Chapter 4

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


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,\(^4\) the Marburg I variant has been shown to be a risk factor for late complications of carotid stenosis\(^7\) 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.\textsuperscript{13}

\textbf{Study design and follow-up of the GENDER study}

The present study sample has been described previously.\textsuperscript{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.

\textbf{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.

\textbf{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
The FSAP polymorphism (G534E) is associated with increased risk for stroke and mortality. 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

<table>
<thead>
<tr>
<th></th>
<th>PROSPER N=5804</th>
<th>GENDER N=3104</th>
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<tbody>
<tr>
<td>Continuous variates (mean, SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>75.3 (3.3)</td>
<td>62.1 (10.7)</td>
</tr>
<tr>
<td>Body mass index, (kg/m²)</td>
<td>26.8 (4.2)</td>
<td>27.0 (3.9)</td>
</tr>
<tr>
<td>Categorical variates (n, %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male sex</td>
<td>2804 (48)</td>
<td>2216 (71)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>1558 (27)</td>
<td>762 (25)</td>
</tr>
<tr>
<td>History of diabetes</td>
<td>623 (11)</td>
<td>453 (15)</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>3592 (62)</td>
<td>1259 (41)</td>
</tr>
<tr>
<td>History of myocardial infarction</td>
<td>776 (13)</td>
<td>1239 (40)</td>
</tr>
<tr>
<td>History of stable angina</td>
<td>1559 (27)</td>
<td>2079 (67)</td>
</tr>
<tr>
<td>Genotype, minor allele frequency (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marburg I G/A *</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Marburg II G/C **</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

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.
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 polymorphism.

![Figure 1. Marburg I hazard ratios for vascular endpoints in GENDER and PROSPER](image1)

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.

![Figure 2. Marburg II hazard ratios for vascular endpoints in GENDER and PROSPER](image2)

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. Moreover, earlier studies showed that the Marburg I polymorphism does not seem to influence the risk for venous thrombosis. However, FSAP cleaves PDGF-BB and has been shown to inhibit vascular smooth muscle cell proliferation and migration *in vitro* and *in vivo*. 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. 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. 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.\textsuperscript{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.\textsuperscript{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),\textsuperscript{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.
REFERENCES