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(Epi)genetic factors in vascular disease

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(Epi)genetic Factors in Vascular Disease

Douwe Pons

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(Epi)genetic Factors in Vascular Disease

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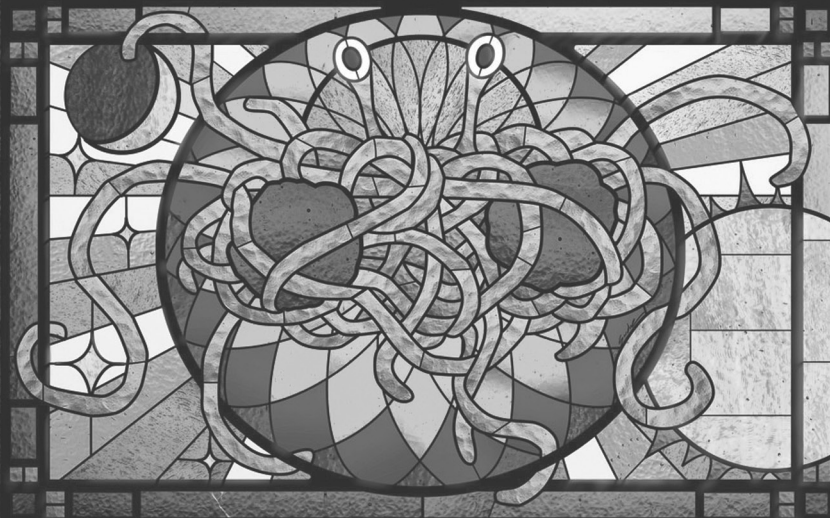
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Chapter 1

General introduction and outline of the thesis

PART 1

Restenosis, stent malapposition and other aspects of vascular disease.

After a percutaneous coronary intervention (PCI) with stent placement re-endothelialization should occur as part of a normal wound healing response. In a considerable amount of patients treated with a bare metal stent (BMS) a dysregulation of this response leads to neointimal hyperplasia and partial reocclusion of the intervention site, which is known as restenosis. As nowadays the use of stents, either bare metal or drug-eluting, has become standard, which largely rules out restenosis due to vascular recoil, we will focus on in-stent restenosis, which is mainly due to neointimal proliferation. After bare metal stenting, the incidence of target vessel revascularization, which is considered to be the most important endpoint by regulatory agencies, is approximately 10%. From the combined data of randomized controlled trials we know that patients treated with a drug eluting stent (DES) have approximately half this risk.¹ However, disadvantages related to late acquired stent malapposition^{2,3} and delayed endothelialization with longer required use of P2Y12 antagonists, do not favour the use of DES in every patient. Taking into account that DES have not eradicated restenosis completely and that at least certain groups of patients benefit more from BMS, it is important to improve risk stratification and to tailor individual therapy. However, only few clinical and lesion-related risk factors have been found to predict the development of restenosis. From many studies, with different indications for PCI and different endpoint definitions, we have learned that Diabetes Mellitus is the only strong and consistent clinical predictor of restenosis.⁴⁻⁶ Hypertension has also been reported to increase the risk for restenosis.^{6,7} In addition, several lesion-related and procedural characteristics such as stenosis severity (before stenting) and residual stenosis (after stenting), which were regularly reported to be associated with restenosis risk,^{6,8,9} can be used as clues to select the appropriate treatment.

Clinical risk factors such as diabetes and hypertension are also since long known to play a role in the development of atherosclerosis, a disease process with several similarities to restenosis, leading to world-wide frequently occurring diseases such as angina pectoris, myocardial infarction and stroke. Restenosis, coronary atherosclerosis and also atherosclerosis in other arteries are proliferative processes driven by inflammation. However, the relative importance of risk factors differs between these diseases. In contrast to restenosis, the development of atherosclerosis is strongly influenced by circulating lipids and smoking. And the precise risk profile in atherosclerosis even differs depending on the location of the plaque. Stroke is relatively more determined by hypertension, whereas plasma cholesterol is more important in coronary disease.

The importance of genetics

The different aspects of vascular disease have in common that multiple genetic factors play an important role in their development. The actual impact of any clinical risk factor on an individual depends for a large part on his/her genetic susceptibility to this factor. The value of genetics in cardiovascular disease is corroborated by the strong predictive value of a positive family history and further confirmed by twin studies showing that death from coronary artery disease at an early age of one twin is a strong predictor of the risk in the other twin.¹⁰ Many genes in inflammatory and proliferative processes, but also in processes important in hemostasis, cell signaling, lipid metabolism and endothelial function, have already been found to play a role in vascular disease.^{11, 12}

The first chapter of part 1 of this thesis (chapter 2) will review current views on the role of genetics in restenosis after BMS placement and acquired malapposition after treatment with a DES. The remainder of part 1 (chapter 3-7) will discuss new data further establishing the important influence of genetic factors in the development of adverse events after PCI and other aspects of vascular disease such as stroke. Each chapter addresses a specific process and its relative importance in one of these vascular diseases, mostly restenosis after PCI in the GENDER-study, which included 3104 patients after successful PCI for stable angina pectoris or non-ST-elevation myocardial infarction.

Part 1	Process	Endpoint	Population
Chapter 3	Hemostasis	Restenosis	GENDER
Chapter 4	VSMC prol./hemostasis	Restenosis/stroke	GENDER/PROSPER
Chapter 5	Matrix formation	Restenosis	GENDER
Chapter 6	VSMC function	Restenosis	GENDER
Chapter 7	Endothelial function	Restenosis	GENDER
Chapter 8	Inflammation	Stent malapposition	MISSION

Considering that a reliable risk estimate cannot be made on the basis of clinical factors, genetic epidemiology can provide new risk markers to improve risk stratification. It can also lead to new insights in the pathophysiology and thereby provide new targets for therapy.

PART 2

Epigenetics

Since long we know that the final phenotype of an organism, and also its tendency to develop disease, is the result of the interplay between nature and nurture. It has now become clear that environmental influence (nurture) can exert its effect not only by

influencing the code of DNA, but also (far more easily) by regulating gene expression without changing the code.^{13, 14}

Early research by Kaati et al., investigating early nutritional influences on cardiovascular mortality, already demonstrated heritability of environmental effects.¹⁵ They exploited records of annual harvests from an isolated community in northern Sweden that go back as far as 1799 to explore the effects of food availability across three generations. Kaati and coworkers showed a remarkable effect of food availability during the slow growth period (SGP) just before puberty of the paternal grandfather on the longevity of the probands. Scarcity of food in grandfather's SGP was associated with a significantly extended survival of his grandchildren for many years, whilst food abundance was associated with a greatly

shortened life span of the grandchildren.¹⁵ These findings are most probably an example of non-DNA sequence-related heredity, which we now refer to as "epigenetics". In contrast to classical mendelian views on inheritance, epigenetics focuses on the heredity of environmental effects, a phenomenon that is called 'epigenetic inheritance'. Although the precise mechanism remains unknown, it seems likely that the phenomenon observed by Kaati et al. is the result of DNA-methylation, the best understood example of epigenetic modification which is known to be involved in 'genetic imprinting'.¹⁶ Methylation of DNA leads to silencing of genes and is maintained during cell division by virtue of the enzyme DNA methyltransferase I.

Several other findings implicate genetic imprinting in similar transgenerational effects. Mice experiments with the Agouti-allele, which normally leads to a yellow phenotype, have shown that a methyl-rich diet, when given to pregnant mice not carrying the Agouti-allele, could silence the Agouti-gene in their offspring.¹⁷ Especially interesting was the finding that methylation of the Agouti-allele was more likely to be maintained when the allele was maternally inherited. In humans, investigations into the underlying cause of the Prader-Willi and Angelman syndromes have led to the discovery of a similar example of epigenetic inheritance. Due to a different DNA-methylation imprint, loss of the paternal copy of 15q11-q13 was found to lead to Prader-Willi syndrome, which is characterized by obesity, a short stature, extreme flexibility and delayed puberty, whereas maternal deletion of the same region on chromosome 15 has been shown to lead to Angelman syndrome,¹⁸ a neuro-genetic disorder characterized by intellectual and developmental delay, sleep disturbance, seizures, jerky movements (especially hand-flapping) and frequent laughter or smiling.

A second well-studied example of epigenetic change is chromatin modification; rearrangement of nucleosomes, which include covalent post-translational modifications of histone tails. Of several types of chemical modification, also including methylation, phosphorylation and ubiquitinylation, especially acetylation of lysine residues in the histone tails is considered a key process in gene regulation and is the main subject of part

II of this thesis. The histone acetylation status is regulated by two sets of enzymes: lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). KATs acetylate histones by transfer of an acetyl-group to the ϵ -portion of lysine residues, which results in an open modification of chromatin structure and in accessibility of DNA to transcription factors and recruitment of the basal transcription initiation machinery.^{19, 20} Conversely, gene repression is mediated via KDACs, which remove acetyl groups and counteract the activity of KATs resulting in a closed chromatin structure. Unlike DNA methylation, a possible mechanism of maintaining histone acetylation through generations is not well understood. However, the modern definition of epigenetics does not require meiotic heritability, but should mention DNA modifications, other than DNA sequence variation, that carry information content during cell division.¹⁴ Although a replicating enzyme has yet to be discovered, histone acetylation changes might turn out to be self-perpetuating.²¹ A possible mechanism is suggested by the phenomenon of ‘spreading’ of silencing in yeast which is mediated by the histone deacetylase activity of Sir2p.²² Sir2p-induced hypoacetylation of nucleosomes attracts other Sir proteins and leads to spreading of silent chromatin along the chromosome in *S. cerevisiae*. Irrespective of these findings the process of histone acetylation/deacetylation is generally accepted as one of the pillars in epigenetic research.

Epigenetic regulation of gene expression is also known to be important for cell differentiation. In every cell two thirds of our more than 25.000 genes are repressed by epigenetic mechanisms and every cell-type expresses a totally different set of genes. Furthermore, epigenetic mechanisms have been found to play a role in the development of human complex diseases such as cancer.^{23, 24} Chapter 9 of Part 2 of this thesis will discuss new insights in the role of epigenetic gene regulation (chromatin remodeling) also in determining susceptibility to cardiovascular disease, a new area of research. This chapter will also discuss the reversibility of epigenetic changes and the promising role of these mechanisms in the development of future therapy.

Epigenetic epidemiology

Thus far, the main focus has been to investigate the environmental influence on epigenetic processes. From literature we know that epigenetic differences arise during the lifetime of monozygotic twins²⁵ and that oxidative stress influences the balance between KATs and KDACs in favour of KATs, leading to an increase in inflammation.²⁶ Part 2 of this thesis introduces the concept that epigenetic processes are also under genetic control and that, besides the environment, genetic variation in genes encoding KATs and KDACs could also be an important determinant of susceptibility to complex human diseases such as cardiovascular disease.

It has already been shown that single gene disorders of the epigenetic machinery also impair normal gene expression. Lack of the MeCP2 protein, which recognizes

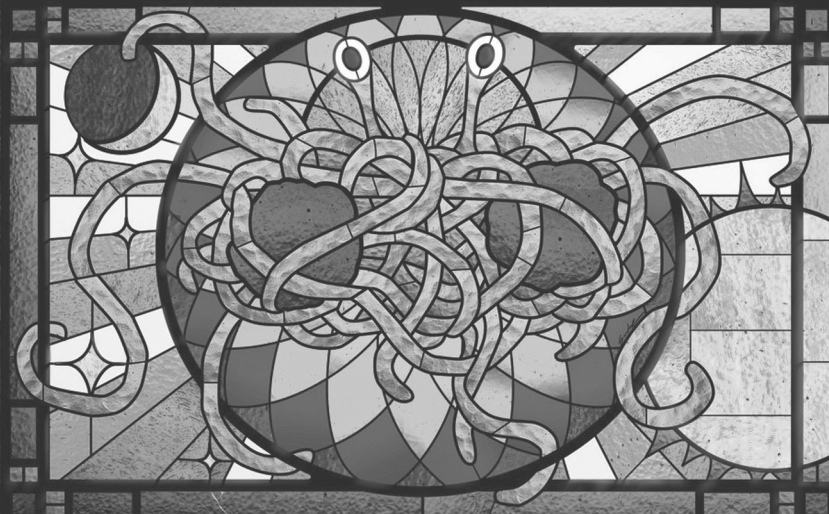
methyalted DNA and helps to repress gene expression, is known to lead to the Rett syndrome.²⁷ Similarly, loss of one functional copy of the CREB-binding protein (CBP), a transcriptional co-activator with intrinsic KAT-activity, underlies all abnormalities in patients with the Rubinstein-Taybi Syndrome.^{28, 29} Single nucleotide polymorphisms in the sequence of these epigenetic genes could act generally on disease susceptibility by affecting the fidelity of the histone acetylation machinery. In the worm *C. Elegans*, the genetic variants which were found to have the broadest influence on gene expression, affecting many signaling pathways, were found to be present in chromatin-modifying genes.³⁰ Furthermore, in humans, recent finding demonstrate that common genetic variants in the CBP gene are associated with altered cognitive function in the PROSPER-study, which included 5804 elderly patients at risk for vascular disease.³¹

Chapter 10 of this thesis will focus on this relatively new area of research, which we call 'epigenetic epidemiology'. In this chapter the PCAF gene will be introduced, encoding a co-activator with intrinsic KAT-activity and a broad influence on inflammatory and proliferative gene expression. This chapter addresses its newly identified role in cardiovascular disease and the significance of common genetic variation in epigenetic genes in determining coronary heart disease mortality.

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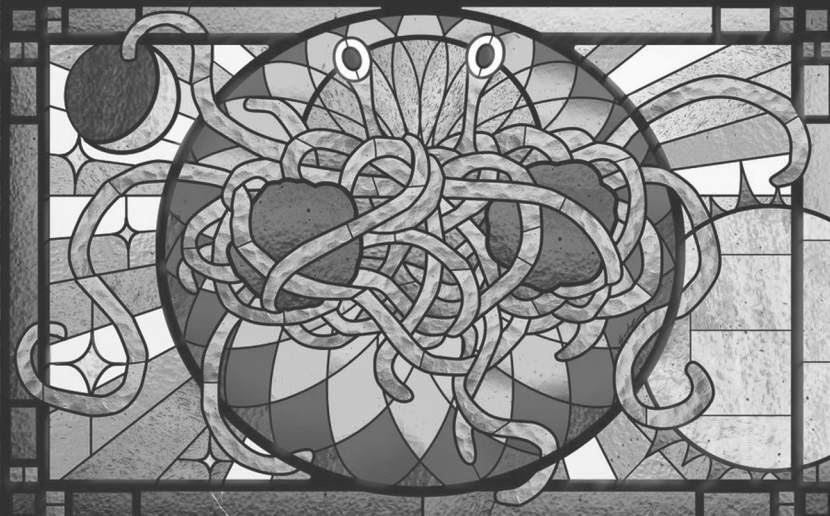
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Part 1

Genetic determinants of adverse outcome
after stent implantation



Chapter 2

Potential genetic determinants of adverse outcome after stent implantation: results, limitations and perspectives

Sandrin C Bergheanu, Douwe Pons, Ioannis Karalis, Orçun Özsoy, Jeffrey JW Verschuren, Mark M Ewing, Paul HA Quax and J Wouter Jukema

Future medicine - Interv cardiol 2011;2:147-157.

ABSTRACT

Despite its unequivocal superiority compared with balloon angioplasty, coronary stenting did not abolish the restenosis problem and even brought along a completely new type of pathology. Bare-metal stents still associate with around 20-30% in-stent restenosis rate and need for repeat revascularization. Drug-eluting stents (which unfortunately did not completely prevent restenosis either) sometimes determine late-acquired stent malapposition in a significant number of patients. This is followed occasionally by a very serious event – stent thrombosis. Patient co-morbidities, stent design, procedural characteristics and anti-platelet therapy influence the risk of post-stenting complications. Research in the recent years has revealed that also individual genetic profile plays an important role in adverse outcome after stent implantation. This manuscript reviews the evidence of genetic variations associated with stent restenosis, late-acquired stent malapposition and stent thrombosis.

INTRODUCTION

The era of percutaneous coronary intervention (PCI) began with the first balloon angioplasty performed by Andreas Gruentzig in 1977.¹ Although this technique provided impressive immediate results, mid and long term follow up was characterized by high restenosis rates and need for repeat revascularization.^{1,2} Evolving our techniques, bare-metal prosthetic devices (stents) were designed to act as a barrier against intima growth and recoil, assuring long-time patency of the coronary vessel. In 1986 Sigwart and Puel implanted the first coronary stent in a human patient.³ Superior to balloon angioplasty alone (32-42% restenosis rate), bare-metal stent (BMS) implantation remains however vulnerable to restenosis (22-32% of cases)^{4,5,6} and often requires re-intervention. Drug-eluting stents (DES) were conceived as an answer to this problem. They, for the majority, consist of a metallic platform covered with a combination of polymer and cellular proliferation inhibitor. The antiproliferative agent is gradually released in the arterial wall at the site of stent deployment preventing restenosis. The first successful DES trials were with sirolimus stents and led to their approval for use in 2002 in Europe and 2003 in USA.^{7,8} Currently, other DES based upon paclitaxel, everolimus, zotarolimus, biolimus and tacrolimus are available. DES have successfully achieved their task of preventing restenosis but the experience of the last years revealed an increased incidence of stent malapposition and stent thrombosis associated with their use.⁹ The aim of this article is to briefly present incidence and mechanisms of 1) stent restenosis, 2) stent malapposition and 3) stent thrombosis and to focus on potential genetic factors related to these complications. The majority of available data is retrieved from candidate gene approach studies, limiting thus the results to specific pre-targeted pathophysiologic sequences. Further novel pharmacogenomic approaches such as GWAS (genome wide association studies) may be able to identify new genetic factors for a better prediction of outcome after coronary stent deployment.

IN-STENT RESTENOSIS

In-stent restenosis (ISR) is defined angiographically when neo-formation tissue represents more than 50% of the lumen diameter at the site of the stented vessel (Figure 1). The clinical confirmation of ISR is the recurrence of angina pectoris, which further requires intervention: TLR (target lesion revascularization) or TVR (target vessel revascularization). Although the severity of angiographic stenosis correlates with the need for TLR, half of the patients with angiographically confirmed ISR do not manifest clinical complains.^{6,10} For this reason authors generally prefer to conduct their research in relation to angiographically documented ISR when an insight in the mechanism of

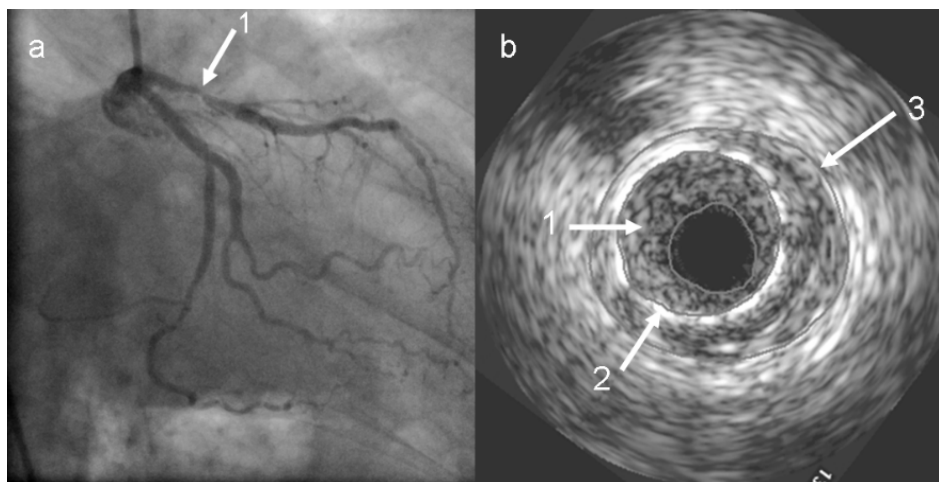


Figure 1. In-stent restenosis

Fig. 1 a) Angiographically documented in-stent restenosis; b) IVUS documented in-stent restenosis. 1 – neointima; 2 – stent contour; 3 – vessel contour.

restenosis is aimed, while studies comparing different stents are in relation to clinically-driven TLR or TVR.

ISR is the result of in-stent cellular proliferation and migration along with extracellular matrix accumulation.¹¹ Classic predictors of angiographic ISR (both in BMS and DES) include diabetes, renal failure, lesion length, reference vessel diameter and post-intervention lumen area.^{12,13} Inflammation plays a pivotal role in ISR and it is triggered by the vascular injury during the stent deployment and by the presence of stent struts within the vessel wall.^{14,15} Together with inflammation, major contributors are smooth muscle cell migration and proliferation but the process of restenosis involves many different cell-types, among which platelets and endothelial cells, and is also characterized by thrombus formation and to a lesser extent by matrix remodelling.

GENETIC FACTORS RELATED TO IN-STENT RESTENOSIS

Genetic variations in thrombus formation

In principle, any vascular intervention initiates the formation of a thrombus. Initial studies have shown associations of only a few polymorphisms in the hemostatic system with the risk for adverse events following a PCI. These early reports showed significant associations of the PLA1/A2 polymorphism with acute stent-thrombosis and coronary restenosis.^{16,17} However, other studies in this field could not confirm these associations.^{18,19} On grounds of the hypothesis that carriers of the PLA2 allele have a more intense binding of fibrinogen and vitronectin and thus a higher risk of platelet-rich white thrombus

formation, the PLA2 allele can be expected to lead to an increased risk for acute stent thrombosis. However, as platelet inhibition by IIb/IIIa and P2Y12 antagonists does reduce acute stent thrombosis, but not in-stent restenosis rates,²⁰ thrombus formation is probably not a main player in the development of restenosis. This hypothesis is further confirmed by findings showing that especially the strong pro-thrombotic genetic risk factors for venous thrombosis do not increase the risk for restenosis.²¹ Moreover, results from the GENDER study²¹ have shown that the Factor V Leiden polymorphism (a well-known prothrombotic risk factor) was even found to reduce the risk for restenosis after PCI. A total of 3104 consecutive patients with stable angina pectoris or non-STEMI, of whom 2309 (74.4%) received stents, were included.²¹ The factor V (1691 G>A or factor V Leiden) amino acid substitution was associated with a decreased risk of TVR (HR=0.41, 95%CI 0.19–0.86). The Factor V allele, which is known to lead to increased activation of protein C, might therefore influence restenosis risk by mechanisms not involved in coagulation, but in processes that have a more prominent role in neointimal growth, such as inflammation. Even though in another study of the same patient sample, associations were found between P2Y12 receptor haplotypes and restenosis,²² fewer and smaller effects were present in the stented subgroup. The decrease of the effects in this group could be due to inhibition of this receptor by clopidogrel (although several studies^{23,24,25,26} failed to demonstrate a functional role of the P2Y12 receptor polymorphism in patients receiving dual antiplatelet therapy). Therefore, the genetic variation in this receptor, and also in many other genes with a role in the hemostatic system, may have been more important at a time in which not every patient was receiving a stent and concomitant platelet inhibition.

The 4G/4G genotype of the PAI-1 4G/5G polymorphism determines higher PAI-1 levels in plasma^{27,28,29} and tissue.^{30,31,32} The PAI-1 4G allele was associated with an increased risk of restenosis after PCI in the GENDER study.²¹ When compared to 5G/5G homozygotes, heterozygous patients were at higher risk for clinically-driven TVR (HR=1.46, 95%CI 1.05–2.03), whereas patients with the 4G/4G genotype had an even further increased risk (HR=1.69, 95%CI 1.19 – 2.41). Although one smaller study could not confirm this association,³³ many reports found a positive correlation between post-PCI PAI-1 levels or activity and restenosis.^{34,35} Nevertheless, PAI-1 has a diverse role in several processes involved in restenosis, also in inflammation and proliferation.³⁶ Even if the 4G allele would increase the risk for restenosis, this could be mediated by a mechanism not related to fibrinolysis inhibition. Taking these findings together, we suggest that coagulation is not a main determinant of the long-term process that leads to restenosis.

Genetic variations in inflammatory factors

Early studies investigating the role of genetics in restenosis showed associations between variants in genes encoding cytokines³⁷ and selectins,³⁸ important mediators of inflam-

mation, and suggested a role for inflammation in restenosis. One of these studies was performed by Kastrati et al.,³⁷ and included 1850 consecutive stented patients. They demonstrated a protective effect of allele 2 of a polymorphism in exon 2 of the gene encoding the IL-1 receptor antagonist (IL-1ra), an anti-inflammatory interleukin, on both angiographic and clinical restenosis (OR=0.78, 95%CI 0.63-0.97 and OR=0.73, 95%CI 0.58-0.92, respectively). Monraats et al. have further established the important role of inflammatory genes in the development of restenosis. In the GENDER study, the rare alleles of the -260 C/T polymorphism in the CD14 gene, the 117 Ile/Thr polymorphism in the colony stimulating factor 2 gene (also known as granulocyte-macrophage

colony stimulating factor, GM-CSF) and the -1328 G/A polymorphism in the eotaxin gene were associated with decreased risk of TVR.³⁹ Eotaxin is a chemokine which selectively recruits eosinophils and was previously reported to be elevated in plasma of patients with advanced atherosclerosis. After coronary interventions, eotaxin levels increase and remain high for at least 24 hours but no longer than 3 months.⁴⁰

Furthermore, the variant alleles of two promoter polymorphisms in the Tumor Necrosis Factor alpha (TNF- α) gene have been shown to protect against the development of restenosis.⁴¹ Stented patients with the -238A/A genotype (HR=0.44, 95%CI 0.23-0.83) and patients with the -1031C/C genotype (HR=0.72, 95%CI 0.52-1.00) needed TVR less frequently. Several other inflammatory genes were shown to be involved in the process of restenosis in this cohort, among which interleukin 10 and caspase-1 (IL-1 β converting enzyme).^{42,43} All these findings support the hypothesis that restenosis is largely (albeit not solely) determined by inflammation.

Genes involved in smooth muscle cell proliferation

Stents specifically aiming to inhibit inflammation (dexamethasone eluting stents) were not proven as effective as stents inhibiting both inflammation and cell proliferation.⁴⁴ Despite the fact that restenosis is mainly determined by proliferation and migration of vascular smooth muscle cells (VSMCs), relatively few studies investigated genes involved in proliferation, such as cell-cycle regulatory genes. A recent important finding in this field by Van Tiel et al.⁴⁵ was an association between the -838 G/A polymorphism in the cyclin-dependent kinase inhibitor p27(kip1) (a key regulator of SMC proliferation) with ISR. Three polymorphisms concerning the p27(kip1) gene (-838C>A; -79C>T; +326G>T) were determined in a cohort of 715 patients undergoing coronary angioplasty and stent placement. Patients with the p27(kip1) -838AA genotype had a decreased risk of ISR (HR=0.28, 95%CI 0.10-0.77). This finding was replicated in another cohort study of 2309 patients (HR= 0.61, 95%CI 0.40-0.93). The -838 A allele corresponded to enhanced promoter activity which in turn may explain decreased SMC proliferation.

Genetic variations in matrix metalloproteinases

Matrix metalloproteinases (MMPs) are Zn^{2+} -requiring proteases capable of degrading a variety of extracellular matrix components. Due to their significance in vascular remodeling, MMPs are suspected to play an (important) role in the pathogenesis of atherosclerosis and restenosis.⁴⁶ Especially MMP2, MMP3 and MMP9 are potential players in the process of restenosis after PCI. MMP2 and MMP9 (the gelatinases) are produced by vascular VSMCs and degrade basement membrane components and other matrix proteins to allow migration and proliferation of vascular smooth muscle cells (VSMCs).⁴⁷ They are upregulated and activated in VSMCs during intima formation in many different animal models for restenosis involving balloon angioplasty.⁴⁷ An increase in MMP2 levels and activity was demonstrated in human coronary sinus blood samples 4 and 24 hours after elective coronary angioplasty.⁴⁸ This small study, in which only 21 of 47 patients were stented, also showed an association between MMP2 levels and restenosis. MMP3 (stromelysin-1) expression has been found to be related to plaque-instability in pathological studies.⁴⁹ MMP3 reduces the matrix content of the vascular wall and is therefore expected to protect against restenosis.⁴⁹ Functional studies have shown that the MMP3 -1612 5A/6A promoter polymorphism is associated with altered MMP3 expression. Carriers of the 6A/6A genotype were found to have a reduced MMP3 expression^{50,51,52,53} and were at increased risk of developing restenosis in a subset of the REGRESS study, in which stents were not yet frequently used,⁵⁴ and in two other studies with luminal narrowing after plain balloon angioplasty.^{55,56} However, an association between the MMP3 5A/6A polymorphism could not be confirmed in a study which included 217 stented patients. Unpublished results from the GENDER study indeed show no association between this polymorphism and clinical restenosis in stented patients. Therefore, even though matrix formation is an important process in the development of restenosis, variations in genes involved in matrix remodeling were infrequently investigated or studies yielding negative results and were not published.

STENT MALAPPOSITION

Stent malapposition (SM), commonly detected by intravascular ultrasonography (IVUS), represents a separation of the stent struts from the intimal surface of the arterial wall (in the absence of a side branch) with evidence of blood speckles behind the struts⁵⁷ (Figure 2a). SM may be acute (present immediately after implantation), persistent (present both immediately after implantation and at follow-up) or late-acquired (present only at follow-up). Acute and persistent SM are mainly procedure-driven while late-acquired stent malapposition (LASM) is a consequence of positive remodelling of the vessel wall and and/or of plaque volume decrease behind the stent (including clot lysis or plaque

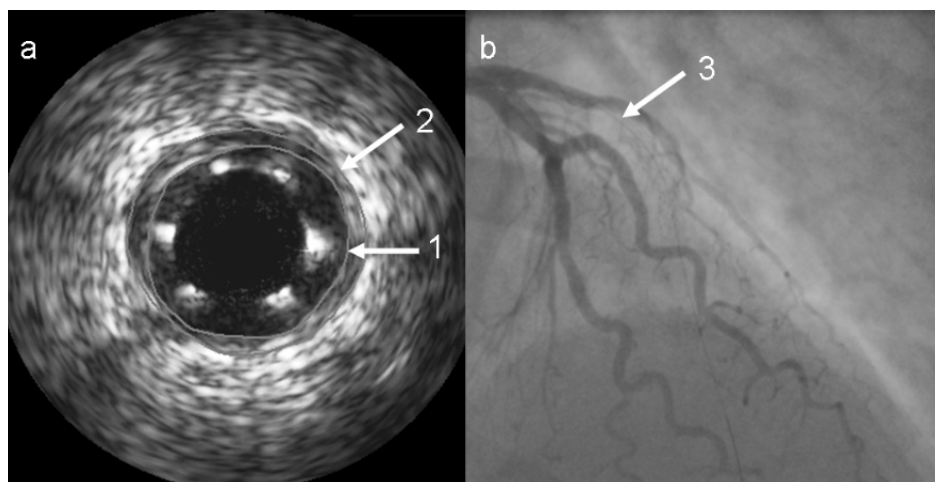


Figure 2. Stent malapposition and thrombosis

Fig. 2 a) IVUS documented stent malapposition; b) angiographically documented stent thrombosis. 1 – lumen contour behind stent struts; 2 – vessel contour; 3 – stent thrombosis.

regression).^{58,59,60,61,62} The main repercussion of late SM (persistent or acquired) is stent thrombosis (ST).⁹ Independent predictors of LASM include lesion length, unstable angina, absence of diabetes and primary stenting in acute MI.^{63,59} The risk of LASM in patients with DES is approximately 4 times higher compared to those with BMS.⁹ This is due to the fact that in BMS, hypersensitivity to the metallic stent is mostly associated with restenosis, whereas in DES, the hypersensitivity to the metallic stent, the polymer or to the drug is associated with positive remodelling and excessive inflammation in the vessel wall.⁶⁴

GENETIC FACTORS RELATED TO STENT MALAPPOSITION

We have previously investigated 7 polymorphisms (involved in inflammatory processes and related to restenosis) on the risk of LASM in SES patients.⁶⁵ In total, 104 STEMI patients from the MISSION! intervention study⁶² were genotyped for the caspase-1 5352 G/A, eotaxin 1382 A/G, CD14 260 A/G, colony stimulating factor 2 1943 C/T, IL10 -1117 C/T, IL10 4251 C/T and the TNF- α 1211 C/T polymorphisms. LASM occurred in 26/104 (25%) of patients. We found a significantly higher risk for LASM in patients carrying the caspase-1 (CASP1) 5352 A allele (RR= 2.32, 95% CI 1.22-4.42). In addition, mean neointimal growth was significantly lower in patients carrying this LASM risk allele (1.6 vs 4.1%, $p=0.014$). The other 6 polymorphisms related to inflammation were not significantly related to the risk of LASM. Given the limited number of patients included in the study, similar reports are needed to confirm our findings. Moreover, a direct rela-

tion between the CASP1 5352 A allele and the risk of ST was not investigated. To our knowledge, no other studies yet scrutinized the role of genetic variations in LASM.

STENT THROMBOSIS

Stent thrombosis (ST) (Figure 2b) is a complication which occurs in 0.8-2% of patients undergoing PCI and is associated with large MI and death.⁶⁶ ST is categorized into “acute” ST (within 24 hours from stent implantation), “subacute” ST (within 1 – 30 days from stent implantation), “late” ST (within 30 days – 1 year) and “very late” ST (> 1 year after stent implantation). Subacute and acute ST are classically related to procedure parameters such as stent underdeployment (acute SM)^{67,68} or procedure related complications such as coronary dissections.^{69,70} In contrast, (very) late ST appears to be an active phenomenon associated with late SM (persistent or acquired),^{9,71} stent type⁹ duration of dual anti-platelet therapy⁶⁶ and inflammation.⁵⁸ Gene variations in the platelet aggregation pathway, responsiveness to clopidogrel or presence of inherited thrombophilic disorders were associated with both acute and late ST.

GENETIC FACTORS RELATED TO STENT THROMBOSIS

Platelet receptor gene polymorphism

Platelet aggregation involves the binding of fibrinogen to the glycoprotein (GP) IIb/IIIa receptor on the platelet surface. One polymorphism of the GP IIIa gene (PLA1/A2 or HPA – 1a/1b) has been related to the inherited risk of coronary thrombosis.⁷² Of importance, the same polymorphism had no influence on the degree of myocardial salvage achieved in 133 acute MI patients undergoing coronary stenting and abciximab administration.⁷³ The PLA2 polymorphism is a substitution of cytosine for thymidine at position 1565 in exon 2. Walter et al.⁷⁴ investigated the association of PLA2 allele with acute and subacute stent thrombosis in 318 consecutive BMS patients stented for coronary dissection, acute occlusion or high residual restenosis after PTCA lesions in by-pass grafts, and restenotic lesions. They found that patients with the PLA2 allele had an increased risk of stent thrombosis compared with patients homozygous for PLA1 (OR=5.26, 95%CI 1.55-17.85). Kastrati et al.⁷⁵ confirmed these findings partially in their prospective study including 1759 patients with stable and unstable angina pectoris. No difference was seen at 30 days after stent placement in terms of ST or a composite end-point of death, MI or urgent revascularization between PLA1/A1 and PLA1/A2 carriers. However, the incidence of ST and the composite end-point were higher in the PLA2

homozygotes versus PLA1 homozygotes (8.7% vs. 1.7%, $p=0.002$ and 13.0% vs. 5.4%, $p=0.06$, respectively).

More recently, Sucker et al.⁷⁶ assessed the relevance of prothrombotic platelet receptor polymorphisms for the onset of coronary stent thrombosis in 316 patients. They compared the prevalence of GP Iba, GP IIb, GP IIIa (including PLA1/A2) and GP Ia prothrombotic polymorphisms in patients with coronary stent thrombosis occurring in the first 6 month after stent implantation and healthy control subjects. Carriers of the above mentioned prothrombotic versions did not appear to be at any increased risk for stent thrombosis. Selection of patients (differences in number of elective and acute stent implantations) and the treatment of more complex coronary lesions in the latter study or the limited power might explain these discrepancies.⁷⁶ Angiolillo et al.⁷⁷ have investigated the differential platelet sensitivity between PLA1 homozygotes and PLA2 carriers in 38 patients undergoing coronary stent implantation and receiving a 300 mg clopidogrel loading dose. They have shown that PLA2 carriers have a lower inhibition of platelet reactivity following the standard clopidogrel loading dose, which might finally lead to stent thrombosis.

Genetic variations in response to clopidogrel

In current practice, patients undergoing PCI and stent deployment are given 300-600 mg clopidogrel as a loading dose followed by 1 year dual anti-platelet therapy (aspirin 80-325 mg and clopidogrel 75 mg daily) and continued with life-long aspirin intake.

A good responsiveness to clopidogrel is therefore crucial in order to prevent thrombotic events after stent deployment.

Clopidogrel is an inactive prodrug which requires a two-step oxidation by the hepatic cytochrome P450 (CYP) enzymes to transform into an active metabolite which further inhibits the ADP P2Y₁₂ receptor producing the anti-aggregation effect. The genes encoding the CYP enzymes are polymorphic and several variants were related to a decreased catalytic activity and subsequent attenuated effect of the drug.

The CYP3A5 gene has a functional polymorphism which includes the expressor (*1) and non-expressor (*3) alleles.^{78,79} Suh et al.⁷⁹ compared clinical outcome in 348 patients (with stable angina, unstable angina or non-STEMI) who had PCI with BMS implantation. Antiplatelet therapy consisted of aspirin (100-300 mg daily, prescribed indefinitely) and clopidogrel (75 mg daily after 300 mg loading dose) administered for at least 4 weeks after the procedure. Atherothrombotic events (a composite of cardiac death, MI and non-hemorrhagic stroke) occurred more frequently within 6 months after stent implantation among the patients with the non-expressor genotype than among those with the expressor genotype (14/193 vs. 3/155, $p=0.023$). Moreover, the CYP3A5 polymorphism was a predictor of atherothrombotic events in clopidogrel users.

These findings are interesting especially since a number of studies (which did not aim at clinical end-points) found no association between the CYP3A5 variants and clopidogrel response and/or residual platelet aggregation (RPA)^{80,81,82} nor did a number of studies with clinical end-points.^{83,84}

Trenk et al.⁸⁵ investigated whether the CYP2C19 681G>A *2 polymorphism was associated with high (>14%) RPA on clopidogrel and whether high on-clopidogrel RPA affects clinical outcome after elective coronary stent placement. RPA was assessed in 797 consecutive patients after a 600 mg loading dose and after the first 75 mg maintenance dose of clopidogrel before discharge. Patients were followed-up for 1 year. Between the *2 carriers and *1/*1 carriers (wild-type) the authors found significant ($p<0.001$) differences in the proportion of patients with RPA>14%, both after loading (62.4% vs. 43.4%) and at pre-discharge (41.3% vs. 22.5%). RPA >14% at discharge was associated with a 3-fold increase (95%CI 1.4-6.8, $p=0.004$) in the 1-year incidence of death and myocardial infarction. However, authors could not show a direct relation between the CYP2C19*2 allele and clinical outcome.

This relation was demonstrated by Giusti et al.⁸⁶ in a subanalysis of the RECLOSE trial. The role of the CYP2C19*2 polymorphism in the occurrence of DES ST (definite or probable) or the composite end-point of ST (definite or probable) and cardiac mortality within 6-month follow-up was assessed in 772 patients undergoing PCI and receiving either sirolimus or paclitaxel DES. Patients with ACS and STEMI were included as well as patients with left main disease, chronic total occlusions, bifurcation lesions or diffuse disease. All patients received aspirin (325 mg) and a loading dose of clopidogrel 600 mg before the procedure followed by a maintenance dose of clopidogrel 75 mg and aspirin 325 mg daily. Patients with ST or ST and cardiac mortality end-point had a higher prevalence of the *2 allele (54.1% vs. 31.3%; $p=0.025$ and 51.7% vs. 31.2%; $p=0.020$, respectively). At multivariate logistic regression analysis, the CYP2C19*2 allele was an independent risk factor for ST (OR=3.43, 95%CI 1.01-12.78, $p=0.047$) and ST and cardiac mortality (OR=2.7, 95%CI 1.00-8.42, $p=0.049$).

Mega et al.⁸³ reconfirmed these findings on long term assessment of patients from TRITON-TIMI 38 study. A number of 1389 patients treated with clopidogrel who underwent PCI and stenting were followed-up for 15 months. Patients were initially admitted with non-STEMI (71%) and STEMI (29%). They received a 300 mg clopidogrel loading dose, followed by 75 mg daily maintenance dose for up to 15 months. For the CYP2C19, the presence of at least one copy of the *2 allele was associated with a higher rate of composite death from cardiovascular causes, non-fatal MI, non-fatal stroke (HR=1.42, 95%CI 0.98-2.05) and of definite/probable ST (HR=3.33, 95%CI 1.28-8.62) than did non-carriers.

Sibbing et al.⁸⁷ assessed the role of the mutant *2 allele of the CYP2C19 polymorphism on the 30-day incidence of definite ST in 2485 consecutive patients undergoing coronary

stent placement. There are a number of differences with regard to the previous study⁸³: (1) STEMI patients were excluded, (2) the end-point was acute and subacute definite ST and (3) patients received 600 mg clopidogrel loading dose. DES were used in 25% and BMS in 75% of the patients. Of the patients studied, 73% were CYP2C19 wild-type homozygotes (*1/*1) and 27% carried at least one of the *2 allele. The cumulative 30-day incidence of ST was significantly higher in CYP2C19*2 allele carriers vs. wild-type homozygotes (1.5% vs. 0.4%, HR=3.81, 95%CI 1.45-10.02, P=0.006). The risk of ST was highest (2.1%) in patients carrying the CYP2C19 *2/*2 genotype (p=0.002).

Recently, Collet et al.⁸⁸ demonstrated the role of the CYP2C19*2 allele in 259 young patients (aged <45 years) who survived a first MI and received clopidogrel treatment for at least a month. The primary endpoint was a composite of death, MI, and urgent coronary revascularization occurring during exposure to clopidogrel. The secondary endpoint was angiography-documented stent thrombosis. Median clopidogrel treatment duration was approximately one year. The primary endpoint occurred more frequently in carriers than in non-carriers (15 vs. 11 events; HR=3.69, 95%CI 1.69-8.05, P=0.0005), as did stent thrombosis (8 vs. 4 events; HR=6.02, 95%CI 1.81-20.04, P=0.0009). The effect of the CYP2C19*2 genetic variant persisted from 6 months after clopidogrel initiation up to the end of follow-up (HR=3.00, 95%CI 1.27-7.10, p=0.009). The CYP2C19*2 genetic variant appeared the only independent predictor of cardiovascular events (HR=4.04, 95%CI 1.81-9.02, P=0.0006).

In a study⁸⁴ of 2208 patients presenting with acute MI (among which 1535 underwent PCI), patients carrying any two CYP2C19 loss-of-function alleles (*2, *3, *4, or *5), had a higher rate of death from any cause, nonfatal stroke, or myocardial infarction during 1 year of follow-up than patients with none (21.5% vs. 13.3%; adjusted HR=1.98; 95%CI 1.10-3.58). Among the patients who underwent PCI during hospitalization, the rate of cardiovascular events among carriers of CYP2C19 loss-of-function alleles was 3.58 (95%CI 1.71-7.51).times higher than among those with none.

For the development of a risk score for better prediction of RPA, Geisler et al.⁸¹ analyzed the CYP2C19*2 genotype and previously identified non-genetic risk factors (age >65 years, type 2 diabetes mellitus, decreased left ventricular function, renal failure and acute coronary syndrome). They demonstrated a significant correlation of the non-genetic factors ($\chi^2 = 5.32$; $P = 0.021$) and CYP2C19*2 ($\chi^2 = 21.31$; $P < 0.0001$) with high RPA, and the highest association for the combination of both ($\chi^2 = 25.85$; $P < 0.0001$). This was the first study to show that prediction of clopidogrel responsiveness may substantially be improved by adding CYP2C19*2 genotype to non-genetic risk factors. The important influence of the CYP2C19*2 genotype over platelet function and cardiovascular outcomes was recently confirmed by Shuldiner et al.⁸⁹ in the first GWAS paper identifying CYP2C19 as a candidate gene. In the Pharmacogenomics of Antiplatelet Intervention (PAPI) Study, clopidogrel was administered for 7 days to 429 healthy

individuals and the response was measured by ex vivo platelet aggregometry. A GWAS was performed followed by genotyping the loss-of-function cytochrome CYP2C19*2 variant. The relation between CYP2C19*2 genotype and platelet aggregation was replicated in 227 clopidogrel-treated patients undergoing PCI ($P = 0.02$). Patients with the CYP2C19*2 variant were more likely (20.9% vs 10.0%) to have a cardiovascular ischemic event or death during 1 year of follow-up ($HR=2.42$, 95%CI 1.18-4.99, $P = 0.02$).

Factor V Leiden mutation

Factor V Leiden is the most common inherited thrombophilic disorder, resulting from a single mutation (1691 G>A) in the factor V gene. Individual heterozygous for this mutation are at increased risk for venous thrombosis, and in homozygous the risk becomes extremely high. Although conceivable, there is only one case report to document a possible relation between a factor V Leiden heterozygous patient and stent thrombosis (simultaneous occlusion of two stents, one in left anterior descending artery and one in the right coronary artery at 4 days after implantation in a patient receiving standard dual anti-platelet therapy).⁹⁰ Further larger studies are therefore needed before factor V Leiden may be linked to ST.

LIMITATIONS

Many studies have managed to identify genes and polymorphisms involved in the post-stenting outcome after scrutinizing various plausible pathophysiologic mechanisms. However, to predict an accurate scale of adverse effects, an interaction assessment between genetic, non genetic (traditional risk factors) as well as epigenetic factors is of extreme importance. This information remains momentarily scarce. .

Also of importance, findings from certain studies cannot sometimes be confirmed by other studies. This is largely explained by variation in study settings and therefore the replication of findings in independent studies needs to be further emphasized.

The candidate gene approach used to date in the majority of investigations narrows the results to specific areas of interest.

CONCLUSION

In-stent restenosis and stent thrombosis remain important limitations of the current PCI practice. Besides the procedure-related risk factors and medication, solid evidence shows that patient's own response to stent implantation influences the outcome. Individual genetic response involves inflammation, cellular proliferation, platelet receptors

and drug metabolism pathways. A better understanding of the stent pathology has led to the identification of new important genes and genetic polymorphisms. They may help us better identify the vulnerable patients who need extraordinary therapeutic measures. Conversely, genetic-epidemiologic studies have identified genes which subsequently have revealed important pathophysiologic mechanisms.

FUTURE PERSPECTIVES

The speed by which new genes are being related to stent pathology is matched by the speed of new developments in stent technology and medication. Novel pharmacogenomic approaches (such as GWAS, 1000 genome project⁹¹ etc) may help to identify unknown genetic factors for a better prediction of outcome after stent implantation.

It is however difficult to estimate whether screening for established polymorphisms will prove in the future a cost-effective method for a better stent type selection or medication in the daily routine.

The classic stents seem to rapidly make place to new and complex body-polymer-drug constructions that address most, if not all, of the current problems. The new generation of stents may appear capable of modulating local inflammation, to permit a good re-endothelization, to prevent stent thrombosis, to reduce the duration of anti-platelet medication and, if necessary, even to degrade after local healing is achieved.

New drugs such as prasugrel, ticagrelor and cangrelor seem to effectively inhibit platelet aggregation with no or little inter-individual response variability. The combination of lessons learned from genetic and pathophysiologic studies, the newly available resources (stents, antiplatelet drugs, imaging) and refined implantation techniques will definitely improve PCI performances and extend its use.

EXECUTIVE SUMMARY

Genetic variants associated with an increased or decreased risk of in-stent restenosis (ISR) and stent thrombosis (ST)

In-stent restenosis (ISR)

- *Genetic variations in thrombus formation*
Associated with decreased risk:
Factor V 1691G>A (factor V Leiden) amino acid substitution
Associated with increased risk:
4G allele of the PAI-1 4G/5G polymorphism

- *Genetic variations in inflammatory factors*
Associated with decreased risk:
*2 allele of the IL-1ra gene
T /T genotype of the CD14-260 C/T polymorphism
Thr allele of the CSF2-117 Ile/Thr polymorphism
A allele of the CCL 11 (Eotaxin) 1328 G/A polymorphism
A/A genotype of the TNF -238 G/A polymorphism
C/C genotype of the TNF -1031 T/C polymorphism
Associated with increased risk:
A/A genotype of the IL-10 -2849 G/A polymorphism
A/A genotype of the IL-10 -1082 G/A polymorphism
G/G genotype of the IL-10 +4259 A/G polymorphism
A/A genotype of the Caspase-1 5352 G/A polymorphism

- *Genes involved in smooth muscle cell proliferation*
Associated with decreased risk:
A/A genotype of the p27(kip1)-838G/A polymorphism

Stent thrombosis (ST)

- *Platelet receptor gene polymorphism*
Associated with increased risk:
PLA2 allele of the GP IIIa PLA1/A2

- *Genetic variations in response to clopidogrel*
Associated with increased risk:
*3 allele of the CYP3A5 gene (encodes hepatic cytochrome P450 CYP enzymes)
*2 allele of the CYP2C19 gene (encodes hepatic cytochrome P450 CYP enzymes)

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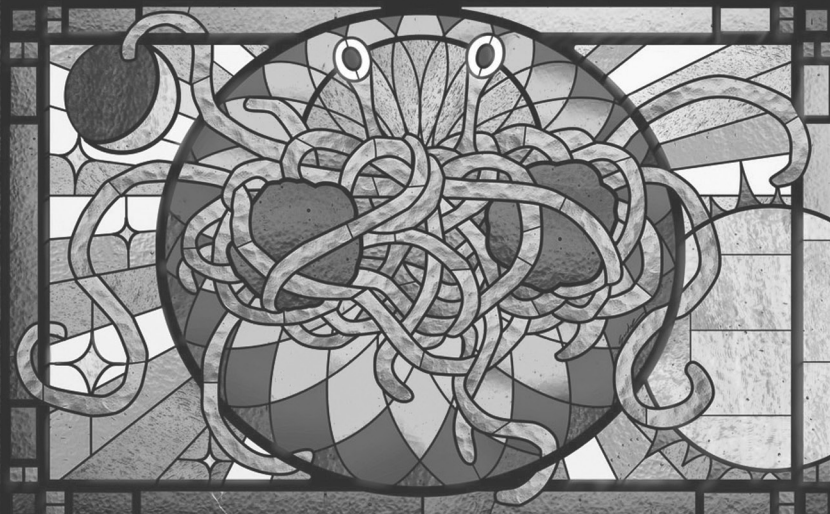
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Chapter 3

The influence of established genetic variation
in the hemostatic system on clinical restenosis
after percutaneous coronary interventions

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ABSTRACT

Since activation of the hemostatic system is an important feature of the wound healing response triggered by arterial injury, variations in genes involved in thrombus formation may play a role in restenosis after percutaneous coronary interventions (PCI).

Therefore, our aim was to examine the relationship between polymorphisms that are known to play a role in the hemostatic system and the risk of clinical restenosis in the GENetic DEterminants of Restenosis (GENDER) study, a multicenter prospective study design that enrolled 3,104 consecutive patients after successful PCI. Target Vessel Revascularization (TVR) was the primary endpoint. All patients were genotyped for 6 polymorphisms in the Factor II, Factor V, Factor VII and PAI-1 genes.

The PAI-1 4G variant was associated with an increased risk of TVR. When compared to 5G/5G homozygotes, heterozygous patients were at higher risk for TVR (HR: 1.46, 95%CI: 1.05-2.03), whereas patients with the 4G/4G genotype had an even further increased risk (HR: 1.69, 95%CI: 1.19-2.41). In contrast, the factor V 506Gln (Factor V Leiden) amino acid substitution was associated with a decreased risk of TVR (HR: 0.41, 95%CI: 0.19-0.86).

Our findings indicate that polymorphisms in the Factor V and PAI-1 genes may play a role in the process of restenosis.

INTRODUCTION

Restenosis has always been a major limitation to the long term benefit of percutaneous coronary intervention (PCI). Despite the reduction in restenosis rates obtained by stenting and the use of drug-eluting stents, the incidence of restenosis remains a significant problem. Moreover, the development of these increasingly protective, but more expensive treatment options raises the need to stratify patients with regard to their risk for restenosis and to tailor individual treatment. Thus far, it has proven insufficient to make this stratification on the basis of clinical or procedural risk factors.¹ A promising tool to improve risk stratification may be found in a search for genetic risk factors, which may also provide targets for more effective therapy.

Injury to the vessel wall after PCI causes thrombus formation and subsequent invasion of leucocytes, followed by expression of growth factors and cytokines, leading to alterations in matrix composition and migration and proliferation of vascular smooth muscle cells (VSMCs). There is increasing evidence that polymorphisms in genes involved in inflammation and matrix formation are associated with the risk of restenosis.² However, the relevance of factors involved in thrombus formation remains uncertain, also because high doses of platelet-inhibitors are given at the time of the PCI to prevent acute stent thrombosis. The purpose of this study was to evaluate in a large unselected patient population whether polymorphisms in genes known to be of clinical importance with respect to thrombosis, predict the risk of clinical restenosis after PCI.

METHODS

Study design

The present study sample has been described previously.³⁻⁵ In brief, the GENetic DEterminants of Restenosis project (GENDER) was designed to study the association between various gene polymorphisms and clinical restenosis, defined in our study by Target Vessel Revascularization (TVR). Patients were eligible for inclusion if they were successfully treated by PCI for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia. Patients treated for acute ST elevation myocardial infarction (MI) were excluded. All patients were treated in four referral centers for interventional cardiology in the Netherlands (Academic Medical Center Amsterdam, University Medical Center Groningen, Leiden University Medical Center and Academic Hospital Maastricht). The inclusion period lasted from March 1999 until June 2001. In total, 3177 consecutive patients were included in this prospective multicenter follow-up study. Patients were considered to be diabetic if they were using oral anti-diabetic medication (N=303) or

insulin (N=150) at study entry. Smokers were defined as individuals who smoked within the month preceding the PCI.

The study protocol conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure.

PCI procedure

Standard angioplasty and stent placement were performed by experienced operators using a radial or femoral approach. Before the procedure patients received aspirin (300 mg) and heparin (7500 IU). The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, was at the discretion of the operator. When a stent was implanted, patients received either ticlopidine or clopidogrel for at least one month following the procedure depending on local practice. During the study no drug-eluting stents were used.

Follow-up and study endpoints

Follow-up lasted at least nine months or until a coronary event occurred. Patients were either seen in the outpatient clinic or contacted by telephone. TVR, either by PCI or coronary artery bypass grafting (CABG), was considered as the primary endpoint, since it is considered most relevant for clinical practice by regulatory agencies. An independent clinical events committee adjudicated the clinical events. Since the purpose of this study was to examine the risk of restenosis, and not sub-acute stent thrombosis, events occurring within one month after the procedure were excluded from the analysis. Data were collected with standardized case-report forms that were completed by the research coordinator at each site who was blinded to the genotype of the patients. Representatives from the data-coordinating center monitored the sites.

Genetic methodology

Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. Six polymorphisms; PAI-1 -695 4G/5G, PAI-1 11053 T/G (rs7242), Factor II (prothrombin) 20210 G/A, Factor V G1691A Arg506Gln (Factor V Leiden, rs6025), Factor VII -323 del/ins (CCTATATCCT) and Factor VII Arg353Gln (rs6046) were determined by a validated multilocus genotyping assay (Roche Molecular Systems). All polymorphisms were selected for their previously described association with thrombotic cardiovascular disease or with the concentration of proteins involved in hemostasis.

Each DNA sample was amplified in multiple polymerase chain reactions (PCRs) using biotinylated primers. The PCR product was then hybridised to a corresponding panel of sequence-specific oligonucleotide probes that had been immobilized in a linear

array on nylon membrane strips.⁶ A colorimetric detection method based on incubation with streptavidin-horseradish peroxidase conjugate, using hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine as substrates, was used. Operators blinded to patient data performed genotyping. To confirm genotype assignments, PCR analysis was randomly replicated on 10% of the samples. Two independent observers carried out scoring. Disagreements (<1%) were resolved by further joint reading, and when necessary a repeat genotyping reaction was performed.

Statistical methodology

Allele frequencies were determined by gene counting. The 95% confidence intervals of the allele frequencies were calculated from sample allele frequencies based on the approximation of the binominal and normal distributions in large sample sizes.

In the first stage we determined the association between each of the 6 polymorphisms and TVR using a Cox proportional regression model. If less than 50 patients were homozygous for the minor allele, the homozygotes and heterozygotes were combined. No adjustment for covariates was performed at this stage to allow for the assessment of the possible involvement of the polymorphisms in the causal pathway of TVR.

Finally, polymorphisms with independent prognostic value were selected in a multivariable regression model, also containing clinical and procedural risk factors. Statistical analyses were carried out using SPSS 12 (SPSS Inc., Chicago, IL).

RESULTS

Patient characteristics

The characteristics of this patient sample have been described previously.³ In summary, 3509 patients were eligible for the study of which 3177 were included. A total of 3146 patients had a complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9). Out of 3146 patients 42 had an event in the first 30 days. These patients were excluded from further analysis, according to the protocol. The remaining 3104 patients had a mean age of 62.1 ± 10.7 years. Of the patients 888 (28.6%) were women, and 453 (14.6%) had diabetes mellitus. The majority of the patients were Caucasian (97%) and stents were used in 2309 (74.4%) patients. Nearly all patients received either clopidogrel (n=2076) or ticlopidin (n=1016) and 812 patients (26.2%) received glycoprotein IIb/IIIa inhibitors. A total of 4061 lesions were treated in this unselected patient sample. Complex (type C) lesions, classified according to the modified American College of Cardiology and American Heart Association Task Force classification, were treated in 802 patients (25.8%).

Follow-up

Of the 3104 patients, 304 (9.8%) patients underwent TVR during follow-up. Fifty-one patients died (1.6%) and 22 (0.7%) suffered from MI. Genotype distributions and minor allele frequencies of the polymorphisms are shown in table 1. For the PAI-1 4G/5G, PAI-1 11053 T/G, Factor II 20210 G/A, Factor V Arg506Gln, Factor VII -323 del/ins and Factor VII Arg353Gln polymorphisms genotyping was successful in 3018, 3063, 3059, 3044, 3053 and 3045 patients, respectively. Patients with unknown genotype, due to unavailable DNA or inconclusive genotyping, did not differ in any characteristic from patients who were successfully genotyped. All genotype distributions were consistent with HWE ($p>0.05$). The low frequency of the Factor II 20210A, the Factor V Leiden 506Gln, the Factor VII -323ins and the Factor VII 353Gln alleles has prompted us to pool heterozygotes and homozygotes for these variants as specified in the statistical approach.

Table 1. Allele frequencies, genotype distributions and individual analysis of polymorphisms in association with TVR

Polymorphisms	Minor allele frequency (%)	Genotype counts N (%)	TVR (%)	HR (95%CI)
FII G20210A				
G/G	0.02	2925 (96.9)	9.7	1.12 (0.60-2.11)
G/A		92 (3.0)	10.8	
A/A		1		
FV Arg506Gln				
Arg/Arg	0.03	2896 (94.5)	10.0	0.41 (0.19-0.86)
Arg/Gln		164 (5.4)	4.2	
Gln/Gln		3 (0.1)		
FVII -323 del/ins				
D/D	0.13	2338 (76.4)	9.9	0.94 (0.72-1.24)
D/I		673 (22.0)	9.1	
I/I		48 (1.6)	12.5	
FVII Arg353Gln				
Arg/Arg	0.11	2414 (79.3)	9.9	0.89 (0.67-1.20)
Arg/Gln		587 (19.3)	8.3	
Gln/Gln		43 (1.4)	16.3	
PAI-1 4G/5G				
5G/5G	0.47	641 (21.0)	7.0	1.0 (reference)
5G/4G		1561 (51.1)	10.0	1.46 (1.05-2.03)
4G/4G		851 (27.9)	11.5	1.69 (1.19-2.41)
PAI-1 11053 T/G				
T/T	0.44	917 (30.1)	8.6	1.0 (reference)
T/G		1554 (51.0)	10.3	1.21 (0.92-1.58)
G/G		574 (18.9)	10.1	1.18 (0.84-1.65)

Univariate Cox regression shows that the PAI-1 4G/5G polymorphism and the Factor V Arg506Gln polymorphism were significantly associated with TVR. When compared to 5G/5G homozygotes, heterozygous patients were at higher risk for TVR (HR: 1.46, 95%CI: 1.05-2.03), whereas patients with the 4G/4G genotype had an even further increased risk (HR: 1.69, 95%CI: 1.19-2.41). In contrast, TVR was less frequent in carriers of the Factor V Leiden 506Gln variant, than in patients without the Factor V Leiden mutation (HR: 0.41, 95%CI: 0.19-0.86). None of the other variants were associated with the risk for TVR (Table 1).

After adjustment for possible confounders, such as age, sex, hypertension, diabetes, current smoking, stenting, total occlusion and residual stenosis>20%, both the PAI-1 4G/5G and the Factor V Leiden polymorphisms remained predictors of TVR (HR: 1.26, 95%CI: 1.07-1.49 and HR: 0.40, 95%CI: 0.19-0.85, respectively) (Table 2).

Table 2. Multivariable Cox regression of polymorphisms associated with TVR, including clinical factors

	HR	95% CI	
Age	0.99	0.98	1.00
Sex	0.96	0.74	1.25
Diabetes	1.51	1.14	2.01
Hypertension	1.21	0.96	1.53
Current smoker	0.71	0.53	0.96
Stenting	0.78	0.59	1.02
Total occlusion	1.46	1.09	1.97
Residual stenosis>20%	1.36	0.97	1.90
FV Arg506Gln	0.40	0.19	0.85
PAI-1 4G/5G	1.26	1.07	1.49

Diabetes

Diabetic patients are known to have increased levels of PAI-1^{7,8} and PAI-1 dependent mechanisms have been implicated in the pathogenesis of insulin resistance and type 2 diabetes.⁹ Therefore, we performed separate analyses in a prespecified subgroup of patients with diabetes. Mean glucose levels were 9.4 (sd 4.3) mmol/L in diabetic patients versus 5.8 (sd 1.4) in patients without diabetes ($p<0.001$). At follow-up 63 patients (13.9%) in the diabetic group (N=543) versus 241 (9.1%) in the non-diabetic group (N=2651) had to undergo TVR. Cox regression showed an association of diabetes with TVR (HR: 1.6, 95%CI: 1.2-2.1).

Despite clues for an interaction between insulin levels and the PAI-1 4G/5G polymorphism in risk of myocardial infarction,¹⁰ the effect of this polymorphism on TVR risk was similar in patients with and without diabetes (Table 3). However, there was a remarkable difference for the PAI-1 11053 T/G polymorphism. The hazard ratio for this polymorphism was higher in the group of patients with diabetes (HR: 1.41, 95%CI:

Table 3. Cox regression of polymorphisms in association with TVR in subgroups of patients with diabetes and smokers

Subgroup	Polymorphism	Genotype counts	TVR%	HR (95%CI)
Diabetes N=453	PAI-1 4G/5G			1.29 (0.89-1.88)
	5G/5G	88	12.5	
	5G/4G	234	12.0	
	4G/4G	122	18.0	1.41 (0.98-2.04)
	PAI-1 11053			
	T/T	129	11.6	
	T/G	231	12.1	
No diabetes N=2651	PAI-1 4G/5G			1.27 (1.05-1.52)
	5G/5G	553	6.1	
	5G/4G	1327	9.6	
	4G/4G	729	10.4	1.03 (0.85-1.23)
	PAI-1 11053			
	T/T	788	8.1	
	T/G	1323	10.0	
Smokers N=762	PAI-1 4G/5G			1.52 (1.05-2.20)
	5G/5G	168	3.6	
	5G/4G	368	8.7	
	4G/4G	216	10.2	1.25 (0.87-1.80)
	PAI-1 11053			
	T/T	223	6.3	
	T/G	375	8.0	
Non-smokers N=2342	PAI-1 4G/5G			1.21 (1.01-1.46)
	5G/5G	473	8.2	
	5G/4G	1193	10.4	
	4G/4G	635	12.0	1.06 (0.88-1.28)
	PAI-1 11053			
	T/T	694	9.4	
	T/G	1179	11.0	
	GG	423	10.2	

0.98-2.04) than in the group of patients without diabetes (HR: 1.03, 95%CI: 0.85-1.23) (Table 3), but did not reach statistical significance ($p=0.066$).

Smoking

Since it was suggested that smoking carriers of the PAI-1 5G/5G genotype are predisposed to develop restenosis,¹¹ we stratified our population in current smokers and non-smokers. From the literature it is known that smokers have a decreased risk of restenosis. Also in our study, current smokers were at reduced risk to develop restenosis (HR 0.77, 95%CI: 0.6-1.0). At follow-up 62 (8.1%) smokers (N=762) versus 242 (10.3%) non-smokers (N=2342) had to undergo TVR. Carriership of PAI-4G was associated with TVR occurrence in smokers and non-smokers alike, with a slightly higher hazard ratio

in smokers (HR: 1.5, 95%CI: 1.1-2.2) than in non-smokers (HR: 1.2, 95%CI: 1.0-1.5) (Table 3).

DISCUSSION

In a large prospective multicenter follow-up study, we investigated 6 polymorphisms in hemostatic genes that may also play a role in clinical restenosis. In our study, the PAI-1 4G variant is associated with an increased risk of TVR after PCI, and carriership of the Factor V Leiden 506Gln amino acid substitution is associated with a decreased risk of TVR.

Since the PAI-1 4G variant has been shown to be associated with higher PAI-1 levels in plasma¹²⁻¹⁴ and in tissue,¹⁵⁻¹⁷ our data suggest that PAI-1 promotes neointima proliferation. This hypothesis for the role of PAI-1 is supported by a number of reports on a positive correlation between post-PTCA PAI-1 levels or activity and restenosis,¹⁸⁻²³ but contradicted by one study which could not find an association between the PAI-1 4G/5G polymorphism and restenosis.²⁴

Several underlying mechanisms by which PAI-1 could influence the process of restenosis have been reported. Firstly, PAI-1 prevents the degradation of fibrin by inhibiting the formation of plasmin. Since fibrin is a provisional matrix for the invasion of VSMCs and may directly stimulate VSMC proliferation,²⁵ inhibition of fibrin degradation could promote restenosis. On the other hand, it has been suggested that PAI-1 has a protective role because of its inhibitory actions in the migration and proliferation of VSMCs and the invasion of inflammatory cells.²⁶ However, data on the role of PAI-1 in cell migration are conflicting.^{25,27} PAI-1 can regulate cell migration by several mechanisms and the net effect has been suggested to depend on the composition of the local environment and PAI-1 levels.²⁷ Furthermore, aortic VSMCs from PAI-1 knockout mice have been shown to be more susceptible to plasmin-induced apoptosis than VSMCs from wild type mice,²⁸ which also fits into the hypothesis that PAI-1 promotes neointima formation.

Factor V Leiden was associated in our study with protection against clinical restenosis. In a study by Volzke et al.,²⁹ this association was not observed, but their study had limited statistical power and the genotype distribution of the Factor V Leiden polymorphism was not in Hardy-Weinberg equilibrium. The Factor V Leiden polymorphism is a well-known risk factor for venous thrombosis. The Arg506Gln amino acid substitution at the APC cleavage site in the heavy chain of Factor V leads to APC resistance and subsequently to increased production of thrombin and fibrin. Apart from its role in venous thrombosis, Factor V Leiden has also been found to be moderately associated with the risk of myocardial infarction.^{30, 31} Then, what could be the explanation for a protective effect of a well established prothrombotic factor in the process of restenosis?

Carriers of the Factor V Leiden mutation were found to have a survival advantage when suffering from severe sepsis.³² The underlying mechanism may be an increased production of thrombin and activated protein C (APC) in Factor V Leiden carriers.³² In concordance with this hypothesis, the PROWESS trial demonstrated that treatment with human recombinant APC reduced mortality in patients with severe sepsis.³³

The beneficial effect of APC in sepsis may in part be explained by its strong anti-inflammatory properties.³⁴ It inhibits the release of TNE, blocks leukocyte adhesion and interferes with monocyte activation.³⁵ Since inflammation is a main determinant of neointima formation,^{36,37} APC could protect against the development of restenosis via similar mechanisms.

Diabetes

Although diabetic patients are known to have an increased risk of developing restenosis, there is not yet a genetic explanation for their enhanced susceptibility. Polymorphisms can be responsible for differences in gene expression in response to their altered metabolic state. In order to identify polymorphisms that selectively increase the risk of TVR in diabetics, we repeated the analysis of the PAI-1 polymorphisms in a prespecified subpopulation of diabetics. Although differences did not reach statistical significance, our data show a tendency towards a higher TVR risk associated with the 11053G allele, only in patients with diabetes. Although there is no evidence yet on the functionality of the 11053 T/G polymorphism, we do know that metabolic circumstances can induce PAI-1 expression. High glucose has been shown to induce PAI-1 expression in human adipose tissue.^{38,39} Furthermore, PAI-1 plasma levels seem to be determined by glucose in humans.^{7,8} Polymorphisms in the PAI-1 gene could alter gene expression in response to these factors.

Smoking

In agreement with our results, Ortlepp et al. have demonstrated that the PAI-1 5G allele lowered the risk of restenosis in non-smokers.¹¹ However, we could not confirm their finding that the 5G allele increased the risk for restenosis in smokers. We found similar hazard ratios in smokers and non-smokers. The discrepancy could be due to the small population of smoking 5G homozygotes in their study (N=42).

Limitations of the study

In our study we lack data on PAI-1 and APC concentration and activity in plasma. However, we believe that our data do not suffer from this due to a number of reasons. In the first place, the PAI-1 4G/5G polymorphism is known to be functional. The functionality of PAI-1 has been extensively investigated and the majority of results indicate that the 4G variant leads to higher expression levels.^{12,13, 15-17} In contrast, little evidence is avail-

able regarding APC levels in Factor V Leiden carriers and carriers of the PAI-1 4G/5G genotypes. P. Weiler *et al.* observed only marginal augmentation of protein C activation in Factor V Leiden-mice.⁴⁰ In their human studies, augmentation of APC levels was not detectable with the sensitivity of the employed assay. In our study, circulating APC protein levels were not assessed since basal (pre-PCI) plasma measurements of the gene product are not likely to reflect the genetically determined differences in reaction to a local trauma such as PCI. Furthermore, local differences in PAI-1 and APC sensitive reactions, such as occurring in the vessel wall at the site of PCI, cannot be measured systemically, as it is not yet possible to measure gene products in the vessel wall locally in the acute phase of treatment and the following days.

Conclusion

In conclusion, our data suggest that the PAI-1 4G allele, which is known to be associated with higher PAI-1 levels, increases the risk of clinical restenosis, while carriership of the Factor V Leiden allele reduces the risk of clinical restenosis after PCI. As Factor V Leiden, an established prothrombotic risk factor, seems associated with a decreased risk of restenosis, it may be considered that this polymorphism may also play a role in restenosis by mechanisms not involved in coagulation.

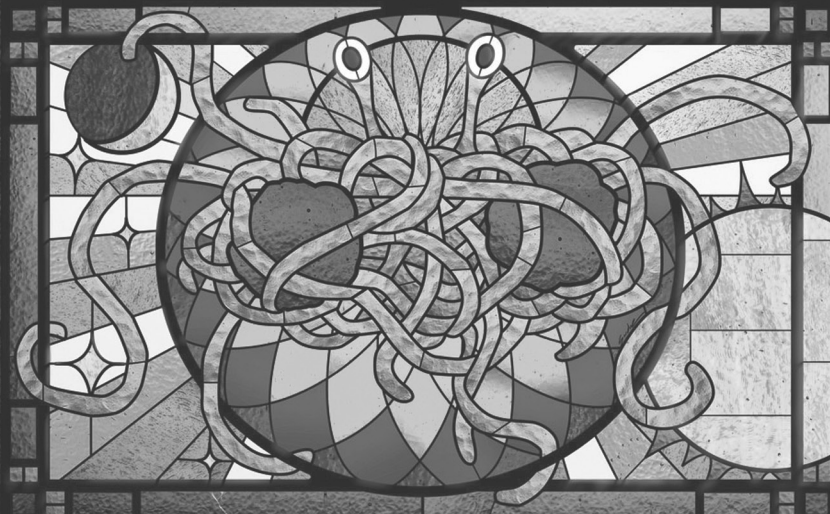
After confirmation of our results, genotyping for the Factor V Leiden and PAI-1 4G/5G polymorphisms may lead to better risk stratification and more tailored therapy in the prevention of restenosis after PCI. Furthermore, our results could contribute to the unravelling of the restenotic process and may provide targets for future therapy.

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Chapter 4

The factor VII activating protease (FSAP)
polymorphism (G534E) is associated with
increased risk for stroke and mortality

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ABSTRACT

Introduction: FSAP is a plasma serine-protease that is localized within atherosclerotic lesions. The FSAP-Marburg I polymorphism (G534E), which reduces FSAP activity, has been shown to be associated with late complications of carotid stenosis in humans and with a reduced capability to suppress vascular smooth muscle cell proliferation and neointima formation in an animal model. Therefore we investigated the influence of the Marburg I polymorphism and the closely linked Marburg II polymorphism (E393Q) on various cardiovascular outcomes in two independent large study populations.

Methods: The Marburg I and Marburg II polymorphisms in the HABP2 gene encoding FSAP, were genotyped in a large population of elderly patients at risk for vascular disease (the PROSPER-study, n=5804) and in a study population treated with a percutaneous coronary intervention (the GENDER-study, n=3104). Associations between the Marburg I and II polymorphisms and various cardiovascular outcomes were assessed with Cox-regression adjusted for sex and age.

Results: The Marburg I polymorphism was associated with an increased risk of clinical stroke (HR: 1.60, 95%CI: 1.13-2.28) and all-cause mortality (HR: 1.33, 95%CI: 1.04-1.71) in the PROSPER study. On the other hand, carriers of this variant seemed at lower risk of developing restenosis (HR: 0.59, 95%CI: 0.34-1.01) in the GENDER study. The Marburg II polymorphism is in linkage disequilibrium with the Marburg I polymorphism and indeed showed therefore similar, although weaker results.

Discussion: The observed increase in stroke risk in Marburg I carriers could be a consequence of differential effects on smooth muscle cells as well as on matrix metalloproteinases, thereby influencing plaque stability. The possible protective effect on restenosis could be the result of reduced activation of zymogens such as pro-urokinase or matrix metalloproteinases which are involved in haemostasis and matrix remodeling.

INTRODUCTION

Factor seven activating protease (FSAP) is a plasma serine-protease which is known to activate factor VII (FVII)¹ and pro-urokinase (pro-uPA).² Despite these actions, it is unclear if endogenous FSAP has a relevant role in haemostasis. The Marburg I (G534E) polymorphism in the HABP2 gene encoding FSAP, which leads to an amino-acid change in the protease domain of this protein, may lead to a pro-thrombotic phenotype when it is associated with reduced activation of pro-uPA, but unchanged activation of FVII.³ Although its possible association with venous thrombosis remains controversial,⁴⁻⁶ the Marburg I variant has been shown to be a risk factor for late complications of carotid stenosis⁷ and coronary heart disease.⁸

Furthermore, FSAP has been identified as a potent inhibitor of smooth muscle cell proliferation and migration,⁹ specifically through its ability to cleave platelet derived growth factor-BB (PDGF-BB). The FSAP-Marburg I variant, which also has reduced proteolytic activity towards PDGF-BB, has been shown to be associated with a reduced capability to suppress neointima formation in an animal model.¹⁰ This might be another mechanism by which the Marburg I polymorphism could play a role in carotid stenosis and many other aspects of cardiovascular disease.¹¹

Therefore, we investigated the influence of this polymorphism on clinical stroke, coronary events, vascular mortality and all-cause mortality in a large population of elderly patients at risk for vascular disease (the PROSPER-study, n=5804) and on clinical restenosis after a percutaneous coronary intervention (the GENDER-study, n=3104). Although it is not associated with altered enzymatic activity, we also investigated the Marburg II variant (E393Q), a closely linked polymorphism that leads to an amino acid change in the protease domain of FSAP.

METHODS

Study design and follow-up of the PROSPER study

The protocol of PROSPER has been described in more detail elsewhere.¹² 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, we screened and enrolled subjects 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 genetic sub-study, we evaluated the predefined endpoints all-cause mortality, vascular mortality, and the secondary

endpoints fatal or non-fatal coronary events and fatal or non-fatal clinical stroke. Mean follow-up was 3.2 years (range 2.8-4.0) and 604 (10.4%) patients died during the study.¹³

Study design and follow-up of the GENDER study

The present study sample has been described previously.¹⁴ In brief, the GENetic DEterminants of Restenosis project (GENDER) was a multicenter follow-up study designed to study the association between various gene polymorphisms and clinical restenosis. A total number of 3104 patients eligible for inclusion in the GENDER-study were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in four out of 13-referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST elevation myocardial infarction were excluded. Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. During the study, no drug-eluting stents were used. Follow-up lasted for at least nine months, except when a coronary event occurred. The primary endpoint was clinical restenosis, defined as target vessel revascularization (TVR), either by PCI or coronary artery bypass grafting (CABG). Median follow-up duration was 9.6 months (interquartile range 3.9) and 304 (9.8%) patients underwent TVR during follow-up.

For both studies, all endpoints were adjudicated by independent clinical events committees. The protocols meet the criteria of the Declaration of Helsinki and were approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating patients.

Genotyping

Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. The Marburg I and II polymorphisms were determined using the Sequenom Massarray genotyping platform. A multiplex assay was designed using Assay designer software (Sequenom). As quality controls, 5-10% of the samples were genotyped in duplo. No inconsistencies were observed. Cluster plots were made of the signals from the low and the high mass allele. Two independent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Genotyper 4.0 (Sequenom Inc.) were left out of the results.

Statistical analysis

Allele frequencies were determined by gene counting. The Chi-squared test was used to test the consistency of the genotype frequencies at the SNP locus with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses were adjusted for sex and age. The analyses with PROSPER data were additionally adjusted for pravastatin use and

country. In the GENDER-study, polymorphisms were included in a multivariable model containing clinical and procedural risk factors for restenosis, such as diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. The SPSS software (version 12.0.1, SPSS Inc, Chicago, ILL) was used for all statistical analyses.

RESULTS

The PROSPER Study

Patient characteristics and minor allele frequencies are presented in table 1. Genotyping success rates were 98 % and 97 % for the Marburg I and II polymorphisms respectively and there were no significant deviations from Hardy-Weinberg equilibrium.

Using a Cox proportional hazards model, which included the variables sex, age, pravastatin use, and country, we found a significant association of the Marburg I polymorphism with clinical stroke. As presented in figure 1, the combined group of heterozygotes (n=518) and homozygotes (n=17) was at increased risk for clinical stroke (HR: 1.6, 95%CI: 1.13-2.28, p=0.009) when compared to the wild type group (n=5161). Also all-cause mortality was significantly higher in patients carrying one or two copies of this variant (HR: 1.33, 95%CI: 1.04-1.71, p=0.025). The increased mortality was mainly a result of an increase in vascular mortality (HR: 1.37, 95%CI: 0.96-1.97, p=0.082), whereas vascular mortality was mainly determined by death from stroke. The Marburg I

Table 1. Baseline characteristics of the PROSPER and the GENDER study

	PROSPER N=5804	GENDER N=3104
Continuous variates (mean, SD)		
Age (years)	75.3 (3.3)	62.1 (10.7)
Body mass index, (kg/m ²)	26.8 (4.2)	27.0 (3.9)
Categorical variates (n, %)		
Male sex	2804 (48)	2216 (71)
Current smoker	1558 (27)	762 (25)
History of diabetes	623 (11)	453 (15)
History of hypertension	3592 (62)	1259 (41)
History of myocardial infarction	776 (13)	1239 (40)
History of stable angina	1559 (27)	2079 (67)
Genotype, minor allele frequency (%)		
Marburg I G/A *	5	5
Marburg II G/C **	2	2

All data are presented in number (%) unless otherwise stated.

* In PROSPER and GENDER measured in 5583 and 2949 participants, respectively.

** In PROSPER and GENDER measured in 5595 and 2953 participants, respectively.

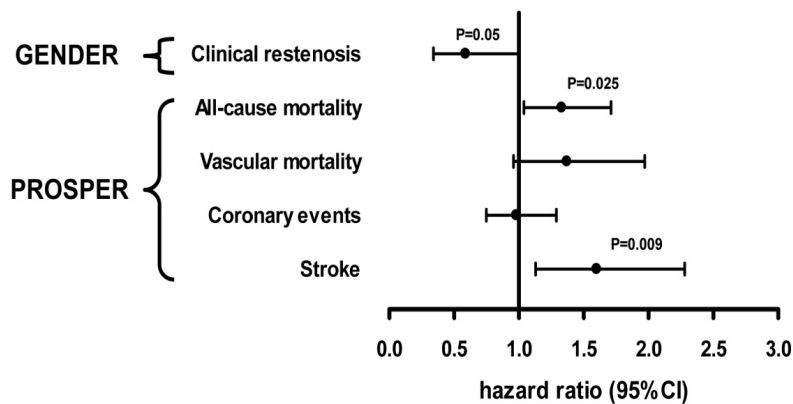


Figure 1. Marburg I hazard ratios for vascular endpoints in GENDER and PROSPER
The Marburg I (G534E) polymorphism is associated with an increased risk for stroke and mortality in the PROSPER study whereas it tends to reduce the risk for clinical restenosis in the GENDER study.

polymorphism did not seem to influence the risk for coronary events (HR: 0.98, 95%CI: 0.75-1.29).

Due to its location in the same coding sequence and its proximity to the Marburg I polymorphism we also present data of the Marburg II polymorphism which show significant effects on mortality (Figure 2). The similar trends that were observed for this polymorphism are probably a result of the high linkage disequilibrium between the

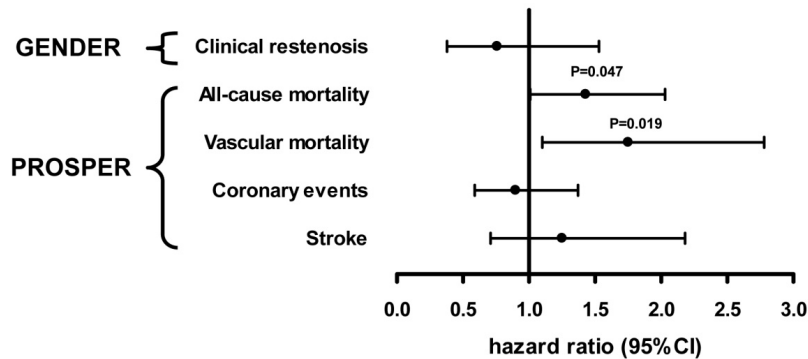


Figure 2. Marburg II hazard ratios for vascular endpoints in GENDER and PROSPER
As the Marburg II polymorphism does not lead to altered enzymatic activity, the similar trends that were observed for this variant are probably a result of the high linkage disequilibrium between the Marburg variants. Despite high confidence intervals, a significant effect was observed on mortality.

Marburg variants. The LD-coefficient was 0.79 and 62% of Marburg II carriers were also genotyped with the Marburg I allele. Due to a lower allele frequency of the Marburg II polymorphism there were only 234 heterozygotes and 4 homozygotes with the variant allele. The large confidence intervals indeed indicate that these results are less reliable than the Marburg I findings.

The GENDER Study

Patient characteristics and minor allele frequencies are presented in table 1. Genotyping success rates were 95 % for both polymorphisms and there were no significant deviations from Hardy-Weinberg equilibrium.

In contrast with the results from the PROSPER study, the Marburg I polymorphism tended to reduce the risk for clinical restenosis in the GENDER study (Figure 1, HR: 0.59, 95%CI: 0.34-1.01, $p=0.061$). The protective effect was borderline significant. Carriership of the Marburg II variant only slightly and non-significantly reduced the risk of TVR (Figure 2).

DISCUSSION

Our data suggest that the Marburg I polymorphism, which leads to less FSAP-activity, increases stroke risk and mortality. The observed Marburg I associated increase in mortality was mainly due to an increased risk of fatal stroke. Surprisingly, carriers of this variant seemed at lower risk of developing restenosis.

Although FSAP was originally identified as a potential activator of Factor VII and pro-urokinase (pro-uPA), its role in haemostasis remains unclear. Endogenous FSAP, of which intravascular levels are low, has not been clearly shown to influence blood coagulation or fibrinolysis.¹¹ Moreover, earlier studies showed that the Marburg I polymorphism does not seem to influence the risk for venous thrombosis.^{6, 15} However, FSAP cleaves PDGF-BB and has been shown to inhibit vascular smooth muscle cell proliferation and migration *in vitro*⁹ and *in vivo*.¹⁰ The activity of FSAP in Marburg carriers is low and could therefore be a risk factor for atherosclerosis and restenosis, processes which are known to be determined by vascular smooth muscle cell proliferation.

A possible role for FSAP in human atherosclerosis was suggested by a study showing an association of the Marburg I polymorphism with advanced atherogenesis in the carotid arteries.⁷ Despite the low number of carriers among cases ($n=8$) and controls ($n=2$), their findings suggest a role for Marburg I in carotid plaque formation. Another study, investigating the effect of Marburg I on coronary heart disease, could not find a significant effect in the whole population, but observed an interactive effect on risk between the Marburg I variant and elevated levels of cholesterol and triglyceride.⁸ The

primary endpoint in this study was a composite of myocardial infarction and the need for a PCI.

In agreement with these previous studies we also found no association of Marburg I with myocardial infarction in the whole population of patients taking part in PROSPER study, whereas we did find a significant association with stroke, which is known to be related to carotid plaque formation. The observed increase in stroke risk in Marburg I carriers could be a consequence of hyper-proliferation of smooth muscle cells, due to a reduced proteolytic activity of Marburg I-FSAP towards PDGF-BB.

The possible protective effect of Marburg I on clinical restenosis in patients treated for stable angina pectoris is difficult to explain. Despite some similarities between atherosclerosis and restenosis, such as the involvement of inflammation and smooth muscle cell proliferation, it is well known that there are important mechanistic differences between these processes. In contrast with atherosclerosis, which develops partly in response to elevated lipoprotein levels and cigarette smoke, the restenotic process is not particularly sensitive to circulating lipids and smokers even seem to have a reduced risk for restenosis.¹⁴ It is therefore not unlikely that a genetic risk factor would have opposite effects on stroke and clinical restenosis after PCI.

However, as opposed to WT-FSAP, Marburg I-FSAP was less effective in preventing neointima formation in an animal model for restenosis.¹⁰ Based on these findings, the Marburg I polymorphism was expected to increase the risk for restenosis in humans. Although the protective effect observed in the GENDER study was just not significant, there was a strong trend towards protection. The contradicting results could relate to the subjects (mice vs. humans) or the intervention site (femoral artery vs. coronary arteries), but could also be the result of differences in the exact location and concentration of the Marburg I-FSAP-protein, which was locally applied in high concentrations to injured arteries in the mouse model. Furthermore, FSAP plays a role in many different processes which are known to be important in vascular remodeling. FSAP has recently been shown to activate the matrix metalloproteinases MMP2 and MMP9 (gelatinases),¹⁶ which are important in matrix remodeling. Further research is needed to understand the precise role of FSAP in vascular remodeling and the pathogenesis of stroke.

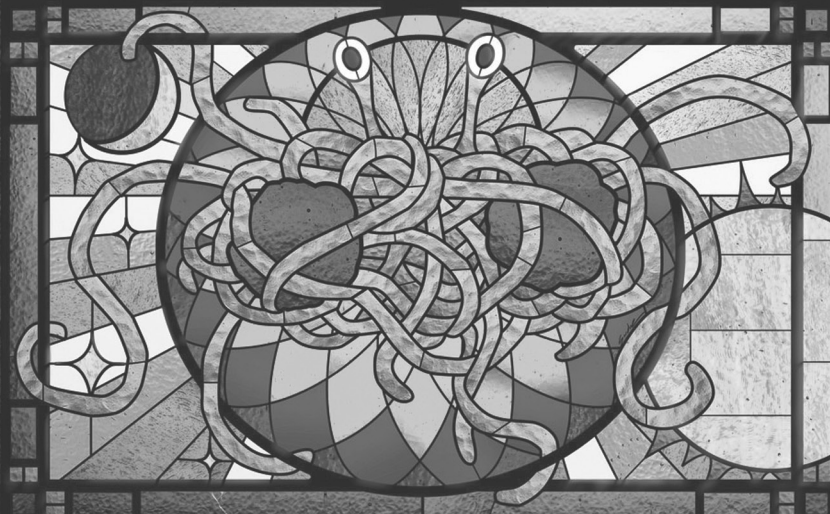
The Marburg II polymorphism, also located in the protease domain of FSAP, has no known functional implications and is not associated with altered catalytic activity.

Due to its low allele frequency (2%), the Marburg II findings are less reliable. The similar, but much weaker trends that were observed for this polymorphism could be a result of the linkage disequilibrium with the Marburg I variant. Large confidence intervals indicate that the observed significant association with vascular mortality, which is slightly stronger than the effect of Marburg I on vascular mortality, probably occurred by chance.

In conclusion, we demonstrated that carriers of the Marburg I polymorphism are at increased risk for clinical stroke and stroke related mortality. Furthermore, a strong trend towards a reduced risk for clinical restenosis was observed in Marburg I carriers. As Marburg I-FSAP has reduced proteolytic activity towards PDGF-BB, the increase in stroke risk could be a consequence of hyper-proliferation of smooth muscle cells.

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Chapter 5

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

*Is there still room for determining genetic variations of MMPs
for assessment of an increased risk of restenosis?*

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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 were 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 6A/6A 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 mul-

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 4.1²¹). 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 MassARRAY[™] 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 ($\pm$ 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 $p\text{-value} < 0.0017$.

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 cal-

culated 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 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 restenosis 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 1. Demographic, clinical and lesion characteristics of the total GENDER population composed of 3104 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).

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

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	Promoter	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.

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.

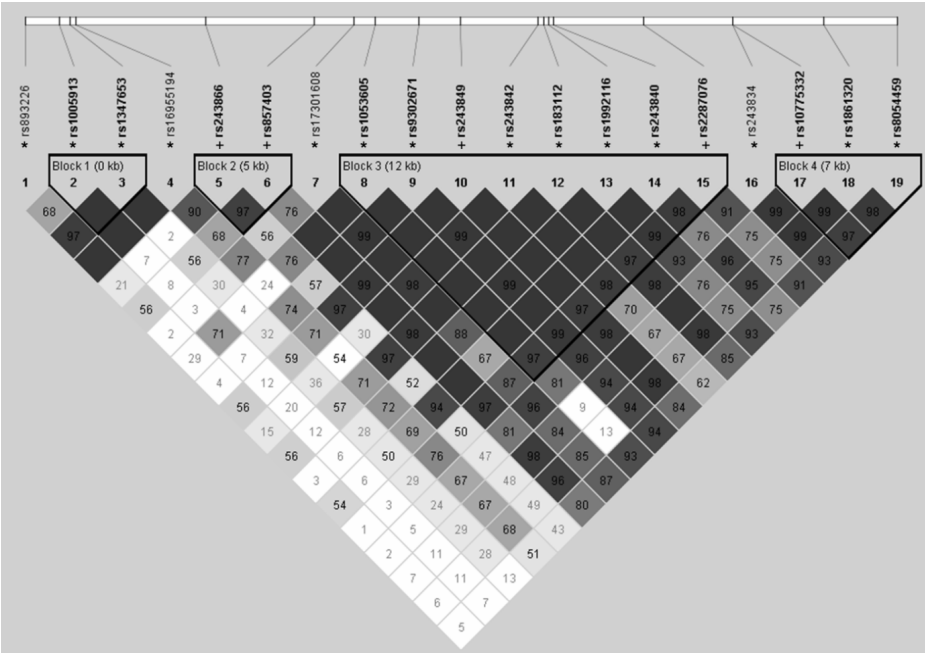


Figure 1. Linkage disequilibrium structure of 19 MMP2 SNPs on chromosome 16
Values shown in D'; * SNP from GWAS, + SNP from candidate approach

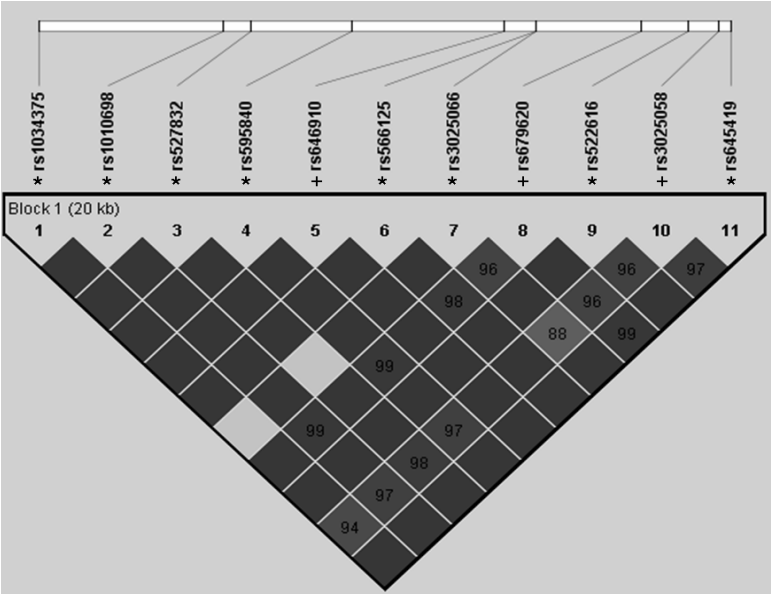


Figure 2. Linkage disequilibrium structure of 11 MMP3 SNPs on chromosome 11
Values shown in D'; * SNP from GWAS, + SNP from candidate approach

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.

Table 5. Haplotype analysis of MMP3

Haplotype	Frequency	HR	95% CI	p-value
AACTTCAAAAA*	42.43%			
ACTCTCAGGCG	12.84%	1.157	(0.84 – 1.59)	0.37
ACCCATAGACG	11.99%	0.994	(0.80 – 1.24)	0.95
ACCCTCAGGCG	9.35%	1.112	(0.87 – 1.42)	0.40
CCCCTCAGACG	7.71%	1.212	(0.93 – 1.58)	0.16
ACCCTCAGACG	5.72%	1.059	(0.78 – 1.44)	0.72
AACTTCGAAAA	5.59%	1.105	(0.87 – 1.41)	0.42

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

approaches strengthened our findings of the lack of association between the two MMP genes and coronary restenosis after successful PCI.

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.⁹ 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.

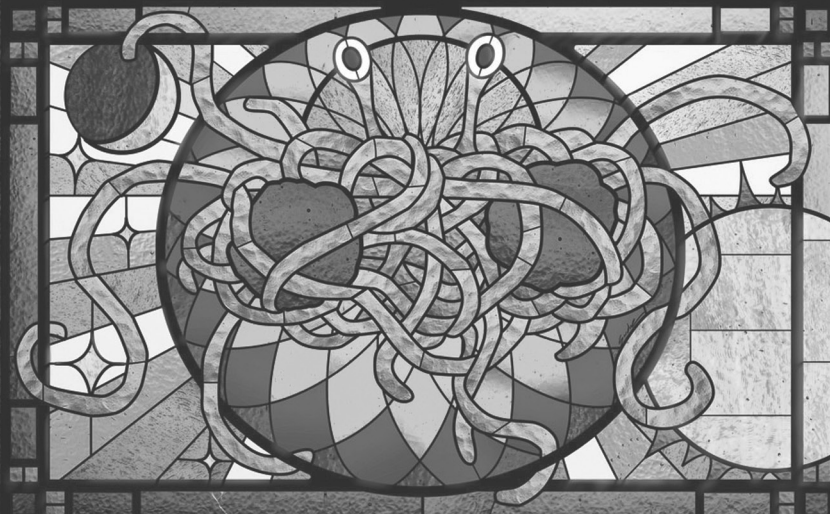
A limitation of our study is that the patients included in the GENDER study were mostly patients with a Caucasian ancestry (96.9%). Different results may be obtained in other ethnic groups. Furthermore, we did not test all known polymorphisms in the two genes of interest, but since SNPs were selected 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

Genetic variation at the Quaking locus associates
with clinical restenosis after PCI and induces
vascular smooth muscle cell dysfunction

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ABSTRACT

Introduction: The mRNA binding protein Quaking (Qk) is a key regulator of neuron myelination, while more recent data point to a pivotal role in embryonic arteriogenesis. Therefore, we hypothesized that Qk might exert regulatory functions in vascular patency responses such as restenosis, a frequent complication of percutaneous coronary intervention (PCI).

Methods and results: We addressed the role of Qk in in-stent restenosis by analysis for association of single nucleotide polymorphisms (SNPs) throughout the Qk gene and the risk of restenosis in the prospective GENetic DEterminants of Restenosis (GENDER) study (N=3,104 patients). Seven out of twelve tested SNPs associated with risk for or protection against restenosis. The strongest associations were found for SNPs located close to the Qk transcriptional start site (2786 T/C: HR: 2.14, 95%CI: 1.24-3.68, P=0.006) and in intron 3 (57896 A/G: HR: 0.74, 95%CI: 0.6-0.92, P=0.005 and 65752 A/G: HR: 1.5, 95%CI: 1.14-1.98, P=0.003). Importantly, patients carrying the 65752G risk allele but lacking the protective 57896G allele were at a high risk to develop restenosis (HR: 1.8, 95%CI: 1.3-2.5, P<0.001). In vitro characterization of Qk deficient vascular smooth muscle cells (VSMC) revealed severely compromised cell viability, as evidenced by a decreased resistance to stress, decreased cellular proliferation, migration and extracellular matrix production. In keeping with this notion, Qk deficient Qk^v mice showed markedly decreased neointima formation in a mouse model of in-stent restenosis.

Conclusions: We are the first to identify a link between Qk and the risk for in-stent restenosis after PCI, which may be attributable to its regulatory role in VSMC function. Therefore, Qk could serve as a marker for TVR risk prediction as well as a novel target to reduce restenotic risk.

INTRODUCTION

The mRNA-binding protein Quaking (Qk) is a member of the signal transduction and activation of RNA (STAR) family of transcription factors. It is located on chromosome 6 in humans and chromosome 17 in mice and is highly conserved during mammalian evolution.¹ As a result of alternative splicing, Qk has three main isoforms derived from mRNA transcripts of 5, 6 and 7 kb, notably Qk-5, Qk-6 and Qk-7.² Qk-5 is the most abundantly expressed isoform and is essential during embryogenesis,² whereas Qk-6 and Qk-7 are primarily expressed post-natally.³ Qk has been found to play a critical role in driving oligodendrocyte differentiation, positioning Qk as a central regulator of myelination of both the central and peripheral neural network.⁴ Illustratively, the Qk viable mouse mutant (Qk^v), which carries a 1 MB deletion in the promoter region resulting in Qk-knockdown, displays severe developmental defects, characterized by a phenotype of seizures and movement-dependent tremors.⁴ However, its high expression levels in heart, brain, lung and testis as well as its broad range of putative Qk response genes points to a more general regulatory function of Qk, including vascular function.³ Support for this notion came from a study showing a pivotal role of Qk in embryonic vascular development, as N-ethyl-N-nitrosourea (ENU) induced Qk as well as Qk-null mouse mutants were seen to die during development because of a lack of embryonic vascular remodelling.^{5,6} This lethality was found to be due to a lack of local pericyte differentiation into vascular smooth muscle cells (VSMC), preventing the maturation of the yolk sac vasculature. Furthermore, Qk expression has been detected in murine VSMC of the aorta and coronary arteries,^{6,7} suggesting that Qk may also influence VSMC function after gestation.

In-stent restenosis after percutaneous coronary intervention (PCI) is largely dependent on VSMC proliferation. This process, harbouring potentially serious clinical consequences, is characterized by proliferation of cellular components of the vascular wall, in particular VSMCs in response to vascular injury, leading to re-occlusion of the stented arterial segment.^{8,9} Previous studies have established the significant contribution of various genetic factors to the occurrence of restenosis.^{8,10} Given the proposed regulatory role of Qk in arteriogenesis and VSMC function, we hypothesized that single nucleotide polymorphisms (SNPs) in both the promoter and coding region of the Qk gene could influence Qk expression and function, and could therefore potentially influence the occurrence of restenosis in patients who underwent PCI. Furthermore, we assessed the effects of reduced Qk expression on neo-intimal hyperplasia *in vivo* and studied the specific effects of Qk abrogation in aortic-derived VSMCs of Qk^v mice *in vitro*. We demonstrate strong two-directional associations of various Qk SNPs with the risk of restenosis after PCI and also, based on direct *in vivo* and *in vitro* data, propose a mechanistic explanation for this intriguing observation.

METHODS

GENDER project study design

The present study population has been described previously.¹¹ In brief, the GENDER project was designed to study the association between various gene polymorphisms and clinical restenosis, defined by target vessel revascularization (TVR). Patients eligible for inclusion were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in 4 out of 13 referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST elevation myocardial infarction were excluded. Patients suffering from events occurring within 1 month after PCI were also excluded from analysis, as these events were attributable predominantly to sub-acute stent thrombosis or occluding dissections, rather than to restenosis.

PCI procedure

Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. Before the procedure, patients received aspirin (300mg) and heparin (7500 IU). The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, were carried out at discretion of the operator. In case a stent was implanted, patients received either ticlopidin or clopidogrel for at least 1 month following the procedure depending on local practice. During the study, no drug-eluting stents were used.

Follow-up and study end points

Follow-up lasted for at least 9 months, except when a coronary event occurred. TVR, either by PCI or coronary artery bypass grafting (CABG), was designated the primary end point. An independent clinical events committee evaluated the clinical events. The study protocol meets the criteria of the Declaration of Helsinki and was approved by the Medical Ethics Committees of each of the participating institutions. Written informed consent was obtained from all participating patients before the PCI procedure.

Genotyping

Blood was collected in EDTA tubes upon admission to hospital and genomic DNA was extracted following standard procedures. Validated polymorphisms in the promoter and in each linkage disequilibrium (LD)-block in the Qk gene, as defined by the algorithm of Gabriel et al.¹², were selected using the program Haploview with genotype data from the International HapMap Consortium. The -7528 C/T (rs3857503), -4539 T/C (rs3904720), -2616 C/T (rs3857504), 2786 A/G (rs3763197), 34084 C/A (rs2759393), 57896 A/G (rs1737603), 65752 A/G (rs6937072), 71659 T/C (rs1016502), 106999 A/G (rs9458849), 120103 G/A (rs9364692), 150256 C/T (rs715020) and 161241 G/T (rs1047944) poly-

morphisms were selected on the basis of their high minor allele frequency (>5%) and measured using the Sequenom Massarray genotyping platform (Sequenom, San Diego, CA, USA). A multiplex assay was designed using Assay designer software (Sequenom). All PCR reactions had a final volume of 5 μ l and contained standard reagents and 5 ng of genomic DNA. After PCR a primer extension reaction was performed to introduce mass-differences between alleles and, after removing salts by adding resin, approximately 15 nl of the product was spotted onto a target chip with a 384 patches containing matrix. Mass differences were detected by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF; Autoflex; Bruker Daltonics, Billerica, MA, USA) and genotypes were assigned real-time using Typer 4.0 software (Sequenom). As quality controls, 5-10% of the samples were genotyped in duplo. Cluster plots were made of the signals from the low and the high mass allele. Scoring was performed by two independent researchers. Disagreements or vaguely positioned dots produced by Genotyper 4.0 (Sequenom) were left out of the results.

Femoral cuff induced vascular hyperplasia

Male Qk^v mice (N=6) on a C57Bl6-J background together with wild-type age and sex-matched C57Bl6-J mice (N=6) were used (Jackson Laboratories, Bar Harbor, ME, USA). Mice were fed with water and chow diet ad libitum. At the age of 12 weeks, mice were anaesthetized with an intraperitoneal injection of 5 mg/kg Midazolam (Roche, Basel, Switzerland), 0.5 mg/kg Medetomidine (Orion, Helsinki, Finland) and 0.05 mg/kg Fentanyl (Janssen, Geel, Belgium). The femoral artery was dissected from its surroundings and sheathed with a non-constrictive cuff.¹³ Animals were sacrificed 21 days after cuff placement. Preparation for histological analyses was performed as described previously.^{13, 14} All animal work was approved by institutional regulatory authority and carried out in compliance with guidelines issued by the Dutch government.

Temporal gene expression cuff model

15 C57Bl6-J mice were maintained and provided with a femoral cuff as described above. At 0, 1, 2, 3, 7 and 14 days after placement of the femoral cuff, the mice were sacrificed and cuffed arteries were dissected and stored in liquid nitrogen. Trizol (Invitrogen) was used to extract total RNA from the femoral arteries, and the samples were subjected to DNase I treatment (Promega, Madison, WS) to ensure that the samples were rid of genomic DNA. Subsequently, cDNA was generated using RevertAid M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) according to manufacturer's protocol.² Semi-quantitative expression of the QKI gene was performed using the Assays-on-Demand primer and probe set for QKI (Mm 01318927_m1) and SYBR-Green (Eurogentec, Liege, Belgium) on a Bio-Rad iCycler (Bio-Rad, Hercules, CA). GAPDH was utilized as

housekeeping gene. Relative gene expression was calculated by determining the $\Delta\Delta C_t$ value of QKI versus GAPDH.

Histological analysis and quantification of cuffed femoral artery lesions

All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert's elastin staining was used to visualize elastic laminae. VSMCs were visualized with α -SM actin staining (DakoCytomation, Glostrup, Denmark). Collagen content was determined using a 0.1% (w/v) solution of Sirius Red F3B dye (BDH Laboratory Supplies, Poole, UK) in aqueous picric acid for 1h. Unbound dye was removed by washing the sections with 0.01M HCl. Subsequently, the collagen-bound dye was dissolved by incubating the sections with 0.1M NaOH for 30 minutes. The amount of medial VSMC and collagen content was determined by morphometry (Leica Qwin, Wetzlar, Germany) and expressed as the percentage of total medial area consisting of α -SM actin- or Sirius red-positive area in six equally spaced serial cross-sections in all animals. Monocytes were detected with the use of an anti-CD45 antibody (PharMingen, San Diego, CA, USA). Incorporation of 5-bromo-2'-deoxyuridine (BrdU, Sigma) into DNA as a marker of DNA synthesis was used to determine the rate of cell proliferation in cuffed vessel segments. Mice were injected intraperitoneally with 25 mg/kg BrdU three times at 72, 48, and 24 hours prior to sacrifice. Sections were incubated with a mouse monoclonal anti-BrdU antibody (DakoCytomation). Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining according to manufacturers protocol (Roche). The number of labeled nuclei per cuffed artery were counted in six equally spaced cross-sections and expressed as a percentage of the total number of nuclei. For all immunohistochemical stainings, sections were incubated with an isotype-matched IgG at an equal titer as the primary antibody to serve as a control.

VSMC isolation and culture

Experiments were performed using primary mouse vascular smooth muscle cells (mVSMCs) derived by outgrowth from fragments of mouse aorta from C57Bl/6-J and Qk(v) mice seeded on fibronectin-coated plates (1 mg fibronectin dissolved in 10 mL PBS; solution left on culture dish for approximately 15 minutes). The tissue fragments and outgrowth cells were maintained in DMEM culture medium containing 20% fetal calf serum and penicillin/streptomycin (0.01 Units/ml and 0.01 μ g/mL, respectively), at 37°C and 5% CO₂ unless specified otherwise. The purity of VSMC outgrowth was assessed immunohistochemically using anti-mouse smooth muscle α -actin and peroxidase-conjugated goat-anti-mouse immunoglobulins, generally yielding 90-95% smooth muscle α -actin positive cells. In designated experiments, VSMCs were treated

with freshly isolated oxLDL (20 µg/mL), VLDL (50 µg/mL), and lipopolysaccharide (50 ng/mL) for the specified times. Experiments were performed with cells at passages 3-4.

Extracellular matrix production assay

Primary wild-type and Qk^v mVSMCs were seeded at a density of 1.5×10^4 cells/cm² and grown for 24h in culture medium described above. Subsequently, the cells were cultured in DMEM containing 1% FCS and P/S and Gln for 16h. Next, the cells were extensively washed with PBS and fixed for 30 minutes using Formalfix (Thermo Scientific, Waltham, MA, USA). To assess total cellular collagen production, the cells were stained with Sirius Red F3B dye as described above. Concomitantly, a calibration curve was prepared by plating increasing concentrations of gelatin in PBS, which were air-dried and fixed in Formalfix. Total collagen content was determined spectrometrically at an optical density of 550nm on a microplate reader.

DNA synthesis assay

To quantify DNA synthesis, explant-derived mVSMCs from wild-type and Qk^v aortic explants were seeded at a density of 1×10^4 cells/cm² and cultured in medium described above, after which the cells were cultured for 24h in serum-free DMEM. Subsequently, the cells were incubated for 12h with [³H]-thymidine (10µCi/mL) in DMEM containing 10% FCS, after which cell associated [³H]-thymidine was determined by assaying the trichloroacetic acid precipitable counts with Aquasol-2 (PerkinElmer, Waltham, MA, USA) on a Beckman LS3801 scintillation counter (Beckman, Fullerton, CA, USA).

Cellular migration assay

Primary mVSMCs were cultured in medium described above, and 24h prior to seeding into a transwell plate, cells were incubated in culture medium containing 1% FCS and Gln. DMEM containing 10% FCS, P/S, Gln as well as the chemotactic peptide n-formyl-methionine-leucine-phenylalanine (fMLP; 0.1nM) was added to the basolateral side of a semi-permeable membrane with a pore size of 12 µm (ChemoProbe, UK). Subsequently, the cells were detached from the culture dish using trypsin-EDTA and 2×10^4 cells were seeded onto the membrane and allowed to migrate for 24h. After centrifugation, adherent mVSMCs were fixed, stained with Mayer's hematoxylin, and the number of cells that had completely migrated to the basolateral side of the chamber was assessed microscopically and scored manually.

Cellular outgrowth assay

Aortas were harvested from both wild-type and Qk^v mice after sacrificing. The aortas were excised from the base of the aortic arch until the iliac bifurcation. Subsequently, the aortas were cut longitudinally to expose the luminal portion of the vessel and the

endothelial cells were removed by gently scraping. After this, the aortas were cut into approximately 0.5 cm² pieces, and placed on fibronectin-coated culture dishes. The explants were cultured in DMEM containing 20% FCS, P/S and Gln. After 7 days, phase-contrast photomicrographs of cellular outgrowth from individual pieces of aortic tissue were taken. Using Leica QWin software, the photomicrographs were used to quantitate both the total area of the designated piece of tissue and its corresponding cellular outgrowth was calculated based on: $(A_{\text{outgrowth}} = (\text{Total Area } (A_{\text{outgrowth}} + A_{\text{tissue}})) - A_{\text{tissue}})$.

Western blot analysis

Protein expression was assessed by Western blot analysis with chemiluminescence detection. Qk-5 was detected using a rabbit polyclonal anti-human Qk antibody (Bethyl Laboratories, Montgomery, TX, USA). As a reference α -tubulin was detected using a monoclonal antibody (Clone B-5-1-1, Sigma). Horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG F(ab')₂ (Amersham Pharmacia, Roosendaal, the Netherlands) were used as secondary antibodies. Bound Fab fragment was detected with enhanced chemiluminescent reagents (Boehringer, Ingelheim, Germany) and exposed on Kodak X-Omat blue XB-1 film.

Statistics

Allele frequencies were determined by gene counting. The Chi-squared test was used to test whether genotype frequencies at the SNP locus complied with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses were adjusted for clinical and procedural risk factors for restenosis, such as diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. All polymorphisms were also assessed using dominant and recessive models, and the model with the lowest Akaike information criteria was used in the multivariable regression analysis¹⁵. SPSS software (version 12.0.1, SPSS Inc, Chicago, IL, USA) was used for all GENDER analyses. All *in vitro* and *in vivo* results were analysed via a 2-tailed Student's t-test and ANOVA when appropriate using Graphpad Prism 4.0 Software (Graphpad software, La Jolla, CA, USA). P-values <0.05 were considered significant.

RESULTS

Patient characteristics

Characteristics of the GENDER cohort have previously been described in detail¹¹. In brief, 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 months). 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. Bare metal stents were used in 2309 patients (74.4%), whereas the rest of the population was treated with plain balloon angioplasty. Of all patients, 304 patients (9.8%) underwent TVR during follow-up, 51 patients (1.6%) died and 22 (0.7%) had a myocardial infarction.

Quaking genotyping

Due to lack of genomic DNA or inconclusive genotyping (vaguely positioned dots or genotyping errors), not all 3,104 patients could be successfully genotyped. For each SNP, genotype counts and minor allele frequencies are presented in Table 2 and figure 1A. There were no significant deviations from Hardy-Weinberg equilibrium. Out of the 12 SNPs we assessed, 4 were found to significantly increase the risk of TVR using multiple regression analysis: -2616 T/T (promoter region; HR 1.91, 95% CI 1.11-3.28), 2786 G/G (intron 1; HR 2.14, 95% CI 1.24-3.68), 65752 G/G (intron 3; HR 1.57, 95% CI 0.87-2.8) and 106999 G/G (intron 3; HR 1.51, 95% CI 1.15-1.98) (Table 2 and figure 1A).

Table 1. Demographic, clinical and lesion characteristics of patients with TVR and without TVR during follow-up.

	TVR (n=304)	No TVR (n=2800)	Total (n=3104)
Age (years)	61.7 ± 10.1	62.2 ± 10.8	62.1 ± 10.7
BMI (kg.m ⁻²)	26.9 ± 3.7	27.0 ± 3.9	27.0 ± 3.9
Male sex	220 (72.4%)	1996 (71.3%)	2216 (71.4 %)
Diabetes	63 (20.7%)	390 (13.9%)	453 (14.6%)
Hypercholesterolemia	188 (61.8%)	1702 (60.8%)	1890 (60.9%)
Hypertension	138 (45.4%)	1121 (40.0%)	1259 (40.6%)
Current smoker	62 (20.4%)	700 (25.0%)	762 (24.5%)
Family history of MI	121 (39.8%)	977 (34.9%)	1098 (35.4%)
Previous MI	109 (35.9%)	1130 (40.4%)	1239 (39.9%)
Previous PCI	64 (21.1%)	493 (17.6%)	557 (17.9%)
Previous CABG	36 (11.8%)	340 (12.1%)	376 (12.1%)
Stable angina	198 (65.1%)	1881 (67.2%)	2079 (67.0%)
Multivessel disease	148 (48.7%)	1284 (45.9%)	1432 (46.1%)
Peripheral vessel disease	12 (3.9%)	92 (3.3%)	104 (3.4%)
Lipid lowering medication	171 (56.3%)	1516 (54.1%)	1687 (54.3%)
Restenotic lesions	27 (8.9%)	181 (6.5%)	208 (6.7%)
Total occlusions	56 (18.4%)	372 (13.3%)	428 (13.8%)
Type C lesion	94 (30.9%)	708 (25.3%)	802 (25.8%)
Proximal LAD	70 (23.0%)	619 (22.1%)	689 (22.2%)
RCX	75 (24.7%)	764 (27.3%)	839 (27.0%)

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

Table 2. Distribution and multivariate analysis of 12 Qk polymorphisms in association with TVR.

Polymorphisms	Minor allele frequency	Genotype Counts	TVR risk (%)	Genetic model	HR (95%CI)	P-value
rs3857503 -7528						
CC	0.43	948	9.5	Additive	0.94 (0.79-1.11)	0.45
CT		1393	10.4			
TT		555	7.9			
rs3904720 -4539						
TT	0.15	2090	9.2	Recessive	1.57 (0.87-2.8)	0.13
TC		734	9.7			
CC		75	16.0			
rs3857504 -2616						
CC	0.15	2094	9.4	Recessive	1.91 (1.11-3.28)	0.019
CT		734	9.8			
TT		74	18.9			
rs3763197 2786						
AA	0.15	2064	9.3	Recessive	2.14 (1.24-3.68)	0.006
AG		695	8.9			
GG		69	20.3			
rs2759393 34084						
CC	0.23	1553	11.1	Dominant	0.68 (0.52-0.88)	0.004
CA		928	7.8			
AA		151	7.9			
rs1737603 57896						
AA	0.24	1650	11.0	Additive	0.74 (0.6-0.92)	0.005
AG		1054	8.1			
GG		166	6.6			
rs6937072 65752						
AA	0.07	2534	9.1	Additive	1.57 (0.87-2.8)	0.003
AG		396	12.6			
GG		16	25			
rs1016502 71659						
TT	0.24	1663	10.9	Additive	0.76 (0.61-0.94)	0.01
TC		1045	8.2			
CC		161	6.8			
rs9458849 106999						
AA	0.07	2534	9.2	Additive	1.51 (1.15-1.98)	0.003
AG		396	13.1			
GG		14	21.4			
rs9364692 120103						
GG	0.46	859	9.4	Additive	0.97 (0.82-1.14)	0.72
GA		1435	10.5			
AA		629	8.4			
rs715020 150256						
CC	0.46	865	9.1	Additive	0.98 (0.83-1.16)	0.82
CT		1447	10.6			
TT		615	8.3			
rs1047944 161241						
GG	0.32	1351	9.9	Additive	0.91 (0.76-1.09)	0.30
GT		1252	10.0			
TT		283	7.1			

Values are corrected for diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. TVR = target vessel revascularization; HR = hazard ratio; CI = confidence interval

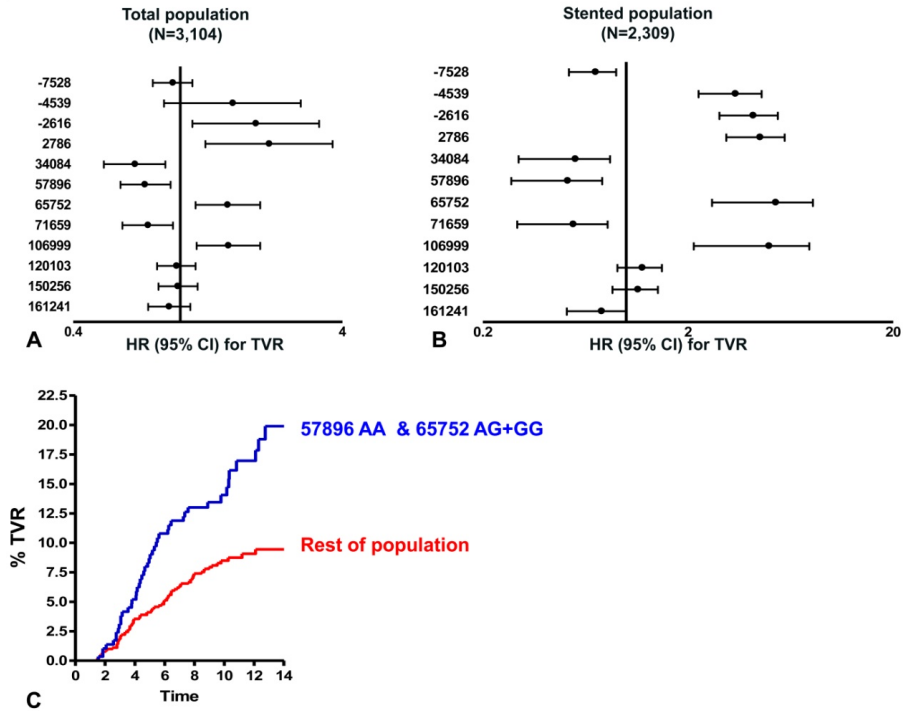


Figure 1.

Hazard ratio distribution for all 12 Qk SNPs for the total GENDER cohort (N=3,104) as well as for a sub-cohort of all stented patients (N=2,309). Three SNPs are located within the promoter region and 9 within the coding region. Panel A shows the hazard ratio's (HR) for all 12 SNPs in the total population with multivariate regression analysis. Four SNPs are associated with a significant increased HR for TVR, whereas three SNPs have a protective effect. The highest hazard ratio's are located within the promoter region and the first intron. Panel B shows HRs for all 12 SNPs in stented patients. The same distribution of risk per SNP is observed, although with larger 95% CI. Patients who carry the 65,752 A/G and G/G alleles together with 57,896 A/A allele are at higher risk of restenosis compared to the rest of the study population (C). This genotype is observed in 10% of the total population. TVR = target vessel revascularisation. 95% CI = 95% confidence interval.

In contrast, three SNPs were seen to significantly protect against TVR: 34084 A/A (intron 1; HR 0.68, 95% CI 0.52-0.88), 57896 G/G (intron 2; HR 0.74, 95% CI 0.6-0.92) and 71659 C/C (intron 3; HR 0.76, 95% CI 0.61-0.94). Only 5 SNPs were not significantly associated with TVR in the multiple regression analysis model. Importantly, analysis of the sub-cohort of patients who received one or more coronary stents did not reveal any major differences compared to results the total population (table 3 and figure 1B). Therefore, our findings can be extrapolated to the current PCI era in which plain balloon angioplasty is hardly being used anymore.

Interestingly, patients who carry the 65752 A/G and G/G alleles together with the 57896 A/A allele were at high risk of restenosis compared to the rest of the population (HR 1.77, 95% CI 1.29-2.44; $P < 0.001$) during 15 months of follow-up (figure 1C). This particular genotype was observed in 10% of the total population. This demonstrates that

Table 3. Distribution and multivariate analysis of 12 Qk polymorphisms in association with TVR in the stented subgroup.

Polymorphisms	Minor allele frequency	Genotype Counts	Risk for TVR (%)	HR (95%CI)	P-value
rs3857503 -7528					
CC	0.43	694	8.9	0.85 (0.69-1.05)	0.13
CT		1029	9.3		
TT		423	5.9		
rs3904720 -4539					
TT	0.15	1560	7.8	2.67 (1.45-4.94)	0.002
TC		539	8.9		
CC		50	22.0		
rs3857504 -2616					
CC	0.15	1567	7.9	3.32 (1.88-5.85)	<0.001
CT		538	9.1		
TT		49	26.5		
rs3763197 2786					
AA	0.15	1548	7.8	3.66 (2.08-6.47)	<0.001
AG		509	8.6		
GG		46	28.3		
rs2759393 34084					
CC	0.23	1150	10.0	0.7 (0.51-0.96)	0.027
CA		684	7.6		
AA		109	4.6		
rs1737603 57896					
AA	0.24	1220	9.8	0.72 (0.55-0.93)	0.014
AG		793	7.6		
GG		122	4.1		
rs6937072 65752					
AA	0.07	1890	8.1	1.56 (1.13-2.17)	0.008
AG		284	10.9		
GG		14	28.6		
rs1016502 71659					
TT	0.24	1228	9.9	0.75 (0.58-0.97)	0.03
TC		793	7.8		
CC		116	4.3		
rs9458849 106999					
AA	0.07	1890	8.1	1.59 (1.15-2.22)	0.006
AG		283	11.7		
GG		12	25.0		
rs9364692 120103					
GG	0.46	651	7.1	1.09 (0.89-1.34)	0.39
GA		1053	10.0		
AA		461	8.0		
rs715020 150256					
CC	0.46	660	7.0	1.07 (0.88-1.31)	0.50
CT		1064	10.1		
TT		450	7.6		
rs1047944 161241					
GG	0.32	1001	8.7	0.92 (0.73-1.15)	0.44
GT		924	9.0		
TT		213	6.1		

Values are corrected for diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%. TVR = target vessel revascularization; HR = hazard ratio; CI = confidence interval

specific combinations of protective and hazardous genotypes can have additive effects on outcome in this high risk patient group.

Effect of Qk abrogation on VSMC function

Since post-operative restenosis is a fibrocellular hyperplastic response to vascular injury mediated by VSMC dedifferentiation, migration and proliferation, we sought to determine whether altered Qk expression could affect VSMC function. For this, we utilized Qk^v mice, which contain a 1 Mb deletion in the Qk promoter region resulting in ablated transcription. Primary mouse VSMC cultures were established by aortic outgrowth and shown to be SM- α -actin positive by immunohistochemistry. Western blot analysis revealed a marked decrease in Qk-5 expression, the most prominent isoform (figure 2A). Next, we assessed the effect of serum starvation as well as proatherogenic stimuli on Qk-5 expression. We observed a striking down-regulation of Qk-5 in 1% FBS, suggesting that a shift to a non-proliferative phenotype is accompanied by reduced Qk-5 expression (figure 2B). Treatment of VSMC for 24h with either oxLDL, VLDL or LPS did not influence Qk-5 expression, whereas remarkably that of Qk-6 was markedly decreased upon stimulation with oxLDL and VLDL, suggesting differential responses of VSMC Qk isoforms to pro-inflammatory stimuli (figure 2C).

Primary WT and Qk^v VSMCs displayed pronounced phenotypic differences even under non-stimulated conditions. Qk^v cells were characterized by a flattened, enlarged morphology with prominent stress-fibers as opposed to WT cells which displayed a healthy phenotype (figure 3A,B). To gain further insight into the consequences of decreased Qk-5 expression for VSMC phenotype, we assessed cellular proliferation, migration and extracellular matrix production in primary WT and Qk^v VSMC. Qk^v cells displayed a 45% decrease in outgrowth from aortic explants 7d after plating (WT

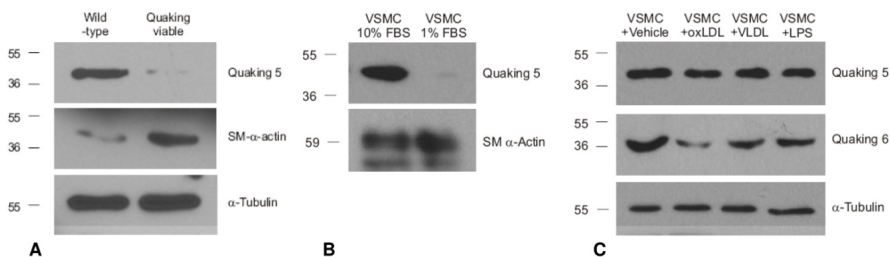


Figure 2.

Qk protein expression analysis in primary mVSMCs. Decreased Qk-5 expression is confirmed in Qk^v cells (A). In primary WT mVSMCs, Qk-5 protein expression is markedly reduced upon serum starvation, suggesting a decreased Qk-5 functionality in non-proliferative mVSMCs (B). No differences are observed for Qk-5 expression upon incubation with pro-atherogenic stressors such as oxLDL, VLDL and LPS, but incubation with oxLDL and to a lesser degree VLDL caused a decrease in the Qk-6 isoform protein expression (C). α -tubulin is used as housekeeping control.

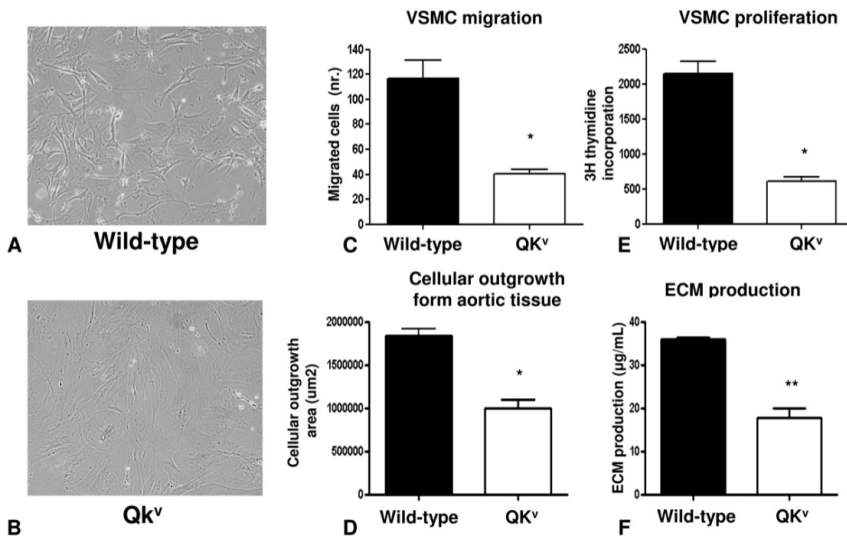


Figure 3.

Cultured primary mVSMCs, derived from WT-mice, display a healthy spindle-shaped VSMC-like phenotype, whereas Qk^v derived mVSMCs display the cobblestone morphology typically seen in disturbed VSMC homeostasis (A,B). Primary Qk^v mVSMCs show a marked, 3-fold decrease in migratory response (C). Qk mVSMC outgrowth form aortic tissue is nearly 50% reduced as compared to controls (D). Qk mVSMC show a 3.5-fold decrease in proliferative response (E). Decreased viability of primary Qk^v mVSMCs also affects the synthesis of extracellular matrix, as witnessed by a 50% reduction in collagen production (E). Results are mean $\pm$ SEM, * $P < 0.001$, ** $P < 0.05$

$1.8 \times 10^6 \pm 5.6 \times 10^5 \mu\text{m}^2$ vs. Qk^v $1.0 \times 10^6 \pm 6.0 \times 10^5 \mu\text{m}^2$ VSMC area; $N=34$; $P < 0.001$; figure 3D). The proliferation rate of primary Qk^v VSMC was 3.5 fold lower than that observed in WT mice as assessed by [³H]-thymidine incorporation (WT $2,149 \pm 806$ vs. Qk^v 607 ± 292 DPM/well; $N=25$; $P < 0.001$; figure 3E). Moreover, transwell cellular migration was significantly decreased with Qk^v cells (WT 116 ± 32 vs. Qk^v 40 ± 7 migrated cells; $N=5$; $P < 0.001$; figure 3C). Finally, Qk^v cells showed a 50% reduction in extracellular matrix production than their WT counterparts (36 ± 1 vs. $18 \pm 5 \mu\text{g/mL}$; $N=6$; $P < 0.05$; figure 3F). Altogether, these data point to a significant role of Qk-5 in VSMC homeostasis, underscoring a causal relationship of Qk SNPs and the occurrence of clinical restenosis.

Restenosis in vivo model analyses

As a next step we have studied the implication of Qk dependent impairment of VSMC function on restenosis in vivo. Firstly, total vessel area of cuffed femoral arteries in Qk^v and WT mice did not differ at 2 weeks post surgery (WT $19,458 \pm 4,942$ vs. Qk^v $17,593 \pm 4,433 \mu\text{m}^2$; $P=0.42$; figure 4A). However, in keeping with our *in vitro* finding that both the proliferative capacity and production of ECM is reduced in Qk^v VSMC, both medial area (WT $9,870 \pm 1,798$ vs. Qk^v $7,670 \pm 931 \mu\text{m}^2$; $P < 0.01$) and intimal hyperplasia (WT

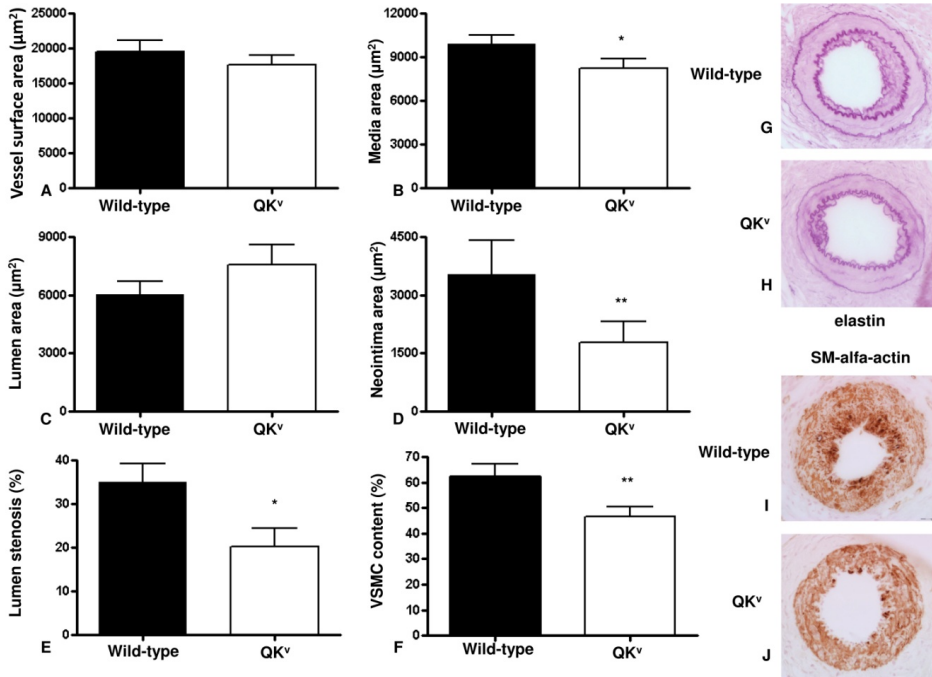


Figure 4.

Morphometric analyses of murine femoral artery lesions 3 weeks after non-constricting cuff placement in WT and Qk^v mice. No difference is seen in vessel surface area (A). Interestingly, Qk^v mice show decreased medial expansion, with an even more attenuated intimal hyperplasia (B,C). This results in reduced luminal stenosis in the Qk^v group (D,E). VSMC content is significantly decreased in Qk^v mice (F). Representative pictures of control en Qk^v femoral artery sections, stained with Weigert's elastin staining (G,H) and α-SM actin (I,J). Magnification 200x. Results are mean ± SEM, * P<0.01, ** P<0.05

3,537 ± 1,147 vs. Qk^v 1,779 ± 758 μm²; P<0.05; figure 4B,D) were significantly lower in cuff induced femoral artery lesions of Qk^v mice. Of note, luminal stenosis of the cuffed femoral artery segment was considerably lower in Qk^v than in WT mice (20% and 34%, respectively P<0.05; figure 4C,E). These findings suggest that attenuation of Qk gene and protein expression not only affects VSMC function but also results in a clinically relevant decreased fibroproliferative response upon vascular injury. Immunohistochemical analysis of the VSMC content of the neointimal lesions revealed that lesions in Qk^v mice contained a significantly lower percentage of SM-α-actin staining (WT 62.5 ± 4.7% vs. Qk^v 46.5 ± 4.1%; P<0.05; figure 4F). Although not significant, Qk^v mice also tended to display a decreased neo-intimal proliferative response, as assessed by cellular BrdU incorporation (WT 1.30 ± 0.5% vs. Qk^v 0.45 ± 0.15%; P=0.14). No significant differences were seen in apoptosis (TUNEL staining), nor in collagen and CD45⁺ leukocyte content in Qk^v vs. WT lesions (data not shown).

To assess normal Qk gene expression development following cuff placement in WT mice, we performed a temporal Qk gene profile analysis as shown in figure 5. Following

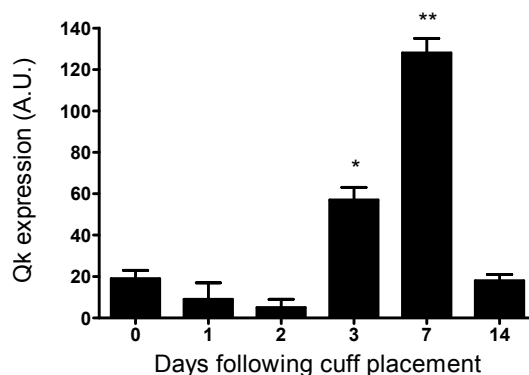


Figure 5.

Temporal Qk gene expression in femoral arteries following cuff placement. A significant increase in vascular Qk expression is observed during neointimal hyperplasia, peaking 7 days post cuff placement. Results are mean $\pm$ SEM, * $P < 0.01$, ** $P < 0.001$.

cuff placement, Qk gene expression is unchanged the first 2 days. However, starting at day three until day seven, a significant increase in Qk expression was observed in femoral arteries, coinciding with neointimal hyperplasia. Two weeks later, Qk expression falls back to basal levels. These data indicate that Qk has a regulatory role in VSMC differentiation in response to neointimal proliferative triggers.

DISCUSSION

In the present study we demonstrate the association of Qk SNPs with clinical restenosis and suggest that this association is the result of Qk regulated VSMC dysfunction.

To date, the majority of studies pertaining to Qk have assessed its role in central nervous system development, studies that have primarily been performed in a murine setting. More recently, human studies and clinical data have started to emerge, and provide links between Qk and human diseases such as Canavan disease and schizophrenia.^{16, 17} In the latter case, SNPs in the Qk gene were seen to associate with the occurrence of schizophrenia in a Swedish cohort, possibly caused by aberrant Qk splicing.^{17, 18} Furthermore, previous work has identified that alterations in Qk isoform expression are linked to the development of glioblastoma multiforme and ataxia.^{19, 20} Our study is the first to assess the impact of Qk SNPs on clinical restenosis and cardiovascular disease. We have identified 7 SNPs that conferred enhanced risk of or protection against TVR, with the most significant associations for SNPs located in the promoter region and introns 1,2 and 3 of Qk. Importantly, these analyses included a correction for possible confounding factors, thereby increasing the strength of the current analysis. Furthermore, separate analysis of the stented population revealed similar significant outcomes, which

is particularly relevant for current clinical practice in which plain balloon angioplasty is only rarely employed because of the higher risk of restenosis of this procedure. Of note, only bare metal stents were applied, thereby excluding bias due to interference of Qk with the activity of stent-coated drugs. Remarkably, subjects with both the 57896AA protective allele and 65752AG and GG risk alleles, a genotype prevalent in 10% of the total population, exhibited a 2 fold enhanced risk for restenosis, the strongest of all associations observed before in previous analyses of the GENDER study. Cloning and functional characterization of individual SNPs *in vitro* will likely clarify the effects of each SNP. Further studies are needed to examine whether protective SNPs confer reduced Qk expression or activity and if deleterious SNPs enhance Qk expression or vice versa.

The *in vitro* analyses point to a causal involvement of Qk in restenosis. In fact reduced Qk expression results in VSMC dysfunction. Qk-5 protein expression was significantly reduced in Qk^v derived VSMCs, whereas expression of Qk-6 and Qk-7 did not appear to be affected in Qk^v mice. It has previously been shown that Qk isoforms show differential patterns of temporal expression during differentiation in various cell types.^{3,21} Interestingly, Qk^{-/-} mice have previously been found to be embryonically lethal, an effect that was attributed to a loss of Qk-5 expression during vascular development and maturation.^{5,6,22} Our findings suggest that as a result of decreased Qk-5 expression, Qk^v VSMCs lose their capacity to function properly, displaying many of the hallmarks of stressed cells, highlighted by a senescent morphology. This was substantiated in four functional assays, all pointing to decreased VSMC function as indicated by altered phenotype and decreased migration, proliferation, outgrowth and extracellular matrix production, features of the cobble-stone shaped synthetic phenotype. Therefore, Qk is likely to have a critical regulatory role in VSMC function and/or differentiation. Li et al. demonstrated that vitelline arteries of Qk null mice, which die at day 10 after gestation, are essentially devoid of α -actin⁺ VSMCs, a feature that was attributed to an inability of endothelial cells to differentiate into VSMCs pointing to a vital role of Qk in VSMC differentiation.⁶ The defective VSMC phenotype could be rescued *in vitro* by adding Qk and PECAM-1 positive cells to a culture of para-aortic splanchnopleural cells derived from Qk^{-/-} mice, suggesting the presence of a paracrine factor mediating the differentiation of endothelial cells into VSMCs.⁶ In addition, Bohnsack et al. demonstrated the paracrine effects of Qk-mediated release of retinoic acid regulating visceral endoderm survival and endothelial cell function.²² We extend these observations by showing that even decreased, not completely abrogated Qk expression has serious implications on VSMC function *in vitro*, without requiring paracrine effects of neighboring endothelial cells.

Although we observed both protective and enhanced risk ratios of restenosis within our Qk SNP analysis, Qk^v mice displayed a decreased neo-intimal response after femoral cuff placement which was in line with our *in vitro* observations. This is likely to be caused by decreased VSMC migration and proliferation, as SM- α -actin and, to a lesser extent,

BrdU staining was decreased in neo-intimal lesions of Qk^v mice. The temporal Qk gene expression analysis strengthens this hypothesis, while Qk expression was significantly raised seven days following femoral cuff placement in wild type mice. Therefore, Qk is imperative for the physiological neo-intimal proliferative response. It should be noted that the 1-MB deletion in the Qk gene in Qk^v mice not only affects Qk expression, but also that of Parkin as well as of the Parkin co-regulate gene (Pacrg).²³ Although we have solid evidence that underscores a direct relationship of Qk with VSMC function, and while others have previously shown that expression of Qk-6 rescues the dysmyelination phenotype of Qk^v mice,²² it cannot be excluded that parkin or Pacrg are partly responsible for VSMC dysfunction and/or restenosis as well.

The actual downstream effectors in this regulatory role of Qk on VSMC function are hitherto unknown. Recently, Galarneau and Richard, based on a computational analysis for Qk response elements, composed a list of 1,433 putative downstream targets of Qk.³ These targets included genes involved in development, cell adhesion, morphogenesis, organogenesis, transport, cell differentiation, cell growth and/or maintenance, and cell communication, implicating Qk proteins as regulators of cell fate and proliferation. Many of these putative targets are implicated in cancer, a disease process heavily dependent on (neo)vascularization. Extending these observations, we hypothesize based on these putative targets that Qk has additional functions in cardiovascular disease next to restenosis, such as atherogenesis, vascular and cardiac remodeling.

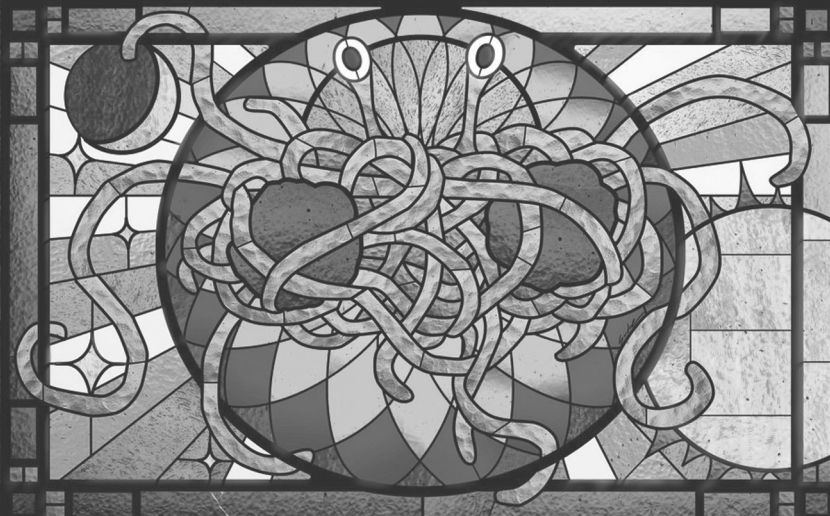
Finally, our observations strengthen the hypothesis that proteins which are originally associated with central nervous system development and maintenance also play a crucial role in vascular homeostasis.²⁴ In this regard, Nogo-B, a protein involved in the inhibition of axonal growth and repair, was shown to regulate VSMC as well as endothelial cell migration *in vitro* and vascular remodeling *in vivo*.²⁵ Furthermore, Nogo-B overexpression was recently shown to reduce neo-intima formation following vascular injury.²⁶ Although these effects are opposite to the effects of Qk, it shows that genes involved in neural cell maintenance may have substantial influence on vascular cells as well.

In conclusion, Qk SNPs are associated with the occurrence of restenosis in a large prospective cohort. As in this study decreased Qk activity could be linked to VSMC dysfunction, we propose that Qk may serve as a future genetic marker for restenosis risk, as well as a target for anti-restenosis therapy. The development of compounds which directly target the Qk transcriptional route responsible for regulation of VSMC function would be a feasible next step towards direct *in vivo* therapeutic targeting.

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Chapter 7

Metabolic background determines
the importance of NOS3 polymorphisms
in restenosis after percutaneous
coronary intervention

A study in patients with and without the metabolic syndrome

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ABSTRACT

Variation in the *NOS3* gene has been related to the development of restenosis. The Glu298Asp polymorphism has previously been investigated for its effect on NO levels and the development of restenosis. However, the variability of findings gave rise to the hypothesis that the functional significance of this polymorphism may only become manifest under conditions of endothelial dysfunction. Since patients with the metabolic syndrome are known to have endothelial dysfunction, we aimed to investigate if the significance of *NOS3* polymorphisms may depend on the presence of the metabolic syndrome.

We examined the impact of the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms in the *NOS3* gene on the risk of clinical restenosis in a previously described subpopulation of the GENDER-study, a multicenter prospective study design that enrolled consecutive patients after successful PCI. This subpopulation consisted of 901 patients of whom sufficient data were available to establish absence or presence of the metabolic syndrome. Of these patients, 448 had the metabolic syndrome. Clinical restenosis, defined as target vessel revascularization (TVR), was the primary endpoint.

We demonstrated that the minor -949G, -716T and 298Asp alleles were associated with a significantly increased risk of TVR in patients with the metabolic syndrome (HR: 1.58 [95%CI: 1.04-2.40], HR: 1.95 [95%CI: 1.02-3.70] and HR: 1.67 [95%CI: 1.09-2.54], respectively). In the group without the metabolic syndrome we observed no association between the three polymorphisms and TVR.

We suggest that the metabolic alterations present in patients with the metabolic syndrome influence the functional significance of these polymorphisms and increase the susceptibility of carriers of one of these variations in the *NOS3* gene to develop restenosis after PCI.

INTRODUCTION

The frequent occurrence of restenosis is a major limitation to percutaneous coronary intervention (PCI) in patients with coronary artery disease. Patients at increased risk for restenosis could benefit from alternative treatment options such as drug-eluting stents or coronary artery bypass surgery. To be able to stratify patients with regard to their risk of developing restenosis, it is important to determine both inherited and clinical factors influencing this complex and multifactorial process.

Nitric oxide (NO) is a vasodilatory molecule known to inhibit adhesion of platelets¹ and leukocytes² to the vessel wall and to decrease proliferation and migration of vascular smooth muscle cells (VSMCs),³ which are all processes known to play an important role in the development of restenosis.⁴ Due to its favourable actions in these processes, NO is likely to play a protective role in the formation of restenosis. Restenosis has already been related to alterations in nitric oxide synthase (NOS) expression⁵ and impaired NO-dependent endothelial vasodilation.⁶ In support of these observations, Piatti *et al.* showed a lack of NO release after an oral glucose tolerance test (OGTT) in patients with restenosis.⁷ Since endothelial NOS catalyses the formation of NO from L-arginine in endothelial cells, polymorphisms in the NOS3 gene could influence NO formation and thereby play a role in the process of restenosis. The Glu298Asp polymorphism in the NOS3 gene has previously been investigated. However, data on its influence on NO levels and the development of restenosis are still conflicting. Sofowora *et al.* suggested that the functional significance of this polymorphism may only become manifest under conditions of endothelial dysfunction.⁸ Since endothelial dysfunction is an important characteristic of the metabolic syndrome, we hypothesize that the metabolic alterations present in patients with the metabolic syndrome may influence the functional significance of NOS3 polymorphisms. Therefore, the aim of this study was to examine whether the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms in the NOS3 gene are related to the risk of restenosis after PCI in patients with and without the metabolic syndrome. The GENDER protocol was designed to allow separate analyses in a prespecified subgroup of patients with the metabolic syndrome.

PATIENTS AND METHODS

The present study population has been described previously.⁹ In brief, the GENetic DEterminants of Restenosis project (GENDER) was designed to study the association between genetic polymorphisms and clinical restenosis. Patients were eligible for inclusion if they were successfully treated for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI. Patients treated for acute ST elevation myocardial

infarction were excluded. All patients were treated in 4 of the 13 referral centers for interventional cardiology in the Netherlands. The overall inclusion period lasted from March 1999 until June 2001. We determined the lipid profile (serum triglycerides, serum total cholesterol, serum HDL-cholesterol) and fasting serum glucose in a subpopulation of patients of whom plasma was collected. The study protocol conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure.

PCI procedure

Standard angioplasty and stent placement were performed by experienced operators using a radial or femoral approach. Before the procedure, patients received aspirin 300 mg and heparin 7500 IU. The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, was at the discretion of the operator. In case of stent implantation, patients received either ticlopidin or clopidogrel for at least one month following the procedure depending on local practice. During the study, no drug-eluting stents were used.

Follow-up and study endpoints

Follow-up lasted at least nine months, except when a coronary event occurred. Patients were either seen in the outpatient clinic or contacted by telephone. Restenosis was defined by target vessel revascularization (TVR), either by PCI or coronary artery bypass grafting (CABG). An independent clinical events committee adjudicated the clinical events.

Events occurring within the first month were excluded from the analysis, since these events were attributable mainly to sub-acute stent thrombosis or occluding dissections, and less likely to restenosis. Data were collected with standardized case-report forms that were completed by the research coordinator at each site. Representatives from the data-coordinating center monitored all sites.

Genotyping

Blood was collected in EDTA tubes at baseline and DNA was extracted following standard procedures. The NOS3 -949 A/G (rs1800779), -716 C/T (rs3918226) and the 5219 G/T (rs1799983) polymorphism, resulting in the amino acid substitution Glu298Asp, were determined by a validated multilocus genotyping assay (Roche Molecular Systems).¹⁰ The Glu298Asp polymorphism has already been implicated in coronary artery disease¹¹ and restenosis.^{12, 13} The -949 A/G polymorphism was selected on the basis of its involvement in atherosclerotic disease¹⁴ and the -716 C/T polymorphism is located between the

cAMP regulatory element (nt -726 to -732) and an activator protein-1 binding domain (nt -655 to -661),¹⁵ which suggests a potential involvement in gene regulation.

In short, each DNA sample was amplified in a multiple polymerase chain reaction (PCR) using biotinylated primers. The PCR product pool was then hybridized to a matching panel of sequence-specific oligonucleotide probes, immobilized in a linear array on nylon membrane strips. A colorimetric detection method based on incubation with streptavidin-horseradish peroxidase conjugate, using hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine as substrates, was used. Operators blinded to restenosis status performed genotyping.

To confirm genotype assignments, the PCR-procedure was performed in replicate on 10% of the samples. Two independent observers carried out scoring. Disagreements (<1%) were resolved by further joint reading, and when necessary, genotyping was repeated.

Definitions

Patients were defined as having metabolic syndrome by presence of three or more of the following criteria: 1) triglyceride level ≥ 1.7 mmol/l (150 mg/dl); 2) HDL cholesterol level < 1.04 mmol/l (40 mg/dl) in men and < 1.3 mmol/l (50 mg/dl) in women; 3) systolic blood pressure ≥ 130 mmHg and/or diastolic blood pressure ≥ 85 mmHg; 4) obesity, defined as BMI > 28.8 kg/m², which was equivalent to a waist circumference of 102 cm in a cross-sectional study and similar to the BMI value (28.2 kg/m²) calculated in a regression of BMI on waist circumference in a large population of Scottish men;^{16, 17} and 5) fasting glucose level ≥ 5.55 mmol/l (100 mg/dl), above which patients have either pre-diabetes (impaired fasting glucose) or diabetes. This level was established by the American Diabetes Association and suggested as the new cut point for identifying the lower boundary to define an elevated glucose level as one criterion for the metabolic syndrome.¹⁸⁻²⁰

Statistical methodology

Allele frequencies were determined by gene counting, the 95% confidence intervals of the allele frequencies were calculated from sample allele frequencies, based on the approximation of the binominal and normal distributions in large sample sizes. Continuous variables are expressed as mean $\pm$ standard deviation and were compared by means of an unpaired, two-sided *t* test. Discrete variables are expressed as counts or percentages and were compared with a chi-square test.

The association between each of the 3 polymorphisms and TVR was determined using a Cox proportional regression model. If less than 10 patients were homozygous for a particular allele, the homozygotes and heterozygotes were combined. Polymorphisms

with independent prognostic value were selected in a multivariable regression model, also containing clinical and procedural risk factors.

To investigate possible additive effects of haplotypes of the *NOS3* polymorphisms on TVR risk, we analyzed haplotype effects using the THESIAS program (www.genecanvas.org), which implements the stochastic-EM (Expectation–Maximization) algorithm and allows the use of Cox proportional hazards survival regression.²¹ Statistical analyses were carried out using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The characteristics of this patient sample have been described previously.⁹ In summary, a total of 3,146 patients had complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9 months). Out of 3,146 patients 42 experienced an event in the first 30 days and were therefore excluded from further analysis, according to the protocol. In a previously described subpopulation of 901 patients,²² data were sufficient to establish absence or presence of metabolic syndrome. Of these patients, 448 (49.7%) had metabolic syndrome (≥ 3 of the 5 characteristics). The prevalence of metabolic syndrome characteristics in patients with and without the metabolic syndrome, as well as demographic, clinical and lesion characteristics, are presented in table 1. The presence of one or more components of the metabolic syndrome was common in both sexes: 16.3% had one component, 29.6% had two components, 26.9% had three components, 15.9% had four components, and 7.0% had all five components. Except for the presence of diabetes, the prevalence of factors that were previously found to be related to restenosis did not differ between the two groups. However, patients with the metabolic syndrome had higher insulin levels and were more frequently treated with statins or insulin.

At follow-up 47 patients (10.5%) with the metabolic syndrome versus 45 (9.9%) without the metabolic syndrome underwent TVR. On multivariable Cox regression, there was no significant increase in risk of TVR in patients with the metabolic syndrome (HR: 1.03 [95%CI: 0.68- 1.57]).

Genotyping of the -949 A/G, the -716 C/T and the Glu298Asp (G/T) polymorphisms was successful in 96.9%, 96.7% and 96.9% of patients with the metabolic syndrome and in 98.7%, 98.2% and 98.2% of those without the metabolic syndrome, respectively. Results of the remaining patients were missing due to lack of DNA or inconclusive genotyping. In both groups, the frequencies of the minor -949G, -716T and 298Asp alleles were 0.37, 0.09 and 0.31, respectively. The genotype distributions were consistent with Hardy-Weinberg equilibrium and did not differ significantly between patients with the metabolic syndrome and controls ($p>0.05$).

Table 1. Baseline and metabolic syndrome characteristics by metabolic syndrome status (N=901)

	Metabolic syndrome		P value ^a
	Absent N=453	Present N=448	
Baseline Characteristics:			
Age (y± SD)	63 (±10)	61 (±11)	0.003
BMI (kg/m ² ± SD)	25.4 (±3.0)	28.7 (±4.1)	<0.001
Female Sex (%)	127 (28)	131 (29)	0.69
Diabetes Mellitus (%)	32 (7)	108 (24)	<0.001
Current Smoking (%)	80 (18)	94 (21)	0.21
Stent placement (%)	376 (80)	373 (83)	0.92
Residual stenosis (%)	51 (11)	44 (10)	0.52
Total occlusion (%)	32 (7)	41 (9)	0.27
Total cholesterol (mmol/L, ±SD)	4.91 (±1.05)	5.01 (±1.12)	0.32
Statins (%)	231 (51)	265 (59)	0.014
Insulin ^b (mU/l, IQR)	12 (9)	20 (20)	<0.001
Insulin Therapy (%)	13 (3)	32 (7)	0.003
Metabolic Syndrome Characteristics			
BMI >28.8 kg/m ²	37 (8.2%)	207 (46.2%)	
Triglycerides ≥ 1.7 mmol/L	128 (28.3%)	370 (82.6%)	
HDL cholesterol < 1.04 mmol/L men and < 1.3 mmol/L women	128 (28.3%)	324 (72.3%)	
Systolic BP ≥130 and diastolic BP ≥85 mm Hg	273 (60.3%)	383 (85.5%)	
Fasting glucose ≥ 5.55 mmol/L	115 (25.4%)	329 (73.4%)	

^a p-value of the nonparametric Mann-Whitney test, or chi-square test^b Insulin levels are presented as median with interquartile range (IQR)

Univariate Cox regression in patients with the metabolic syndrome revealed a significant association between the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms and the rate of TVR (HR: 1.58 [95%CI: 1.04-2.40] $p=0.034$, HR: 1.95 [95%CI: 1.02-3.70] $p=0.042$ and HR: 1.67 [95%CI: 1.09-2.54] $p=0.018$, respectively) (Table 2). TVR occurred more often in -949 GG homozygotes (18.2%) than in AG heterozygotes (11.0%) or AA homozygotes (7.6%). Since only one patient was homozygous for the -716T allele, we combined homozygous and heterozygous patients for this polymorphism. This combined group had an increased risk of TVR (17.6%) when compared to the group with the -716 CC genotype (9.2%). Homozygotes and heterozygotes for the 298Asp allele also had a higher risk of TVR (21.1% and 11.5%, respectively) when compared to the Glu/Glu genotype (7.8%). In contrast, in patients without the metabolic syndrome, the -949 A/G, -716 C/T and the Glu298Asp polymorphisms were not associated with TVR (HR: 0.90 [95%CI: 0.57-1.43] $p=0.65$, HR: 0.33 [95%CI: 0.10-1.08] $p=0.067$ and HR: 1.02 [95%CI: 0.66-1.59] $p=0.93$, respectively). For each polymorphism, TVR-risks were equal in the different genotype groups (Table 2).

Table 2. Cox regression of NOS3 polymorphisms in association with TVR in patients with and without the metabolic syndrome

Metabolic Syndrome	Polymorphisms	Number of patients	TVR (%)	HR (95%CI)
Present (N=448)	-949 A/G			
	AA	170	7.6	1.58 (1.04-2.40)
	AG	209	11.0	
	GG	55	18.2	
	-716 C/T			
	CC	359	9.2	1.95 (1.02-3.70)
	CT	73	17.6	
	TT	1		
	Glu298Asp (G/T)			
	GG	204	7.8	1.67 (1.09-2.54)
	GT	192	11.5	
	TT	38	21.1	
Absent (N=453)	-949 A/G			
	AA	167	10.8	0.90 (0.57-1.43)
	AG	226	8.4	
	GG	54	11.1	
	-716 C/T			
	CC	374	10.7	0.33 (0.10-1.08)
	CT	63	4.2	
	TT	8		
	Glu298Asp (G/T)			
	GG	219	10.0	1.02 (0.66-1.59)
	GT	174	8.0	
	TT	52	13.5	

After adjustment for patient and intervention-related characteristics that were previously described to be related to TVR risk, such as age, sex, hypertension, diabetes, current smoking, stenting, total occlusion and residual stenosis >20%, the -949 A/G, the -716 C/T and the Glu298Asp polymorphisms were predictors of TVR (HR: 1.55 [95%CI: 1.01-2.37] $p=0.043$, HR: 2.05 [95%CI: 1.05-4.00] $p=0.034$ and HR: 1.68 [95%CI: 1.09-2.59] $p=0.019$, respectively).

As determined with haploview, the -949 A/G and -716 C/T polymorphisms revealed significant linkage disequilibrium (D' : 0.95) and were combined into haplotypes for further analysis. The Glu298Asp polymorphism was excluded from this analysis due to the lack of linkage disequilibrium between this polymorphism and the -949 A/G polymorphism (D' : 0.45). In both groups, frequencies of the -949A/-716C, -949G/-716C and -949G/-716T haplotypes were 0.62, 0.29 and 0.08, respectively. Due to its low

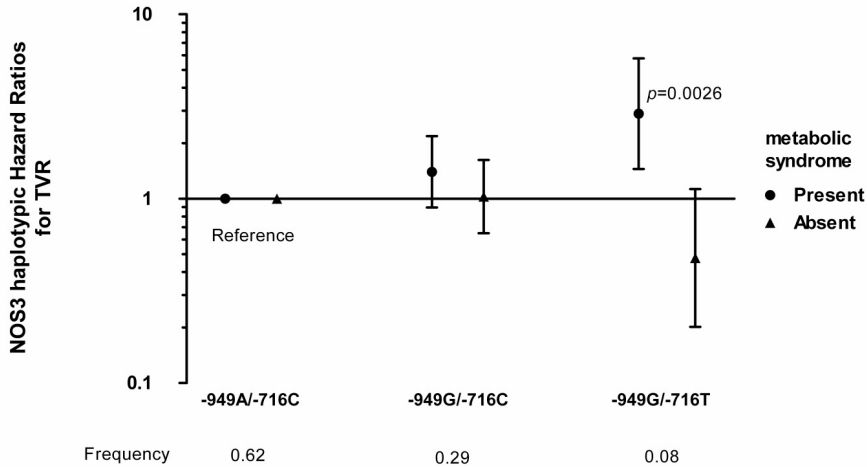


Figure 1.

NOS3 haplotype effects on the risk for target vessel revascularization (TVR) in patients with (circles) and without (triangles) the metabolic syndrome. Hazard ratios by reference to the most frequent haplotype were calculated using the THESIAS software. The -949G/-716T promoter haplotype showed a strong association with TVR (HR: 2.89 [95%CI: 1.45-5.76], $p=0.0026$), which indicates that both polymorphisms contribute separately to the risk of TVR.

frequency (0.007 in patients with the metabolic syndrome and 0.008 in patients without the metabolic syndrome), the -949A/-716T haplotype was excluded from the analysis. A haplotype-based risk analysis in patients with the metabolic syndrome revealed a significant association of the -949G/-716T haplotype with the risk for TVR when compared to -949A/-716C, the most frequent reference haplotype (HR: 2.89 [95%CI: 1.45-5.76], $p=0.0026$ (Figure 1)).

DISCUSSION

To test our hypothesis that the functional significance of *NOS3* polymorphisms may depend on the presence of the metabolic syndrome, we studied the relationship between these polymorphisms and the risk of restenosis in a subpopulation of patients with the metabolic syndrome that took part in a large follow-up study of unselected, consecutive patients undergoing PCI. We demonstrated that the -949G, -716T and 298Asp variants in the *NOS3* gene were associated with a highly increased risk of TVR after PCI in patients with the metabolic syndrome, but not in patients without the metabolic syndrome.

Of the -949 A/G, -716 C/T and Glu298Asp polymorphisms, only the Glu298Asp polymorphism has previously been investigated for its involvement in restenosis. Suzuki *et al.* have demonstrated that carriers of the 298Asp allele were at increased risk of devel-

oping angiographic restenosis after stent placement (OR: 3.9 [95%CI: 1.09-13.95], after multivariate analysis).¹² However, their population was very small (n=85) and consisted of relatively many patients with diabetes (27%). In support of their results, Gomma *et al.* demonstrated in a still relatively small and selected group of 205 stented patients, that possession of one or more alleles of the 298Asp variant was associated with an increased rate of angiographic restenosis after adjusting for clinical factors (OR: 2.47 [95% CI: 1.22-5.00]).¹³ However, Gorchakowa *et al.* could not confirm these findings and demonstrated in a similar study, with a much larger patient number (n=1850), that rates of angiographic restenosis or TVR did not differ significantly between the Glu298Asp genotypes.²³

The mechanism by which this polymorphism would promote neointima formation remains subject to discussion. It was originally suggested that NO would protect against restenosis because of its inhibitory role in VSMC proliferation, adhesion of leukocytes to the vessel wall and coagulation,¹⁻³ processes known to be involved in restenosis.⁴ In addition, NO has been shown to suppress the production of the potent vasoconstrictors endothelin and angiotensin II, that also induce VSMC proliferation.^{24,25} Its role in restenosis was further established by the observation that L-arginine, the precursor to NO, decreases restenotic lesion formation in the rat,²⁶ whereas L-NAME (N^G-nitro-L-arginine methyl ester), a non-selective NOS-inhibitor, stimulates neo-intimal hyperplasia in the rabbit after balloon angioplasty.²⁷ In humans, intramural administration of L-arginine has been shown to reduce neointimal hyperplasia at 6 months after stent placement.²⁸ Furthermore, patients with in-stent restenosis had impaired endothelial vasodilation and a lack of NO-release after an OGTT when compared to patients without restenosis.⁷

Although several *in vitro*²⁹ and *in vivo*^{8, 30} studies showed reduced levels of NO in Glu298Asp homozygotes, data on the functional significance of this polymorphism are still conflicting. The 298Asp variant has been shown to result in protein with twofold less activity³¹ and an increased susceptibility to proteolytic cleavage.³² However, Sessa *et al.* demonstrated that the observed fragmentation was an artifact of sample preparation for gel electrophoresis³³ and several authors reported similar activity of NOS3 in the different Glu298Asp genotypes.^{34,35} Studies examining vasodilation in response to intrabrachial injection of acetylcholine produced conflicting results.^{36,37} Furthermore, flow-mediated dilation of the brachial artery was not affected by the Glu298Asp polymorphism,^{38,39} whereas Naber *et al.* demonstrated a higher coronary vascular resistance in 298Asp homozygotes compared to that of 298Glu homozygotes at rest.⁴⁰ These discrepancies might reflect the impact of interactions with environmental factors, such as smoking.¹¹

To explain this variability, Sofowora *et al.* put forward an interesting hypothesis. In spite of the observed reduction in urinary excretion of NO in 298Asp homozygotes, they could not demonstrate differences between 298Asp homozygotes and 298Glu homozygotes as to vascular responses to acetylcholine, glyceryl trinitrate or phenylephrine

in a healthy population.⁸ Since, in contrast to their results, 298Asp homozygous patients with ischemic heart disease were found to be more sensitive to the vasoconstrictive effects of phenylephrine, they suggested that the functional significance of the Glu298Asp polymorphism may only become manifest under conditions of endothelial dysfunction. In support of this possibility, Leeson *et al.* demonstrated that 298Asp homozygotes had significantly lower flow-mediated dilation than 298Glu homozygotes, only in smokers, a group known to have endothelial dysfunction.³⁸ Recently, smoking carriers of the 298Asp allele were also found to have an increased risk of coronary heart disease relative to nonsmokers carrying two 298Glu alleles.⁴¹

Endothelial dysfunction is an important characteristic of the metabolic syndrome. A study by Lteif *et al.* showed that insulin resistance and systolic blood pressure seem to be the most important determinants of endothelial dysfunction in patients with the metabolic syndrome.⁴² Furthermore, high glucose levels are also known to determine endothelial dysfunction. A recent study showed that glucose downregulates NOS3 mRNA and protein expression through regulation of mitochondrial production of reactive oxygen species (ROS) with subsequent activation of AP-1.⁴³ The inhibitory effect of glucose on NOS3 gene transcription was reversed by mutation of the AP-1 binding site in the promoter region. These findings indicate a mechanism by which hyperglycemia could increase the significance of NOS3 promoter polymorphisms.

Thus, if we assume that the functional significance of NOS3 polymorphisms depends on the presence of endothelial dysfunction, an explanation of our results may be that patients with the metabolic syndrome which carry the -949G, -716T or 298Asp alleles produce less NO than patients without the metabolic syndrome or carriers of the -949C, -716C or 298Glu alleles. Since the -949G/-716T haplotype showed a stronger association with TVR than both polymorphisms individually, these alleles could even interact to reduce NOS3 activity in patients with the metabolic syndrome. Further research has to be performed to test these hypotheses.

LIMITATIONS OF THE STUDY

One of the limitations of our study is that we did not have waist circumference as per criteria of ATP-III. We therefore substituted waist circumference with variable of obesity as defined by BMI of $>28.8 \text{ kg/m}^2$, this cutoff was equivalent to a waist circumference of 102 cm in a cross-sectional study and similar to the BMI value (28.2 kg/m^2) calculated in a regression of BMI on waist circumference in a large population of Scottish men.^{16, 17}

In our study we lack data on NOS3 activity or NO concentration. Abundant data is present on the functionality of the Glu298Asp polymorphism. However, little is known about a difference of NOS3 activity between patients with and without the metabolic

syndrome. We would like to promote research that compares *NOS3* activity and local NO concentrations between patients with the metabolic syndrome and controls having specific genotypes of the -949 A/G, -716 C/T and Glu298Asp polymorphisms.

Conclusions

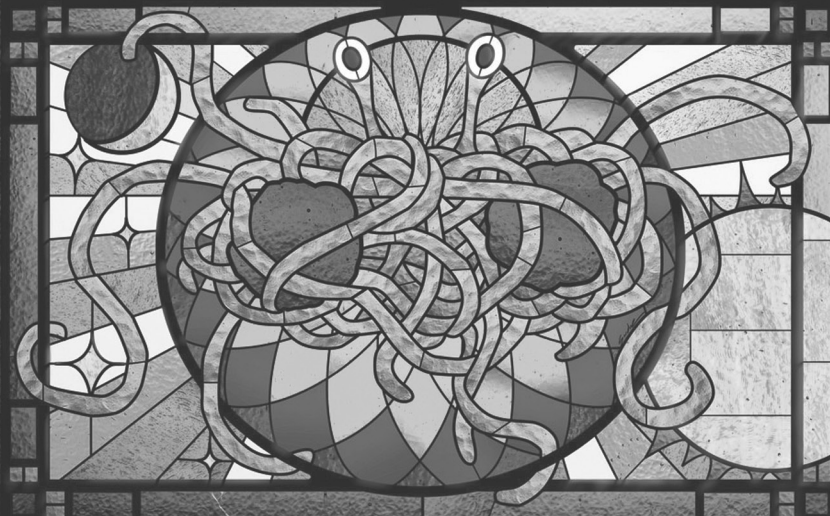
In conclusion, we have demonstrated that the variant alleles of the -949 A/G, -716 C/T and Glu298Asp polymorphisms in the *NOS3* gene increase the risk of TVR in patients with the metabolic syndrome, but not in patients without the metabolic syndrome. We suggest that the metabolic alterations in these patients influence the functional significance of the three *NOS3* polymorphisms and increase the susceptibility of carriers of these variations in the *NOS3* gene to develop restenosis after PCI. Genotyping for these polymorphisms may lead to better risk stratification and more tailored therapy in patients with the metabolic syndrome to prevent TVR after PCI.

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Chapter 8

The 5352 A allele of the pro-inflammatory
Caspase-1 gene predicts late acquired
stent malapposition in STEMI patients
treated with sirolimus stents

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ABSTRACT

Late acquired stent malapposition (LASM) is a common finding after sirolimus-eluting stent (SES) implantation and may be the cause for late stent thrombosis. Inflammation may play a pivotal role in LASM just as it plays in stent restenosis. We have therefore investigated 7 polymorphisms involved in inflammatory processes, related in previous reports to restenosis, on the risk of LASM in SES patients. Patients with ST-elevation myocardial infarction who underwent SES implantation and had intravascular ultrasonography (IVUS) data available for both immediate post-intervention and 9-month follow-up were included in the present study. In total, 104 patients from the MISSION! intervention study were genotyped for the caspase-1 5352 G/A, eotaxin 1382 A/G, CD14 260 A/G, colony stimulating factor 2 1943 C/T, IL10 -1117 C/T, IL10 4251 C/T and the tumor necrosis factor alpha 1211 C/T polymorphisms. LASM occurred in 26/104 (25%) of patients. We found a significantly higher risk for LASM in patients carrying the caspase-1 (CASP1) 5352 A allele (RR= 2.32; 95% CI 1.22-4.42). In addition, mean neointimal growth was significantly lower in patients carrying this LASM risk allele (1.6 vs 4.1%, $p=0.014$). The other 6 polymorphisms related to inflammation were not significantly related to the risk of LASM. In conclusion, carriers of the 5352 A allele in the caspase-1 gene are at increased risk of developing LASM after SES implantation. If this is confirmed in larger studies, screening for this polymorphism in patients undergoing percutaneous coronary interventions could eventually help cardiologists to better select between commercially available stents.

INTRODUCTION

Over the past years concerns were raised with regard to the long-term safety of coronary stents, mostly related to the fearful complication of late stent thrombosis. Several studies reported stent thrombosis to occur more frequently after drug-eluting stents (DES) than bare-metal stents (BMS) implantation¹⁻³ and to be associated with underlying stent malapposition.⁴⁻⁶ Stent malapposition, as detected by intravascular ultrasonography (IVUS), represents a separation of the stent struts from the intimal surface of the arterial wall (in the absence of a side branch) with evidence of blood speckles behind the struts.⁷ Stent malapposition may be acute (present immediately after implantation), persistent (present both immediately after implantation and at follow-up) or late acquired (present only at follow-up).

Intravascular ultrasonography (IVUS) studies have repeatedly identified positive vessel remodeling as the main mechanism of late acquired stent malapposition (LASM).^{6,8-10} Positive remodeling is a complex process characterized by a regional increase in the vessel wall diameter.⁷ A hypersensitivity reaction to the polymer coating of DES⁹ and induction of apoptosis by sirolimus may lead to positive remodeling.^{11,12}

In the GENDER study,¹³ 7 polymorphisms located in the caspase-1,¹⁴ eotaxin, CD14, colony stimulating factor 2 (CSF2),¹⁵ interleukin 10 (IL10)¹⁶ and tumor necrosis factor alpha (TNF α) genes,¹⁷ were found significantly associated with clinical restenosis after a percutaneous coronary intervention (PCI) for stable angina pectoris. Besides its established role in restenosis, inflammation may also play an important role in LASM after DES implantation. On the long term, stent malapposition may be the consequence of chronic inflammation and delayed healing, resulting in tissue necrosis and erosion around the stent.¹⁸

In the present study we aimed at investigating these 7 inflammatory polymorphisms with regard to LASM and neointimal growth in a population of ST-elevation myocardial infarction patients treated by primary PCI with sirolimus-eluting stents (SES) in the prospective MISSION! Intervention Study.¹⁹

METHODS

IVUS measurements

Details about the MISSION! protocol and the MISSION! Intervention Study have been published elsewhere.^{19,20} In brief, MISSION! Intervention Study was a single center, single blind, randomized prospective study to evaluate clinical, angiographic and IVUS results in documented acute STEMI patients treated with either BMS (Vision™, Guidant Corp. Indianapolis, Indiana, USA) or SES (Cypher™, Cordis Corp. Miami Lakes, Florida,

USA). The study protocol was approved by the institutional ethical committee. Patients and operators performing the follow-up angiography were blinded to the treatment assignment. The study was conducted from February 2004 to October 2006. Patients had an IVUS examination immediately after the stent deployment and at nine-month follow-up. LASM at 9 months was common after SES implantation (26/104 patients, 25.0 %) but rare after BMS implantation (4/80 patients, 5.0%).⁶ As a consequence, in the present study an analysis on LASM was conducted only for the SES group.

IVUS images were analyzed off-line, using quantitative IVUS analysis software (QCU-CMS 4.14, Medis, Leiden, The Netherlands).²¹ After determining vessel, stent and lumen contours, their volumes were automatically calculated.

LASM was defined as stent malapposition present at 9-month follow-up IVUS examination but absent at immediate post-intervention IVUS examination. We have therefore examined first the follow-up IVUS images in order to identify cases of stent malapposition. After identification, IVUS images of follow-up and immediate post-intervention procedures were analyzed side-by-side in order to discriminate the late-acquired stent malapposition cases.

The neointima growth (%) at nine months was calculated with the formula: [stent volume(mm³) – lumen volume(mm³) in the stented region]/stent volume(mm³) and represents the amount of new intimal tissue growth inside the stent as a fraction of the stent volume.

All patients gave informed consent before the procedure. An additional informed consent was obtained for follow-up angiography and IVUS at nine months. Patients were considered to have dislipidemia, hypertension and diabetes if they had been diagnosed such by a physician previous to the present myocardial infarction admission. IVUS imaging was performed with motorized pull-back (0.5mm/s) starting at least 10 mm distal to the stent and ending at the coronary ostium, using a 2.9F 20MHz catheter and a dedicated IVUS console (Eagle Eye, Volcano Corp. Rancho Cordova, California, USA).²² Each angiogram and ultrasound sequence was preceded by 200-300µg of intracoronary nitroglycerin. After the initial procedure aspirin (100 mg/day) was prescribed indefinitely and clopidogrel (75 mg/day) for twelve months. During follow-up, patients were treated with beta-blockers, statins and ACE-inhibitors or ATII-blockers, according to current guidelines.²⁰

Genotyping

Blood was collected in EDTA tubes upon admission to hospital and genomic DNA was extracted following standard procedures. A multiplex assay which included the caspase 1 (CASP1) 5352 A/G (rs580253), eotaxin (*CCL11*) -1382 A/G (rs4795895), CD14 -260 A/G (rs2569190), colony stimulating factor 2 (*CSF2*)1943 C/T (rs25882), IL10 -1117 C/T (rs1800896), IL10 4251 C/T (rs3024498) and the tumor necrosis factor alpha (*TNFα*)

-1211 C/T (rs1799964) polymorphisms, was designed using Assay designer software (Sequenom). All PCR reactions had a final volume of 5 µl and contained standard reagents and 5 ng of genomic DNA. After PCR a primer extension reaction was performed to introduce mass-differences between alleles and, after removing salts by adding resin, approximately 15 nl of the product was spotted onto a target chip with a 384 patches containing matrix. Mass differences were detected using an Autoflex (Bruker) matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) and genotypes were assigned real-time using Typer 4.0 software (Sequenom). Cluster plots were made of the signals from the low and the high mass allele. Two independent researchers carried out the scoring. There were neither disagreements nor vaguely positioned dots produced by Genotyper 3.0 (Sequenom Inc.).

Data analysis

Allele frequencies were determined by gene counting. The Chi-squared test was used to test the consistency of the genotype frequencies at the SNP locus with Hardy-Weinberg equilibrium. Relative risks (RR) with 95% confidence intervals (CI) were calculated with the Chi-Square test. If less than 10 patients were homozygous for a particular allele, the homozygotes and heterozygotes were combined. Differences in neointima formation, as measured by IVUS, were calculated and compared using a Student's t-test. Differences in baseline characteristics between genotypes were assessed with Student's t-test (for continuous variables) and Chi-Square test (for categorical variables). Population attributable risk was calculated as $[f(RR-1)]/[1+f(RR-1)]$, where f is the frequency of the genotype(s) of interest in the present SES population and RR is the relative risk for LASM. A p-value <0.05 was considered statistically significant. The SPSS software (version 15.0, SPSS Inc, Chicago, IL) was used for all statistical analyses.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

A total number of 104 SES patients with complete IVUS data were analyzed from our MISSION! Intervention Study database. The baseline characteristics are presented in Table 1. Of all clinical and procedural factors analyzed, only the absence of diabetes seemed to predict LASM: none of the 10 diabetic patients and 26 of the 93 non-diabetic patients presented LASM. The 10 diabetic patients in our SES population also had more neointimal growth when compared to non-diabetics (8.9 vs. 2.7%, $p=0.014$).

The RR for LASM of all 7 polymorphisms are presented in table 2. The CASP1 5352 G/A polymorphism was significantly associated with the risk for LASM: carriers of the

Table 1. Baseline clinical and angiographic characteristics. Adapted from van der Hoeven et al.⁶

Characteristic	Sirolimus-eluting stents (N=104)
Age (yrs)	58.6±11.5
Male sex – No.(%)	76 (73.1)
Diabetes mellitus – No.(%)	10 (9.6)
Current smoker – No.(%)	62 (59.6)
Hypercholesterolemia – No.(%)	22 (21.2)
Hypertension – No.(%)	36 (34.6)
Family history of CAD – No.(%)	45 (43.3)
Prior myocardial infarction – No.(%)	5 (4.8)
Prior PCI or CABG – No.(%)	2 (1.9)
Target vessel – No.(%)	
LAD	60 (57.7)
RCA	25 (24.0)
LCX	19 (18.3)
Multivessel disease – No.(%)	37 (35.6)
TIMI flow – No.(%)	
0-1	73 (70.2)
2-3	31 (29.8)
Vessel reference diameter (mm)	2.81±0.56
Minimal luminal diameter (mm)	0.23±0.36
Diameter stenosis (%)	92.0±12.4

Values are means (±SD) or numbers (percentages). CABG = coronary artery bypass graft; CAD = coronary artery disease; LAD = left anterior descending, LCX = left circumflex, PCI = percutaneous coronary artery, RCA = right coronary artery.

Table 2. Relative risk for late acquired stent malapposition in sirolimus-eluting stent patients.

SNP	N	LASM N (%)	RR (95%CI) for LASM
CASP1 5352 G/A	103	26	
GG	72	13(18.1)	1.0 (ref)
GA	30	13(43.3)	2.4 (1.27-4.55)
AA	1	0(0)	NA
GA+AA*	31	13(41.9)	2.32 (1.22-4.42)
CCL11-1382 A/G	104	26	
AA	78	21(26.9)	1.0 (ref)
AG	25	5(20)	0.74 (0.31-1.67)
GG	1	0(0)	NA
AG+GG*	26	5(19.2)	0.71 (0.3-1.7)
CD14 -260 A/G	104	26	
AA	32	12(37.5)	1.0 (ref)
AG	50	7(14.0)	0.37 (0.16-0.85)
GG	22	7(31.8)	0.85 (0.4-1.8)

Table 2. Continued.

CSF2 1943 C/T	104	26	
CC	60	13(21.7)	1.0 (ref)
CT	35	10(28.6)	1.32 (0.65-2.69)
TT	9	3(33.3)	1.54 (0.54-4.36)
CT+TT*	44	13(29.5)	1.36 (0.7-2.65)
IL10 -1117 C/T	104	26	
CC	29	9(31.0)	1.0 (ref)
CT	49	11(22.4)	0.72 (0.34-1.53)
TT	26	6(23.1)	0.74 (0.3-1.8)
IL10 4251 C/T	104	26	
CC	59	18(30.5)	1.0 (ref)
CT	36	7(19.4)	0.64 (0.3-1.37)
TT	9	1(11.1)	0.36 (0.06-2.4)
CT+TT*	45	8(17.8)	0.58 (0.28-1.22)
TNF -1211 C/T	103	26	
CC	65	19(29.2)	1.0 (ref)
CT	31	5(16.1)	0.55 (0.23-1.34)
TT	7	2(28.6)	0.98 (0.29-3.35)
CT+TT*	38	7(18.4)	0.63 (0.29-1.36)

*A dominant model was used. LASM = late-acquired stent malapposition. NA = not applicable. RR = relative risk. SNP = single nucleotide polymorphism.

CASP1 A allele were at clearly higher risk of developing LASM than homozygous wild type patients (41.9% vs 18.1, RR=2.3, 95%CI: 1.22-4.42). They also had less neointimal growth than patients not carrying the CASP1 A risk allele (1.6 vs. 4.1%, $p=0.014$). Only

Table 3. Baseline characteristics by CASP1 5352 G/A genotype

Characteristic	GG (%)	GA+AA (%)	p-value
Male sex	73.6	71	0.78
Caucasians	83.3	90.3	0.63
Age (yrs)	57.4±11.7	60.7±10.7	0.18
BMI (Kg/m ²)	26.9±3.6	28.6±5.6	0.11
Diabetes	9.7	9.7	0.99
Current smokers	61.1	58.1	0.77
Hyperlipidemia	18.1	29	0.21
Hypertension	31.9	38.7	0.51
Family history of CAD	44.4	41.9	0.81
Previous MI	4.2	6.5	0.62
Previous PCI	1.4	0	0.51
Previous CABG	0	3.2	0.13

Values are percentages or means (±SD). BMI = body mass index (kg/m²), CAD = coronary artery disease, CABG = coronary artery bypass graft, MI = myocardial infarction, PCI = percutaneous coronary intervention

one patient was homozygous for the CASP1 A allele and was included in the heterozygous group in all analyses.

There were no significant differences in baseline characteristics in the GG vs. (AA+AG) genotypes of the CASP1 5352 G/A polymorphism (Table 3). In the present sirolimus-eluting stent population the frequency of the AA+AG genotypes was (31/103) =30%. Population attributable risk (PAR) in sirolimus-eluting stent population was therefore $[0.3 \times (2.3-1)]/[1+0.3 \times (2.3-1)] = 28\%$.

DISCUSSION

In this study we investigated the influence of 7 polymorphisms involved in inflammatory processes on LASM and neointimal growth in SES patients. We found that of these polymorphisms, the caspase-1 5352 G/A polymorphism was a strong predictor of LASM. Moreover, there was a clear inverse relation between LASM risk and the amount of neointimal growth. This polymorphism has previously been identified to play a role in clinical restenosis after PCI.¹⁴

The caspase family of cysteine proteases is a key component in signal transduction cascades leading to inflammatory responses.²³ Caspase-1 (CASP1 or interleukin-1 β -converting enzyme, ICE) transforms immature IL-1 β to the mature form which can then be secreted by monocytes and macrophages.²³ CASP1 is also required for the production of IL-1 α and activation of IL-18.²³ The other members of the caspase family, namely caspases 2, 3, 6-10 are mainly involved in apoptosis. Because caspases share similarities in structure and substrate specificity,²³ CASP1 was initially thought to have a pronounced apoptotic effect. Initial studies showed that overexpression of CASP1 could promote apoptosis in rat fibroblasts.²⁴ However, other studies showed that CASP1 null mice exhibit defects in IL-1 and IL-18 production but did not clearly present defects in the regulation of apoptosis.^{25,26}

One previous study¹⁴ found the 5352 AA genotype of CASP1 to be associated with the higher risk for target vessel revascularization (TVR) and angiographic restenosis in patients undergoing percutaneous transluminal coronary intervention (PTCA) and BMS deployment.

In the present study we found that patients carrying the 5352 A allele had a significantly higher risk for LASM. Furthermore, with IVUS analysis we found the 5352 A allele to be associated with significantly less neointimal growth at 9-month follow-up.

In the above mentioned study,¹⁴ authors investigated the role of the CASP1 5352 G/A polymorphism in patients treated by PTCA, in the majority followed by BMS implantation. In our study all patients received SES. Sirolimus (rapamycin) was initially described as a cytostatic anti-tumoral and immunosuppressant agent.²⁷ Experiments on various

cell lines showed that in some cases they may develop resistance and continue to grow while in others sirolimus may even induce apoptosis.²⁷⁻²⁹ In current practice, SES perform better than BMS (and some other DES³⁰) in respect to restenosis but are frequently associated with LASM.^{6,31} Conversely, LASM is mostly absent after BMS implantation.^{6,32} It is not clear to date whether sirolimus-induced apoptosis is the main mechanism for LASM or local inflammation plays the major role. Ozer et al.³³ showed that systemic inflammatory markers display different patterns according to the type of implanted stent: SES associate with a lower plasma increase in high-sensitivity C-reactive protein (hsCRP) when compared with BMS, but with a slightly higher interleukin 6 (IL-6) increase; tumor necrosis factor alpha (TNF- α) showed a similar trend in DES and BMS. Although the precise mechanism remains to be revealed, we speculate that sirolimus is responsible for the differential effect of the CASP 1 5352 G/A polymorphism in the BMS and SES population.

In our sirolimus-eluting stent population with a frequency of approximately 30% for 5352 AA/AG we calculated a population attributable risk for LASM of 28%. In other words if the 5352 AA/AG genotypes could hypothetically be eliminated, then this would reduce LASM risk with 28% in the sirolimus-stented patients.

According to existing data, in case of the patients with one or two A allele of the CASP1 5352 G/A polymorphism, cardiologists might need to choose between a greater risk of LASM potentially triggering stent thrombosis (in SES) or a greater risk of stent restenosis (in BMS). Therefore, cardiologists may need to carefully assess risks and benefits related to patient's condition, previous coronary interventions and associated pathology before making this choice. In future, a better tailored dual-antiplatelet therapy as well as the new generation of stents may drastically decrease the risks associated with DES implantation. At the same time, further research is needed to optimize the approach of patients with documented stent thrombosis³⁴.

In our study, none of the 10 diabetic patients and 26 of the 93 non-diabetic presented LASM. Diabetes is a predictor of restenosis after PCI in general³⁵ and higher glucose levels have been associated with diminished efficacy of sirolimus on smooth muscle cell proliferation in particular³⁶. This may explain a lower incidence of LASM in diabetic compared to non-diabetic SES patients. In the larger DIABETES trial³⁷, IVUS analysis with a 40 MHz catheter identified at 9 month LASM in 11 out of the 75 (14.7%) SES treated lesions in diabetes patients.

The present study investigated inflammatory polymorphisms in STEMI patients. Although late (acquired) stent malapposition incidence is higher in STEMI versus non-STEMI patients, inconclusive data are available about the incidence of late (acquired) stent malapposition in STEMI patients receiving SES. Results from previous studies³⁸⁻⁴¹ vary according to the DES type, the length of the follow-up and the use of either IVUS or of the much higher resolution optical coherence tomography (OCT) for assessment.

It is therefore unclear to what extent the present findings may be extrapolated to the wide category of non-STEMI patients, including those who receive SES during elective procedures or to patients receiving a different type of DES. To date there is no relation known between unstable plaque and CASP1. Moreover, there are yet no arguments for a differential effect of CASP1 in SES versus non-SES which may limit the outcome of this study.

Our findings do not prove a causal relationship between CASP1 polymorphism and stent malapposition. The relation between 5352 G/A polymorphism and cytokine expression needs further investigation. In a subset of 69 patients, Monraats et al.¹⁴ examined the functional role of the 5352 G/A polymorphism by measuring mature IL-1 β levels. IL-1 β levels were found numerically higher in individuals with the 5352 AA genotype compared with 5352 GA and 5352 GG genotypes but the difference did not reach statistical significance. In our analysis, a policy of not making adjustments for multiple comparisons was preferred since, in our view leads to fewer errors of data interpretation⁴².

Conclusions

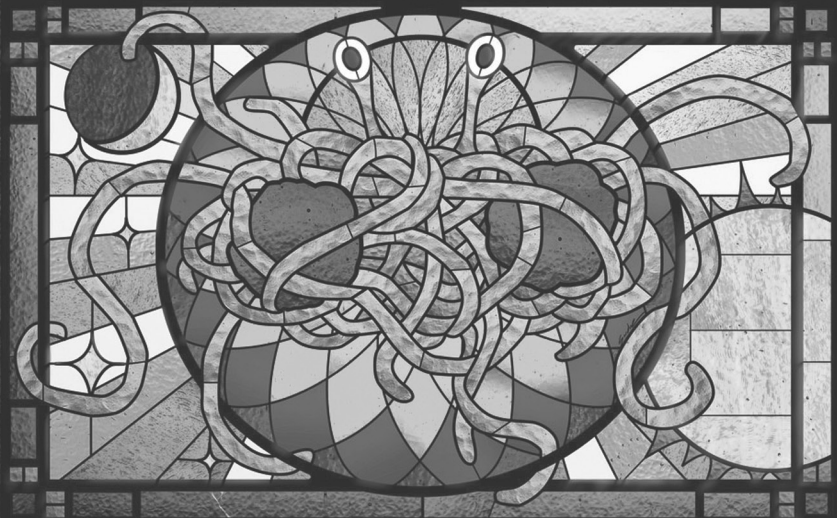
Our findings sustain the important role of inflammation (and possibly apoptosis) in the mechanism of LASM. The A allele of the 5352 G/A polymorphism is associated with higher risk of LASM in SES. If this is confirmed in larger studies, genotyping for CASP1 5352 G/A polymorphism may help cardiologists to make a better decision between SES and BMS implantation and may guide the (duration of) anti-platelet therapy in the future.

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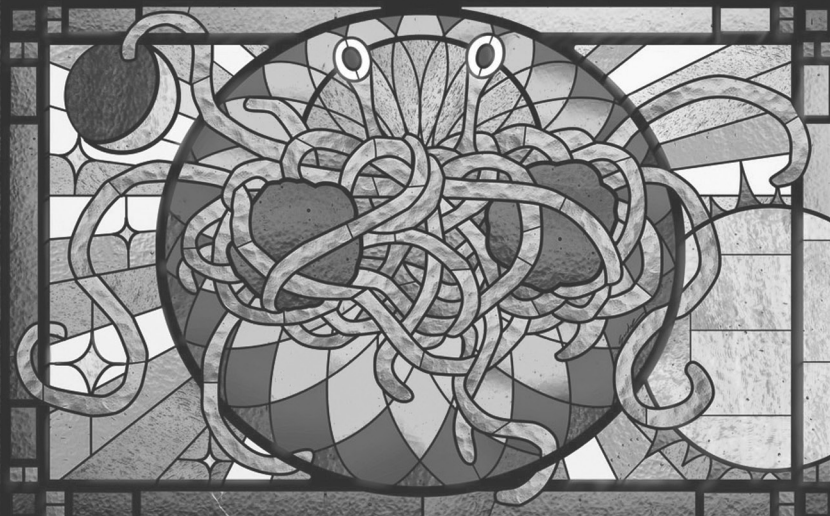
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Part 2

Epigenetic determinants in vascular disease



Chapter 9

Epigenetic histone acetylation modifiers
in vascular remodeling – new targets
for therapy in cardiovascular disease

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ABSTRACT

Significant progress has been made in the clinical management of a variety of cardiovascular diseases. Nevertheless, the therapeutic efficacy of the current treatment modalities for atherosclerosis and restenosis is not fully sufficient in a large proportion of patients. One of the major contributing factors is the clinical and biological heterogeneity of these still life-threatening diseases, which involve processes that we do not fully understand at the moment. Over the past decades it has become increasingly clear that part of the gene-environmental interactions relevant for complex diseases is regulated by epigenetic mechanisms such as histone acetylation and DNA methylation. Epigenetic processes modulate gene expression patterns without modifying the actual DNA sequence and have profound effects on the cellular repertoire of expressed genes. They contribute to the expression of genes that play a key role in extracellular matrix formation, inflammation and proliferation, processes involved in cardiovascular pathologies such as atherosclerosis and restenosis.

Therefore, in this review we argue that epigenetic regulators involved in histone acetylating and deacetylating activities, contribute to the pathogenesis of atherosclerosis and restenosis. Furthermore, as alterations in chromatin structure are reversible, these epigenetic modifications are amendable to pharmacological intervention, which may prove to be an effective treatment modality for management of cardiovascular diseases.

1. INTRODUCTION

1.1 Atherosclerosis and restenosis

Atherosclerosis and restenosis are multifactorial processes, which have several corresponding features and share some risk factors such as diabetes and hypertension. Both processes are in a significant part characterized by an inflammatory response to injury of the endothelium, which causes reshaping of the vessel wall in size and composition, called vascular remodeling. Furthermore, they share proliferation and migration of vascular smooth muscle cells (VSMCs) and elaboration of extracellular matrix, leading to accumulation of collagen and proteoglycans.

Despite these similarities, there also are important differences in the cause and progression of atherosclerosis and restenosis. Atherosclerosis develops partly in response to elevated lipoprotein levels and cigarette smoke, whereas restenosis is mainly an overshooting wound healing response to vascular injury by balloon dilatation or stent placement. This restenotic process is not particularly sensitive to circulating lipids and smokers even seem to have a reduced risk for restenosis.¹ Furthermore, atherosclerosis is, in contrast to restenosis, characterized by accumulation of oxidized lipoproteins, both within foam cells and extracellularly. While development and progression of atherosclerosis is associated with aging, the restenotic process is relative rapidly induced after revascularization interventions such as balloon dilatation or stent placement.

Currently, atherosclerosis and restenosis are still serious health problems, which we do not yet fully understand. It is therefore important to search for factors that cause and contribute to the development of the processes involved in these diseases. Investigation into the exact mechanisms of the disturbed lipoprotein metabolism, inflammation, proliferation and migration of smooth muscle cells and vascular remodeling, will offer us more opportunities for prediction, diagnosis and finally treatment.

Because clinical risk factors do not fully predict the development of atherosclerosis and restenosis, it is important to search for genes that determine susceptibility to these risk factors. A positive family history is a major risk factor for cardiovascular disease and twin studies have shown that death from coronary artery disease at an early age of one twin is a strong predictor of the risk in the other twin.²

As reviewed by Nordlie *et al.*, many genes in lipid metabolism, vascular homeostasis, hemostasis and inflammation have been found to be related to coronary artery disease.³ Although observed associations were not always replicated by other studies, their review emphasizes the importance of genetics in atherosclerotic disease. Moreover, the multifactorial nature of the disease suggests a role for many other, yet uninvestigated genes.

Previous research from our department has demonstrated the importance of genetics in restenosis after percutaneous coronary interventions (PCI). Polymorphisms in several inflammatory genes, such as tumour necrosis factor α (TNF- α), eotaxin, CD14,

granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin 10 (IL-10), caspase-1, but also non-inflammatory genes, such as lipoprotein lipase (LPL), stromelysin-1 (MMP3) and the β adrenergic receptor have been found to be associated with the risk for restenosis.⁴⁻⁷

In the past decade, research into cardiovascular diseases has been focused on the identification of these genetic factors. It has become clear, however, that part of the gene-environmental interactions relevant for complex diseases in which inflammation, proliferation and remodeling play an important role, is regulated by epigenetic mechanisms such as histone acetylation and DNA methylation.

Epigenetics, the study of non-DNA sequence-related heredity, can help to explain the relationship between an individual's genetic background, the environment, aging and disease. Thus, a review of the possible role of epigenetics in complex diseases like atherosclerosis and restenosis seems appropriate and is presented here.

1.2 Epigenetics

Epigenetic processes modulate gene expression patterns without modifying the actual DNA sequence and have profound effects on the cellular repertoire of expressed genes.⁸ It is well known that epigenetic processes can lead to meiotically and mitotically heritable changes in gene expression and play an important role in control of cell identity. Two well-known examples of epigenetic mechanisms are methylation of DNA at CpG dinucleotides and modification or rearrangement of nucleosomes, which include covalent post-translational modifications of histone tails.^{9,10} Both processes can be influenced by environmental factors and affect gene function without changing the DNA sequence.

In contrast with classical mendelian views on inheritance, epigenetics also focuses on heredity of environmental effects which lead to DNA modifications other than DNA sequence variation, a phenomenon which is called 'epigenetic inheritance'. An increasing number of findings from both human and animal studies support the existence of epigenetic inheritance, and show that DNA-methylation is the main responsible mechanism.^{11, 12} Inheritance of DNA-methylation is regulated by the enzyme DNA methyltransferase 1 (Dnmt I), which is known to copy methylation patterns during semiconservative DNA replication.¹³

Although a similar mechanism has not yet been discovered for post-translational histone modifications, they are likely to be maintained through cell-division. Not only methylation of DNA, but also acetylation of histones, has been shown to accumulate in time.⁸ Older monozygotic twins were found to have larger differences in DNA methylation and histone acetylation when compared to younger twin pairs. This finding also demonstrates that post-translational histone modifications can be influenced by environmental factors.

Despite the lack of an evident mechanism of maintaining post-translational histone modifications during cell division, modification of histone tails is by many considered an epigenetic process.^{14, 15}

Epigenetic modifications of histone tails include acetylation, methylation, ubiquitination and SUMOylation of lysine residues, phosphorylation of serine residues and methylation of arginines. The various histone modifications form a code which is read by non-histone proteins and have varying effects on chromatin structure and gene accessibility.⁹ As a rule of thumb, conformationally relaxed chromatin (euchromatin) is a hallmark of potentially active genes and is associated with hypomethylation of CpG dinucleotides in DNA and acetylated histones. Compact chromatin (heterochromatin) is associated with transcriptionally silent genes and is associated with DNA hypermethylation at CpG dinucleotides and nonacetylated histones. These chromatin modifications are exerted by epigenetic regulators such as DNA methyltransferases (Dnmts), histone (lysine) acetyltransferases (KATs) and histone methyltransferases (HMTs), which are increasingly being implicated as direct or indirect components in the regulation of expression of vascular, immune, and other (tissue)-specific genes.

A great promise of epigenetics is that it offers new targets for therapy in cardiovascular disease. Epigenetic processes are reversible by nature, which is underscored by the counterbalancing action of KATs and KDACs (lysine deacetylases), and of HMTs and HDMs (histone demethylases), and is required for fine-tuning of gene expression for fundamental cellular processes such as cell proliferation and differentiation. This offers the prospect of pharmacological inhibition of the various enzymatic activities involved in epigenetic DNA and histone modifications, which is aimed at the induction or silencing of transcription of disease relevant genes. In addition to the possibility of modifying the effects of deleterious genes, it might be possible to influence the effects of environmental risk factors.

Since KATs and KDACs are involved in fundamental processes that regulate the expression of multiple genes, they most likely play an important role in the multifactorial processes that lead to atherosclerosis and restenosis. Therefore, it is important to identify those KATs and KDACs that play a role in the transcriptional regulation of genes, the products of which contribute to the processes involved in neointima formation and atherogenesis, such as inflammation, smooth muscle cell proliferation and matrix formation.

Single gene disorders in the histone acetylation machinery have already been shown to cause clinical syndromes. For example, patients with Rubinstein-Taybi syndrome are known to have mutations in the gene encoding CREB binding protein (CBP), a transcriptional coactivator with KAT-activity.¹⁶ Therefore it seems likely that also polymorphisms in genes encoding

KATs or KDACs, provided that they have functional implications, may influence cardiovascular disease susceptibility by affecting the fidelity of these enzymes. This hypothesis was recently confirmed by research from our department showing that the -2481 G/C polymorphism in the promoter of the gene encoding p300/CBP-associated factor (PCAF), a KAT with an important role in inflammatory gene activation, is significantly associated with all-cause mortality in the PROSPER-study (n=5804) and with clinical restenosis after a percutaneous coronary intervention in the GENDER-study (n=3104). Genetic epidemiology may lead to the identification of more risk markers in genes encoding KATs or KDACs and provide new insights into the pathophysiology of atherosclerosis and restenosis, which could contribute to the development of better therapy.

In this review we will discuss the role of KATs and KDACs in the transcriptional regulation of genes that play a critical role in inflammation, proliferation and matrix formation, processes which are thought to be pathognomonic for atherosclerosis and restenosis development.

2. KATS AND KDACS

Gene expression is regulated by acetylation of core histones through the action of KATs by transfer of an acetyl group to the ϵ -portion of lysine residues. Hyperacetylation of histones results in an open modification of chromatin structure and affects gene transcription through accessibility of DNA to the basal transcription initiation machinery (figure 1).¹⁷⁻¹⁹ Conversely, gene repression is mediated via KDACs and other corepressors, which remove the acetyl groups from hyperacetylated histones and counteract the activity of KATs resulting in a closed chromatin structure. These nuclear enzymes have been shown to regulate the expression of inflammatory genes by modifying chromatin structure.²⁰ KATs are recruited to promoters by gene regulatory proteins that interact with specific recognition sequences in DNA, and in this way control gene transcription. This is exemplified by the transcriptional control of MHC-II genes in which the class II transactivator (CIITA), a component of the promoter assembled enhanceosome, provides the platform for recruitment of KAT and KDAC activities.²¹⁻²³

At the moment a large variety of KATs have been identified, which can be divided in several families, including the PCAF/Gcn5, p300/CBP, MYST, SRC, TAF_{II}250, KAT1 and ATF-2 families. The KATs within these families show high sequence similarity, but there is poor similarity between these families.²⁴ All KAT proteins vary in their KAT-domains and their substrate-specificity, but have the common feature that they require the assembly of multiprotein complexes for nucleosomal acetylation.²⁵ Their precise substrate-specificity even depends on the context of these multisubunit KAT-complexes.²⁶

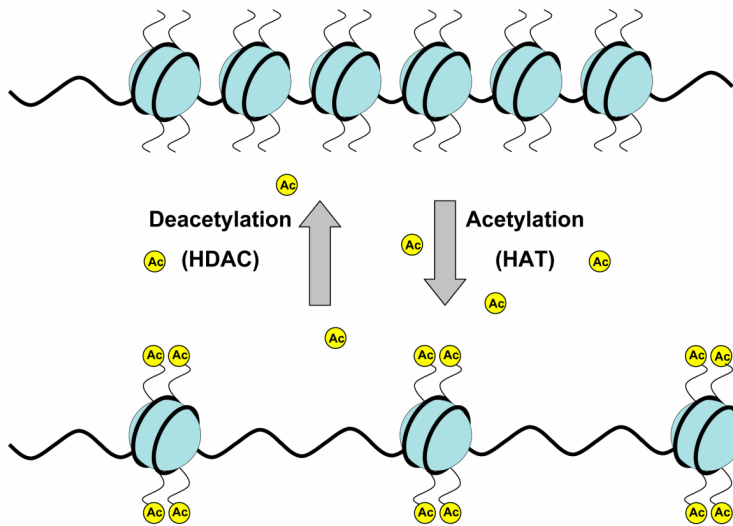


Figure 1. Histone acetylation

Acetylation of histone-tails is mediated by histone (lysine) acetyltransferases (KATs) and results in an open modification of chromatin structure. It allows transcriptions factors to access the DNA and initiate gene transcription. Conversely, gene repression is mediated via lysine deacetylases (KDACs), which remove the acetyl groups from the histone-tails, resulting in a closed chromatin structure.

The KDAC enzymes are zinc-dependent aminohydrolases which can be grouped into different classes.²⁷ Class I KDACs (KDAC 1-3 and 8) are widely expressed, reside almost exclusively in the nucleus and are known to modulate cell proliferation and survival. Class II KDACs (KDAC 4-7, 9 and 10) have a more restricted distribution, are able to move between the nucleus and cytoplasm in response to certain cellular signals and may be involved in cellular differentiation.^{27, 28} Class II KDACs can be further divided in subclasses IIa (KDAC 4, 5, 7 and 9) and IIb (KDAC 6 and 10). Class IIa members form a distinct group because of the presence of an extended N-terminal regulatory domain,²⁹ whereas KDAC 6 and 10 contain an extra catalytic domain.³⁰ The most recently discovered isoform is KDAC11, which constitutes class IV.³¹ A different NAD⁺-dependent family consists of sirtuins (SIRT 1-7) which are thought to be involved in apoptosis of mononuclear cells.^{32, 33}

The different KDACs are likely to be regulated differently. KDACs interact with corepressor molecules, such as nuclear receptor corepressor and ligand-dependent corepressor, NuRD and mSin3, all of which aid KDACs in gene repression and may provide specificity by selecting which genes are switched off by KDACs.^{34, 35} Furthermore, KDAC activity is associated with the action of Polycomb Group Proteins,³⁶ which play a key role in gene silencing and maintenance of cellular identity.

Besides their role in histone acetylation, KATs are also found to act as factor acetyltransferases (FATs), which acetylate many non-histone proteins,³⁷ such as p53, which

is directly acetylated by PCAF,^{38, 39} CREB binding protein (CBP) and p300,^{40, 41} and nuclear factor kappa B (NFκB), which is acetylated by CBP and p300.^{42, 43} Acetylation of these proteins often results in increased DNA-binding and transcriptional activity.³⁷ Accumulating evidence suggests that many KDACs can deacetylate these non-histone proteins. For example, KDAC3 is known to deacetylate the p65 subunit of NFκB, promoting association with IκBα, which leads to IκBα-dependent nuclear export of NFκB.⁴⁴ Furthermore, KDAC1 has been shown to deacetylate p53 *in vitro* and *in vivo*.⁴⁵ As many other non-histone proteins are also known to be targeted by KATs and KDACs, their influence on gene expression is not necessarily dependent on chromatin remodeling. Changes in gene expression which are caused by alterations in the activity of KATs or KDACs can therefore not automatically be regarded as epigenetic.

Over the past years several KAT and KDAC inhibitors have been identified. These inhibitors mechanistically affect the action of KATs and KDACs. The large variety of KDAC-inhibitors that have been discovered or developed can be divided in structural classes (table 1).^{27, 46, 47} Early phase clinical trials have demonstrated that inhibitors from all different classes seem to be well tolerated and exhibit clinical activity against several human neoplasms.²⁷

In the next paragraphs we will discuss the role of KATs and KDACs in inflammatory, proliferative and remodeling processes associated with atherosclerosis and restenosis. Furthermore, we will discuss the potential applicability of KAT and KDAC inhibitors in disease management.

Table 1. Structural classes of KDAC inhibitors

Short-chain fatty acids	Sodium butyrate, Sodium phenylbutyrate, Valproic acid (VPA), Pivaloyloxy-methylbutyrate (AN-9)
Hydroxamic acids	Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA), Azelaic bishydroxamic acid (ABHA), Aroyl pyrrolyl hydroxy-propenamides (APHA), carboxycinnamic acid bishydroxamide (CBHA), Suberoyl bishydroxamic acid (SBHA), Oxamflatin, Scriptaid, Pyroxamide, Tubacin, ITF2357, LBH589, NVP-LAQ824, 3-CI-UCHA, A-161906, JNJ16241199, CRA-024781, PXD101
Benzamides	MS-275 (MS-27-275), MGCD0103, CI-994
Epoxyketone-containing cyclic tetrapeptides	Trapoxins, Chlamydocin, 2-Amino-8-oxo-9,10-epoxydecanoic acid (AOE)
Non-epoxyketone-containing cyclic tetrapeptides	Depsipeptide, FR901228 (FK228), Apicidin
Hybrid molecules	Cyclic hydroxamic acid-containing peptides (CHAPs): CHAP31, CHAP50

Adapted from: Santini *et al.* (2007), Drummond *et al.* (2005) and Monneret *et al.* (2005).²

3. INFLAMMATION, THE ROLE OF KATS AND KDACS

3.1 Nuclear Factor kappa B

Atherosclerosis and restenosis are for a major part determined by inflammation.^{5, 48} The expression of multiple inflammatory genes is regulated by pro-inflammatory transcription factors, such as NFκB. The transcription factor NFκB plays a role in the orchestration, amplification and perpetuation of the inflammatory response and forms the molecular basis of chronic inflammation.⁴⁹⁻⁵¹ KATs and KDACs have been implicated in modulating NFκB activity. In unstimulated cells NFκB is found in the cytoplasm associated with its inhibitor protein called IκB, which masks the nuclear translocation signal and prevents NFκB from entering the nucleus.⁵² Upon cell stimulation with various NFκB inducers, including reactive oxygen species (ROS) and lipid peroxidation products, IκB is rapidly phosphorylated and thereby targeted for ubiquitination.^{53, 54} The released NFκB dimer is translocated to the nucleus and activates target genes by binding with high affinity to κB elements in their promoters.^{55, 56} This process is known to be activated by KATs and repressed by KDACs.⁵⁷ IL-1β and TNF-α have been shown to stimulate the binding of the NFκB p65 subunit to CBP and to induce histone acetylation, thus leading to an increase in NFκB-mediated expression of inflammatory genes, such as GM-CSF and IL-8 (figure 2).^{58, 59} Furthermore, p300 has been shown to enhance angiotensin II-induced IL-6 expression in rat VSMCs, a process which is also mediated by NFκB.⁶⁰ Also PCAF is known to be required to coactivate p65-dependent transcription and has been shown to activate the transcription of several NFκB-regulated genes known to be involved in cardiovascular disease. Spermidine/Spermine N1-Acetyltransferase 2 (SSAT2) has been found to cooperate with CBP and PCAF to enhance TNF-α-induced NFκB activity.⁶¹ Furthermore, Miao *et al.* demonstrated that PCAF could enhance the p65 mediated increase in TNF-α promoter activity and that high glucose increased the recruitment of PCAF to the TNF-α and cyclooxygenase 2 (COX-2) promoters.⁶² They demonstrated concomitant acetylation of specific lysine residues of histone H3 and H4 at these promoters. Since TNF-α and COX-2 have been implicated in the development of both atherosclerosis^{63, 64} and restenosis,^{4, 65} PCAF may play a role in the development of these diseases. A role for PCAF in vascular disease was recently confirmed by findings from our department showing that the -2481 G/C polymorphism in the promoter of the PCAF gene is significantly associated with all-cause mortality in the PROSPER-study (manuscript in preparation, abstract published in *Circulation*, Oct 2007; 116: II_40). The minor C allele was associated with a significant survival advantage, while this allele was also found to protect against clinical restenosis after a percutaneous coronary intervention in the GENDER-study.

In line with the findings showing a role for KATs in NFκB-mediated transcriptional activation, KDACs have been shown to reverse this process and to repress NFκB-mediated

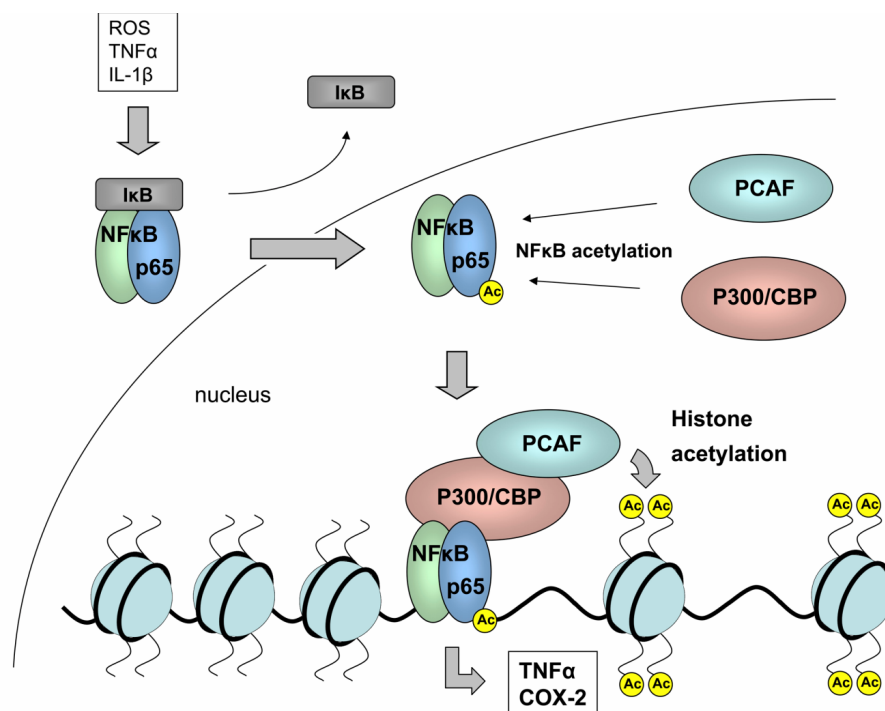


Figure 2. The role of PCAF in the activation of NFκB-mediated transcription.

The figure illustrates the dual role of p300, CBP and PCAF in the activation of NFκB-mediated transcription upon cell stimulation with NFκB-inducers, such as Reactive Oxygen Species (ROS), TNF-α or IL-1β. These coactivators act as lysine acetyltransferases (KATs) at the site of NFκB-regulated genes. Furthermore, they act as factor acetyltransferases (FATs) and appear to acetylate the p65 subunit of NFκB, increasing its DNA-binding and causing transcriptional activation also by this mechanism.

gene expression.^{66, 67} Furthermore, KDAC-inhibition by trichostatin A (TSA) was found to increase basal and TNF-α-induced expression of the NFκB-regulated IL-8 gene, whereas expression of KDAC1 and KDAC2 repressed TNF-induced NFκB-dependent gene expression.⁶⁷

In addition to its ability to associate with KATs and KDACs, the p65 component of NFκB is also a direct target for acetylation and deacetylation. PCAF and CBP/p300 appear to acetylate specific lysine residues on the p65 subunit of NFκB, increasing its DNA binding and causing transcriptional activation (figure 2), whereas KDAC3 has been shown to deacetylate NFκB and promote its association with the inhibitor IκBα within the nucleus, in order to promote export into the cytoplasm and thus terminate the activity of NFκB.^{42, 44, 68, 69}

3.2 Granulocyte Macrophage – Colony Stimulating Factor

Several findings indicate the importance of GM-CSF in atherosclerosis and restenosis. Administration of GM-CSF has been shown to prevent the progression of athero-

sclerosis in WHHL (Watanabe heritable hyperlipidemic) rabbits.⁷⁰ Furthermore, in a rabbit model of restenosis, GM-CSF injections have been shown to reduce neointima formation.⁷¹ In further support of a role for GM-CSF in the development of restenosis, variation in the GM-CSF gene has been shown to be related to the risk of restenosis in a large patient population undergoing PCI.⁵ Because of its role in atherosclerosis and restenosis it is of interest to note that the NFκB-mediated, inflammatory signalling induced hyperacetylation of histone H4 also correlated with an increased expression of GM-CSF. The KDAC inhibitor TSA was found to lead to an increased expression of GM-CSF and IL-8 in alveolar macrophages and airway epithelial cell lines after activation with inflammatory stimuli.^{58, 72, 73}

3.3 Eotaxin

Eotaxin, an eosinophil chemoattractant important in inflammatory responses, has already been shown to play a role in atherosclerotic disease. Eotaxin plasma levels were found to be associated with angiographic coronary artery disease⁷⁴ and human atherosclerotic plaques were found to express increased quantities of eotaxin mRNA.⁷⁵ Furthermore, the A23T polymorphism in the eotaxin gene is associated with the risk for myocardial infarction⁷⁶ and the -1328 A allele was found to decrease the risk for restenosis after PCI.⁵

Inflammatory signalling by TNF-α induces selective histone H4 acetylation and binding of p65 to the eotaxin promoter as determined by chromatin immunoprecipitation (ChIP), resulting in eotaxin gene transcription.⁷⁷ Notably ChIP analysis also revealed that Beta(2)-agonists and glucocorticoids were able to reduce transcription of eotaxin by inhibiting both TNF-α-induced histone H4 acetylation and recruitment of p65 to the eotaxin promoter, without altering the capability of NFκB to translocate to the nucleus.

3.4 Cyclooxygenase-2

Several proteins that have been implicated in the development of cardiovascular disease have been studied more extensively in cancer and inflammatory diseases. This is exemplified by Cox-2, a protein that may be associated with the development of atherosclerosis⁶⁴ and restenosis,⁶⁵ which plays an important role in colon carcinogenesis and inflammation.⁷⁸ Cox-2 expression can be induced by several cytokines, including TNF-α, and the KDAC-inhibitors sodium butyrate (NaBu) and TSA were found to repress Cox-2 mRNA and resulting protein synthesis in a colon cancer cell line (HT-29).⁷⁹ Although KDAC inhibition by NaBu is known to suppress cytokine-induced NFκB activation, and NFκB binding sites exist within the Cox-2 promoter, this repression of Cox-2 expression in HT-29 cells was not mediated by a lower Cox-2 promoter activity, but was related to mRNA stabilization.⁷⁹ These observations infer that besides a direct effect of KDAC

inhibitors on gene promoter activity, additional cellular mechanisms exist which contribute to modulation of mRNA expression and protein levels of Cox-2.

3.5 Reactive Oxygen Species

Activated inflammatory cells and a perturbation of lipoprotein metabolism are key factors in atherogenesis. The oxidative modification of lipids is involved in the recruitment of mononuclear leukocytes to the arterial intima. Furthermore, activated leukocytes, as well as endothelial mitochondria, can produce reactive oxygen species (ROS) that are associated with endothelial dysfunction.⁸⁰ Elevated intracellular ROS are generated under various physiological and pathological conditions involving oxidative stress, including inflammation, ischemia, reperfusion, and sepsis. The biological effects resulting from oxidative stress are in part mediated by modifications in KAT and KDAC activities, which lead to alterations in histone acetylation levels and resulting NF κ B activation.^{28, 52, 81} Several *in vitro* experiments have shown that hydrogen peroxide increases KAT-activity⁷³ and decreases KDAC-activity,^{57, 82} leading to an increase in histone acetylation and expression of inflammatory mediators.

The role of ROS in chromatin remodeling is further illustrated by the observed imbalance in expression levels of KATs and KDACs in bronchial biopsies from patients with asthma, when compared with airways of non-asthmatic subjects.⁸³ Notably, treatment with inhaled steroids resulted in increased KDAC activity and reduced KAT activity in subjects with asthma thereby influencing this imbalance. This is in accordance with other studies describing that recruitment of KDAC activity to activated transcriptional complexes was required for glucocorticoid repression of inflammatory genes.^{58, 84} In addition, the glucocorticoid receptor also acts as a direct inhibitor of CBP-associated KAT activity.⁵⁸ This could be a mechanism that explains the effectiveness of glucocorticoids in suppressing inflammation in asthma.

Together there are several lines of evidence that suggest an important role of altered histone acetylation levels in inflammatory processes, which are associated with atherosclerosis and restenosis.

4. PROLIFERATION OF (V)SMCS AND HISTONE ACETYLATION

4.1 Serum Response Factor

Precise control of smooth muscle cell (SMC) gene transcription plays a major role in vascular development and pathophysiology. Vascular SMCs undergo profound changes in their phenotype during neointima formation in response to vessel injury or within atherosclerotic plaques.⁸⁵ Serum Response Factor (SRF) is an important mediator in transcriptional activation of genes that exhibit SMC-restricted expression. SRF exerts

its activation through binding to CArG box DNA sequences found within regulatory elements of these genes. Recent findings indicate that binding of this factor is influenced by chromatin structure. *In vitro* and *in vivo* experiments have shown that SRF-binding is associated with patterns of posttranslational histone modifications that are specific to the SMC lineage, including di-methylation of lysine residues 4 and 79 in histone H3, acetylation of lysine 9 in histone H3 and acetylation of histone H4. In particular, deacetylation of histone H4 was coupled with loss of SRF binding to CArG box DNA during suppression of SMC differentiation in response to vascular injury.⁸⁶ SRF-mediated transcriptional activation is enhanced by myocardin, which acts as a co-activator, through interaction with the MADS box of SRF. Myocardin is specifically expressed in cardiac and smooth muscle cells, and plays a critical role in the differentiation of SMCs through activation of SMC-specific genes by tethering to CArG boxes with SRF.⁸⁷ The promyogenic activity of myocardin is enhanced by p300, a lysine acetyltransferase that associates with the transcription activation domain of myocardin.⁸⁷ Conversely, class II lysine deacetylases interact with a domain of myocardin distinct from the p300-binding domain and suppress smooth muscle gene activation by myocardin.

4.2 Hyper nuclear acetylation

Hyper nuclear acetylation (HNA) plays a role in proliferation, differentiation and apoptosis.⁸⁸ Using an antibody against ϵ -acetylated lysine, increased histone acetylation was observed in VSMCs in coronary atherosclerotic lesions, whereas it was not present in normal coronary arteries. Furthermore, thrombin, a humoral factor known to cause activation and proliferation of VSMCs, strongly potentiates HNA in cultured VSMCs. In thrombin-induced HNA of VSMCs, the MAP kinase pathway and the CREB binding protein (CBP) are implicated.⁸⁹ It suggests that coactivators cooperating with signal-dependent transcription activators play a role in atherosclerogenesis via HNA in VSMCs.

4.3 Cell-cycle regulators

Because KDACs also modulate histone acetylation levels at the site of genes involved in cell cycle control, they have also been implicated in the proliferation of SMCs.^{90, 91} This is illustrated by the observation that TSA was found to inhibit SMC proliferation via induction of p21waf1.⁹² Activity of p21waf1 is known to induce cell-cycle arrest in vascular smooth muscle cells,⁹³⁻⁹⁶ and A20, a NF κ B-dependent gene that has been shown to inhibit proliferation of VSMCs via increased expression of p21waf1, has been shown to prevent neointima formation after balloon angioplasty in a rat model of carotid artery stenosis.⁹⁷

Also PCAF could play a role in the suppression of VSMC proliferation by its ability to directly acetylate p53.^{38, 39} PCAF has been shown to activate p53-responsive enhancer

elements within the p21waf1 promoter.³⁹ Moreover, KDAC8 was found to be exclusively expressed in cells showing smooth muscle differentiation, including visceral and vascular smooth muscle cells, myoepithelial cells and myofibroblasts, and is mainly detected in their cytosol.⁹⁸ Until recently, it was thought that its activity was restricted to the nucleus, with histones as a unique substrate.

5. REMODELING: EXTRACELLULAR MATRIX FORMATION AND CHROMATIN DYNAMICS

Inappropriate remodeling of the vessel wall in response to a variety of stimuli, including hemodynamic changes, inflammation and tissue injury, is currently thought to be causative to the development of atherosclerosis and restenosis. The matrix metalloproteinases (MMPs) comprise a family of structurally related proteins, which includes collagenases, stromelysin and gelatinases. They have the collective capability to degrade components of the extracellular matrix and to act as mediators in a variety of cell signalling pathways through degradation of non-matrix proteins. MMP expression is strongly enhanced in vascular pathologies and is therefore thought to contribute to the disease process. This is illustrated by the observation that enhanced expression of MMP2 and MMP9 (also referred to as Gelatinase A and Gelatinase B) is noted in vulnerable regions of human carotid plaques in association with macrophages.⁹⁹ Furthermore, as reviewed by Newby, induction of MMP9 and activation of MMP2 occur rapidly after balloon injury in multiple animal models.¹⁰⁰ Additional evidence for an important role of MMP2 and MMP9 in vascular remodeling comes from knockout mouse-models of these gelatinases which showed reduced VSMC migration and less neointima formation after arterial injury.¹⁰¹⁻¹⁰⁴ Increased MMP3 (also referred to as stromelysin-1) expression has been linked to plaque rupture.^{99,105} In addition, several studies investigating the MMP3 5A/6A promoter polymorphism have shown that the risk of myocardial infarction is higher in patients carrying the 5A allele, which is associated with increased MMP3 levels.¹⁰⁶⁻¹¹⁰ MMP1 and MMP13 are also associated with cardiovascular disease. MMP1 was found expressed in human atherosclerotic lesions and not in unaffected blood vessel walls.¹¹¹ Moreover, the MMP1 1G/2G polymorphism at position -1607 of the transcriptional start site, which is associated with a higher promoter activity, reduces the risk for coronary artery disease.¹¹¹ Likewise, the MMP13 -77 A/G polymorphism is associated with atherosclerosis in the abdominal aorta of black males.¹¹²

Due to their essential role in vessel wall remodeling, MMPs could become prime targets for pharmacological interference in various pathological conditions that exhibit increased or decreased MMP levels and resulting excessive or reduced matrix degradation.

In the next sections we will discuss the role of the chromatin environment in expression of the various MMPs in vascular pathologies.

5.1 MMP2 and MMP9 (Gelatinase A and Gelatinase B)

Epigenetic mechanisms have been implicated in regulation of transcription and/or functional expression of MMP2 and MMP9. This is underscored by several observations showing modulation in expression levels of MMPs following exposure of cells to different KDAC inhibitors in various cell types. Treatment of different tumour cell-lines with NaBu resulted in modulation of secreted expression levels of MMP2 and MMP9, which was accompanied by global histone H4 hyperacetylation.¹¹³ On the other hand it was demonstrated that increased histone acetylation by TSA-treated 3T3 cells decreases mRNA as well as zymographic activity of MMP2.¹¹⁴ This TSA-induced inhibition of MMP2 activation is mediated via upregulation of the glycoprotein RECK.¹¹⁵ With respect to MMP9, it has been demonstrated that IFN- γ induced a reduction in MMP9 expression, which is mediated by CIITA. CIITA is induced by IFN- γ through the JAK/Stat signalling pathway and downregulation of MMP9 is exerted by CIITA through sequestering of CBP from the MMP9 promoter, effectively reducing histone H3 acetylation, explaining the inhibition in MMP9 transcription by IFN- γ .¹¹⁶ Further support for a role of epigenetic mechanisms in the regulation of MMP9 expression comes from the observation that the metastases associated gene MTA1 represses MMP9 expression, in part by the recruitment of KDAC2 to the distal MMP9 promoter-region¹¹⁷ and modulation of the activity of MMP9 by depsipeptide in uveal melanoma cells.¹¹⁸

5.2 MMP3 (Stromelysin-1)

Besides MMP2 and MMP9, several findings also indicate the involvement of epigenetic mechanisms in the expression of MMP3. Overexpression of the TEL (Translocation-ETS-Leukemia)-protein, which specifically associates with KDAC3, represses transcription of the stromelysin-1 gene. A role for KDAC3 in the TEL-mediated repression was supported by the observations that histone H3 was underacetylated near the TEL binding sites in the stromelysin-1 promoter and that administration of TSA impaired TEL-dependent repression of the stromelysin-1 promoter.¹¹⁹ A role for histone acetylation in MMP3 expression is further supported by the finding that NaBu selectively enhanced protein production and mRNA expression of stromelysin-1 in TNF- α or IL-1 β stimulated mesenchymal cells.¹²⁰ Furthermore, it has been demonstrated that the transcription factors Ets-1 and Ets-2 recruit p300 and CBP to the human stromelysin-1 promoter to activate transcription.¹²¹

5.3 MMP1 and MMP13 (Collagenase 1 and collagenase 3)

Histone acetylation modifications are also implicated in the expression of MMP1 and MMP13. This notion is derived from studies in chondrocytes which revealed that exposure of cells to TSA or NaBu blocks the induction of MMP1 and MMP13 by pro-inflammatory cytokines at both the mRNA and protein level.¹²² Furthermore, NaBu treatment of liver cancer cells results in decreased transcription of MMP1.¹²³ Additional evidence for a role of histone acetylation modifications in the control of MMP1 expression is derived from the observation that the co-activator p300 has been shown to relieve the Smad-mediated inhibitory effect of TGF- β in cytokine-induced MMP1 expression in dermal fibroblasts.¹²⁴ Expression of MMP1 is also indirectly affected by TSA and SAHA (Suberoylanilide hydroxamic acid) through inhibition of c-jun.¹²⁵

Together, histone acetylation modifications have been shown to play a direct and indirect role in the regulation of MMP expression, critical mediators in vascular remodeling and as such implicated in the pathology of atherosclerosis and restenosis.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Besides genetic control, epigenetic mechanisms contribute to expression at the transcriptional level of genes, the products of which play a key role in extracellular matrix formation, inflammation and proliferation as discussed in this review. Altered expression levels of these genes have been observed in cardiovascular pathologies and therefore it is thought that these aberrant expression patterns contribute to the processes associated with atherosclerosis and restenosis. As the nucleotide composition of genes is not altered by these epigenetic processes and alterations in chromatin structure are reversible, these epigenetic modifications are amendable to pharmacological intervention, which may prove to be an effective treatment modality for the management of cardiovascular diseases.

Although chromatin modifications exist at the level of DNA methylation at CpG dinucleotides and a variety of post-translational histone modifications, we have focussed on the role of histone acetylation modifications exerted by KATs and KDACs in cardiovascular disease. Besides accumulation of epigenetic modifications in aging, histone acetylation modifications are also dynamic: rapid alterations in the levels of histone acetylation modifications can be measured after cell activation by a variety of external stimuli. It can therefore be envisioned that similar alterations in histone acetylation modifications are induced in the response to cellular injury such as inflicted by e.g. flow shear stress or by balloon angioplasty. As detailed in the various preceding sections of this review, there is ample experimental evidence from different cellular models that indeed the activities of KATs and KDACs are key elements, either directly or indirectly,

in the transcriptional control of vascular disease-associated genes. Aberrant expression of these chromatin modifying activities therefore has a profound effect on the overall level of transcription of disease-associated genes and function of their products in the processes involved in extracellular matrix formation, inflammation and proliferation.

Clinical feasibility of applying drugs which inhibit the activity of KATs and KDACs is demonstrated in the application of these compounds in the treatment of cancer and neurological disorders.²⁷ However, it should be realized that application of these compounds can also result in non-specific activation of genes and other genomic elements not only in diseased cells but also in normal cells. Especially the use of non-specific inhibitors that target many different KATs or KDACs can be expected to have broad effects on gene expression and may inflict potential harmful side-effects. It is therefore important to identify those KATs and KDACs that play a role in the transcriptional regulation of cardiovascular disease-associated genes and to develop specific inhibitors that target these KATs and KDACs. As also a single KAT or KDAC family-member can be involved in the transcription of multiple genes, even specific inhibitors could have unwanted effects on gene expression. However, each of these family-members could have some degree of specificity to a set of genes acting together in a vascular disease-associated process. Although KATs and KDACs are known to be substrate-specific, it is thus far not known if they can also be specific to certain processes.

Nevertheless, many KDAC-inhibitors appear to be well tolerated in patients and local application of these epigenetic modifiers would overcome the side-effects which are inheritably linked with systemic application.

Several recent investigations indeed indicate that KDAC-inhibition may be beneficial in vascular disease-associated processes. Transcription of the *CYP7A1* gene encoding cholesterol 7 α -hydroxylase, an enzyme involved in the intra-hepatic conversion of cholesterol to bile acids, is known to be repressed by bile acids as part of a feedback regulatory mechanism. Recent findings demonstrate that the bile acid-mediated repression of this gene was caused by an increase in the nuclear concentration of KDAC7, which promoted the assembly of a repressive complex at the *CYP7A1* promoter.¹²⁶ Notably, treatment of LDL-receptor knockout mice with the KDAC-inhibitors TSA and valproic acid elevated Cyp7a1 mRNA levels and consequently reduced total plasma and LDL-cholesterol dramatically. In addition to these findings, *in vitro* experiments showed that TSA strongly inhibited SMC proliferation and TNF- α production by activated macrophages, processes with an important role in atherogenesis.¹²⁷ Interesting in this context is the recent finding that also statins inhibit KDAC-activity. Lovastatin was found to induce p21 expression through dissociation of KDAC1/2 and association of CBP, leading to histone-H3 acetylation at the site of the p21 promoter.¹²⁸ Therefore, part of the beneficial effect of statins in vascular disease might be due to its role in these mechanisms.

Recent findings from Granger *et al.* also identify KDACs as possible targets for therapy in cardiovascular disease. They showed, utilizing a standard murine model of ischemia-reperfusion, that ischemia induces KDAC activity in the heart resulting in deacetylation of histones H3 and H4.¹²⁹ Within a treatment window from 1 hour before ischemia until 45 minutes after reperfusion, the KDAC inhibitors TSA and Scriptaid were able to reverse the induction of ischemia-induced KDAC activity *in vivo* and to reduce myocardial infarct size by approximately 50%. Furthermore, a 5 hour period of hypoxia induced a strong decrease in acetylated histones H3 and H4 in mouse cardiomyocytes *in vitro*, which could be completely blocked by TSA. The finding that KDAC-inhibition can reduce the size of myocardial infarction in mice by half, even when treatment is applied after reperfusion, has important implications for the potential application of these compounds in the context of acute coronary syndromes.

In conclusion, over the past years it has become increasingly clear that besides genetic also epigenetic components contribute to the etiology of cardiovascular disease. Dynamic interactions of the environment and the epigenome determine accessibility of genes which have profound effects on their transcription and function. A further understanding on the role of epigenetic processes in cardiovascular disease processes such as atherosclerosis and restenosis might provide novel opportunities to understand disease pathology. This would provide the necessary knowledge platform for design of alternative treatment strategies, which are aimed at interfering in these epigenetic processes for the management of atherosclerosis and restenosis.

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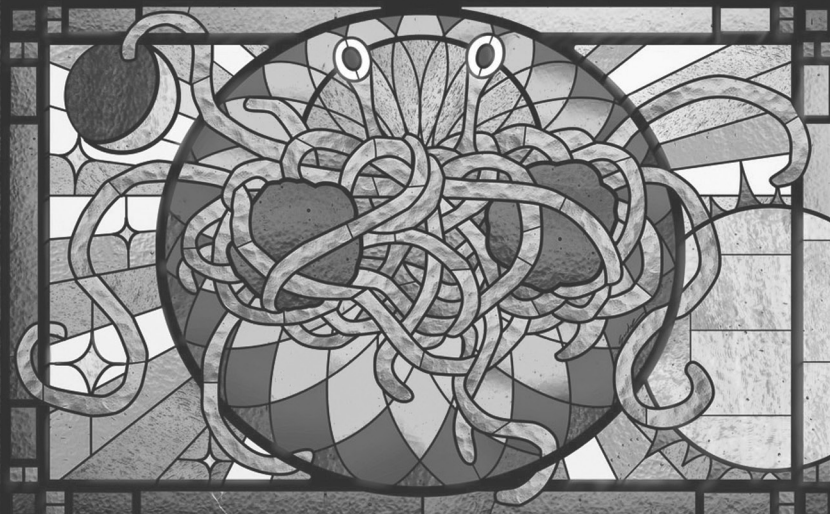
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Chapter 10

Genetic variation in PCAF, a key mediator
in epigenetics, is associated with reduced
vascular morbidity and mortality.

Evidence for a new concept from three
independent prospective studies

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ABSTRACT

Aims: A major influence on gene expression is attributed to the counterbalancing action of lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). This study was designed to investigate the influence of genetic variation in the promoter of the gene encoding P300/CBP associated factor (PCAF), a lysine acetyltransferase (KAT) on coronary heart disease (CHD) and mortality.

Methods and results: We investigated the association of genetic variation in the promoter region of the PCAF-gene on CHD and mortality in two statin trials (PROSPER and WOSCOPS) and on restenosis risk in a third study of percutaneous coronary intervention (GENDER). We combined the results from these cohorts to examine 1) overall effects on CHD mortality and on 2) restenosis risk and determined the contribution of PCAF in an animal model of reactive stenosis. Compared with the homozygous -2481G allele in the PCAF promoter, we observed a significant reduction in CHD mortality risk with the homozygous -2481C PCAF promoter allele in PROSPER (risk reduction 22%; 2% to 37%), a non-significant trend towards reduction in WOSCOPS (risk reduction 17%; -14% to 39%), and a significant reduction of restenosis in GENDER (20%; 3% to 33%). A combined risk reduction for CHD death/ restenosis for the three studies was 21% (15%-26%; $p = 8.1 \times 10^{-4}$). In elderly patients (>58 years) the effects were stronger and significant in all three studies. Furthermore, this PCAF allele was significantly associated with all cause mortality in PROSPER ($p=0.001$). Functional analysis showed that nuclear factors interact in vitro with the oligonucleotides encompassing this -2481 G/C polymorphism and that this interaction might be influenced in some cell types by this polymorphism in the PCAF promoter, and modulation of PCAF gene expression was detectable upon cuff-placement in an animal model of reactive stenosis.

Conclusion: We showed in three large prospective studies that the -2481C allele in the PCAF promoter is associated with a significant survival advantage in elderly patients while also protecting against clinical and angiographic restenosis after PCI. Our observations promote the concept that epigenetic processes are under genetic control and that, other than environment, genetic variation in genes encoding KATs may also determine susceptibility to CHD outcomes and mortality.

INTRODUCTION

Investigations into the pathogenetic mechanisms of human complex disease, such as cardiovascular disease and cancer, may lead to better risk prediction, treatment and new targets for future therapy. Cell proliferation regulatory pathways and pro-inflammatory transcription factors, such as NF κ B, have been associated with progression of these diseases.¹ In the past decade, research into cardiovascular diseases such as atherosclerosis and restenosis, has been focused on the identification of genetic factors that determine disease risk. Several genes involved in inflammation and cell proliferation appeared to be common denominators of these diseases.²⁻⁴ It has become clear, however, that part of the gene-environmental interactions relevant for complex diseases is regulated by epigenetic mechanisms such as histone acetylation and DNA methylation.⁵ Epigenetic processes modulate gene expression patterns without modifying the actual DNA sequence and have profound effects on the cellular repertoire of expressed genes.⁶ Evidence is growing that epigenetic mechanisms also regulate the expression of genes in the inflammatory and cell proliferation pathways^{7,8} and may therefore also play a role in cardiovascular disease.⁹⁻¹¹

A major influence on gene expression is attributed to the counterbalancing action of lysine acetyltransferases (KATs) and lysine deacetylases (KDACs).¹² KATs acetylate histones by transfer of an acetyl-group to the ϵ -portion of lysine residues, which results in an open modification of chromatin structure and in accessibility of DNA to transcription factors and recruitment of the basal transcription initiation machinery. Conversely, gene repression is mediated via KDACs, which remove acetyl groups and counteract the activity of KATs resulting in a closed chromatin structure. Thus far, the main focus has been to investigate the environmental influence on epigenetic processes. Research in this field has shown that epigenetic differences arise during the lifetime of monozygotic twins.⁶ Furthermore, oxidative stress has been shown to influence the balance between KATs and KDACs in favour of KATs, leading to an increase in inflammation.¹³ Notably, genetic variations in the genes encoding KATs and KDACs, which affect the activities of the enzymes they encode, have a bearing on the global and gene-specific levels of histone acetylation. As such, these genetic variations in the genes encoding KATs and KDACs could also be important determinants contributing to susceptibility to major human diseases.

P300/CBP associated factor (PCAF) is a transcriptional co-activator with intrinsic KAT-activity. Besides its role in lysine acetylation of histones at the site of NF κ B-regulated genes and the resultant inflammatory gene activation,^{14,15} PCAF is also found to act as a factor acetyltransferase (FAT) that acetylates non-histone proteins, including several tumor-suppressor proteins, such as p53^{16,17} and the phosphatase and tensin homolog (PTEN).¹⁸ Because PCAF is involved in proliferation and inflammation, common

denominators of the major diseases determining human mortality, with clear evidence for inflammatory factors predicting incident CVD events,^{19,20} incident cancers²¹ and mortality, we hypothesized that the PCAF-gene could be of major importance in the development of cardiovascular disease and cancer, and death from such disease.

We investigated the impact of genetic variation in the promoter region of the PCAF-gene on all-cause mortality and mortality due to coronary heart disease and cancer in the PROSPER-study, a randomized controlled trial in which 5804 elderly patients (age 70-82) at risk for vascular disease were randomized to pravastatin or placebo.²² In order to validate the observed effects and to be able to extrapolate our findings to a younger population, we investigated the PCAF gene in the WOSCOPS study, a randomized controlled trial similar to the PROSPER-study, designed to determine the effect of pravastatin in middle-aged men with hypercholesterolemia without a history of cardiovascular disease. Finally, in order to further test the validity of results in the two statin trials, and to get insights in the mode of action, we investigated these variants in another large prospective study, the GENDER-study, a prospective follow-up study that included 3104 patients undergoing percutaneous coronary intervention (PCI).²³ The primary endpoint in this study was clinical restenosis, a process that is known to be mainly determined by inflammation and proliferation.²

All participants in these three study groups were analyzed for 2 polymorphisms (SNPs) in the promoter region of the PCAF-gene. Of these polymorphisms, the -2481G/C SNP was found significantly associated with CHD mortality in elderly patients in PROSPER and WOSCOPS, and, in addition, with differential risk for restenosis in the GENDER study.

METHODS

Study design and follow-up of the PROSPER study

The protocol of PROSPER has been described elsewhere.²⁴ 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, we screened and enrolled subjects 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 genetic sub-study, we evaluated the predefined endpoints all-cause mortality and mortality due to vascular events and cancer. Mean follow-up was 3.2 years (range 2.8-4.0) and 604 (10.4%) patients died

during the study.²² Of these patients, 292 (48%) died from vascular disease and 206 (31%) from cancer.

Study design and follow-up of the WOSCOPS study

The WOSCOPS study (the West of Scotland Coronary Prevention Study), a primary prevention trial, included 6595 men, aged between 45 and 64 years old, who had LDL cholesterol levels between 174 and 232 mg/dL (4.5 and 6.0 mmol/L), who had no history of myocardial infarction, but were considered to be at enhanced risk for developing CHD.²⁵ The first patient was enrolled on February 1, 1989, and the study ended on May 15, 1995. Mean follow up duration was 4.9 years. All participants were randomly assigned to receive 40 mg of pravastatin or placebo daily.

The present genetic study was performed in a previously described nested case-control cohort.²⁶ In brief, the prospective nested case-control study included all of the 580 on-trial CHD events (death from CHD, nonfatal MI, or revascularization procedures) from the WOSCOPS cohort as case subjects and 1,160 control subjects matched to case subjects by age and smoking. In the present genetic study we used death from coronary heart disease as our primary endpoint.

Study design and follow-up of the GENDER study

The present study sample has been described previously.²³ In brief, the GENetic DEterminants of Restenosis project (GENDER) was a multicenter follow-up study designed to study the association between various gene polymorphisms and clinical restenosis. Patients eligible for inclusion in the GENDER-study were treated successfully for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI in four out of 13-referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST-elevation myocardial infarction were excluded. Experienced operators, using a radial or femoral approach, performed standard angioplasty and stent placement. 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 TVR, either by PCI or coronary artery bypass grafting (CABG), was the primary endpoint. Median follow-up duration was 9.6 months (interquartile range 3.9) and 304 (9.8%) patients underwent TVR during follow-up. A prespecified subpopulation of 478 patients was scheduled for re-angiography at 6 months, according to standard procedures as described previously.²⁷ Identical projections were used before, during and 6 months after the PCI for all assessed angiograms. Quantitative Computer Analyses (QCA) were independently performed by Heartcore (Leiden, the Netherlands).

For all three studies, all endpoints were adjudicated by independent clinical events committees. The protocols meet the criteria of the Declaration of Helsinki and were

approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating patients.

Genotyping

Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. As a first step to investigate this gene, we selected 2 validated polymorphisms in the PCAF-promoter. The -4556 C/T (rs2623074) and the -2481 G/C (rs2948080) polymorphisms were selected on the basis of their high minor allele frequency (>5%) and measured using the Sequenom Massarray genotyping platform. A multiplex assay was designed using Assay designer software (Sequenom). As quality controls, 5-10% of the samples were genotyped in duplo. No inconsistencies were observed. Cluster plots of the signals from the low and the high mass allele were drawn. Two independent researchers carried out scoring. Disagreements or vaguely positioned dots produced by Genotyper 4.0 (Sequenom Inc.) were left out of the results.

Cells and cell culture

The cell lines (HeLa, U251, Raji) used in this study were obtained through the ATCC (Rockville, MD, USA) and were cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker Europe, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Greiner, Alphen a/d Rijn, The Netherlands), 100 IU/mL streptomycin and 100 IU/mL penicillin. For interferon- γ (IFN- γ) induction, cells were treated with 500U/mL of IFN- γ (Boehringer-Ingelheim, Alkmaar, The Netherlands) for 4 hours, hereafter nuclear extracts were prepared (see below). HUVECs were cultured in Medium 199 with Earl's salt and L-glutamine (Life Technologies, Breda, The Netherlands), supplemented with 20% (v/v) FCS (PAA, Pasching, Austria), 100 IU/mL streptomycin and 100 IU/mL penicillin, 10 IU/mL heparine (Leo Pharma, Breda, The Netherlands) and 25 mg bovine pituitary extract (BPE; Life Technologies).

Transcription factor binding site search

Potential transcription factor binding sites were identified using the TFSEARCH program (<http://www.cbrc.jp/research/db/TFSEARCH.html>), which searches the TRANSFAC database²⁸. Cutoff was set at 75% of the consensus TF binding site.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts and EMSAs were performed as described previously.²⁹ In brief, 2 μ L of nuclear extracts (HeLa, HUVEC, U251, Raji) in binding buffer were incubated for 30 min on ice, with 2 ng of a [³²P]-labeled dsDNA probe. The probe sequences were similar to either the C or G promoter variants of the PCAF-gene (PCAF-C:

5'-GCAATAAGCCTCCTCAATCCTTTGCCCTTG-3'; PCAF-G:

5'-GCAATAAGCCTCCTGAATCCTTTGCCCTTG-3'). Probe sequences for transcription factors MZF1 and GATA1-3 were similar to their previously described consensus sequence (MZF1 (zinc fingers 1-4): 5'- GATCTAAAAGTGGGGAGAAAA-3'; MZF1 (zinc fingers 5-13): 5'- GATCCGGCTGGTGAGGGGGAATCG-3'; GATA:

5'- GGACCTTGATCTTATCTT-3').^{30,31}

For competition assays, nuclear extracts from IFN- γ stimulated HeLa cells were incubated with unlabelled oligonucleotides in 100- and 200-fold excess for 30 min on ice, prior to incubation with the labeled probe. In case of IFN- γ treated samples, cells from five different cell types were stimulated with IFN- γ (500U/mL; Boehringer-Ingelheim) for 4-hours prior to preparing nuclear extracts. Samples were run on a 6% polyacrylamide gel in 0.25x TBE-buffer. Gels were densitometrically analyzed using the ImageJ software.³²

Mouse model for reactive stenosis

The institutional committee on animal welfare approved all animal experiments. For all experiments hyperlipidemic male ApoE*3-Leiden mice³³ were fed a high-cholesterol diet (ArieBlok, Woerden, The Netherlands). Blood samples to determine plasma cholesterol were collected at time of surgery. After 3 weeks on diet, a non-constrictive polyethylene cuff was placed loosely around one femoral artery and mice were sacrificed at several time points after surgery. After sacrifice, at t=0 hours (control, no cuff placement), 6 hours, 24 hours, 2 days, 3 days, 7 days and 14 days, both femoral arteries were isolated and snap-frozen in liquid nitrogen (n=6 mice for each timepoint).

RNA isolation and cDNA synthesis from femoral artery tissue

Per time point, cuffed segments of three femoral arteries were pooled to enable isolation of suitable amount of RNA, resulting in 4 pooled RNA samples obtained from n=6 mice, cuffed at two limbs. After RNA isolation, cDNA was synthesized and RT-PCR analysis was performed as previously described.³⁴

PCAF mRNA quantification

Expression levels of PCAF were measured by virtue of quantitative RT-PCR using TaqMan® gene expression assay (Mm00451387_m1). PCR runs were carried out in the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). HPRT was assayed as control gene and its cycle threshold (Ct) was subtracted from the Ct of the gene of interest, yielding Δ Ct. For each timepoint, $\Delta\Delta$ Ct was determined by subtracting the average Δ Ct at timepoint 0 hours from the Δ Ct at each other timepoint. This $\Delta\Delta$ Ct was used to calculate the displayed fold increase for each gene.³⁵

Statistical analysis

Allele frequencies were determined by gene counting. The Chi-squared test was used to test the consistency of the genotype frequencies at the SNP locus with Hardy-Weinberg equilibrium. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated using a Cox-proportional hazards model. All analyses with PROSPER and WOSCOPS data were adjusted for sex, age and pravastatin use. The analyses with PROSPER data were additionally adjusted for country. In the GENDER-study, polymorphisms were included in a multivariable model containing clinical and procedural risk factors for restenosis, such as diabetes, smoking, hypertension, stenting, total occlusion and residual stenosis >20%.

To reach statistical significance with an alpha of 0.05 and a beta of 0.8 in the association between the -2481 polymorphism and coronary heart disease death in this population, we need 168 cases of coronary heart disease death, for the -4556 we need 379 cases of coronary heart disease death. A combined-effect analysis was performed to pool the results of the effect of the -2481G/C polymorphism on study endpoints (all-cause mortality, CHD death and clinical restenosis) and coronary endpoints (CHD death in two studies and clinical restenosis in the third study) in the three separate studies at old age. The random-effects model was used to consider both the between-study and within-study variability. The pooled hazard ratio over the genotypes was assessed with ordinary logistic regression. The SPSS software (version 12.0.1, SPSS Inc, Chicago, IL) was used for all statistical analyses.

RESULTS

PROSPER study

Participant characteristics are presented in table 1. Genotyping success rates were higher than 96% for all polymorphisms and there were no significant deviations from Hardy-Weinberg equilibrium.

Using a Cox proportional hazards model, which included several clinical variables such as sex, age, pravastatin use, and country, we found a significant association of the -2481 G/C promoter polymorphism with all-cause mortality in PROSPER. As presented in table 2 and figure 1, heterozygotes had a reduced mortality risk by 17% (2% to 30%, $p=0.03$), whereas individuals homozygous for the -2481C allele had a 39% lower mortality (16% to 56%, $p=0.003$). The effect of PCAF is more profound in the association with coronary heart disease (CHD) death (risk reduction 22% (2% to 37%), $p=0.03$) compared to the association with cancer mortality (risk reduction 13% (9% to 30%), $p=0.22$). Due to its proximity to the -2481 G/C polymorphism, we also present the data of the -4556 C/T polymorphism (table 2). It shows a small and non-significant trend

Table 1. Baseline characteristics of the PROSPER, the GENDER and the WOSCOPS study

	PROSPER N=5595	WOSCOPS N=1092	GENDER N=2852
Continuous variates (mean, SD)			
Age (years)	75.3 (3.4)	56.8 (5.2)	62.1 (10.7)
Body mass index, (kg/m ²)	26.8 (4.2)	26.0 (3.2)	27.0 (3.9)
Categorical variates (%)			
Male sex	48	100	71
Current smoker	27	54	25
History of diabetes	11	2	15
History of hypertension	62	18	40
History of myocardial infarction	13	0	40
History of stable angina	27	8	67
Statins	50	48	55
Genotype, minor allele frequency (%)			
PCAF -4556 C/T	9	9	11
PCAF -2481 G/C	33	33	33

All data are presented in % unless otherwise stated.

Table 2. Results of the association between two promoter polymorphisms in the PCAF gene and mortality endpoints within the PROSPER and WOSCOPS studies.

	All cause mortality in PROSPER			CHD death in PROSPER			CHD death in WOSCOPS		
	N (%)*	HR (95%CI)	p-value	N (%)*	HR (95%CI)	p-value	N (%)*	HR (95%CI)	p-value
PCAF -4556 C/T									
C/C	491 (11)	1.0 (ref)		168 (4)	1.0 (ref)		83 (9)	1.0 (ref)	
C/T	81 (9)	0.81 (0.64-1.02)	0.09	32 (3)	0.94 (0.64-1.37)	0.75	11 (6)	0.65 (0.34-1.25)	0.20
T/T	4 (7)	0.63 (0.24-1.69)	0.38	3 (5)	1.31 (0.42-4.10)	0.65	2 (13)	1.4 (0.31-6.28)	0.66
Trend	576 (10)	0.80 (0.65-0.99)	0.04	203 (4)	0.99 (0.71-1.38)	0.95	96 (9)	0.80 (0.47-1.36)	0.41
PCAF -2481 G/C									
G/G	290 (12)	1.0 (ref)		103 (4)	1.0 (ref)		49 (10)	1.0 (ref)	
G/C	246 (10)	0.83 (0.70-0.98)	0.03	86 (3)	0.81 (0.61-1.08)	0.16	40 (9)	0.89 (0.58-1.39)	0.62
C/C	41 (7)	0.61 (0.44-0.84)	0.003	14 (3)	0.58 (0.33-1.00)	0.05	8 (6)	0.63 (0.29-1.36)	0.24
Trend	577 (10)	0.80 (0.70-0.91)	0.001	203 (4)	0.78 (0.63-0.98)	0.03	97 (9)	0.83 (0.61-1.14)	0.26

HR=Hazard ratio. CI=Confidence interval.

All hazard ratios and p-values were assessed with a Cox-proportional hazards model and adjusted for sex, age, country, and use of pravastatin.

*Calculated for 5595 subjects in the PROSPER study and for 1092 in the WOSCOPS study.

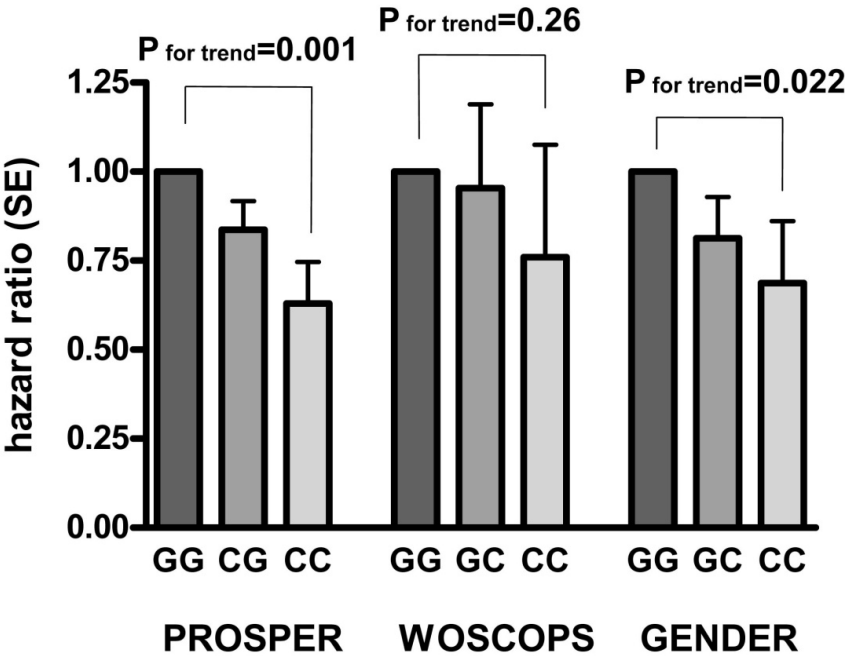


Figure 1. Hazard ratios for all-cause mortality in PROSPER, for coronary heart disease death in WOSCOPS, and for clinical restenosis in GENDER by PCAF -2481 G/C genotype at all ages.
The PCAF -2481 G/C polymorphism is associated with mortality in the PROSPER study, with coronary heart disease death in the WOSCOPS study, and with clinical restenosis in the GENDER study in all age groups

towards a decrease in all-cause mortality. The linkage disequilibrium (LD)-coefficient between these promoter polymorphisms was 0.79.

WOSCOPS study

To validate the association of the -2481 G/C polymorphism with CHD mortality, and to investigate whether its effect is also present in a population largely without established vascular disease, we aimed to replicate our findings in a previously described²⁶ nested case-control cohort of the WOSCOPS study. Baseline characteristics of the WOSCOPS study are presented in table 1.

In this case, the -2481 G/C polymorphism was associated with a non-significant trend towards protection against death from coronary heart disease (CHD) (risk reduction 17% (-14% to 39%), $p=0.26$) at all ages. Heterozygotes (risk reduction 11% (-39% to 42%), $p=0.62$) and homozygotes (risk reduction 37% (-36% to 71%), $p=0.24$) had a non-significantly lower mortality risk. Although these data did not reach statistical significance, the point estimate for CHD mortality in WOSCOPS was similar to that observed

in the PROSPER study (figure 1). The -4556 C/T polymorphism was not significantly associated with the risk of CHD death (risk reduction 20% (-36% to 53%), $p=0.40$).

GENDER study

To extent the observed results in the PROSPER study, we tested the relevance of the gene to clinical conditions in which cell proliferation and inflammation play a role in a large patient population included in the GENDER-study. In this study patients were followed for at least 9 months after a PCI to determine absence or presence of clinical restenosis, as defined by the need for target vessel revascularization (TVR). We considered TVR a suitable intermediate endpoint as it is a direct consequence of mainly proliferative and inflammatory processes.²

In agreement with the results from the PROSPER and WOSCOPS-study, the -4556 C/T polymorphism was not associated with the risk for TVR in the GENDER-study, whereas the

-2481 G/C polymorphism showed a significant association with TVR (p -trend = 0.02) (table 3 and figure 1). Heterozygous patients were at lower risk for TVR (risk reduction 20% (-2% to 38%), $p=0.07$), and patients carrying two -2481C alleles had a greater protection against the development of restenosis (risk reduction 36% (0% to 59%), $p=0.05$).

Table 3. Results of the association between two promoter polymorphisms in the PCAF gene and clinical and angiographic restenosis within the GENDER study.

	Clinical restenosis			Angiographic restenosis ^a			Clinical restenosis in the stented subpopulation ^b		
	N (%)	HR (95%CI)	p-value	N (%)	HR (95%CI)	p-value	N (%)	HR (95%CI)	p-value
PCAF -4556 C/T									
C/C	218 (10)	1.0 (ref)		68 (21)	1.0 (ref)		148 (9)	1.0 (ref)	
C/T	54 (10)	0.98 (0.73-1.32)	0.90	16 (19)	0.80 (0.41-1.53)	0.50	31 (8)	0.86 (0.58-1.26)	0.43
T/T	2 (5)	0.46 (0.11-1.86)	0.28	0 (0)			1 (3)	0.34 (0.05-2.45)	0.34
Trend	274 (10)	0.92 (0.70-1.20)	0.54	84 (21)	0.77 (0.41-1.45)	0.42	180 (9)	0.80 (0.561-1.15)	0.23
PCAF -2481 G/C									
G/G	140 (11)	1.0 (ref)		47 (25)	1.0 (ref)		98 (10)	1.0 (ref)	
G/C	114 (9)	0.80 (0.62-1.02)	0.07	29 (16)	0.56 (0.33-0.96)	0.03	68 (7)	0.70 (0.51-0.95)	0.02
C/C	22 (7)	0.64 (0.41-1.00)	0.05	8 (18)	0.64 (0.27-1.52)	0.31	16 (7)	0.65 (0.38-1.10)	0.11
Trend	276 (10)	0.80 (0.67-0.97)	0.02	84 (21)	0.69 (0.47-1.02)	0.07	182 (9)	0.76 (0.60-0.95)	0.02

^a measured in a subgroup of 478 subjects. ^b measured in a subgroup of 2309 subjects.

HR=Hazard ratio. CI=Confidence interval.

All hazard ratios and p-values were assessed with a Cox-proportional hazards model and adjusted for sex, age, and clinical and procedural risk factors for restenosis.

In analogy of the clinical restenosis (TVR) results, carriers of the -2481C allele also had less angiographic restenosis in a subpopulation of 478 patients undergoing angiography six months after the PCI (risk reduction 44% (4% to 67%), $p=0.03$) (table 3).

Combined effect analysis

Hazard ratios were almost remarkably equal in all three studies (figure 1). We formally test for homogeneity using a standard Olkin-type Q-test and found p -values of 0.91 and 0.77 for heterozygotic and homozygotic carriers of the minor allele of -2481G/C SNP between the three studies. Therefore and since all three studies have comparable endpoints, we conducted a combined effect analysis to show the effect of the -2481C on the study endpoints (CHD death in PROSPER and WOSCOPS, and clinical restenosis in GENDER) at all ages (figure 2, top panel). The pooled hazard ratio for the -2481C allele was 0.79 (95%CI: 0.74-0.85), $p=8.1 \times 10^{-4}$. Heterozygotes had a reduced risk (HR: 0.82, 95%CI: 0.68-0.98, $p=0.03$), and this risk was lower in subjects homozygous for the -2481C allele (HR: 0.61 95%CI: 0.44-0.84, $p=0.002$).

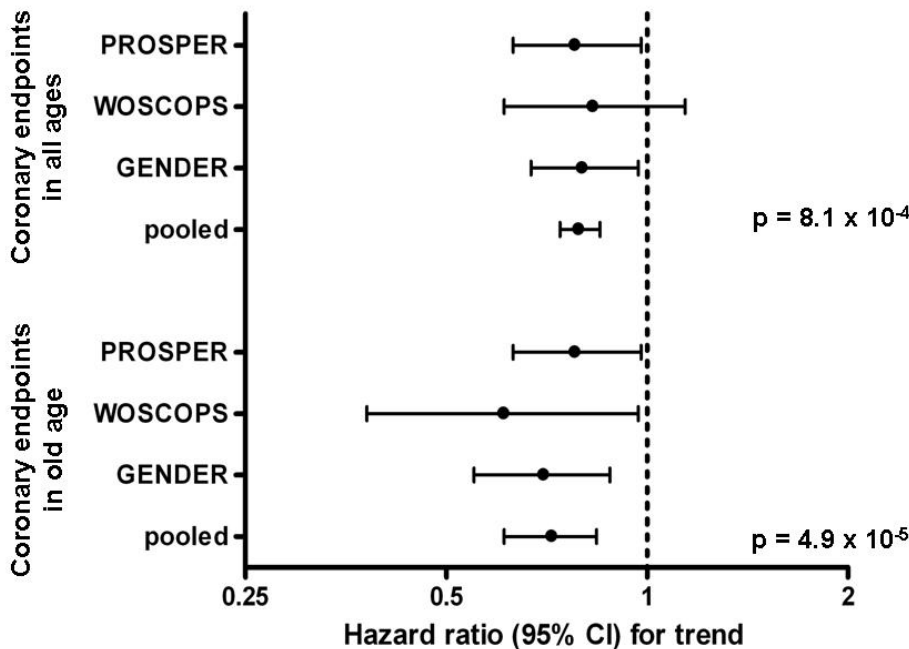


Figure 2. Combined effect estimate of the hazard ratios of the PROSPER, WOSCOPS, and GENDER studies for the PCAF -2481 G/C polymorphism. This figure represents the hazard ratios for the additive model of the PCAF -2481 G/C polymorphism. Coronary endpoints consist of CHD death for the PROSPER and WOSCOPS study, and clinical restenosis for the GENDER study. The top panel is the combined effect analysis for coronary endpoints at all ages, the bottom panel in subgroups with age > 58 years.

Examining for an age effect

We investigated an effect of the -2481C variant allele on vascular mortality in two age strata, older and younger than 58 years old. We could however not perform this analysis in the PROSPER cohort since all subjects were older than 70 years. In the WOSCOPS study, among participants ≥ 58 years old (median age), we found that the -2481C allele significantly reduced the risk of CHD death (risk reduction 39% (3% to 62%), $p = 0.035$), whereas there was no significant effect in the lower age group. We found similar results in the GENDER study. We again observed a strong protective effect of the C-allele in old patients (≥ 58 years, $n=1800$) (risk reduction 30% (12% to 45%), $p=0.003$) whereas there was no significant effect present in young patients (<58 years, $n=1052$).

We also conducted a combined effect analysis to show the effect of the -2481C allele at a high age only (median age >58), shown in figure 2 bottom panel. The pooled hazard ratio for the

-2481C allele on coronary events (CHD death or TVR) in this high age group was 0.72 (95%CI: 0.61-0.84), $p=4.9 \times 10^{-5}$, which is somewhat stronger than the effect at all ages.

Functional involvement: the -2481 region encompassing the C/G PCAF variants

Using the EMSA technique we tested whether the observed polymorphism would lead to differential protein binding in vitro. Using nuclear extracts of different cell types, we studied complex formation at the -2481 region of PCAF. We could detect constitutive protein binding to both PCAF-C and G-variants (figure 3). In both human umbilical vein endothelial cells (HUVECs) and U251 cells nuclear factor binding is slightly enhanced

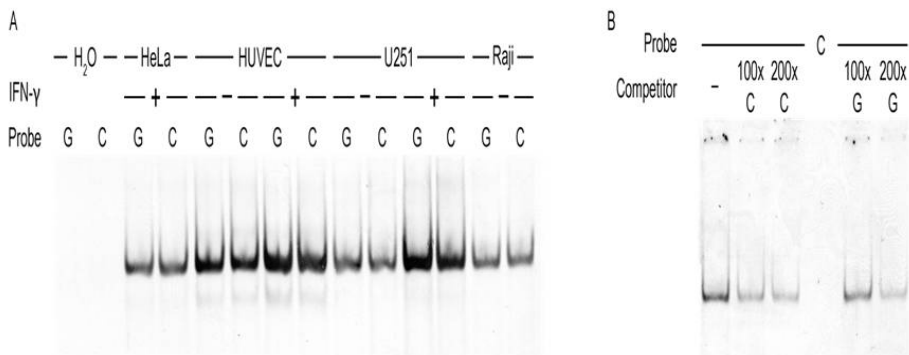


Figure 3. Protein binding to the C- and G-variant of the -2481 region.

(A) EMSA showing binding of protein to the C- and G-variant of the -2481 region using nuclear extracts of various cell-lines. EMSA suggests slightly stronger binding of protein to the G-variant of the -2481 region, most pronounced in HUVECs and U251 cells. (B) Competition assay with nuclear extracts from HeLa cells stimulated with IFN- γ also suggests slightly increased binding affinity for the -2481 G-variant at high concentrations. The difference is best observed when unlabelled probe is added in 200-fold excess, indicating that the observed difference in affinity is weak. Shown are representatives of multiple independent experiments

by IFN- γ stimulation. Densitometric analysis revealed that the PCAF G-variant exhibits slightly stronger protein binding in some cell types than the C-variant, although these differences are not statistically significant. Competition assays also suggest a modest difference in binding affinity (figure 3B).

Using the TFSearch program, we identified possible binding sites for MZF1 and GATA1-3 in the PCAF C-variant promoter. However, ds-oligonucleotides representing binding sites for MZF1 and GATA1-3 did not compete with factor binding to the PCAF C-promoter variant (data not shown).

PCAF mRNA expression in a mouse model of reactive stenosis

Placement of a small non-constrictive cuff around the femoral artery in hyper-cholesterolemic (13.9 ± 3.6 mmol/L) ApoE3Leiden mice results in induction of neointima formation. In the developing neointima a rapid upregulation of the PCAF expression was observed after cuff placement in a time dependent manner (figure 4). PCAF expression showed a peak two days after vascular injury (~1.5 fold increase) and reduces to baseline levels of expression after 7 days. PCAF expression at day 3, 7 and 14 differed significantly from the 24h measurement ($p < 0.05$).

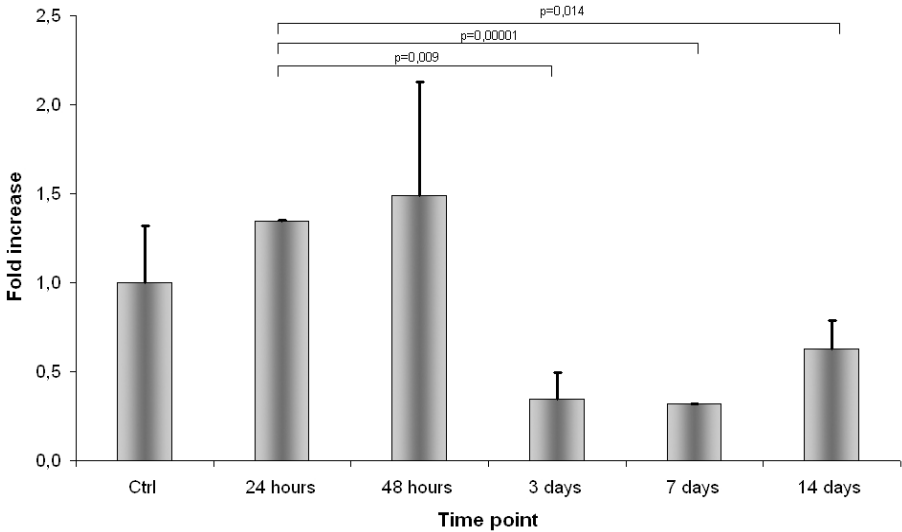


Figure 4. Expression of PCAF mRNA in a cuff-induced reactive stenosis mouse model.

The figure shows PCAF differentially expressed upon activation of the vessel wall in a time dependent manner, with an expression peak 48 hours after vascular injury. The data is presented as fold increase compared to the control arteries, using HPRT as an internal control for cDNA input.

DISCUSSION

Our data indicate that the -2481C allele in the gene encoding PCAF, a protein which has been shown to be a key mediator in epigenetics by acetylating histones and several non-histone proteins, such as the p53 tumor-suppressor proteins, is associated with a significant survival advantage in three independent studies. We found in the PROSPER-study an advantage in survival mainly due to a significant risk reduction in CHD death. In line with this observation we also observed that the -2481C allele was associated with a lower death by CHD in the WOSCOPS study in an age dependent manner. Furthermore, this allele also protects against clinical and angiographic restenosis in the GENDER-study. The effects of the -2481C allele on mortality, CHD death and clinical restenosis were more profound at older age (>58 years). The -2481G/C polymorphism in the PCAF promoter affects transcription factor binding, as was demonstrated by an EMSA band shift analysis. A role for PCAF in vascular disease was further confirmed in a mouse model for reactive stenosis, in which modulation of PCAF expression was detected during vascular remodeling.

Our observation in the PROSPER-study that this promoter variant associates to lower mortality from cardiovascular disease and cancer as well as clinical restenosis after PCI in GENDER, may indicate a role of PCAF in inhibiting cell proliferation also in more general terms. PCAF has been shown, for example, to activate p53-responsive enhancer elements within the p21waf1 promoter¹⁷ and activity of p21waf1 is known to induce cell-cycle arrest in vascular smooth muscle cells.³⁶⁻³⁹ Furthermore, A20, a NFκB-dependent gene that has been shown to inhibit proliferation of VSMCs via increased expression of p21waf1, was able to prevent neointima formation after balloon angioplasty in a rat model of carotid artery stenosis.⁴⁰

Apart from its well-described role in cell-cycle regulation, PCAF is also known to be required to co-activate p65-dependent transcription and has been shown to directly activate the transcription of several NFκB-regulated genes known to be involved in cardiovascular disease.⁴¹ Miao *et al.* has shown that PCAF could enhance the p65 mediated increase in TNF-α promoter activity and that high glucose increased the recruitment of PCAF to the TNF-α and COX-2 promoters.¹⁴ Furthermore, they demonstrated concomitant acetylation of specific lysine residues of histone H3 and H4 at these promoters. Since TNF-α and COX-2 have been implicated in the development of atherosclerosis,^{42;43} and restenosis,^{27;44} and also cancer,^{45;46} our data suggest that PCAF may also play a role in the development of these diseases.

Our finding in the WOSCOPS and GENDER-study that the strong protective effect of the

-2481C allele was more profound in patients older than 58 years old, whereas it seemed not present in young patients (<58 years old) is of particular interest.⁴⁷ It could reveal the combined effect of a life-time dysregulation of expression of the lysine acetyltransferase

-2481C PCAF variant, which affects global levels of histone acetylation, in addition to the accumulating effect of exposure to environmental factors which also affects histone acetylation profiles during life as observed by Fraga *et al.*⁶ In the PROSPER population such an age dependent effect was not observed as this trial included only patients >70 years old. As expected, here the effects on cardiovascular and cancer mortality associated with the PCAF

-2481 G/C polymorphism were evident for the entire population. After observing an age-dependent effect in the WOSCOPS and GENDER study, we suggest that this polymorphism in PCAF is associated with an altered tendency to acetylate histones and non-histone proteins (such as the tumor-suppressor p53 whose function relies on acetylation, reviewed in Spange *et al.*³⁶) and may therefore become important especially in elderly patients, who may have been under the influence of altered PCAF activity for many years. This hypothesis needs however further investigation.

Although our findings in the GENDER-study do not directly replicate the effect of the -2481 G/C polymorphism on CHD mortality in the elderly, they are of much value as this study has a mechanistically linked and better defined concise endpoint. Thus in this way patho-physiologic insights would be obtained and not just replication only. Restenosis after a PCI is very well investigated and is now known to be mainly the consequence of inflammatory and proliferative processes, which is underscored by the fact that stents eluting drugs that suppress these processes are highly efficacious in the prevention of restenosis. Therefore, we believe that our finding that the -2481C allele protects against restenosis in the GENDER-study could possibly confirm its functional significance, but could also provide mechanistic insights in its beneficial role in survival in the PROSPER and WOSCOPS studies. However this could only explain part of the causes by which CHD death risk is decreased in PROSPER and WOSCOPS studies, the exact mechanism is not known. We did not find any association between the strongly linked -4556C/T polymorphism and survival in any of the three studies. The estimated hazard ratios were quite similar as expected, however, due to small numbers since the minor allele frequency of -4556C/T is three to four times smaller than of -2481G/C, no significant results were yielded. This could simply be due to lack of power.

Here we show a strong association between the PCAF locus involved in epigenetic control and clinical conditions in three large follow-up studies with a mechanistically linked endpoint, however our studies warrant further investigation into the influence of the -2481 G/C polymorphism on the activity of the PCAF promoter or expression levels of NFκB-regulated genes. Here we hypothesize a new concept that differential transcription of the PCAF gene leads to differences in gene expression in various path-

ways mechanistically linked to CHD events, such as inflammatory regulatory pathways or pathways involved in proliferation. Hence, further research has to be performed to test this hypothesis.

In a first analysis we were able to demonstrate binding of nuclear factors to the specific region flanking the -2481 G/C polymorphism in the PCAF promoter. EMSA analysis showed that the G-variant possibly exhibits a slightly higher affinity for nuclear factor binding than the C-variant in some cell types tested (e.g. HUVECS and U251), albeit that this was not significant. It should also be noted that IFN- γ stimulation slightly increases nuclear factor binding in HUVECs and U251 cells, suggesting a role for IFN- γ induced nuclear factors. It remains to be established whether these interacting factors play a role in the transcriptional regulation of PCAF. However, provided that this SNP influences PCAF transcription and resulting protein levels, this could have a bearing on the cellular portrait of expressed genes and might lead to a dramatic different outcome if the effects accumulate over years. The fact that nuclear extracts do bind to the same region of the promoter in which the polymorphism is situated does strongly suggest that the SNP might affect the binding of transcription complexes and thus influences gene transcription, however this needs to be confirmed in future studies.

To further illustrate a possible role of PCAF in vascular disease, we quantified PCAF-transcripts in the stenotic vessel wall in a mouse model of cuff induced reactive stenosis in the femoral artery. During the stenotic process, the PCAF gene expression was rapidly upregulated, indicating that PCAF gene expression is activated upon vascular injury and suggesting that this transcriptional coactivator is involved in the development of reactive stenosis, at least in the early stages. Unfortunately it is not possible to measure the PCAF protein levels directly, however the rapid up and down regulation of the mRNA levels suggests that PCAF is not stable. The changes in mRNA levels are likely to reflect the changes in protein levels.

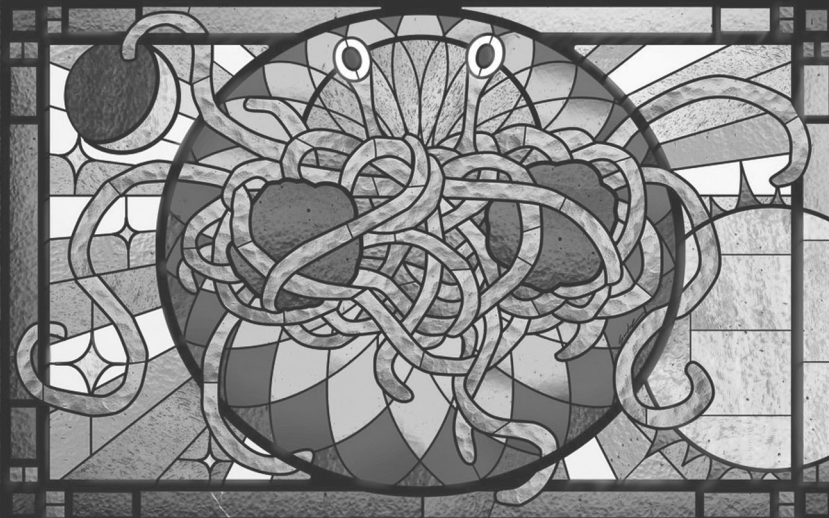
In conclusion, we showed in three large prospective studies that the -2481C allele in the PCAF promoter is associated with a significant survival advantage in elderly patients while also protecting against clinical and angiographic restenosis after PCI. Although the exact mechanisms of these actions are thus far unknown, we suggest that the effect of this allele on these endpoints may be due to the well-known involvement of PCAF in inflammatory and proliferative processes. Our observations promote the concept that epigenetic processes are under genetic control and that, other than environment, genetic variation in genes encoding KATs may also determine susceptibility to CHD outcomes and mortality. Therefore epigenetic histone modification, when our results are confirmed in our studies, might become a target for future therapy.

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Chapter 10

appendix

EDITORIAL: THE GENETICS OF EPIGENETICS: IS THERE A LINK WITH CARDIOVASCULAR DISEASE?

Qingzhong Xiao, Shu Ye

Heart 2011;97: 96-97

There is evidence indicating that altered epigenetic regulation of gene expression in inflammatory and vascular cells plays a role in the development of atherosclerosis and cardiovascular diseases.¹ DNA in the nuclei of eukaryotic cells wraps around histone proteins, forming nucleosomes which are further packaged to form chromosomes, and the combination of DNA, histone and other proteins in chromosomes is referred to as the chromatin. Histones are susceptible to chemical modifications which can alter the chromatin structure, consequently increasing or decreasing the accessibility of DNA elements to transcription factors that regulate gene transcription.

The most extensively studied histone modification is acetylation by the action of histone acetyltransferases.² P300/CBP associated factor (PCAF) is one of a number of proteins with acetyltransferase activity and can acetylate histones as well as many non-histone proteins that regulate the expression of genes involved in inflammation and cell proliferation.³⁻⁶ In this issue of *Heart*, Pons *et al.* provide evidence of an association between a single nucleotide polymorphism (SNP) in the PCAF gene promoter and reduced risk of coronary heart disease (CHD) mortality and restenosis.⁷ The authors studied three population cohorts previously recruited in the PROSPER, WOSCOPS and GENDER studies, the former two being prospective, randomised placebo-controlled pravastatin trials for prevention of cardiovascular events and the latter being a follow-up study of clinical restenosis after percutaneous coronary intervention. An association between the SNP and reduced CHD mortality was detected in PROSPER and a non-significant trend in WOSCOPS, while a relationship between the SNP and reduced risk of clinical restenosis was observed in GENDER. A meta-analysis of the three data sets estimated that individuals who were heterozygous for the low-risk allele had ~20% lower risk of the cardiovascular events (pooled HR of 0.82 (95% CI 0.68 to 0.98)) and individuals who were homozygous for this allele had ~40% lower risk (HR 0.61 (95% CI 0.44 to 0.84)). To provide a possible explanation for the association, the authors show that PCAF expression is increased in a mouse model of mechanical injury-induced neointima formation and provide some evidence suggesting that the SNP associated with reduced risk of cardiovascular events in humans might have an effect on PCAF expression.

A strength of the study of Pons *et al.* is the replication of the relationship between the genetic variation and cardiovascular traits in three independent cohorts. However, before the PCAF gene variant can be reliably regarded as a genetic factor for CHD,

further replications of the association will still be required. A handful of accepted CHD-associated genomic loci have recently been identified via genomewide association studies with subsequent confirmation in replication studies.⁸⁻¹¹ The PCAF gene does not reside at any of these loci. Nevertheless, since the handful of accepted loci identified so far only account for a small proportion of the heritability of the disease, it is likely that many more CHD-associated loci are still yet to be uncovered, and it is possible that the PCAF locus could be one of them.

The SNP (-2481G/C) found to be associated with the cardiovascular traits in the study of Pons *et al.* is located in the promoter region of the PCAF gene. The authors provide results from electrophoretic mobility shift assays which suggest that the SNP might affect binding of a nuclear protein (whose identity is unknown) to the PCAF gene promoter, which could potentially have an effect on PCAF transcription. If this is indeed the case, the association between the SNP and cardiovascular events could be due to altered expression of PCAF and consequently changes in the expression of genes regulated by PCAF. Alternatively, the SNP (-2481G/C) may be just a proxy marker for a functional SNP located elsewhere at the PCAF locus due to linkage disequilibrium. If this is the case, because the 'functional SNP' has not been identified, it is unknown what functional effect it exerts -i.e., it is unknown whether it affects the level of PCAF expression or its acetyltransferase activity, or something else. Despite a number of unanswered questions, the study of Pons *et al.* suggests a potential link between cardiovascular events and genetic variation of PCAF whose acetyltransferase activity renders it as having a role in epigenetic regulation. If confirmed, the finding will add to the growing evidence that altered epigenetic regulation of gene expression plays a part in the development of atherosclerosis and cardiovascular diseases.

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SUMMARY AND CONCLUDING REMARKS

This thesis examines the impact of genetic and epigenetic factors on several aspects of vascular disease. Part 1 has addressed the influence of genetic variation in genes involved in the different processes that lead to the occurrence of adverse events after percutaneous coronary intervention (PCI), mainly restenosis after bare metal stent (BMS) placement, but also late acquired stent malapposition (LASM) after implantation of a drug-eluting stent (DES). The role of a relatively new area of research, which we refer to as ‘epigenetic epidemiology’, in restenosis and other aspects of coronary heart disease has been discussed in part 2.

Traditionally, angiographic restenosis is defined by 50% occlusion of the lumen diameter, which gradually develops in the first year after a PCI. As not all of these angiographic re-occlusions lead to complaints in patients, the clinical definition of restenosis; target vessel revascularization (TVR), is currently regarded the most valuable endpoint. Since long we are aware of the multifactorial nature of the restenotic process. Initially, it develops as a normal wound-healing response after intervention-induced damage to the vessel wall. Thrombus formation is followed by an immune response and migration/proliferation of vascular smooth muscle cells (VSMC's). Previous research by Monraats *et al.* has demonstrated the importance of the relative overactivity of the immune system,¹ which we now consider as one of the driving forces in restenosis development. Restenosis is also known to be caused by VSMC proliferation and matrix formation, although evidence for the influence of genetic polymorphisms in these processes on neointima formation is still lacking.

From this point of view it could be stated that the process has some similarities to the development of cancer, a process which is also largely determined by inflammation, proliferation and matrix remodeling. However, an important exception to this comparison is the self-limiting behavior of the restenotic process, which will never lead to continued growth or even invasive tumor formation after complete occlusion of the vessel.

Since the important role of inflammatory genes has been established by the findings of Monraats *et al.*, part 1 of this thesis has focused on the other processes of which histological and non-genetic findings have shown relevance in neointima-formation, such as hemostasis, matrix formation, VSMC proliferation and endothelial function. Chapter 3 and 4 discuss new insights in the role of hemostasis in restenosis development and describe the influence of established genetic variations in the hemostatic system on the risk for TVR. Coagulation seems important as any vascular intervention leads to the formation of a thrombus. However, many genetic studies have shown associations of only a few polymorphisms in the hemostatic system with the risk for restenosis after PCI.² Furthermore, one strong (Factor V Leiden, chapter 3), and also a more subtle (Marburg 1, chapter 4), pro-thrombotic risk factor were even found to reduce this

risk.³ The first chapters of this thesis therefore reduce the value of thrombotic factors in restenosis after PCI. In contrast with these findings, the PAI-1 4G/5G variant, which is known to be associated with higher PAI-1 levels, was found to increase restenosis risk.³ This might be due to the diverse functional profile of PAI-1, which is also known to have a role in inflammatory pathways. Most of the findings in chapter 3 and 4 suggest that coagulation is not a main determinant of the long-term remodelling process that leads to restenosis. Chapter 4 also describes our finding that the Marburg I polymorphism, which leads to less activity of factor VII activating protease (FSAP), increases stroke risk and mortality. The observed Marburg I associated increase in mortality was mainly due to this increased risk of fatal stroke. Unexpectedly, as mentioned earlier, we also observed a strong trend towards a reduced risk for clinical restenosis in Marburg I carriers. This apparent contradiction might be due to the diverse functional profile of FSAP, which increases proliferation of VSMCs via cleavage of platelet derived growth factor (PDGF), but has also been found to increase activation of matrix metalloproteinases (MMPs), and could thereby also reduce plaque-stability.

Due to their importance in vascular remodeling MMPs are expected to play a role in several aspects of cardiovascular disease, including restenosis. Nearly all MMPs have been found to be elevated in atherosclerotic plaques⁴ and genetic variations in MMP2 and MMP3, two well investigated MMPs, were also found to be associated with myocardial infarction.⁵ The well studied 5A/6A MMP3 promoter polymorphism, of which we know that the 6A insertion leads to less MMP3, was even found to be associated with restenosis after PCI in some, but not all studies investigating this subject.⁶⁻⁹ In chapter 5 of this thesis we combined the results of a candidate gene approach and a whole genome association scan to investigate the influence of genetic variation in the MMP2 and MMP3 genes on restenosis after PCI. In our study we did not find an association of any polymorphism in the MMP2 or MMP3 gene with TVR. Some of these polymorphisms, especially the MMP3 5A/6A polymorphism and two promoter polymorphisms in the MMP2 gene, might still be functional, as was demonstrated in studies investigating their role in myocardial infarction.⁵ Their influence on the risk of myocardial infarction has been hypothesized to be due to their tendency to destabilize atherosclerotic plaques. In restenosis, in which plaque stability is not a issue, subtle effects of the MMP2 and MMP3 polymorphisms on MMP function or levels probably lead to minor changes in total neointima volume, which are not sufficient to cause clinical restenosis.

Although proliferation of VSMCs is by many regarded as one of the main contributors to neointima formation, there is little genetic evidence supporting this hypothesis. Recent findings by Van Tiel *et al.* demonstrated an association of the -838 G/A polymorphism in the cyclin-dependent kinase inhibitor *p27(kip1)*, a key regulator of smooth muscle cell proliferation, with in-stent restenosis in two different studies.¹⁰ In 715 patients undergoing coronary angioplasty and stent placement, patients with the *p27(kip1)* -838

AA genotype had a decreased risk of in stent restenosis (HR: 0.28; 95% CI: 0.10–0.77). This finding was replicated in another cohort study of 2309 patients (HR: 0.61; 95% CI: 0.40–0.93). The -838A allele also corresponded to enhanced promoter activity, which in turn may explain decreased smooth muscle cell proliferation. Chapter 6 of this thesis constitutes an important addition to this field and describes multiple strong associations of polymorphisms in the Quaking gene with the occurrence of clinical restenosis after PCI. The quaking protein, which was thus far known for its role in myelination, has recently gained renewed interest as a possible mediator in vascular disease. Recent work by Van der Veer and Kraaijeveld *et al.*, which is also described in chapter 6, revealed an important role of the protein in proliferation and migration of VSMCs. Furthermore, Quaking viable mice (qk^v) mice showed markedly decreased neointima formation in a mouse model of in-stent restenosis. In the GENDER-study, investigating the effect of SNPs throughout the whole quaking gene, the strongest associations were found for SNPs located upstream of the QKI transcriptional start site (-2616 T/C: HR: 2.14, 95%CI: 1.24-3.68, P=0.006) and within intron 3 (57896 A/G: HR: 0.74, 95%CI: 0.6-0.92, P=0.005 and 65752 A/G: HR: 1.5, 95%CI: 1.14-1.98, P=0.003). Though a small amount of proliferative genes have yet been investigated, the strength of these associations in comparison to those found in other association studies investigating coronary restenosis, emphasizes the importance of proliferative genes in neointima formation. A combined effect of the 5752 GG and GA genotypes together with the 57896 AA genotype, was demonstrated to be even stronger (HR 1.77, 95% CI 1.29-2.44; p<0.001). This particular genotype combination was observed in 10% of the total population.

Due to its inhibitory role in VSMC proliferation, adhesion of leukocytes to the vessel wall and coagulation,¹¹⁻¹³ nitric oxide (NO) has often been suggested to protect against the development of restenosis. Its role in restenosis was further established by the observation that L-arginine, the precursor to NO, reduces neointimal hyperplasia at 6 months after stent placement.¹⁴ Despite these findings, studies investigating the influence of the Glu298Asp polymorphism in the NOS3 gene on restenosis after PCI have yielded conflicting results. Especially the larger studies did not find a significant association of this polymorphism with TVR.¹⁵

After their finding that 298Asp homozygous patients with ischemic heart disease were found to be more sensitive to the vasoconstrictive effects of phenylephrine, Sofowora *et al.* suggested that the functional significance of the Glu298Asp polymorphism may only become manifest under conditions of endothelial dysfunction.¹⁶ In chapter 7 we confirm this hypothesis and demonstrate that the functional significance of NOS3 polymorphisms may depend on the presence of the metabolic syndrome, a syndrome which is characterized by endothelial dysfunction. We studied the relationship between these polymorphisms and the risk of restenosis in a subpopulation of patients with the metabolic syndrome that took part in a large follow-up study of unselected, consecutive

patients undergoing PCI.¹⁷ We demonstrated that the -949G, -716T and 298Asp variants in the *NOS3* gene were associated with a highly increased risk of TVR after PCI in patients with the metabolic syndrome, but not in patients without the metabolic syndrome. We suggest that the metabolic alterations present in patients with the metabolic syndrome influence the functional significance of these polymorphisms and increase the susceptibility of carriers of one of these variations in the *NOS3* gene to develop restenosis after PCI.

As introduced in chapter 2, stent malapposition (SM), commonly detected by intravascular ultrasonography (IVUS), represents a separation of the stent struts from the intimal surface of the arterial wall and has been suggested to be a risk factor for late stent thrombosis. Late-acquired stent malapposition (LASM) is a consequence of positive remodeling of the vessel wall and/or of plaque volume decrease behind the stent (including clot lysis or plaque regression).¹⁸⁻²⁰ The precise mechanisms leading to LASM are thus far unknown. Although a hypersensitivity reaction to the polymer coating has been suggested to play a role,²¹ it may very well be a consequence of increased apoptosis^{22, 23} or chronic inflammation.²⁴ In chapter 8 we found support for the possibility that the process may be determined by inflammation. We found a significantly higher risk for LASM in patients carrying the caspase-1 (*CASP1*) 5352 A allele (RR: 2.32; 95% CI: 1.22–4.42). In addition, mean neointimal growth was significantly lower in patients carrying this allele (1.6 vs 4.1%; $p = 0.014$). The caspase 1 protein, also known as IL-1 β converting enzyme, increases inflammation through activation of IL-1 β and IL-18.²⁵ However, as caspase-1 may also induce apoptosis by the proteolytic cleavage of other caspase family members, caspase-1 may have divergent effects on cell survival, depending on which of its substrates is preferentially processed.²⁶⁻²⁸ Therefore, there is a slight possibility that caspase-1 could also play a role in LASM through its influence on apoptosis. Further research, preferably in larger patient groups, has to be performed to learn which of these processes is predominant in the development of LASM.

Part 2 of this thesis describes a relatively new area of research which we refer to as ‘epigenetics’, the study of non-DNA-sequence related heredity. Chapter 9 summarizes evidence showing that epigenetic mechanisms regulate the expression of genes in the inflammatory and cell proliferation pathways^{29,30} and may therefore play a role in cardiovascular disease.³¹⁻³³ The fact that epigenetic modifications are reversible makes this area of research especially interesting. New compounds which are aimed at interfering in epigenetic processes may prove to be an effective in the management of cardiovascular diseases.

Part 2 has focused on covalent post-translational modifications of histone tails, a well-studied example of epigenetic change that leads to rearrangement of nucleosomes. Of several types of chemical modification, especially acetylation of lysine residues in the histone tails is considered a key process in gene regulation. Lysine acetyltransferases

(KAT) acetylate histones by transfer of an acetyl-group to the ϵ -portion of lysine residues, which results in an open modification of chromatin structure and in accessibility of DNA to transcription factors, which leads to gene activation. Gene repression is mediated via KDACs, which remove acetyl groups and counteract the activity of KATs resulting in a closed chromatin structure. As mentioned in the general introduction, the main focus has thus far been to investigate the environmental influence on epigenetic processes. Part 2 of this thesis has introduced the concept that epigenetic processes are also under genetic control and that, besides the environment, genetic variation in genes encoding KATs and KDACs could also be an important determinant of susceptibility to complex human diseases such as cardiovascular disease.

Our findings in chapter 10 that the -2481C allele in the gene encoding PCAF, a KAT which has been shown to be a key mediator in epigenetics by acetylating histones at the site of important inflammatory genes such as NF κ B, is associated with a significant survival advantage in both the PROSPER and the WOSCOPS studies. The finding that this allele also protects against restenosis, a process which is mainly determined by inflammation and proliferation, supports the hypothesis that the PCAF gene has a role in coronary heart disease mortality through its actions in inflammatory and proliferative processes. The effects of the -2481C allele on mortality, CHD death and clinical restenosis were more profound at older age (>58 years). This age-dependent effect suggests that the polymorphism in PCAF is associated with an altered tendency to acetylate histones and may therefore become important especially in elderly patients, who may have been under the influence of altered PCAF activity for many years.

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SAMENVATTING EN SLOTOPMERKINGEN

Dit proefschrift beschrijft de invloed van genetische en epigenetische factoren op verschillende aspecten van hart- en vaatziekten. Deel 1 behandelt de invloed van genetische varianten op de verschillende processen die kunnen leiden tot complicaties na een percutane coronaire interventie (PCI). De meest voorkomende complicatie hiervan is restenose na plaatsing van een bare metal stent (BMS). Een andere complicatie die in deel 1 besproken wordt is late verworven stent malappositie (LASM) na plaatsing van een drug eluting stent (DES). In deel 2 wordt de rol van 'epigenetische epidemiologie' besproken, een relatief nieuw onderzoeksgebied in atherosclerose en restenose.

Angiografische restenose wordt gedefinieerd als een 50% afname van de lumen diameter, die geleidelijk optreedt in het eerste jaar na een PCI. Omdat niet al deze angiografische re-occlusies tot klachten leiden, wordt de klinische definitie van restenose, target vessel revascularization (TVR) op dit moment beschouwd als het meest waardevolle eindpunt. Sinds lange tijd is duidelijk dat het restenotische proces multifactorieel van aard is. Het begint als een normale wondhelingsreactie na interventie-geïnduceerde schade aan de vaatwand. Vorming van een trombus wordt gevolgd door een inflammatoire reactie en proliferatie/migratie van vasculaire gladde spiercellen (VSMC's). Eerder onderzoek door Monraats *et al.* heeft aangetoond dat met name het immuunsysteem overactief is. Dit wordt nu beschouwd als de drijvende kracht in de ontwikkeling van restenose. Het is ook bekend dat gladde spiercelproliferatie en matrixvorming een rol spelen bij het ontstaan van restenose, hoewel er nog steeds weinig bewijs is voor de invloed van genetische variatie in deze processen op neointima vorming. Vanuit dit oogpunt bekeken heeft het proces dus enige gelijkenis met het ontstaan van kanker, een proces wat ook voor een groot deel bestaat uit inflammatie, proliferatie en veranderingen in de matrix. Echter een belangrijke uitzondering in deze vergelijking is het zelf-begrenzende karakter van restenose: de celgroei zal niet doorgaan nadat een arterie volledig is afgesloten.

Omdat de bevindingen van Monraats *et al.* duidelijk hebben gemaakt dat inflammatoire genen een belangrijke rol spelen, is deel 1 van dit proefschrift vooral gericht op de andere processen die, zoals is gebleken uit histologisch en niet-genetisch onderzoek, relevant kunnen zijn in neointima vorming. Het gaat hier om hemostase, matrix vorming, VSMC proliferatie en endotheel functie. In hoofdstuk 3 en 4 worden nieuwe inzichten in de rol van hemostase in restenose besproken en wordt ook de invloed van bekende genetische varianten in stollingsfactoren op het risico op TVR beschreven. Het lijkt waarschijnlijk dat stolling een rol speelt in restenose omdat iedere vasculaire interventie leidt tot de vorming van een trombus. Echter, uit de vele genetische studies die op dit gebied gedaan zijn, bleek dat slechts enkele stollingsvarianten associeerden met het risico op restenose na een PCI. Bovendien bleek dat een sterke (Factor V Leiden, hoofdstuk 3) en ook een meer subtiele (Marburg 1, hoofdstuk 4) pro-trombotische risicofactor

het risico op restenose zelfs reduceerde. Op basis hiervan lijkt de waarde van trombotische factoren in de ontwikkeling van restenose dus mee te vallen. In tegenstelling tot deze bevindingen, is gebleken dat de PAI-1 4G/5G variant, die geassocieerd is met hogere PAI-1 plasma niveaus, het risico op restenose wel verhoogt. De diversiteit van functies van PAI-1, dat ook inflammatoire effecten kan hebben, vormen een mogelijke verklaring hiervoor. De meeste bevindingen in hoofdstuk 3 en 4 suggereren dat stolling geen belangrijke determinant is van het lange termijn proces wat leidt tot restenose. Hoofdstuk 4 beschrijft ook de bevinding dat het Marburg 1 polymorfisme, wat leidt tot minder activiteit van FSAP, het risico op een CVA en de totale mortaliteit verhoogt. De Marburg 1 geassocieerde toename van de mortaliteit bleek vooral het gevolg van een toename van fatale CVA's. We vonden onverwacht een trend tot een lager risico op restenose in dragers van het Marburg 1 allel. Deze schijnbare tegenstelling zou het gevolg kunnen zijn van het diverse functionele profiel van FSAP, dat enerzijds zorgt voor een toename van de proliferatie van VSMC's via het klieven van Platelet Derived Growth Factor (PDGF), en anderszijds matrix metalloproteinases (MMP's) activeert en daarmee mogelijk de plaque-stabiliteit vermindert.

Door hun belangrijke rol in vasculaire remodeling wordt verwacht dat MMP's een rol spelen bij restenose en andere aspecten van hart- en vaatziekten. Van bijna alle MMP's is een verhoogde expressie gevonden in atherosclerotische plaques. Er werden reeds associaties gevonden tussen genetische varianten in MMP2 en MMP3, twee uitvoerig onderzochte MMP's, en de incidentie van hartinfarcten. Het bekende 5A/6A MMP3 promotor polymorfisme, waarvan we weten dat het 6A allel leidt tot minder MMP3, bleek geassocieerd met restenose na PCI in enkele, maar niet alle studies die dit onderzochten. In hoofdstuk 5 van dit proefschrift hebben we resultaten van de kandidaat gen benadering gecombineerd met een genome wide association scan (GWAS) om de invloed van variatie in de MMP2 en MMP3 genen op restenose na een PCI te onderzoeken. We vonden echter geen significante associatie tussen de MMP2 en MMP3 polymorfismen en restenose. Sommige van deze polymorfismen, zoals het MMP3 5A/6A polymorfisme en de polymorfismen in de MMP2 promotor, zouden nog steeds functioneel kunnen zijn, zoals blijkt uit studies die hun rol in hartinfarcten onderzochten. Hun invloed op het risico op hartinfarcten is mogelijk een gevolg van de neiging van MMP's om atherosclerotische plaques te destabiliseren. Bij restenose, waar plaquestabiliteit geen rol bij speelt, leiden subtiele effecten van MMP2 en MMP3 polymorfismen op MMP functie of expressie waarschijnlijk tot kleine veranderingen in neointima volume, die niet voldoende zijn om klinische restenose te veroorzaken.

Hoewel proliferatie van VSMC's door velen wordt beschouwd als een van de drijvende krachten achter neointima vorming, is er weinig genetisch bewijs voor deze hypothese. Recente bevindingen door Van Tiel *et al.* hebben een associatie laten zien tussen het -838 G/A polymorfisme in de cyclin-dependent kinase inhibitor *p27(kip)*,

een belangrijke regulator van gladde spiercel proliferatie, en in-stent restenose in twee verschillende studies. Bij 715 patiënten die coronaire angioplastiek met stent plaatsing ondergingen hadden patiënten met het *p27(kip1)* -838 AA genotype een lager risico op in-stent restenose (HR: 0.28; 95% CI: 0.10–0.77). Deze bevinding werd gerepliceerd in een andere cohort studie met 2309 patiënten (HR: 0.61; 95% CI: 0.40–0.93). Het -838A allel kwam overeen met een verhoogde promotor activiteit, wat een afname in gladde spiercel proliferatie zou kunnen verklaren. Hoofdstuk 6 van dit proefschrift vormt een belangrijke bijdrage aan dit vakgebied en beschrijft een aantal sterke associaties tussen polymorfismen in het Quaking gen en klinische restenose na PCI. Het Quaking eiwit, dat tot nu toe vooral bekend was om zijn rol in myelinisering, staat nu opnieuw in de belangstelling vanwege een mogelijke rol in hart- en vaatziekten. Recente bevindingen van Van der Veer en Kraaijeveld *et al.*, die ook worden beschreven in hoofdstuk 6, tonen aan dat het eiwit een belangrijke rol speelt bij proliferatie en migratie van VSMC's. Quaking viable (*qk^v*) muizen, die het Quaking eiwit zeer laag tot expressie brengen, lieten sterk gereduceerde neointima vorming zien in een muizenmodel voor restenose. Het effect van single nucleotide polymorphisms (SNP) in het gehele Quaking gen op restenose na PCI hebben we onderzocht in de GENDER-studie. In deze studie werden de sterkste associaties gevonden in de promotor regio (-2616 T/C: HR: 2.14, 95%CI: 1.24-3.68, *P*=0.006) en in intron 3 (57896 A/G: HR: 0.74, 95%CI: 0.6-0.92, *P*=0.005 and 65752 A/G: HR: 1.5, 95%CI: 1.14-1.98, *P*=0.003). Hoewel tot nu toe nog maar enkele proliferatieve genen werden onderzocht, benadrukt de kracht van de gevonden associaties het belang van proliferatieve genen in neointima vorming. Het gecombineerde effect van de 5752 GG/GA genotypen en het 57896 AA genotype was zelfs groter (HR 1.77, 95% CI 1.29-2.44; *p*<0.001). Deze genotype combinatie was aanwezig in 10% van de totale populatie.

Door zijn inhibitoire rol in VSMC proliferatie, adhesie van leukocyten aan de vaatwand en stolling, werd gesuggereerd dat nitric oxide (NO) zou beschermen tegen het ontstaan van restenose. De rol van NO in restenose werd verder ondersteund door de observatie dat L-arginine, de precursor van NO, neointima hyperplasie reduceert 6 maanden na stent plaatsing. Ondanks deze bevindingen hebben studies die de invloed van het Glu298Asp polymorfisme in het *NOS3* gen op restenose onderzochten, tegenstrijdige resultaten geproduceerd. Vooral de grotere studies vonden geen significant effect van dit polymorfisme op TVR.

Na hun bevinding dat 298Asp homozygote patiënten met ischemisch hartlijden gevoeliger waren voor de vasoconstrictieve effecten van phenylephrine, suggereerde Sofowora *et al.* dat het belang van dit polymorfisme alleen duidelijk wordt onder condities van endotheeldysfunctie. In hoofdstuk 7 bevestigen we deze hypothese en geven we aan dat de hazard ratios mogelijk afhankelijk zijn van de aanwezigheid van het metabool syndroom, een syndroom dat gekenmerkt wordt door endotheeldysfunctie. We onderzochten de relatie tussen deze polymorfismen en het risico op restenose in

een subpopulatie van patiënten met het metabool syndroom die deelnamen aan een grote follow-up studie met achtereenvolgende patiënten die een PCI ondergingen, waarop geen selectie werd toegepast. We toonden aan dat de -949G, -716T and 298Asp varianten in het NOS3 gen geassocieerd waren met een hoog risico op TVR in patiënten met het metabool syndroom, terwijl geen associatie gevonden werd in patiënten zonder het metabool syndroom. Onze hypothese is dat de metabole veranderingen in patiënten met het metabool syndroom het belang van deze polymorfismen beïnvloeden en de ontvankelijkheid van dragers van deze varianten in het NOS3 gen om na een PCI restenose te ontwikkelen.

Zoals geïntroduceerd werd in hoofdstuk 2 hebben DES de restenose-incidentie enorm teruggebracht. De ervaring van de laatste jaren toont echter een verhoogde kans op LASM en stent trombose bij gebruik van DES. LASM zou een gevolg kunnen zijn van positieve remodeling van de vaatwand of van een afname van het plaquevolume rondom de stent. De precieze mechanismen die tot LASM leiden zijn tot nu toe niet bekend. Hoewel een hypersensitiviteitsreactie mogelijk een rol speelt, zou het ook een gevolg kunnen zijn van toegenomen apoptose of chronische ontsteking. In hoofdstuk 8 vonden we ondersteuning voor de hypothese dat het proces veroorzaakt zou kunnen worden door ontsteking. We vonden een significant hoger risico op LASM in patiënten die het caspase 1 (*CASP1*) 5352 A allel dragen (RR: 2.32;95% CI: 1.22–4.42). Ook was de gemiddelde neointimagroei significant lager in dragers van dit allel (1.6 vs 4.1%; $p = 0.014$). Het caspase 1 eiwit, ook wel bekend als IL-1 β convertend enzyme, zorgt voor een toename van ontsteking via activatie van IL-1 β and IL-18. Echter, omdat caspase 1 ook apoptose kan induceren door het proteolytisch klieven van andere leden van de caspase groep, zou caspase 1 ook effecten kunnen hebben op celoverleving, afhankelijk van welk substraat bij voorkeur wordt gekleefd. Er bestaat dus een kleine mogelijkheid dat caspase 1 een rol speelt bij LASM via invloed op apoptose. Vervolgonderzoek, bij voorkeur in grotere populaties, is nodig om te ontdekken welke van deze processen overheersend is bij het ontstaan van LASM.

Deel 2 van dit proefschrift introduceert een relatief nieuw onderzoeksgebied, de epigenetica, de studie naar omkeerbare erfelijke veranderingen in genfunctie die optreden zonder wijzigingen in de sequentie van het DNA. Hoofdstuk 9 geeft een overzicht van bevindingen die aantonen dat epigenetische mechanismen de expressie reguleren van proliferatieve en inflammatoire genen en daarmee een rol zouden kunnen spelen bij hart- en vaatziekten. Het feit dat epigenetische modificaties reversibel zijn maakt dit onderzoeksgebied zeer interessant. Nieuwe farmaca die ingrijpen in epigenetische processen zouden effectief kunnen zijn bij de behandeling van hart- en vaatziekten. Deel 2 heeft zich vooral gericht op covalente post-translationele modificaties van histonstaarten, een voorbeeld van een epigenetische verandering die leidt tot herschikking van nucleosomen. Van de verschillende typen chemische modificatie, wordt vooral

acetylatie van lysineresiduen in de histonstaarten beschouwd als een sleutelproces in genregulatie. Lysine acetyltransferases (KAT) acetyleren histonen door een acetyl-groep te plaatsen op het ϵ -gedeelte van lysine residuen, wat resulteert in het openen van de chromatinestructuur. Dit geeft transcriptiefactoren toegang tot het DNA, wat leidt tot gen activatie. Repressie van genen wordt gemedieerd door lysine deacetylases (KDAC's), die een acetyl-groep verwijderen en daarmee de activiteit van HATs antagoneren, wat resulteert in het sluiten van chromatine. Zoals eerder genoemd in de algemene introductie lag tot nu toe het accent vooral op onderzoek naar de invloed van omgevingsfactoren op epigenetische processen. Deel 2 van dit proefschrift introduceert het concept dat epigenetische processen ook onder genetische controle staan. Variatie in genen die coderen voor KAT's en KDAC's kan ook een belangrijke determinant zijn van gevoeligheid voor het krijgen van complexe ziekten zoals hart- en vaatziekten.

In hoofdstuk 10 toonden we aan dat het -2481C allel in het PCAF gen geassocieerd is met een significant overlevingsvoordeel in de PROSPER en de WOSCOPS studies. PCAF is een KAT met een sleutelrol in de epigenetica, die histonen acetyleert op loci waar belangrijke inflammatoire genen zoals NF κ B liggen. De bevinding dat het -2481C allel ook beschermt tegen restenose, een proces wat vooral wordt gedreven door ontsteking en proliferatie, ondersteunt de hypothese dat het PCAF gen effect heeft op de mortaliteit van coronaire hartziekten via zijn rol in inflammatoire en proliferatieve processen. De effecten van het

-2481C allel op de totale mortaliteit, op sterfte aan coronaire hartziekten en op klinische restenose, waren sterker in patiënten met een hogere leeftijd (>58 jaar). Dit leeftijdsafhankelijke effect suggereert dat het polymorfisme geassocieerd is met een veranderde neiging tot histon acetylatie, wat alleen tot uiting komt in oudere patiënten omdat die al hun hele leven de invloed ondervinden van een veranderde PCAF activiteit.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren 8 maart 1979 te Steenberg. In 1997 behaalde hij zijn eindexamen aan gymnasium Juvenaat 't Heilig Hart in Bergen op Zoom. Datzelfde jaar begon hij aan de studie biomedische wetenschappen in Leiden. Interesse voor 'al het medische' was op jonge leeftijd al aanwezig. Toch bestond er ook altijd aantrekkingskracht tot genetica en immunologie. Dit bewoog hem tot stages bij de afdeling medische farmacologie (Gorleus) en de afdeling longzieken (LUMC). Al snel werd hem duidelijk dat hij ook graag geneeskunde wilde gaan studeren. Na 4 maal loten kreeg hij deze kans en kon hij in 2001 eindelijk beginnen. Gedurende de wachttijd voor zijn co-schappen heeft hij een jaartje met plezier gewerkt bij zorgverzekeraar DSW. In december 2005 behaalde hij zijn artsexamen en begon hij aan een promotieonderzoek onder leiding van Prof. Dr. J.W. Jukema. Dit cardiologische onderzoek had een genetische inslag en sloot dus mooi aan bij de gecombineerde biomedische wetenschappen/geneeskunde achtergrond. De resultaten hiervan staan beschreven in dit proefschrift. Naast het promotieonderzoek was hij werkzaam bij de trombosedienst Leiden.