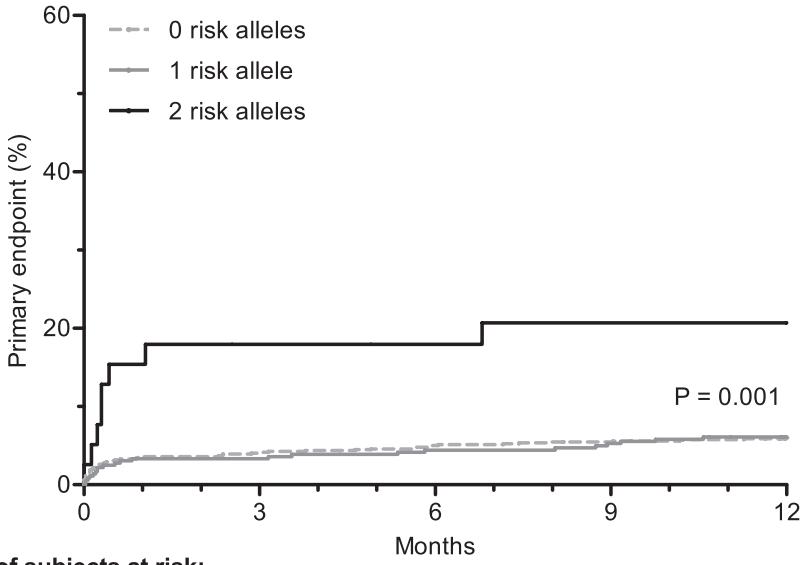
P-values were calculated with a multivariable, stepwise, forward Cox analyses assuming an additive model and including adjustment for age, hypertension, diabetes, family history, previous myocardial infarction, Killip score, culprit lesion, multi vessel disease, stent type, cardiac shock during PCI, final TIMI flow < 3, aspirin at discharge and betablocker at discharge.

netic model, and this model was therefore used for further analysis. Carriage of 2 variant alleles of the CYP2C19*2 polymorphism resulted in a significant increased risk of events during follow-up compared to carriage of 0 or 1 reduced function alleles (20.5% versus 6.0%; adjusted hazard ratio 2.38, 95% CI 1.60-3.56, $P = 2.1 \times 10^{-5}$. The dominant model resulted in a hazard ratio of 1.36, 95% CI 1.03-1.78, P = 0.03. The recessive effect was visually confirmed by the Kaplan-Meier curve (Figure 2). The additive model proved to be the most appropriate for analysis of the COX1 SNP.

When considering both SNPs together, 779 (59%) patients were homozygote for the wild type alleles of both SNPs, 435 (33%) patients had 1 risk allele, 87 (7%) patients had 2 risk alleles and 8 (1%) patients were carrying 3 risk alleles. In our population no patient was homozygote for both variant alleles of both SNPs. Compared to the patients with 0 or 1 risk alleles, the patients who were carrying 2 or 3 risk alleles had an increased risk of the primary endpoint, HR 2.57 (95% CI 1.87-3.54), $P = 6.9 \times 10^{-9}$.

Analysis of the different stent type groups did not result in different results than the analysis of the total study population. Univariable analysis of the patients receiv-

Figure 2 Rates of primary outcome events (cardiac death and recurrent myocardial infarction) according to CYP2C19*2 genotype.



Number of subjects at risk:

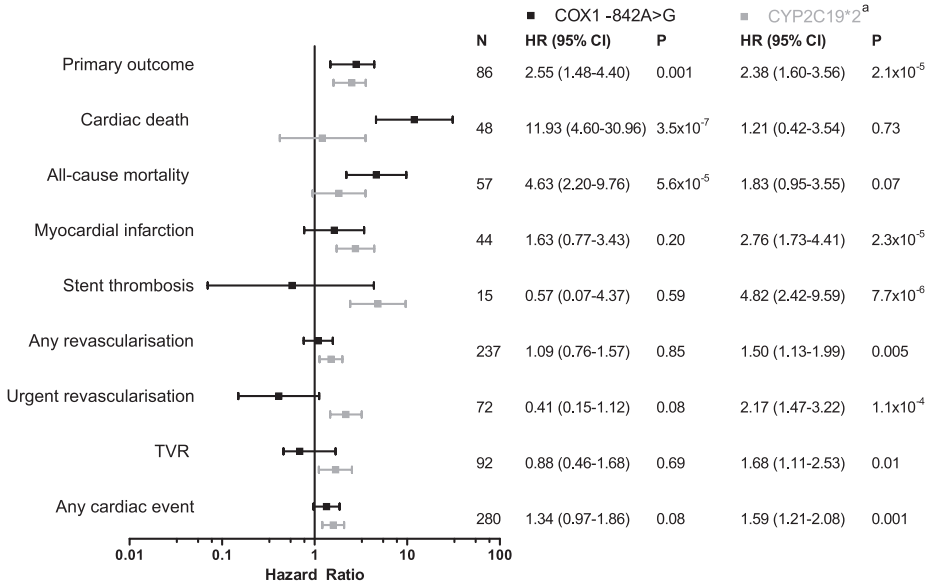
0 risk alleles	916	878	869	864	839
1 risk allele	361	349	344	341	330
2 risk alleles	39	31	30	29	28

P-values were calculated with a multivariable, stepwise, forward Cox analyses assuming an additive model and including adjustment for age, hypertension, diabetes, family history, previous myocardial infarction, Killip score, culprit lesion, multi vessel disease, stent type, cardiac shock during PCI, final TIMI flow < 3, aspirin at discharge and betablocker at discharge.

ing BMS (N = 415) did not result in significant associations of the COX1 SNP (P = 0.093) and CYP2C19*2 (P = 0.13) with the primary endpoint, whereas combination of these 2 SNPs did demonstrate a significant association (P = 0.004). These three analyses did show significant associations in the DES subgroup (N = 843), P = 0.021 for COX1 -846A>G, P = 5.4x10-5 for CYP2C19*2 and P = 9.3x10-5 for the combined analysis.

When analyzing the outcome events such as death, recurrent MI, repeat revascularization and stent thrombosis separately, the COX1 SNP was demonstrated to have a strong association with the mortality related endpoints. However, no significant association was found with the thrombotic endpoints such as myocardial infarction and stent thrombosis, P = 0.20 and P = 0.59 respectively. In contrast, the CYP2C19*2 polymorphism was associated with all endpoints, except for the mortality related endpoints (Figure 3). Patients with the homozygote variant genotype of this SNP were especially at risk for myocardial infarction, HR 2.76 (95% CI 1.73-4.41), $P = 2.3x10^{-5}$ and stent thrombosis, HR 4.82 (95% CI 2.42-9.59), $P = 7.7x10^{-6}$. In addition, a significant relation was also found with the different repeat revascularization related endpoints.

Figure 3 Multivariable Cox analysis of COX1 -842A>G and CYP2C19*2 with the different cardiovascular endpoints



In black are indicated the hazard ratio and 95% confidence interval of the -842A>G COX1 polymorphism and in gray those of CYP2C19*2 with the different secondary endpoints. With urgent revascularization includes the revascularizations performed for the indications unstable angina pectoris and acute myocardial infarction. [a] Analyzed assuming a recessive model. TVR target vessel revascularization.

DISCUSSION

The key finding of this study is that in this unselected real life population of STEMI patients treated with PCI, the homozygous variant genotype of the CYP2C19*2 polymorphism is a strong determinant of the occurrence of thrombotic events during the first year of follow-up. In addition, the -842A>G polymorphism of COX1 is associated with the composite endpoint of cardiac death and recurrent myocardial infarction as well. Especially patients carrying 2 or more risk alleles of these 2 SNPs were at high risk. None of the other previously reported SNPs with proposed pharmacogenetic influence of antiplatelet therapy efficacy were associated with outcome events during 1 year follow-up after STEMI.

The vast amount of pharmacogenetic research of the last years resulted in a tremendous increase of knowledge on pharmacogenetic gene-drug interactions, however inconsistent results prevented reaching consensus and moving the field forward to clinical application. Even regarding the CYP2C19*2 polymorphism, the major enzyme involved in the metabolic conversion of the clopidogrel prodrug into its active form,[2] that was

thought to have a significant role in the outcome of patients treated with clopidogrel, recent meta-analyses report conflicting results.[3,6,7] The current study demonstrates that this SNP and the COX1 polymorphism are significant determinants of thrombotic events in an unselected real life population of patients presenting with STEMI.

Cyclooxygenase 1 (COX1, or prostaglandin endoperoxide G/H synthase) catalyzes the metabolism of arachidonic acid to prostaglandin H_2, which is subsequently metabolized to thromboxane A_2.This enzyme is the therapeutic target of aspirin.[15] The -842A>G polymorphism, that is in complete linkage disequilibrium with 50C>T, was first described by Halushka et al. as a determinant of aspirin responsiveness in healthy individuals. However, subsequent studies investigating patient populations were largely inconsistent.[16-20] To date, the only studies that investigated the association of -842A>G or 50C>T with clinical events did not find a significant relation.[21,22] The association of -842A>G with the primary endpoint of the current study appears to be caused by the strong relation with mortality and not with myocardial infarction. The exact mechanism of this relation is unclear, since a causal relation between mortality and aspirin resistance in the absence of an association with myocardial infarction seems unlikely.

Data on genetic variation associated with aspirin resistance comes only from studies with small sample sizes (< 500 subjects) and when considering the small effect size of the individual SNPs and the variability of laboratory tests for aspirin resistance,[23] it is not surprising that the reported results are largely inconsistent.[17,18,21,24-27] In the current study only COX1 -842A>G was shown to have a significant association with the primary endpoint. But as stated above, this effect seems to be driven by an increased risk of mortality of carriers of this SNP and not due to the more thrombotic specific endpoint myocardial infarction or stent thrombosis. However, even if an effect of a specific SNP on aspirin efficacy exists, it is likely that it will not be detectable in this population with dual antiplatelet therapy since the decreased antiplatelet inhibition will be overcome by the concomitant use of clopidogrel.

In contrast with the COX1 SNP, the association of CYP2C19*2 with the primary endpoint is determined by its effect on the risk of recurrent myocardial infarction and not on cardiac death. Unlike the controversy of its relation with cardiovascular events, the reduced platelet inhibition by clopidogel in carriers of the reduced function alleles is not questioned,[7] making it likely that this is the causal mechanism of the relation with events found in the current study. In addition, patients that carry risk alleles for both of these SNPs are especially at risk as is shown by the combined risk analysis.

Early 2011, a SNP in the paraoxonase-1 gene, 576A>G (R192Q), was described as the major determinant of clopidogrel efficacy in platelet inhibition as well as stent thrombosis,[28] instead of CYP2C19*2. However, since then, several study groups failed to confirm these findings in other cohorts.[29-31] Also in the current STEMI population no association between the PON1 genotype and cardiovascular events could be demonstrated.

The results of the current study demonstrate the strong effect of the CYP2C19*2 polymorphism and the increased risk of thrombotic complications during 1 year of follow-up after presenting with STEMI. The question remains how this knowledge can contribute to improvement of the treatment outcome of these patients. Initially it was thought that increasing the dose of clopidogrel in patients with insufficient platelet inhibition on a normal dose of clopidogrel might improve the outcome.[32] The results of the Gauging Responsiveness with A Verifynow assay-Impact on Thrombosis And Safety (GRAVITAS) trial could not endorse this hypothesis, probably because of the fact that also a higher dose of clopidogrel does not lead to significant platelet inhibition in these patients.[33] In the ELEVATE-TIMI 56 (Escalating Clopidogrel by Involving a Genetic Strategy - Thrombolysis In Myocardial Infarction 56), Mega et al demonstrated that after tripling the clopidogrel dose to 225mg daily in CYP2C19*2 heterozygotes did achieve levels of platelet inhibition comparable to that seen with the standard 75mg dose in non-carriers. However in the *2 homozygotes even 300mg clopidogrel did not result in adequate platelet inhibition.[34] In addition, similar results with respect to clinical events were reported by the RESponsiveness to CLOpidogrel and Stent-related Events-2 in Acute Coronary Syndromes (RECLOSE-2 ACS) study.[35] Patients with high residual platelet activity had an increased event risk compared to those with low platelet activity after clopidogrel loading, despite increasing the clopidogrel dose or switching to ticlopidine.[35] In the current study, most patients (96%) received abciximab before or during the primary intervention. Although we observed no difference of abciximab treatment between cases and controls, it could have influenced our results by decreasing the occurrence of early events. However, since patients only receive abciximab once, it seems unlikely that it has an influence on thrombotic events later during follow up. Moreover, the found effect size of the SNPs in the current study is comparable to other studies,[8,11] decreasing the likelihood of a major impact of abciximab treatment on the presented associations.

Recently, genetic substudies of the TRITON-TIMI-38 (TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet iNhibition with prasugrel–Thrombolysis In Myocardial Infarction 38)[36] and PLATO (PLATelet inhibition and patient Outcomes)[37] trials, investigating the more potent prasugrel and ticagrelor, respectively, have demonstrated that these agents are not significantly affected by the genetic variation in the *CYP2C19* gene nor in other described genes. Prescribing one of these drugs to all patients probably would solve almost all of the pharmacogenetic problems, since the relative small effects of the SNPs are overbalanced by the much stronger platelet inhibition of these drugs. However, subsequent additional bleeding complications should be weighed against the benefit of these drugs, especially in individuals already at risk for bleeding complications. A well-validated score for estimating bleeding risk is however lacking.[38] In the latest guidelines on myocardial revascularization by the European Society of Cardiology the recommended antiplatelet therapy for STEMI patients consists of dual antiplatelet

therapy with aspirin and prasugrel or ticagrelor. In this guideline, clopidogrel should only be used if the more effective platelet inhibitors are contraindicated or unavailable.[38] Nevertheless, an attractive application of the pharmacogenetic knowledge of CYP2C19 could be genotypic guidance in the choice of prescribing clopidogrel to patients with 0 or 1 reduced-function allele of *CYP2C19* and prasugrel or ticagrelor to the carriers of 2 variant alleles.[39] Clinical benefit of this theory as well as economic cost-effectiveness remains to be proven by currently on-going trials (for instance ReAssessment of Anti-Platelet Therapy Using an InDividualized Strategy Based on GENetic Evaluation [RAPID GENE, NCT01184300], Thrombocyte Activity Reassessment and GEnoTyping for PCI [TARGET-PCI, NCT01177592] and Genotyping Infarct patients to Adjust and Normalize Thienopyridine treatment [GIANT, NCT01134380]). The results of the first study were presented at the Transcatheter Cardiovascular Therapeutics (TCT) congress 2011. In the RAPID GENE study 200 patients were randomized to a treatment strategy genotyping and prasugrel prescription for CYP2C19*2 carriers, or to standard therapy with clopidogrel. The authors demonstrated that treatment with prasugrel completely eliminated HPR in the CYP2C19*2 carriers compared to treatment with clopidogrel (unpublished results). Whether this strategy is also cost-effective compared to prasugrel prescription to all patients and if the rate of bleeding complication is lower remains to be shown.

Limitations

Some limitations of this study are worthwhile being mentioned. The fact that all patients in our population received dual antiplatelet therapy limits the possibility to investigate a true pharmacogenetic effect of these SNPs, since there is no placebo controlled group to compare with. Therefore, the relation we detected could theoretically be a direct relation between the SNP and the thrombotic events, independent of the antiplatelet therapy. However a recent meta-analysis of several genome wide associations studies on coronary artery disease did not detect any of the SNPs from the current study,[40] making the chance of an antiplatelet drug independent association unlikely. No platelet function tests were performed in the current study. In the current study three CYP2C19 alleles (*2, *3 and *17 respectively) were analysed. Although, other alleles in this gene and in other CYP genes have also been associated with clopidogrel metabolism,[36] we decided not to analyse these SNPs considering the low allele frequency of some of these SNPs and because the evidence of their involvement is not that solid as compared to the three CYP SNPs tested in the current study.[2] The observational nature of our study could be seen as a limitation. However, to determine the clinical value of findings derived from earlier large trials in an unselected and clinically representative patient population can provide important additional value. Our population is suitable for this purpose for several reasons. First, since we did not handle exclusion criteria on the type or duration of STEMI nor on the subsequent therapy, our cohort is a good representation of common

clinical practice. Second, since all patients were treated according to the standardized guideline-based MISSION! protocol, possible confounding factors during the follow-up period are kept to a minimum.

Conclusion

The contribution to the existing evidence of the current study is that it demonstrates that in daily clinical practice of patients with STEMI, of all the previously proposed candidate SNPs involved in platelet pharmacogenetics, only the influence of the COX1 -842A>G and CYP2C19*2 polymorphisms on the 1 year combined thrombotic outcome could be demonstrated and seems of clinical significance. Considering the population size this does not implicate that the other genetic markers are not associated with antiplatelet treatment failure, but that their possible influence is so small that it is unlikely that they are of clinical relevance. The mortality driven association with COX1 -842A>G deserved further research in a study more specifically dedicated to this endpoint. Ongoing studies on pharmacogenetic guidance of antiplatelet therapy using the CYP2C19 reduced function alleles will provide answers whether this strategy can contribute to finding the balance between adequate platelet inhibition and avoidance of excessive bleeding risk.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *International Journal of Cardiology* online.

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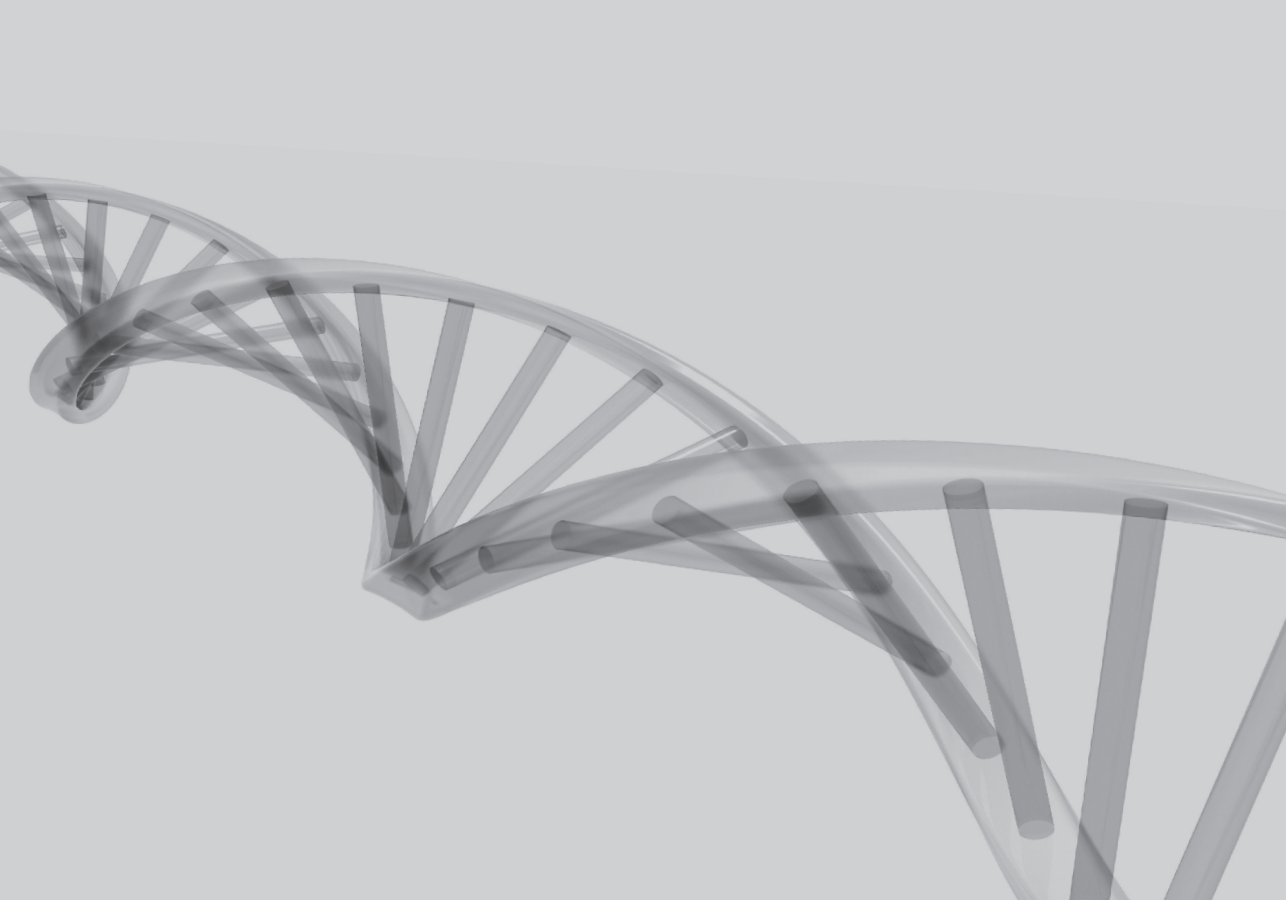
Chapter 13



Summary, conclusions and future perspectives

MAIN FINDINGS

Genetic variation plays an important role in cardiovascular disease development. Due to the complex nature of most cardiovascular diseases (CVD), these genetic factors are part of an intricate interplay with clinical factors and environmental factors, which together determines an individual's risk to develop a certain condition. To date several genetic factors have been associated with different aspects of CVD, however consistent replication has proven to be difficult, often due to a limited sample size.[1-4] The aim of this thesis was to further elucidate the genetic background of coronary restenosis and other cardiovascular diseases using large, well described study populations. Moreover we investigated the role of genetic variation on the individual's response to drug therapy. These three topics are respectively addressed in Part I, II and III of this thesis.

Percutaneous coronary intervention (PCI) is a frequently used therapeutic modality in patients with coronary artery disease. Although the incidence of coronary restenosis after PCI has decreased considerably with improvements of the technique and materials, in particular after the introduction of drug-eluting stents (DES)[5], this complication is still not abolished and remains an important cause of morbidity and need for re-intervention.[6,7] In **chapter 2** and **chapter 3** of Part I, a comprehensive overview of all current knowledge regarding coronary restenosis is given. Despite the vast amount of research on restenosis the causative mechanisms of this complex disease have not yet been fully identified. It is thought that the main contributor to the process is the inflammatory response evoked by vascular damage during angioplasty.[8,9] This inflammatory state subsequently activates several other processes like, proliferation and migration of vascular smooth muscle cells and extracellular matrix (ECM) remodeling, eventually leading to the development of restenosis. Not all individuals have a similar risk of developing restenosis. Diabetes mellitus is the most consistently reported clinical parameter increasing this risk.[10] Moreover, some patients appear to have an inherent predisposition towards developing restenosis, which can be partly explained by their specific genetic background.[11] Many innovative technologies, including DES (with or without specific polymers)[5,12,13] and fully biodegradable stents[14], have been and continue to be developed in the diligent search for an ideal preventive anti-restenosis therapy, that is both effective and safe in the long term. However, if restenosis does occur, patient should receive the most optimal treatment. Whether this treatment should consist of re-implantation of either a bare-metal stent or a DES, a bypass surgery or a newer modality like a drug-eluting balloon should be tailored to the individual.[15,16] Results of ongoing trails might shed more light on which treatment is more suitable in a particular situation. Furthermore, ongoing research on the genetic background of restenosis might make a more personalized approach feasible.

As was described in the general introduction, genetic research has developed from single SNP analysis in candidate genes toward hypothesis-free genome-wide association studies (GWAS). In **chapter 4** we have described the results of the very first GWAS published on restenosis that was performed in the GENetic DEterminants of Restenosis (GENDER) study population. After the GWAS analysis in GENDER, three independent replication steps were performed, leading to the identification of a novel susceptibility locus on chromosome 12. The two associated single nucleotide polymorphisms (SNPs) at the 12q23.3 region, rs10861032 and rs9804922, were also associated with all-cause mortality in GENDER and in the PROspective Study of Pravastatin in the Elderly at Risk study (PROSPER) and also with coronary events in PROSPER, which indicates that this region might play an important role in the broader range of coronary events. Considering the intergenic nature of the locus, we speculate that the SNPs influences a regulatory function of this region. Further research will be needed to disentangle the biological implication of this region in restenosis.

After obtaining the GWAS data we wanted to clarify the previously reported mixed results regarding the association of genetic variation in the ECM remodeling genes, matrix metalloproteinase (MMP) 2 and 3, with restenosis. In **chapter 5** we combined the genotypic data obtained in previous candidate gene studies in GENDER and the available GWAS data from the genetic region of these two genes, together providing a high coverage of their genetic variation. We concluded that a solid association between genetic variation in MMP2 or MMP3 and restenosis was absent, rendering further genetic association studies involving these genes and this endpoint futile.

Alongside the development of new genotyping techniques also novel statistical methods are being developed, better capable of handling the increasing amount of data generated by GWAS. One of these methods is set-based analysis using PLINK software.[17] This analysis is capable of analyzing the joint effect of multiple SNPs. Since a joint effect is biologically more plausible than single SNP associations we decided to apply this analysis in **chapter 6** to examine multiple SNPs in the genomic region of all previously described candidate genes of restenosis. This kind of proof-of-concept study had the aim to explore whether the joint effect of all these SNPs together was associated with restenosis, despite all the inconsistent results from previous studies. In this chapter we demonstrated that the joint effect of 36 candidate genes indeed was associated with restenosis in the GENDER study population.

In **chapter 7** we applied this joint effect analysis to entire biological pathways, to investigate their association with restenosis. In this study we demonstrated a potential role of the cell-ECM interactions pathway in the development of coronary restenosis. Of this pathway the PARVB gene was shown to have the strongest association with restenosis, and several SNPs in this gene replicated in an independent replication cohort. PARVB could therefore be a novel candidate gene for restenosis.

Finally, in the final chapter of part I, **chapter 8**, we present the 10-year follow-up data of the GENDER population. We show that despite previous reports, coronary restenosis after PCI is not associated with an increased risk of mortality, neither on the short term nor on the long term. An attractive conclusion could be that the current treatment of patients with restenosis is so good that patients no longer die from this condition and that we might not concern about restenosis anymore. Although this might be partly true, restenosis still causes considerate morbidity, which still justifies and requires further studies to understand and prevent this phenomenon.

In part II of this thesis the focus shifts to other cardiovascular and vascular conditions related to restenosis. In **chapter 9** we investigated the role of DNA repair mechanisms in the CVD events myocardial infarction and stroke in both GENDER and PROSPER. DNA damage and DNA repair processes have been suggested to play a role in CVD development[18], but only a few studies have explored this possible contributing aspect. We again used the set-based analysis of PLINK to explore the relations of five DNA repair pathways with these endpoints. We demonstrated that the non-homologous end-joining pathway was associated with the occurrence of myocardial infarction in both study populations and that this association was mainly driven by genetic variation in the MRE11A gene. This gene is part of the MRN complex, which has a critical role in the recognition of DNA damage lesions or the chromatin alterations that follow DNA damage[18] and a key role in the cellular response to double strand breaks.[19] To our knowledge, this is the first study to associate the MRE11A with CVD endpoints. Additional studies will be needed to confirm this result and to further explore the implications of this possible relation.

Next, we make a sidestep towards nephrology to study a condition with a likely mechanistic overlap with coronary restenosis, i. e. arteriovenous fistula (AVF) failure in hemodialysis patients. In **chapter 10** we selected a wide range of SNPs in candidate genes for AVF failure, including genes previously related to coronary restenosis and aortic aneurysm formation. We genotyped and analyzed these SNPs in the Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) population and concluded that only the rs1466535 SNP in low density lipoprotein receptor-related protein 1 (LRP1) and the factor V Leiden mutation were significantly associated with AVF failure. Although our SNP selection covered a wide range of candidate genes, the obtained results did not indicate an important role of these SNPs in the development of AVF failure. One explanation for these observations could be that genetic susceptibility does not play an important role in the development of AVF failure. Local factors, such as the abnormal hemodynamics and vascular damage, might have a more important role in the failure of the vascular access required for hemodialysis than the genetic background.

Part III of this thesis focusses on pharmacogenetics of cardiovascular related drugs. Pharmacogenetics is the upcoming field of research that examines genetic variation that influences responses to drug therapy in the individual patient, with the very appealing

goal to realize personalized therapy using genotypic guidance of pharmacotherapy by selecting agents with the greatest potential for efficacy and the least risk of toxicity. In **chapter 11** a comprehensive overview of the available pharmacogenetic evidence of the five major drug classes of cardiovascular diseases is provided. It discusses in detail the variability in efficacy of statins and their risk of myopathy, the genetic determinants of resistance to antiplatelet agents, the dosing issues of oral anticoagulants, genetic variation related to suboptimal responses to β-blockers and the variable response in blood pressure and clinical events in patients taking angiotensin converting enzyme (ACE) inhibitors. The overall conclusion of the review is that there are enough reasons to remain optimistic that, even though we are taking small steps at a time, we are heading to an era where we can finally use pharmacogenetics in clinical practice to optimize treatment for the individual patient.

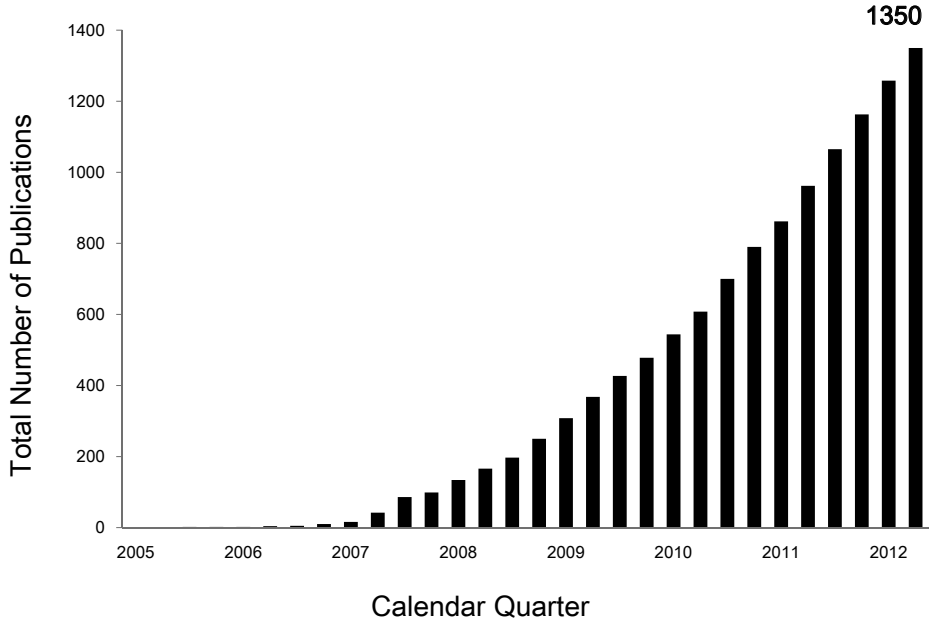
In **chapter 12**, we explored the findings regarding the genetic determinants of antiplatelet drug resistance, obtained by the systematic review described in chapter 11, in a large real-life population of ST-segment elevation myocardial infarction patients. We investigated whether the eight best replicated SNPs, related to aspirin- and clopidogrel efficacy, were associated with recurrent thrombotic events in this unselected patient population and demonstrated that the CYP2C19*2 allele indeed was strongly associated with thrombotic events. In addition, the -842A>G polymorphism of COX1 was associated with the composite endpoint of cardiac death and recurrent myocardial infarction as well. Especially patients carrying two or more risk alleles of these two SNPs were at high risk. We showed that also in a clinical setting, testing for these polymorphisms could be of value in the identification of STEMI patients at risk for recurrent cardiovascular events.

FUTURE PERSPECTIVES

Genetic association studies have come a long way the last decades and our understanding of the genetic background in many different diseases increased substantially. The completion of the Human Genome Project in 2003[20] boosted our knowledge and made the development of GWAS possible.[21] Since then, the scientific world committed itself on this technique, studying all conceivable phenotypes (Figure 1). The National Human Genome Research Institute, part of the American National Institutes of Health, which also performed the Human Genome Projects, keeps record of all the published GWAS in a publicly available database (www.genome.gov/gwastudies).[22] As of 21st of March 2013, this catalog includes 1542 publications and 8828 associated SNPs.

However, the last years we reached a point where we had to broaden our thoughts beyond the traditional GWAS analyses. The single SNP GWAS analyses has resulted in many interesting and novel associations, but considering that common diseases are

Figure 1 Published genome wide association studies up to june 2012



From: http://genome.gov/gwastudies, Hindorff et al. Proc Natl Acad Sci U S A. (2009) 9;106(23):9362-7.

multigenic traits, which involve groups of genes functioning at various stages of disease development, other more advanced statistical methods would be more appropriate to analyze this data. In this thesis we have applied some of these newer statistical methods to account for this joint effect of multiple genetic markers. The genetic research field is however far from the ultimate analyzing tool for handling the large amount of data generated by GWAS.

Future developments will surely result in even better and more sophisticated methods to help us interpret the data. Despite these newer tools, the explained variance remains low and the effect size of the encountered associations remains limited, requiring large study populations. Therefore, the formation of consortia that cooperate in combining data of separate studies will also greatly aid in the generation of more reliable results than the individual studies will do.

Another recent development in the genetic research field is the availability of next-generation sequencing methods.[23] With the falling costs of sequencing technology, the genetic research field is currently shifting from microarray-based genotyping studies to whole genome sequencing.[24,25] However, one you should keep in mind that sequencing studies aim and finding rare variants, present in <1% of the population, that are not present on GWAS-chips consisting mainly of common variants. Whether the missing heritability will be explained by rare variants with large effect sizes of my many common variants with small effect sizes remains to be seen.[26] For our complete understanding of

the complete biological background of CVD genetics alone will however not be enough, systems-level approaches, including the analysis of proteomes, are required for a more comprehensive understanding.[27] An important project, regarding this integrated approach, is the Encyclopedia of DNA Elements (ENCODE) project.[28] The ENCODE project has systematically mapped regions of transcription, transcription factor association, chromatin structure and histone modification. Using this data biochemical functions could be assigned to 80% of the genome.[28] This dataset could be very helpful in understanding the mechanistical implications of genetic associations. A major problem of most genetic association studies is the difficulty of linking the results to a biological plausible mechanism of action. Especially the top hits of the GWAS studies are usually SNPs with an unknown function and are located in intergenic regions or even in so called gene-desserts. It is very easy to conclude that more 'functional' studies should be performed to further elucidate the actual effect of the obtained association, but these studies are often very time consuming, expensive and often fail to obtain the desired result. Therefore the evidence should be convincing enough to pursue a positive hit in more detail. Possible follow up analyses include investigating whether the SNPs affect expression or function of the gene they are located in. If this is not the case, they could influence other (nearby) genes, which could for instance be explored by mRNA expression analyses or allelic expression imbalance.[29] Furthermore, sequencing of the genes of interest could result in identifying functional variants in linkage disequilibrium with the SNPs identified in the current GWAS studies. When a functional gene or region is identified with enough certainty, experimental models *in vitro* or *in vivo* in animal models, if available, could be employed.

In the meantime, clinical application of genetic data is already possible without our complete understanding of their exact effect, in the form of risk prediction. Risk prediction is an important aspect of cardiovascular disease treatment and prevention. Many risk scores for several disease outcomes have been developed over the years, all using clinical parameters for the prediction of the risk of the individual patient.[30-32] Our increasing knowledge of the molecular background of cardiovascular diseases might provide a potential improvement of the current risk models. Several genetic markers have been identified to be associated with the risk of developing cardiovascular events.[2,33,34] To date, several studies explored the added value of genetic polymorphisms to clinical risk scores, reporting mixed results.[35-37] Further studies focusing on the genetic risk scores are needed. Risk scores including high impact genetic markers, like CYP2C19*2 on thrombotic events during clopidogrel treatment as demonstrated in chapter 12, will likely have the highest chance to improve after including these markers.

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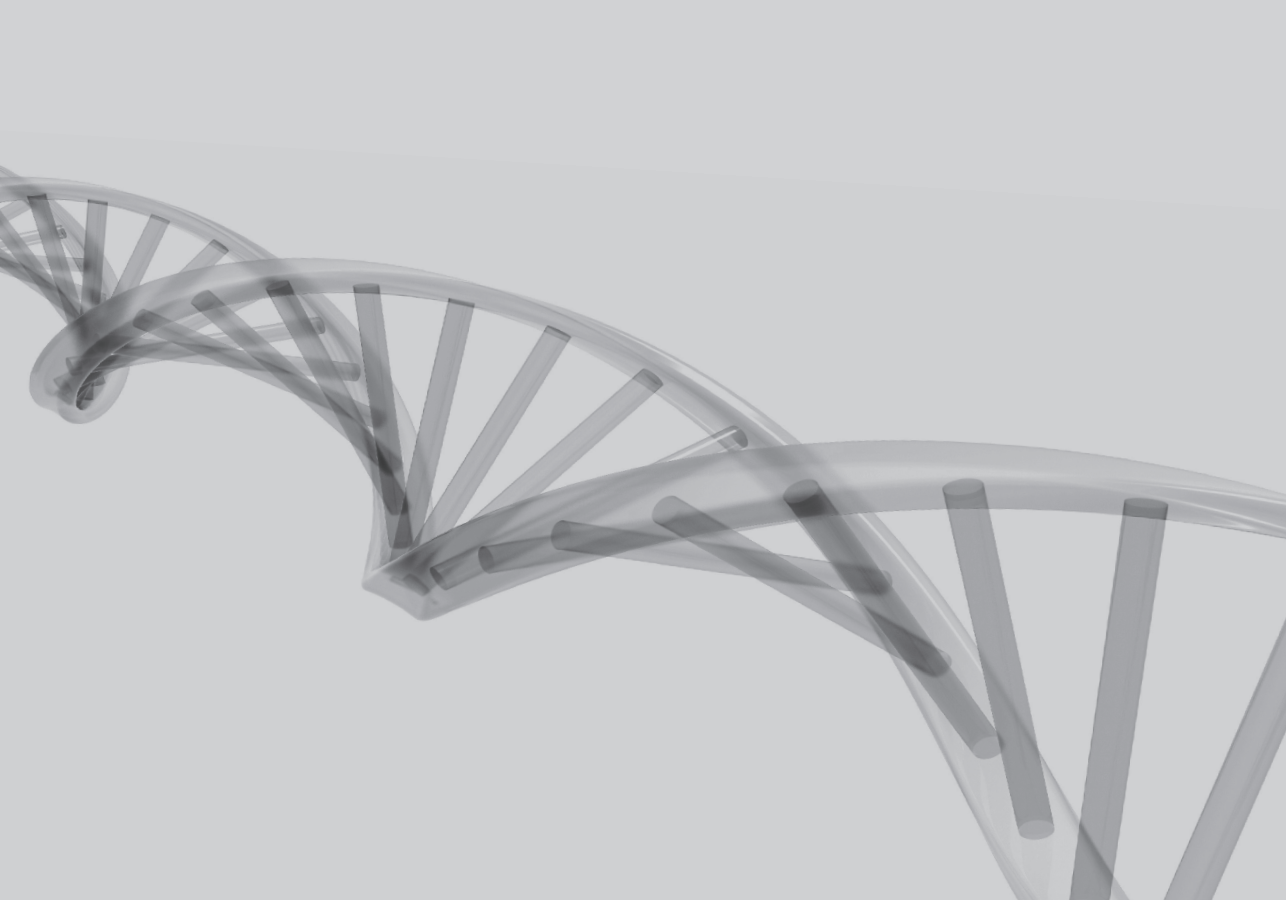


Nederlandse samenvatting en toekomstperspectieven

HOOFDBEVINDINGEN

Genetische variatie beïnvloedt de kans op het krijgen van hart- en vaatziekten (HVZ). Deze genetische factoren bepalen samen met meerdere klinische factoren (bijvoorbeeld diabetes mellitus en een verhoogd cholesterol) en omgevingsfactoren (bijvoorbeeld roken) het risico op het ontwikkelen van HVZ van de individu. Doordat al deze factoren in een ingewikkeld samenspel dit risico beïnvloeden worden HVZ onder de complexe ziektebeelden gerekend. Tot op heden zijn er meerdere genetische factoren in verband gebracht met verschillende aspecten van HVZ, maar consistente bevestiging van deze gerapporteerde bevindingen is schaars. Dit is waarschijnlijk voornamelijk te wijten aan het kleine aantal patiënten dat in de studies is onderzocht. Het doel van dit proefschrift was het verder uitzoeken van de genetische achtergrond van coronaire restenose en andere hart- en vaatziekten met behulp van grote, goed omschreven studie populaties. Bovendien onderzochten we de rol van genetische variatie op het verschil in respons op de behandeling met bepaalde medicijnen. Deze drie thema's worden respectievelijk behandeld in deel I, II en III van dit proefschrift.

Percutane coronaire interventie (PCI) is een veel gebruikte behandeling van patiënten met coronair lijden. Een complicatie van PCI is het opnieuw gaan dichtzitten van het behandelde coronair (kransslagvat), ook wel restenose genoemd. Hoewel de incidentie van coronaire restenose na PCI aanzienlijk is afgenomen de laatste jaren, met name na de introductie van drug-eluting stents (DES), kunnen we deze complicatie nog niet geheel voorkomen en blijft het een belangrijke oorzaak van morbiditeit en behoefte aan re-interventie. In de eerste 2 hoofdstukken van deel I wordt er een uitgebreid overzicht van alle huidige kennis met betrekking tot coronaire restenose beschreven. **Hoofdstuk 2** beschrijft de achtergrond en risicofactoren van restenose. Ondanks het vele onderzoek naar restenose weten we nog steeds niet precies welke mechanismen er ten grondslag van deze complexe ziekte liggen. Men denkt dat de ontstekingsreactie, veroorzaakt door vasculaire schade die tijdens de PCI kan ontstaan, een grote rol speelt. Deze overactieve inflammatoire staat activeert vervolgens verschillende andere processen zoals, proliferatie en migratie van vasculaire gladde spiercellen en extracellulaire matrix (ECM) vorming. Samen kunnen deze processen leiden tot het ontslaan van restenose. Niet iedereen heeft hetzelfde risico om restenose te ontwikkelen. Patiënten met diabetes hebben bijvoorbeeld een verhoogd risico. Verder blijkt uit meerdere studies dat bepaalde genetische factoren dit risico beïnvloeden. In **hoofdstuk 3** wordt met name ingegaan op de praktische kant van het probleem, zoals de verschillende ontwikkelingen om restenose te voorkomen en de behandeling van restenose. Veel innovatieve technologieën, waaronder DES en volledig biologisch afbreekbare stents, zijn reeds ontwikkeld en onderzoek naar de beste preventieve anti-restenose therapie, die zowel effectief en veilig is op de lange termijn, is nog steeds in volle gang. Mocht

dan toch restenose ontstaan, zijn er verschillende behandelopties. Voorbeelden hiervan zijn re-implantatie van ofwel een bare-metal stent of een DES, een bypass operatie of de recent ontwikkelde drug-eluting ballon. Er is vooralsnog geen eenduidig advies over welke behandeling in welke situatie de voorkeur heeft. De resultaten van lopende onderzoeken zullen hier mogelijk meer houvast in gaan bieden.

Zoals werd beschreven in de algemene inleiding, heeft genetisch onderzoek zich ontwikkeld van analyses van losse polymorfismen in kandidaat genen naar hypothesevrije analyses van het hele genoom met *genome wide association studies* (GWAS). In **hoofdstuk 4** beschrijven we de resultaten van de eerste GWAS die is gepubliceerd over restenose. Deze studie werd uitgevoerd in de *GENetic DEterminants of Restenosis* (GENDER) studie populatie. Na de analyses in GENDER werden de bevonden resultaten bevestigd in drie onafhankelijk studies, waarbij we uiteindelijk tot de conclusie kwamen dat er een, niet eerder beschreven, regio op chromosoom 12 geassocieerd was met restenose. De twee polymorfismen in deze regio werden behalve met restenose ook in verband gebracht met een verhoogd risico op sterfte in de GENDER studie en ook in de *PROspective Study of Pravastatin in the Elderly at Risk study* (PROSPER). Mogelijk heeft deze regio dus een bredere rol in HVZ. Omdat deze regio niet op een bepaald gen zit, maar tussen twee genen in ligt, denken wij dat het met name een rol speelt in de regulatie van genen. Verder onderzoek zal nodig zijn om dit uit te zoeken.

Na het verkrijgen van de GWAS data wilden wij dit gebruiken om eerdere tegenstrijdige resultaten uit te zoeken over de rol van genetische variatie in twee ECM vormende genen, matrix metalloproteinase (MMP) 2 en 3. In **hoofdstuk 5** hebben we de genotypische data uit eerdere kandidaat-gen studies in GENDER gecombineerd met de beschikbare GWAS gegevens om zo nog een gedetailleerder overzicht van de genetische variatie binnen deze genen te krijgen. We concludeerden dat er geen duidelijke associatie was tussen de genetische variatie in MMP2 of MMP3 en restenose. Verder onderzoek hiernaar zal dus tot niets zinvols leiden en is dus nu niet langer nodig.

In **hoofdstuk 6** hebben we een andere statistische methode gebruikt om te kijken naar het gezamenlijke effect van meerdere polymorfismen. Aangezien we naar een complexe ziekte onderzoek doen, is het biologisch gezien plausibeler dat meerdere genen samenwerken in het ontstaan van restenose. Deze methode is dus mogelijk een betere manier om naar de relatie met restenose te kijken. In dit hoofdstuk hebben we meerdere polymorfismen, gelegen in alle eerder beschreven kandidaat-genen van restenose, tezamen geanalyseerd. We kwamen tot de conclusie dat het gecombineerde effect van de genetische variatie van 36 kandidaat-genen inderdaad is geassocieerd met restenose in de GENDER studie populatie, ondanks alle inconsistente resultaten uit eerdere studies.

In **hoofdstuk 7** hebben we deze techniek vervolgens toegepast om de invloed van hele biologische pathways op het ontstaan van restenose te onderzoeken. In deze studie

hebben we aangetoond dat er een mogelijke rol is van de cel-ECM interactie pathway in de ontwikkeling van restenose. Van de verschillende genen in deze pathway bleek het PARVB gen het sterkst geassocieerd met restenose. Verschillende polymorfismen in dit gen werden vervolgens ook gerepliceerd in een onafhankelijke studie populatie.

Tot slot presenteren we in het laatste hoofdstuk van deel I, **hoofdstuk 8**, de 10-jaars follow-up van de GENDER populatie. We laten zien dat ondanks eerdere publicaties, coronaire restenose na PCI niet is geassocieerd met een verhoogd risico op sterfte, niet op de korte termijn en ook niet op de lange termijn. Een aantrekkelijke conclusie zou kunnen zijn dat de huidige behandeling van patiënten met restenose is zo goed dat patiënten niet meer sterven aan deze aandoening en dat we ons daarom dus ook niet meer druk hoeven te maken over deze complicatie. Echter, ook al is dit misschien gedeeltelijk waar, restenose veroorzaakt nog steeds aanzienlijke morbiditeit. Dus is er nog steeds meer onderzoek naar restenose nodig.

In deel II van dit proefschrift richten we ons op andere cardiovasculaire en vasculaire aandoeningen. In **hoofdstuk 9** hebben we onderzoek gedaan naar de rol van processen die DNA schade herstellen in het ontstaan van een myocardinfarct of een beroerte. DNA schade en DNA herstel processen spelen mogelijk een rol in HVZ, maar slecht enkele studies hebben hier onderzoek naar gedaan. Ook in deze studie hebben we de analyses naar het gezamenlijke effect van meerdere polymorfismen gebruikt om de relatie van vijf DNA herstel pathways te analyseren in de GENDER en de PROSPER studie populaties. We laten zien dat de non-homologous end-joining pathway geassocieerd is met het optreden van een myocardinfarct in beiden populaties en dat deze associatie vooral werd veroorzaakt door de genetische variatie in het MRE11A gen. Dit gen heeft een belangrijke rol bij de herkenning van DNA schade en tevens een rol in de respons op dubbelstrengs breuken van het DNA. Dit is de eerste studie die een relatie laat zien van dit gen met HVZ. Aanvullende studies zullen nodig zijn om dit resultaat te bevestigen en om vervolgens deze mogelijke relatie verder biologisch uit te zoeken.

In **hoofdstuk 10** maken we een uitstap naar de nierziekten om onderzoek te doen naar het falen van dialyse fistels. Dit probleem lijkt namelijk op veel vlakken op restenose en mogelijk spelen dus ook dezelfde mechanismen een rol in het ontstaan van het falen van deze fistels. In dit onderzoek hebben we diverse polymorfismen onderzocht, gelegen in kandidaat genen voor dialyse fistel falen, maar ook in genen eerder geassocieerd met restenose en aneurysma formatie van de aorta. Voor deze studie hebben we gebruik gemaakt van de *Netherlands Cooperative Study on the Adequacy of Dialysis* (NECOSAD) populatie. Van alle onderzochte polymorfismen waren alleen rs1466535 in het LRP1 gen en de factor V Leiden mutatie significant geassocieerd met fistel falen. In vergelijking met restenose spelen genetische factoren bij fistel falen mogelijk een kleinere rol. Lokale factoren, zoals de abnormale hemodynamiek en vaatschade, zijn mogelijk meer van invloed op het ontslaan van fistel falen.

Deel III van dit proefschrift richt zich op de farmacogenetica van cardiovasculaire medicijnen. Farmacogenetica is het onderzoek naar genetische variatie die de respons beïnvloedt van een individuele patiënt op een bepaald geneesmiddel. De belangrijkste toepassing van deze farmacogenetische kennis is de ontwikkeling van gepersonaliseerde behandeling, doordat op basis van de genetische achtergrond het beste middel geselecteerd zou kunnen worden. In **hoofdstuk 11** wordt een uitgebreid overzicht beschreven van de beschikbare farmacogenetische gegevens van de vijf belangrijkste groepen van cardiovasculaire geneesmiddelen. Er wordt in detail ingegaan op de variabiliteit in het effect van statines en hun risico op myopathie, de genetische determinanten van resistentie tegen plaatjesaggregatieremmers, de dosering problemen van orale anticoagulantia, de genetische variatie met betrekking tot suboptimale reacties op β-blokkers en de variabele respons van de bloeddruk en klinische uitkomstmaten bij patiënten die angiotensine converting enzyme (ACE)-remmers gebruiken. De algemene conclusie van dit overzicht is dat, ook al nemen we steeds kleine stapjes in de vooruitgang, er genoeg redenen zijn om optimistisch te zijn dat de tijd waarin we farmacogenetica kunnen gebruiken in de klinische praktijk om de behandeling van de individuele patiënt te optimaliseren in zicht komt.

Tenslotte hebben we in **hoofdstuk 12** de acht best geschreven polymorfismen, die van invloed zijn op de werking van aspirine en clopidogrel, geanalyseerd in een grote ongeselecteerde populatie van patiënten met ST-segment elevatie myocardinfarct. We hebben onderzocht of deze polymorfismen waren geassocieerd met trombotische uitkomsten en we laten zien dat het *2 risico allel van het CYP2C19 gen inderdaad sterk geassocieerd was met nieuwe trombotische complicaties na het myocardinfarct. Daarnaast werd er ook een relatie gezien met het -842A>G polymorfisme in het COX1 gen. Vooral patiënten die twee of meer risico allelen van deze twee polymorfismen dragen hadden een hoog risico. Met deze studie laten we zien dat in patiënten die gezien worden in de dagelijkse klinische praktijk het testen op deze polymorfismen waardevol kunnen zijn bij de identificatie van de patiënten met een verhoogd risico op nieuwe cardiovasculaire problemen.

TOEKOMSTPERSPECTIEVEN

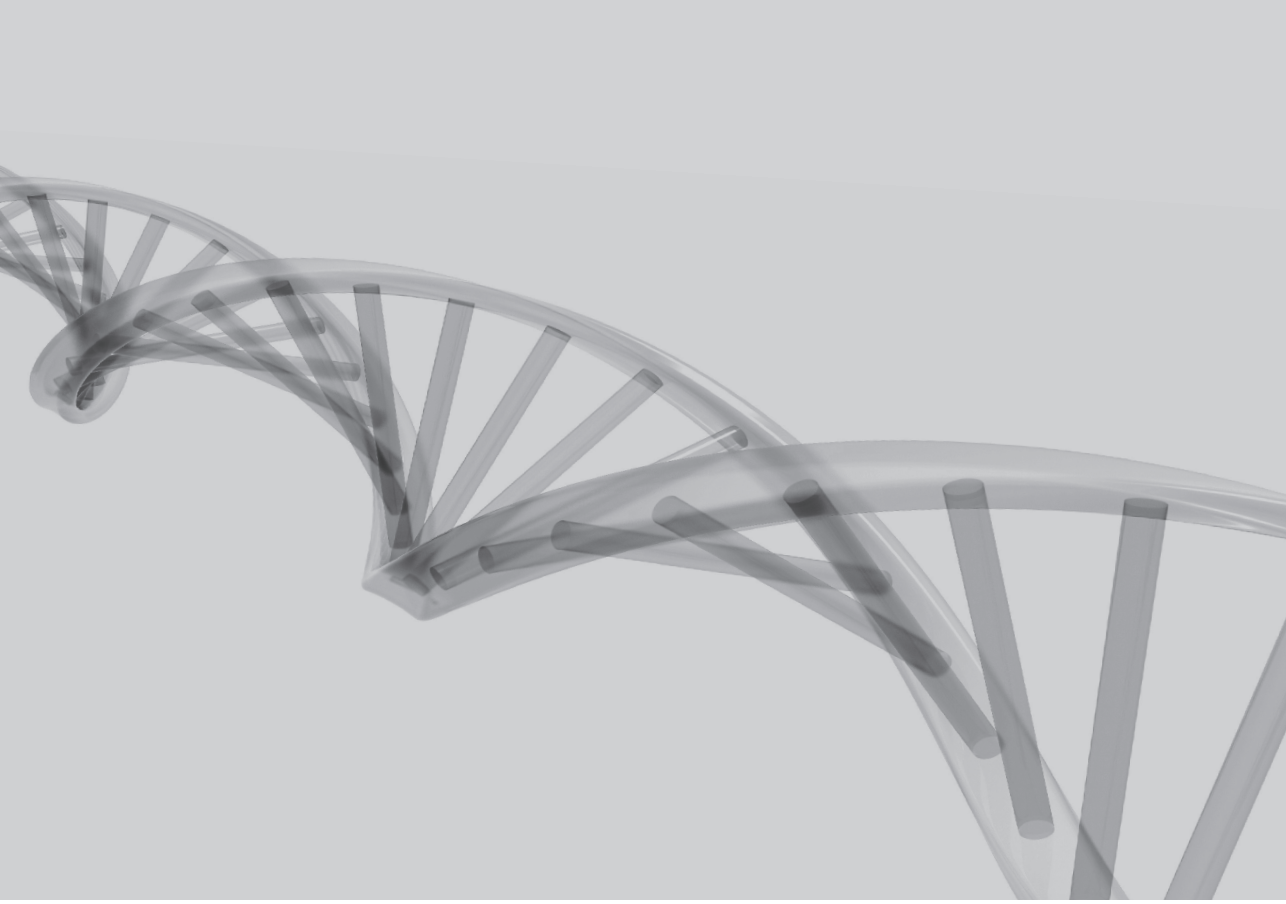
Tijdens de laatste decennia is er enorme progressie geboekt in het genetische onderzoek en onze kennis van de genetische achtergrond van veel ziekten is aanzienlijk toegenomen. Na de voltooiing van het *Human Genome Project* in 2003 en de ontwikkeling van GWAS in 2005 zijn er al ruim 1500 van deze studies gepubliceerd over bijna alle denkbare ziektebeelden. Echter, gedurende laatste jaren hebben we een punt bereikt waarop we onze gedachten weer moeten verbreden buiten de traditionele GWAS ana-

lyses. Deze analyses hebben geleid tot vele interessante en nieuwe bevindingen, maar gezien het feit dat de meeste veelvoorkomende ziekten door meerdere genen worden beïnvloed, zijn andere meer geavanceerde statistische methoden waarschijnlijk meer geschikt om de GWAS data te analyseren. In dit proefschrift hebben we een aantal van deze nieuwere statistische methoden toegepast om naar het gezamenlijke effect van meerdere polymorfismen te kijken. Ook al zijn we nu een stap verder met deze analyses, we zijn nog verre van de ultieme analyse methode om de grote hoeveelheid informatie, die gegenereerd wordt tijdens een GWAS, volledig mee te analyseren. Toekomstige ontwikkelingen zullen zeker nog betere en meer geavanceerde methoden opleveren die ons bij de interpretatie van deze gegevens helpen. Tot nu toe blijft echter de variantie die verklaard wordt met de genetische variatie beperkt en ook is de grootte van het effect van de losse polymorfismen laag waardoor grote studie populaties nodig zijn om relaties aan te tonen. De vorming van consortia waarbinnen de gegevens van meerdere studies gecombineerd worden zullen dan ook meer betrouwbare resultaten opleveren dan de individuele studies.

Een andere recente ontwikkeling in het genetisch onderzoek is de beschikbaarheid van next-generation sequencing, waarmee in nog meer detail het genoom geanalyseerd kan worden. Voor ons volledige begrip van de biologische achtergrond van HVZ zal het analyseren van alleen genetische data echter niet voldoende zijn. Integratie met andere onderzoeksgebieden, zoals eiwitanalyses en epigenetica, zal nodig zijn om daadwerkelijk de biologische processen te begrijpen. Een groot probleem van de meeste genetische associaties studies is dan ook om de gevonden genetische associaties te vertalen naar biologische processen. Vooral de top hits van de GWAS studies zijn van polymorfismen met een onbekende functie, die niet in een gen liggen maar tussen genen in of zelfs gen-*deserts*. Het is heel gemakkelijk om te concluderen dat meer 'functionele' studies moeten worden uitgevoerd om deze relaties verder uit te zoeken. Deze studies zijn echter vaak zeer tijdrovend en duur en leiden vaak niet tot het gewenste resultaat. Daarom zal het bewijs van een genetische associatie overtuigend genoeg moeten zijn voordat er met verder onderzoek gestart wordt. Enkele voorbeelden van mogelijke follow-up analyses omvatten het onderzoeken of de polymorfismen de expressie of functie van een gen beïnvloeden, bijvoorbeeld met mRNA expressie analyses of allelische expressie onbalans. Sequentiebepaling van een regio kan leiden tot de identificatie van een functionele variant die in linkage disequilibrium is met het geassocieerde polymorfisme. Wanneer een functioneel gen of regio met voldoende bewijs is geïdentificeerd kunnen experimentele modellen *in vitro* of *in vivo,* in diermodellen indien beschikbaar, worden toegepast.

Echter ook zonder ons volledige begrip van het exacte effect van een polymorfisme, zouden we deze genetische gegevens al wel in de praktijk kunnen toepassen in de vorm van risicovoorspelling. Risicovoorspelling is een belangrijk aspect tijdens de preventie

en behandeling van HVZ. Door de jaren heen zijn er meerdere risicoscores ontwikkeld voor verschillende ziektebeelden voor het voorspellen van het risico van de individuele patiënt, allemaal met behulp van klinische parameters. Onze kennis van de moleculaire achtergrond van HVZ zou kunnen bijdragen aan de verbetering van de huidige risicomodellen. De tot op heden gepubliceerde studies naar de toegevoegde waarde van genetische polymorfismen op klinische risicoscores, rapporteren echter gemengde resultaten. Er zijn dus meer studies nodig. Met name genetische markers met een groot effect, zoals CYP2C19*2 op trombotische uitkomsten tijdens clopidogrel behandeling, aangetoond in hoofdstuk 12, hebben waarschijnlijk de grootste kans om de klinische risicoscores voor de betreffende eindpunten te verbeteren.

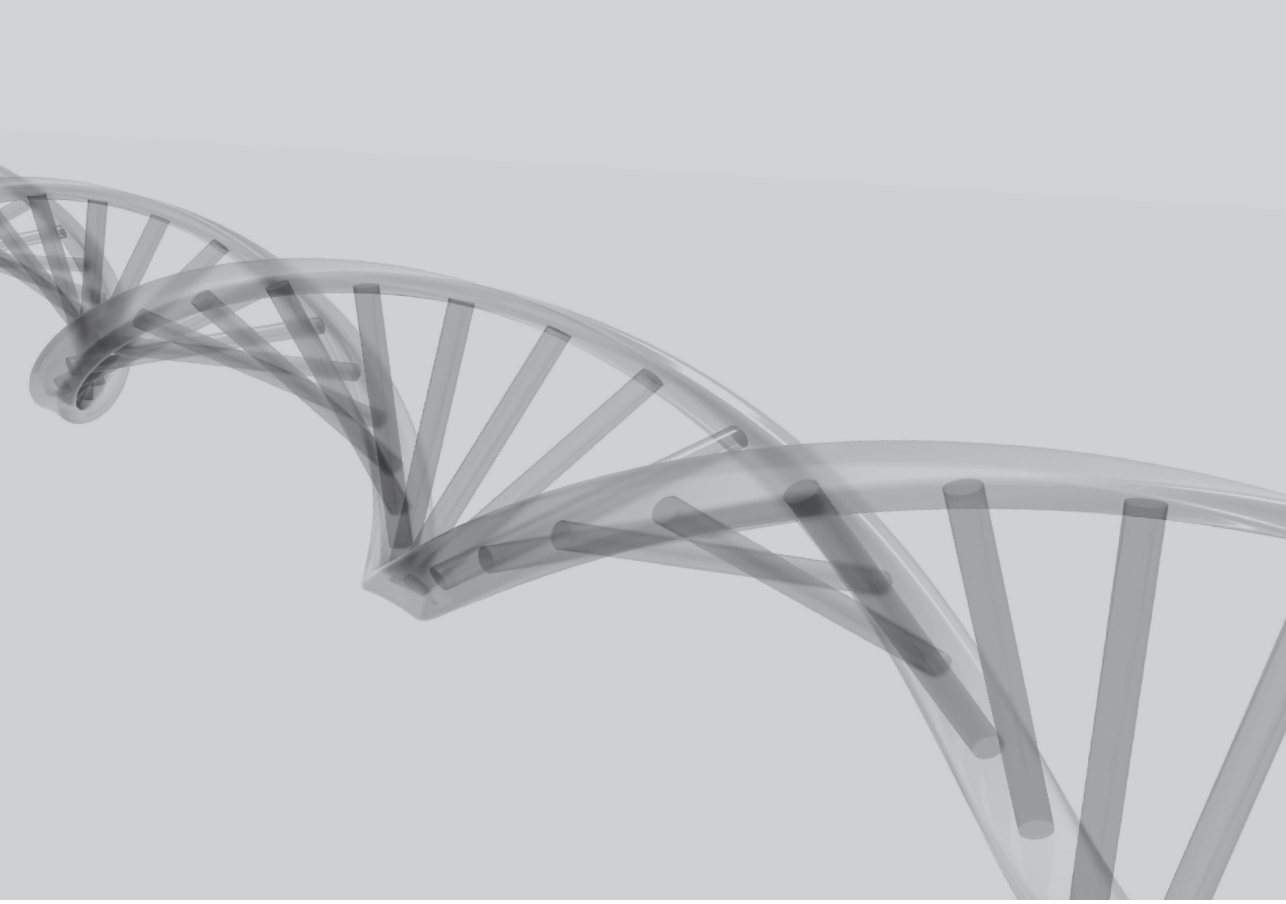


Curriculum Vitae

Jeffrey Johan Willem Verschuren werd geboren op 22 mei 1983 te Lisse. Na het behalen van zijn VWO diploma aan het Fioretti college te Lisse in 2001, begon hij in hetzelfde jaar aan de studie Biomedische wetenschappen aan de Universiteit Leiden. Tijdens het tweede jaar van Biomedische Wetenschappen heeft hij tevens succesvol het 1e jaar van de studie Psychologie aan de Universiteit Leiden gevolgd en dit in 2003 afgesloten met het behalen van de propedeuse van deze studie.

Na het behalen hij zijn Bachelor of Science diploma van Biomedische Wetenschappen in 2004, werd gedurende het eerste jaar van de masteropleiding het duidelijk dat de interesse met name naar de klinische kant van het onderzoek uit ging. Daarom viel dat jaar de beslissing om toch ook Geneeskunde te gaan studeren. Via de zij-instroom mogelijkheid kon hij in 2005 beginnen in het 3e jaar van de studie Geneeskunde. In april 2009 deed hij zijn arts-examen en studeerde hij tevens cum laude af voor de Masteropleiding Biomedical Sciences. Aansluitend werkte hij 8 maanden als arts-assistent-niet-in-opleiding op de afdeling interne geneeskunde in het Bronovo ziekenhuis te Den Haag.

In 2010 begon hij met zijn promotietraject op afdeling hartziekten van het Leids Universitair Medisch Centrum onder leiding van prof. dr. J. Wouter Jukema, waarvan de resultaten zijn beschreven in dit proefschrift. Per 1 december 2012 is hij begonnen aan zijn opleiding tot cardioloog waarvoor hij momenteel werkzaam is op de afdeling Interne Geneeskunde in het Kennemer Gasthuis te Haarlem onder leiding van dr. R.W. ten Kate.



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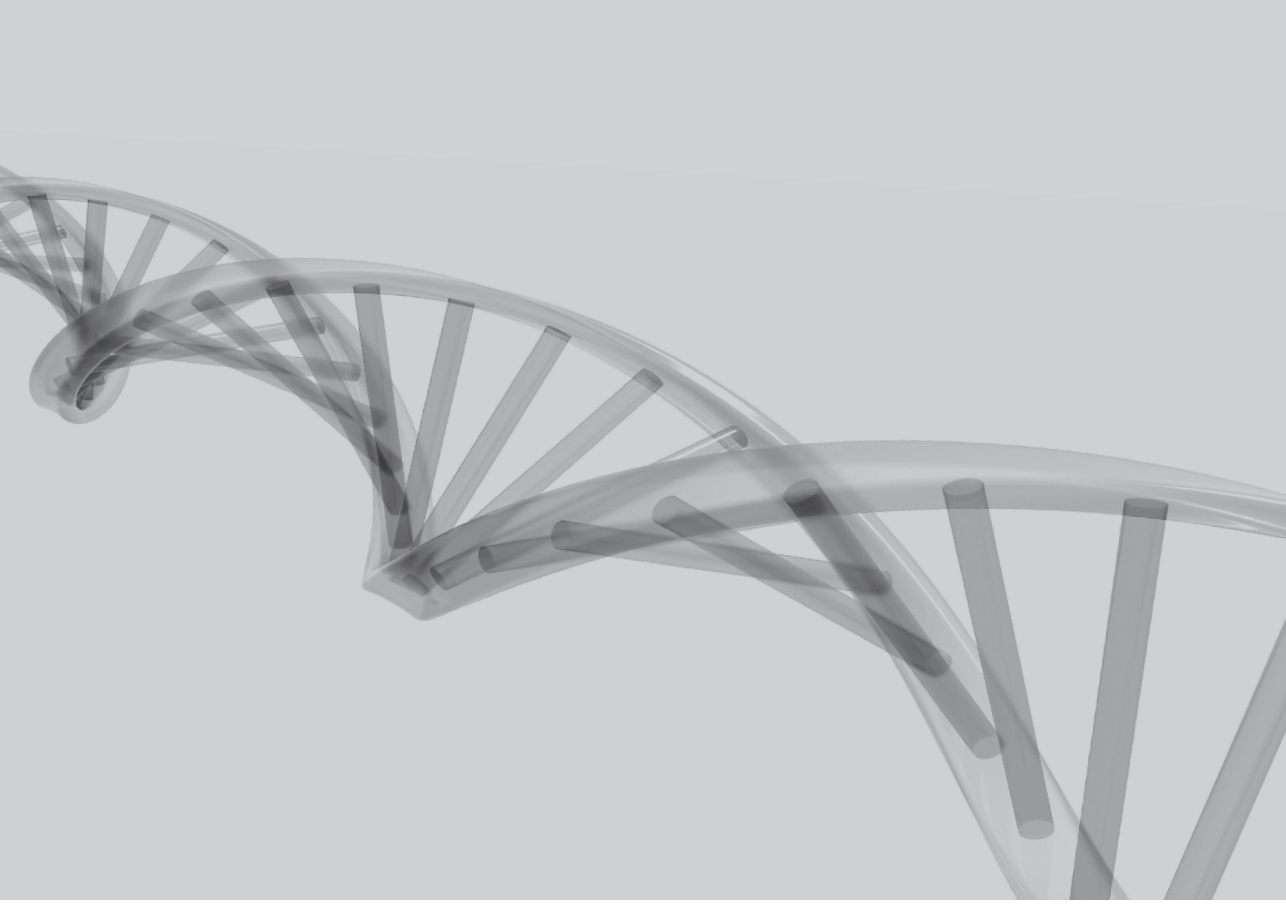

Dankwoord

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