−455G/A Polymorphism of the β-Fibrinogen Gene is Associated With the Progression of Coronary Atherosclerosis in Symptomatic Men

Proposed Role for an Acute-Phase Reaction Pattern of Fibrinogen

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Abstract—Increased plasma fibrinogen levels have been identified as a risk indicator for myocardial infarction, stroke, and thrombosis. Both environmental and genetic factors make an important contribution to plasma fibrinogen levels in humans. In the present study we evaluated, in patients with serum cholesterol levels between 4 and 8 mmol/L, the relation of plasma levels and polymorphisms of fibrinogen with coronary artery disease (CAD), cross-sectionally at baseline and after a 2-year follow-up period in which they received either a placebo or pravastatin. Higher plasma fibrinogen levels (3.9 g/L) were observed at baseline in patients with the −455AA genotype than in patients with the −455GA (3.2 g/L) and −455GG (3.1 g/L) genotypes of the −455G/A fibrinogen β gene polymorphism (P<.05). Plasma levels of fibrinogen were not related to the baseline angiographic variables (mean segment diameter [MSD] and minimum obstruction diameter [MOD]), nor to the quantitative changes in these angiographic variables. However, in the placebo group, patients with the −455AA genotype had more progression of CAD, expressed by a significantly greater decrease of the MSD and MOD, after the 2-year follow-up period than patients with the other genotypes. The −455G/A polymorphism was related to the progression of CAD, and pravastatin therapy seemed to offset this deleterious effect. We hypothesized that the −455A allele may promote a stronger acute-phase response in fibrinogen and that the resulting higher fibrinogen levels may form the pathogenetic basis for the stronger progression of coronary atherosclerosis. Experiments to verify this hypothesis are being proposed and advocated, in view of the possibility of identifying a genetic marker that can recognize a subgroup of patients with an increased risk who may benefit from early treatment with lipid-lowering or anticoagulant drugs.


Key Words: fibrinogen ■ inflammation ■ genetics ■ cardiovascular diseases

There is growing interest in fibrinogen after several epidemiological studies reported a strong association between elevated plasma levels of fibrinogen and an increased risk of myocardial infarction,1–6 stroke,7 thrombotic risk in patients with venous thrombosis,8 and mortality in patients with peripheral arterial disease.9 Since fibrinogen is an acute-phase protein, an increased plasma fibrinogen level may reflect the inflammatory state of the vascular wall and may thus be related to cardiovascular risk. However, a direct association between increased plasma fibrinogen levels and thrombotic events has also been suggested.10–15

The three chains of fibrinogen are encoded by three different genes that are located on the long arm of chromosome 4 in a 50-kb cluster.16 Several DNA polymorphisms of the three genes have been described,17–19 and restriction fragment length polymorphisms of the β gene (Bcl 1 and −455G/A) are associated with differences in the plasma levels of fibrinogen.17,20 Healthy individuals who are homozygous for the rare alleles of β-gene polymorphisms have the highest fibrinogen levels; individuals who are homozygous for the common allele have the lowest plasma fibrinogen levels, while the fibrinogen levels in heterozygotes are intermediate.17,20–22

In the ECTIM study, variation of the β-fibrinogen gene (Bcl I and −455G/A polymorphisms) in the cardiovascular patients was associated with the severity of arterial disease. This finding suggests that individuals with the rare −455A allele may have a greater tendency to develop arterial disease.23,24 In most studies, carriers of the −455A allele have higher plasma fibrinogen levels, which may result in a hypercoagulable state and thereby to increased risk of arterial thrombosis. We hypothesize that CAD patients with the −455A allele may have increased progression of the disease process, because their

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fibrinogen levels increase more when the acute-phase response is triggered. Thus, screening for the $\sim 455A$ allele may offer the possibility to identify a subset of patients with a high risk of increased progression of CAD and its clinical sequelae such as fatal and nonfatal myocardial infarction. Identifying these patients is of great importance because they may benefit from early therapy. Thus far it has been difficult to identify a subgroup with increased risk when lipoprotein disturbances are only moderate.

In the population that was studied within REGRESS,\textsuperscript{25} we evaluated in great detail several aspects of fibrinogen plasma levels and genotypes in relation to quantitative parameters of coronary disease and progression of the disease. REGRESS included patients with symptomatic coronary disease and normal or moderately elevated serum cholesterol levels and therefore represents the majority of cardiac patients seen in clinical practice. First, we evaluated in these patients at baseline the relationships between plasma fibrinogen levels and the state of the disease, as assessed by quantitative coronary angiography. Second, we evaluated the relation between baseline levels and genotypes of fibrinogen and the progression of coronary atherosclerosis in the patients in the placebo group and the group treated with the cholesterol-lowering drug pravastatin. The results are summarized in the formulation of a more detailed hypothesis of the pathogenetic role of fibrinogen in the progression of vessel wall disease.

## Methods

### Study Design

The study design and methodology of REGRESS have been described in detail elsewhere.\textsuperscript{25} Briefly, REGRESS is a double-blind, placebo-controlled, multicenter study to assess the effect of a 2-year treatment with the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor pravastatin on progression and regression of angiographically documented coronary atherosclerosis in 885 male patients undergoing coronary cineangiography to assess anginal complaints. The patients were $\angle 70$ years of age and had normal to moderately raised serum cholesterol levels, i.e., between 4.0 and 8.0 mmol/L, and at least one coronary stenosis causing $\geq 50\%$ diameter reduction (visually assessed). Baseline and follow-up coronary arteriograms were analyzed by quantitative computer analysis. A number of substudies were performed in addition to the angiographic main study. Substudies included B-mode ultrasound studies of the carotid and femoral arteries, ambulatory electrocardiographic monitoring, specialized lipid research, and DNA studies. The study was conducted under the auspices of the Interuniversity Cardiology Institute of the Netherlands, Utrecht, the Netherlands. Written informed consent was obtained from the patients, and the study was performed in accordance with the Declaration of Helsinki.

### Subjects

DNA was available from 679 patients, and from 492 of these patients, plasma was also available. Information about current smoking status and family history of CAD was available from the case record form. The family history was considered positive if one of the parents had had a myocardial infarction before the age of 60 (Table 1). The average MSD, reflecting diffuse atherosclerosis, and the average MOD, reflecting focal atherosclerosis, were calculated from the coronary angiograms at baseline and after 24 months.\textsuperscript{25}

### Blood Assays

Plasma fibrinogen levels were determined with an enzyme immunoassay that uses a monoclonal antibody against the carboxyl-terminal end of the fibrinogen $\alpha$-chain as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the $\alpha$-chain (Y18) as the tagging antibody.\textsuperscript{26} The $\sim 455G/A$ polymorphism of the $\beta$-fibrinogen gene and the Taq I polymorphism of the $\alpha$-fibrinogen gene were assessed as described by Thomas et al.\textsuperscript{18} Briefly, genomic DNA was amplified by polymerase chain reaction and incubated with the appropriate restriction enzymes (Hae III and Taq I, respectively). The DNA fragments were then visualized under UV light after separation on 2% agarose gels containing ethidium bromide. The rare alleles were called the $\sim 455A$ and the T2 allele, respectively. Serum cholesterol, HDL cholesterol, and triglycerides were measured on fasting blood samples by standard techniques as described previously.\textsuperscript{25}

### Statistical Evaluation

The frequencies of the different alleles were assessed by gene counting. Linkage disequilibrium between the $\beta$- and $\alpha$-fibrinogen gene polymorphisms was calculated as described by Chakravarti.\textsuperscript{28} The logarithmically transformed fibrinogen levels nicely followed a normal distribution; therefore, all analyses were done on the (natural) logarithmically transformed fibrinogen data. The relation between genetic polymorphisms and other factors was studied by using analysis of covariance, with age and body mass index as covariables, or a Kruskal-Wallis test. For the analysis of the relation between genetic polymorphisms and changes in other factors, the baseline values were also entered as covariables.

## Results

### Baseline Plasma Levels and Genotype of Fibrinogen in Relation to Environmental Factors and CAD

The plasma fibrinogen levels were not correlated with lipid levels, history of CAD, or history of percutaneous transluminal coronary angioplasty. Comparable plasma fibrinogen levels

### TABLE 1. Baseline Characteristics of the Patients With CAD

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Pravastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>339</td>
<td>343</td>
</tr>
<tr>
<td>age, y (mean (SD))</td>
<td>55.8 (8.2)</td>
<td>56.6 (8.1)</td>
</tr>
<tr>
<td>BMI, kg/m² (mean (SD))</td>
<td>26.2 (2.6)</td>
<td>25.9 (2.6)</td>
</tr>
<tr>
<td>Personal history of myocardial infarction, %</td>
<td>46</td>
<td>51</td>
</tr>
<tr>
<td>Family history of cardiovascular disease, %</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>Nonsmokers, %</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Ex-smokers, %</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>&lt;10 cigarettes/d</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>10–20 cigarettes/d</td>
<td>61</td>
<td>63</td>
</tr>
<tr>
<td>&gt;20 cigarettes/d</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L (mean (SD))</td>
<td>6.07 (0.85)</td>
<td>6.03 (0.86)</td>
</tr>
<tr>
<td>HDL/LDL cholesterol ratio* [geometric mean (CV)]</td>
<td>0.21 (0.27)</td>
<td>0.21 (0.29)</td>
</tr>
<tr>
<td>Triglycerides,* mmol/L [geometric mean (CV)]</td>
<td>1.64 (0.45)</td>
<td>1.62 (0.43)</td>
</tr>
</tbody>
</table>

*BMI indicates body mass index and CV, coefficient of variation.

*Data were logarithmically transformed before analysis.
were observed in patients who did or did not use long-acting nitrate medication, β-blockers, or calcium-channel blockers. No relations were observed between the extent of CAD, the ejection fraction, baseline MSD, baseline MOD and the baseline fibrinogen levels (Table 2).

### Genetic Polymorphisms and Progression of CAD Related to Genetic Polymorphisms and Plasma Fibrinogen Levels

The frequency of the −455A allele of the −455G/A polymorphism was 0.21 (95% CI 0.19 to 0.23) and of the T2 allele of the Taq I polymorphism, 0.31 (95% CI 0.28 to 0.33). The distributions of the −455G/A and Taq I polymorphisms were in Hardy-Weinberg equilibrium, and there was no correlation between the two polymorphisms (Δ=0.02, χ²=0.51, NS).

The individuals who were homozygous for the rare −455A allele had significantly higher fibrinogen levels (3.9 g/L [95% CI 3.2 to 4.8], n=16), while the homozygotes for the common −455G allele and the heterozygotes had comparable plasma fibrinogen levels (3.2 g/L [95% CI 3.0 to 3.3], n=288, and 3.1 g/L [95% CI 2.9 to 3.3], n=154, respectively). In the current smokers with the −455AA genotype, fibrinogen levels were higher than in the nonsmokers (4.65 g/L [95% CI 3.20 to 6.76], n=5, and 3.63 [95% CI 2.85 to 4.61], n=11, respectively). This difference was greater than that in the patients with the −455GG genotype (3.5 g/L [95% CI 3.1 to 3.8], n=71, and 3.1 g/L [95% CI 2.9 to 3.2], n=217, respectively). Only in the current smokers was the relation between −455G/A polymorphism and plasma levels significant (P<.05). No relation was observed between the Taq I polymorphism and the fibrinogen level (3.1 g/L [95% CI 2.7 to 3.7] for T1T1, 3.2 g/L [95% CI 3.1 to 3.4] for T1T2, and 3.0 g/L [95% CI 2.6 to 3.4] for T2T2, NS).

In the evaluation of relationships between baseline disease variables and −455G/A genotypes, no relation was observed between the number of stenosed arteries and the presence of a personal history of ischemic heart disease. However, a significant relation was observed between the −455GA genotype and the quantitative CAD variables MOD and MSD. Patients with the −455AA genotype had greater average baseline MOD and MSD than the other groups (Table 3). This greater average baseline MOD and MSD was observed in both the placebo and pravastatin groups (NS for interaction between treatment and baseline MOD and MSD. MOD in the placebo group was mean (SD) 1.87 mm (0.24) for −455GG, 1.93 mm (0.25) for −455GA, and 2.14 mm (0.32) for −455AA and in the pravastatin group, 1.86 mm (0.26) for −455GG, 1.86 mm (0.19) for −455GA, and 1.97 mm (0.17) for −455AA; MSD in the placebo group was mean (SD) 2.77 mm (0.46) for −455GG, 2.78 mm (0.46) for −455GA, and 3.16 mm (0.56) for −455AA and in the pravastatin group, 2.77 mm (0.47) for −455GG, 2.77 mm (0.44) for −455GA, and 2.95 mm (0.29) for −455AA.

### Change of Angiographic Parameters and Genetic Polymorphism and Plasma Fibrinogen Levels

Patients were stratified at baseline into two groups receiving either placebo or the lipid-lowering drug pravastatin. After 2 years, the progression or regression of coronary atherosclerosis was quantified. The baseline levels of plasma fibrinogen were not related to the changes in coronary atherosclerosis, either in the placebo or in pravastatin group (Table 4). No relations were observed with the risk of coronary events or the occurrence of new lesions. It cannot be excluded that a relation was present, but it may not have been detected due to the small number of events that occurred in these patients (66 coronary events in the placebo group and 36 in the pravastatin group). Remarkably, in the placebo group, the patients with the −455AA genotype, which is associated with the highest fibrinogen levels, had significantly greater progression of CAD, as reflected by a larger reduction of the MSD and MOD, than patients with the −455GG and the −455GA genotypes (interaction tests: P=.024 and P=.024, respectively); see the Figure and Table 5. Thus, in this study, a relation with progression of CAD was observed only with the fibrinogen genotype and not with the plasma fibrinogen levels. In patients receiving pravastatin, this difference was not observed, indicating that the deleterious effects of the −455AA genotype...
could be offset by pravastatin. With respect to the Taq I polymorphism, no differences were found.

**Discussion**

Identifying patients with an increased risk of the progression of CAD is important, because these patients may benefit from early treatment. Thus far, however, it has proved difficult to identify patients at increased risk of progression of CAD when lipoprotein disturbances are only moderate.

In addition to measuring plasma levels of factors that are known to be associated with CAD, determination of genetic polymorphisms may offer a tool for identifying such a subgroup at increased risk of CAD. Besides lipoprotein disturbances, increased fibrinogen levels have also been identified as risk indicators for cardiovascular disease. Therefore, we initiated this study of the relation between fibrinogen plasma levels and fibrinogen DNA polymorphisms in individuals with CAD but without major lipid disturbances.

Herein we report on the relation, in men with CAD with serum cholesterol levels between 4.0 and 8.0 mmol/L, between phenotypic and genotypic information about fibrinogen and quantitative measures of CAD, both at baseline and after 2 years of administration of a placebo or pravastatin. In the placebo group, we evaluated the effect of the natural progression of the disease; in the pravastatin-treated group, the interaction with lipid-lowering therapy was assessed.

### Baseline Plasma Levels in Relation to Environmental Factors and CAD

No significant relations were observed between plasma fibrinogen levels and environmental factors. For example, in most population studies, an association between fibrinogen levels and smoking habits is observed. In this population, the difference is not significant, which may be explained by the relatively small sample size. Furthermore, it is known that there is substantial intraindividual variation in fibrinogen levels in patients with angina pectoris, and this may also explain why associations between plasma fibrinogen levels and environmental factors were not observed in this study.

### Genetic Polymorphisms

The frequencies of the rare alleles of the fibrinogen DNA polymorphisms (−455G/A of the β gene and Taq I of the α gene) are shown in Table 3.

<table>
<thead>
<tr>
<th>Table 3. −455G/A Fibrinogen Polymorphism and Baseline Patient Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>−455G/A Polymorphism of β Gene</td>
</tr>
<tr>
<td>Sample size</td>
</tr>
<tr>
<td>Baseline MSD, mm [mean (SD)]</td>
</tr>
<tr>
<td>Baseline MOD, mm [mean (SD)]</td>
</tr>
<tr>
<td>Extent of CAD, n (%)</td>
</tr>
<tr>
<td>1 Vessel</td>
</tr>
<tr>
<td>2 Vessels</td>
</tr>
<tr>
<td>3 Vessels</td>
</tr>
<tr>
<td>Personal history of MI, n (%)</td>
</tr>
<tr>
<td>Personal history of PTCA, n (%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4. Relation Between Plasma Fibrinogen Levels and Change of Angiographic Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo &amp; Pravastatin</td>
</tr>
<tr>
<td>MS decrease</td>
</tr>
<tr>
<td>MOD decrease</td>
</tr>
<tr>
<td>% Stenosis change</td>
</tr>
<tr>
<td>Events</td>
</tr>
<tr>
<td>New lesions</td>
</tr>
</tbody>
</table>

The MSD and MOD decrease are the baseline to follow-up diameters. Reported are partial correlations adjusted for associated baseline value for quantitative variables (ie, baseline MSD for MSD increase, baseline MOD for MOD increase, baseline stenosis for % stenosis change) and odds ratios (95% CI) for dichotomous variables.

**MI** indicates myocardial infarction and PTCA, percutaneous transluminal coronary angioplasty.

*One-way ANOVA or Kruskal-Wallis test, where appropriate.
The development of the disease was documented after 2 years in both the placebo and the pravastatin-treated group. In the placebo group, a significant reduction of MOD and MSD was observed, while the pravastatin-treated group showed less progression. In the placebo group, the plasma fibrinogen levels were not associated with the development of the disease, but the patients with the −455AA genotype had the largest decrease of both MOD and MSD after adjustment for the baseline MOD or MSD. We conclude from these data that the −455AA genotype, associated with higher plasma fibrinogen levels, is related to the more severe and rapid progression of atherosclerotic narrowing of the lumen. We consider this the major observation in our study.

We further observed that this more rapid progression in the −455AA genotype is not apparent in the pravastatin group. This may be explained by a much larger positive effect of pravastatin treatment than the deleterious influence of the fibrinogen −455G/A polymorphism on the development of the disease. The effect of pravastatin is not likely to be related to the fibrinogen metabolism, since no or only a minor effect of pravastatin on plasma fibrinogen levels has been observed in hyperlipemic patients. However, a decreased thrombotic tendency during pravastatin therapy has been reported by Lacoste et al.32

Our observation that changes of the angiographic parameters are related to the β-fibrinogen gene −455G/A genotype but not to the plasma fibrinogen levels seems to be conflicting. One possible explanation may be the assay we used to determine the fibrinogen levels, which determined the high- and low-molecular-weight fractions, about 95% of the total fibrinogen. The relation between cardiovascular risk and fibrinogen high- and low-molecular-weight fractions has not been studied before, but high-molecular-weight is the fibrinogen form with the highest clotting rate and therefore potentially the most dangerous form.

Another possible explanation may be that the regulatory mechanism of the fibrinogen levels is more important than the actual levels for progression of the cardiovascular disease. We hypothesize that patients with the −455AA genotype will have a greater increase of their fibrinogen levels in an acute-phase situation, and this elevated fibrinogen may then contribute to the disease. Several observations support the possibility that there may indeed be a greater increase of fibrinogen synthesis in acute-phase situations in subjects with the −455AA allele than in those with the −455G allele. First, smoking induces an acute-phase reaction, as in smokers, the levels of inflammatory markers (fibrinogen, C-reactive protein, interleukin-6) are increased. Several studies have shown that the relation between fibrinogen β-gene polymorphisms and fibrinogen levels is stronger in smokers than in nonsmokers, suggesting
that the acute-phase increase of fibrinogen is strongest in carriers of the $-455A$ allele.\textsuperscript{20,24} Also, in this study, in patients with the $-455AA$ genotype, fibrinogen levels were higher in smokers than in nonsmokers; however, the number of patients in this subgroup was very small. Second, Montgomery et al\textsuperscript{13} recently reported that the fibrinogen increase after strenuous exercise was strongest in men with the $-455A$ allele, again suggesting an interaction between the $-455A$ allele and low-grade acute-phase synthesis of fibrinogen. Furthermore, the $-455G/A$ polymorphism is in complete linkage disequilibrium with the $-148C/T$ polymorphism, which is located adjacent to the interleukin-6 responsive element of the fibrinogen $\beta$-gene promoter.\textsuperscript{18} It has been reported\textsuperscript{30,34} that the binding of nuclear proteins to the $-148C$ and $-148T$ alleles is different, which suggests an effect on the interleukin-6--induced fibrinogen synthesis.

The resulting higher fibrinogen levels after synthesis stimulation may result in more rapid progression of the disease in the patients with the $-455AA$ genotype as a direct result of the deleterious role of fibrinogen in the pathogenesis of atherosclerotic vascular disease. Higher fibrinogen levels result in a hypercoagulable state, eg, through the effects of fibrinogen on platelet aggregation\textsuperscript{13} and thrombus size,\textsuperscript{14} and fibrinogen may also contribute to the disease by its role in several pathogenic processes, such as proliferation and migration of smooth muscle cells\textsuperscript{11,12} and growth of atherosclerotic lesions.\textsuperscript{15}

We assume that the genetic information is a better long-term predictor of risk during putative episodes of acute-phase reaction in the follow-up period. The importance of inflammatory episodes in the prognosis of cardiovascular disease has already been described for patients with unstable angina pectoris\textsuperscript{35} and for a group with stable and unstable angina pectoris in the ECAT Angina Pectoris study.\textsuperscript{6,36} Our results support the significance of inflammatory processes.

The above-mentioned hypothetical framework may account for our observations and can form the basis for specific studies to verify our hypothesis. Such verifications will include a longitudinal study on the acute-phase response of plasma fibrinogen levels in patients with different genotypes and the relation to the progression of CAD. We suggest that the $-455G/A$ polymorphism is a potential genetic marker to identify the progression of the disease in patients and therefore may form the basis for future more intensive treatment in a subgroup.

### Acknowledgements

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