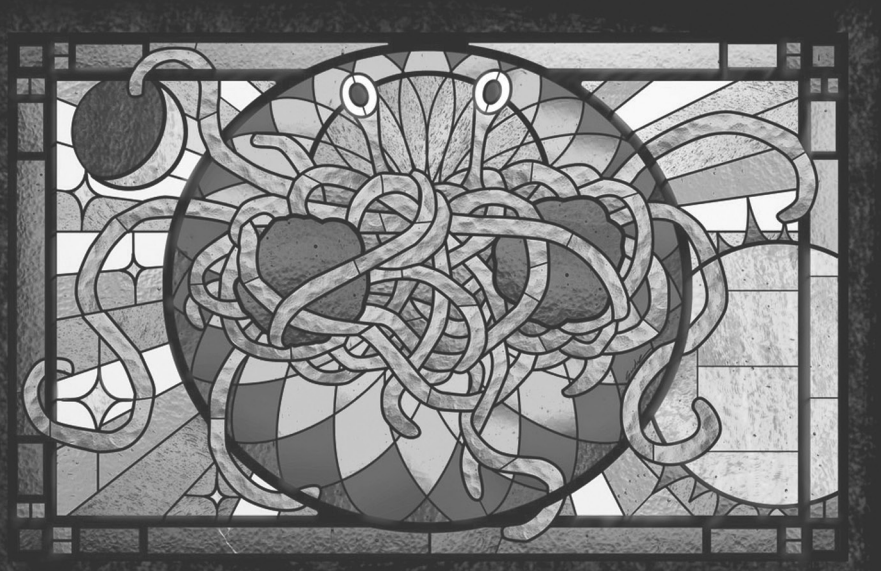

Chapter 3

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	
G/A		92 (3.0)	10.8	1.12 (0.60-2.11)
A/A		1		
FV Arg506Gln				
Arg/Arg	0.03	2896 (94.5)	10.0	
Arg/Gln		164 (5.4)	4.2	0.41 (0.19-0.86)
Gln/Gln		3 (0.1)		
FVII -323 del/ins				
D/D	0.13	2338 (76.4)	9.9	
D/I		673 (22.0)	9.1	0.94 (0.72-1.24)
I/I		48 (1.6)	12.5	
FVII Arg353Gln				
Arg/Arg	0.11	2414 (79.3)	9.9	
Arg/Gln		587 (19.3)	8.3	0.89 (0.67-1.20)
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	**PAI-1 4G/5G**			1.29 (0.89-1.88)
N=453	5G/5G	88	12.5	
	5G/4G	234	12.0	
	4G/4G	122	18.0	
	PAI-1 11053			1.41 (0.98-2.04)
	T/T	129	11.6	
	T/G	231	12.1	
	GG	83	21.7	
No diabetes	**PAI-1 4G/5G**			1.27 (1.05-1.52)
N=2651	5G/5G	553	6.1	
	5G/4G	1327	9.6	
	4G/4G	729	10.4	
	PAI-1 11053			1.03 (0.85-1.23)
	T/T	788	8.1	
	T/G	1323	10.0	
	GG	491	8.1	
Smokers	**PAI-1 4G/5G**			1.52 (1.05-2.20)
N=762	5G/5G	168	3.6	
	5G/4G	368	8.7	
	4G/4G	216	10.2	
	PAI-1 11053			1.25 (0.87-1.80)
	T/T	223	6.3	
	T/G	375	8.0	
	GG	151	9.9	
Non-smokers	**PAI-1 4G/5G**			1.21 (1.01-1.46)
N=2342	5G/5G	473	8.2	
	5G/4G	1193	10.4	
	4G/4G	635	12.0	
	PAI-1 11053			1.06 (0.88-1.28)
	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 plasm[12-14] 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] inhibiton 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 TNF, 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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