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Common Genetic Polymorphisms and Haplotypes of Fibrinogen Alpha, Beta, and Gamma Chains Affect Fibrinogen Levels and the Response to Proinflammatory Stimulation in Myocardial Infarction Survivors

The AIRGENE Study

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Objectives This study was designed to investigate whether single nucleotide polymorphisms (SNPs) and haplotypes of the fibrinogen gene-cluster (fibrinogen chains alpha [FGA], beta [FGB], and gamma [FGG]) could explain the inter- and intraindividual variability of fibrinogen levels in patients with atherosclerosis. We also searched for genetic determinants affecting the responses of fibrinogen genes to proinflammatory stimulation.

Background The mechanisms regulating fibrinogen levels are not fully understood, and they are likely to be regulated by complex gene–environment interactions.

Methods In the AIRGENE study, 895 survivors of myocardial infarction from 5 European cities were followed prospectively for 6 to 8 months, and plasma fibrinogen, interleukin (IL)-6, and C-reactive protein levels were determined monthly. We analyzed 21 SNPs and the corresponding haplotypes in the 3 fibrinogen genes.

Results Eight SNPs in FGA and FGB were significantly associated with fibrinogen levels. Similarly, 2 different haplotypes in FGA and 3 in FGB were also associated with mean fibrinogen levels. The IL-6 levels had a significant impact on the associations between SNPs/haplotypes in FGA/FGB and fibrinogen levels. We also identified SNPs and haplotypes in FGA and FGB with strong impact on the intraindividual variability of fibrinogen during the follow-up period.

Conclusions We identified common SNPs and haplotypes on FGA/FGB genes, explaining the interindividual and intraindividual variability of fibrinogen levels, in patients with a history of myocardial infarction. We have also identified for the first time, SNPs/haplotypes on FGA/FGB whose effects on fibrinogen expression are modified by the underlying IL-6 levels. These findings may have an impact on risk stratification and the design of genetically guided therapeutic approaches in patients with advanced atherosclerosis. (J Am Coll Cardiol 2008;52:941–52)

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Abbreviations and Acronyms

CRP = C-reactive protein
FGA = fibrinogen chain alpha
FGB = fibrinogen chain beta
FGG = fibrinogen chain gamma
IL = interleukin
LD = linkage disequilibrium
MI = myocardial infarction
SNP = single nucleotide polymorphism

Local and systemic low-grade inflammation represent a key feature in atherogenesis, and increased plasma levels of inflammatory biomarkers such as fibrinogen have been associated with increased cardiovascular risk (1). Further to its role as a nonspecific marker of inflammation, fibrinogen may also have a direct role in atherogenesis and thrombogenesis by acting as a bridging molecule for many types of cell-cell adhesion events critical for atherogenesis (2). In addition, it seems to be chemotactic for smooth muscle cells (3) and to affect the stability of atheromatous plaque (4). Fibrinogen plays a central role in the coagulation cascade with a critical impact on the formation of fibrin clots following the rupture of an atherosclerotic plaque (5).

Plasma fibrinogen appears to have high variability in the general population, because in addition to its seasonal variations (6), its expression is affected by the presence of mild inflammatory stimuli induced by smoking (7), aging or severe atherosclerosis (1). However, increasing evidence suggests that ≈50% of the total variability in fibrinogen levels is determined by genetic factors (8). Several single nucleotide polymorphisms (SNPs) have been identified in the genes encoding the 3 fibrinogen chains alpha, beta, and gamma (FGA, FGB, and FGG genes, respectively). Because the synthesis of the beta chain is rate-limiting for fibrinogen's synthesis in vitro (9), recent research has focused mainly on the genetic variability of FGB. Some SNPs in FGB have been associated with variation in plasma fibrinogen levels (10,11). Although sequences responsive to interleukin (IL)-6 (which is the main mediator of acute phase-induced fibrinogen synthesis) are present in the promoter regions of the genes encoding all 3 fibrinogen chains, recent evidence suggests that SNPs on the beta chain have a major regulatory function in fibrinogen synthesis in response to IL-6 (12). This genetically determined sensitivity of fibrinogen expression to IL-6 may also explain previous reports that specific SNPs on fibrinogen genes affect plasma fibrinogen levels only in subgroups with higher IL-6 levels such as smokers (13), or in subjects with an enhanced acute phase response as determined by high C-reactive protein (CRP) levels (14).

In the present study, we hypothesized that SNPs and haplotypes of the fibrinogen genes FGA, FGB, and FGG may affect mean plasma fibrinogen levels in patients with a history of myocardial infarction (MI). We also examined whether these SNPs and the haplotypes defined by them modify the complex relationship between fibrinogen, IL-6, and CRP in this population.

Methods

Study population and protocol. From a total cohort of 1,003 patients with a history of MI participating in the AIRGENE study (15), 895 had suitable blood samples for the present study. The patients were recruited from 5 European cities: Augsburg (Germany), Barcelona (Spain), Helsinki (Finland), Rome (Italy), and Stockholm (Sweden). Details of the design of the study are given elsewhere (15). Subjects were recruited through hospital- (Helsinki, Rome) or population-based registries of patients with MI (Augsburg, Barcelona, Stockholm). The inclusion criteria were survival after MI 3 months to 6 years before entry into the study and age between 35 and 80 years. Because of the rapid changes in the medical management of MI survivors during the last few years, the limit of 6 years after MI was used to increase study group homogeneity. Exclusion criteria were MI or interventional coronary procedure (percutaneous transluminal coronary angioplasty, coronary artery bypass graft) less than 3 months before the beginning of the study; residence outside the defined study area; an extended period of absence from the study area planned during the study period; a major illness that would prevent compliance with the study protocol; and chronic inflammatory diseases and/or anti-inflammatory medication (that might modify the biomarkers considered in the study). Further, samples from visits where the patient indicated having had an infection, surgery, or a major dental intervention in the 3 days before the visit were excluded and subsequently subjects with <2 remaining valid blood samples were completely excluded from the study. Other cardiovascular morbidities, such as heart failure, were not exclusion criteria.

The fieldwork consisted of a baseline visit, where an extensive questionnaire to characterize the patient was administered; blood pressure was measured, the body mass index determined, a 12-lead resting electrocardiogram was recorded; and a venous blood sample was drawn to measure blood count, total cholesterol, high density lipoprotein cholesterol and glycosylated hemoglobin HbA1c, and inflammatory markers (fibrinogen, IL-6, and CRP) and to extract deoxyribonucleic acid. Patients were followed up prospectively for an average of 6 monthly visits. At each monthly visit, the inflammatory markers were measured and a short questionnaire was completed to characterize the 24 h previous to the visit in terms of time spent in traffic, amount of exercise, tea and alcohol consumption, smoking, and exposure to environmental tobacco smoke, to detect an acute infection and to record changes in medication.

Biochemical measurements. Blood samples were stored at 4°C until centrifugation. The resulting plasma samples were stored at −80°C until shipped to the central laboratory in Ulm, Germany, where high-sensitivity CRP and fibrinogen were analyzed by immunonephelometry on a BNII analyzer (Dade Behring Marburg GmbH, Marburg, Germany, for both CRP and fibrinogen). For IL-6, a high-sensitivity

colorimetric sandwich enzyme-linked immunoadsorbent assay (R&D Systems GmbH, Abingdon, United Kingdom) was used. For quality control, 102 fibrinogen duplicates were obtained; the mean of the coefficients of variation in the duplicates was 6.1% (from 2.5% in Augsburg to 11.2% in Rome). The interassay coefficient of variation was 3.6% at 2.35 g/l.

Genotyping. Out of a total of 134 SNPs genotyped for the AIRGENE study, 27 SNPs located in the genes FGA, FGB, and FGG were investigated for the present study. We selected all up-to-then known haplotype tagging SNPs, possibly functional SNPs, as well as DNA variants showing an association in other studies with cardiovascular disease or related phenotypes. For SNP selection, the National Centre for Biotechnology SNP database dbSNP Build 124, based on NCBI Human Genome Build 35.1 (16), the Innate Immunity Program for Genomic Applications database (17), and the SeattleSNPs Program for Genomic Applications (18) were used.

Genotyping was carried out by using the MassARRAY system (Sequenom, San Diego, California) (19). All reactions (PCR amplification, base extension) were carried out in a Tetrad PCR thermal cycler (MJ Research, St. Bruno, Quebec).

Eleven SNPs were genotyped in FGA, 8 in FGB, and 8 in FGG. For 2 SNPs (1 in FGB and 1 in FGG), no assay could be established or the assay did not provide valid results. Two SNPs (1 in FGA and 1 in FGG) were monomorphic in the study population and 2 (both in FGA) had a minor allele frequency below 1%. Finally, 21 fibrinogen gene SNPs were taken into the statistical analyses (rs2070022, rs6050, rs2070018, rs2070016, rs2070014, rs2070011, rs10012555, and rs2070006 in FGA; rs1800791, rs1800790, rs1800788, rs2227399, rs6056, rs4220, and rs2227421 in FGB; and rs2066865, rs1049636, rs2066861, rs2066860, rs1800792, and rs2066854 in FGG). The average success rate for these 21 SNPs in the 3 genes was 93%.

For quality control, sex determination was performed for all samples by amplification of a partial sequence of the amelogenin gene (AMELX) and thus validated genotyping assays. No samples had to be excluded for this analysis.

Negative controls were included on all genotyping plates. For 30% of samples randomly selected and genotyped in duplicate, the replication rate was 99.82%. Each SNP was tested for departures from Hardy-Weinberg equilibrium by means of a chi-square test or Fisher exact test depending on allele frequency. None of the SNPs in the 3 selected genes deviated significantly from Hardy-Weinberg equilibrium.

Estimation of haplotypes. The genewise regions appropriate for haplotype estimation and haplotype association analysis were identified through linkage disequilibrium (LD) analysis. Haplotype blocks were defined as regions with Lewontin's D' >0.8 between consecutive SNPs. Only individuals with complete SNP information were included in the haplotype estimation. To reduce the number of subjects with missing genotypes, highly correlated SNPs were used to impute missing values. In each group of highly correlated SNPs ($r^2 > 0.95$), missing values in the SNP with the highest call rate were imputed from SNPs with lower call rates, which subsequently were excluded from further analysis. In FGA, rs2070022 was complemented using information from rs2070014. In FGB, rs6056 was filled up using rs4220, and missing values in rs2227399 were imputed based on rs1800790. For FGG, a group of 3 highly correlated SNPs was identified (rs2066861, rs2066865, rs2066854) with rs2066861 having the highest call rate. The updated versions of the SNPs rs6056, rs2227399, and rs2066861 entered haplotype estimation, but the 5 SNPs that were used for imputing missing values had to be excluded.

Haplotype estimation was performed for each gene using the expectation-maximization algorithm presented by Schaid et al. (20) with SNPs entered in gene-specific reading direction. To avoid bias resulting from genetic variation between populations, haplotypes were estimated for each center separately. Analysis of individually estimated haplotypes identified only 1 individual with a most probable haplotype pair in gene FGG having a probability of 0.74. All other estimated most probable haplotype pairs in all 3 genes exceeded a probability of 0.94, with an overall ambiguity rate of 8.9%. Additional information obtained through alternative haplotype pairs, therefore, was assumed to be small and only the individually most probable pair of haplotypes was modeled in association analysis.

Statistical analysis. Statistical analysis was performed in 3 steps. In the first step, we identified the nongenetic variables associated with fibrinogen levels; then we identified the SNPs associated with the fibrinogen levels; and finally we created a final model including the SNPs and the variables that fulfilled the confounder definition. This procedure was first done using all the centers and then city by city. Covariates were selected from a list of potential confounders as those found to change the estimated SNP effect by more than 10%. Time invariant (from the baseline questionnaire) as well as time variant (from the short questionnaire) variables with potential effects on fibrinogen levels were entered into the model one by one based on the criterion of showing the minimum Akaike information criterion value. Final adjustment was made for city, gender, age, body mass index, HbA1c, and smoothed trend.

Power calculations suggested that the study sample was able to detect a 16% difference in fibrinogen levels between genotypes, at a genotype frequency of 15% and alpha = 0.05 with a power of 80%.

As the outcome variable, we used the logarithm of the measured fibrinogen values to normalize its distribution. This implies that effects estimated can be expressed as percentage change of the geometric mean in the plasma fibrinogen levels.

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We used mixed effects models with random patient intercepts to account for the serial correlation among repeated measurements within the framework of additive mixed models to allow for nonparametric exposure-response functions (21).

Besides the 21 fibrinogen SNPs, we also tested 6 IL-6 and 8 CRP SNPs. For each SNP, we both calculated the effect only adjusting for city and applied the confounder model to obtain an adjusted effect on the plasma fibrinogen level. For association analyses between the different SNPs and the fibrinogen levels, the original significance level of alpha = 0.05 was corrected for multiple testing following the Sidak method. The number of tests was calculated as the effective number of independent loci among the SNPs analyzed according to Li and Ji (22). Finally, we constructed a mutually adjusted model with SNPs associated to fibrinogen levels in the univariate analysis. In the multiple adjusted model, we included one by one based on the criterion of lowest Akaike information criterion, the SNPs showing a significant association in the previous models; furthermore, we excluded any SNP(s) having a high LD (r^2 >0.8) with the last SNP introduced in the model to avoid collinearity. The common homozygote genotype of each SNP was used as reference. The same confounder model was used to estimate the association between haplotypes and fibrinogen levels. As SNP analyses indicate a generally present additive effect, an additive effect is assumed in haplotype association analysis. Therefore, haplotypes were coded as count variables with the most frequent haplotype of each gene as reference.

For haplotype association analysis, only haplotypes with estimated frequency of at least 0.05 in any center were coded as single variables. All other haplotypes were pooled in a group of rare haplotypes unless this group turned out to be significant. It turned out to be the case for a rare haplotype in FGB gene, and we extracted the most frequent haplotype from this group for separate analysis.

We also investigated interactions between the significant SNPs of the fibrinogen genes and IL-6 or CRP levels in affecting fibrinogen levels. We tested interactions using continuous IL-6 and CRP, and we stratified them by quartiles to obtain the estimations. The same procedure was followed for interactions with haplotypes. Finally, we analyzed the intraindividual variability in fibrinogen levels related to each SNP by using the intraindividual standard deviations of our repeated measurements of the logarithmically transformed fibrinogen in each individual as the outcome variable, adjusting for individual mean logarithmically transformed fibrinogen and the final set of confounder variables described earlier.

Results

Demographic characteristics and fibrinogen levels. The demographic characteristics of the participants are presented in Table 1. To minimize the variability of fibrinogen levels, we performed serial measurements for each patient, across a period of 6 to 8 months (the average number of monthly visits per subject for the participating centers was approximately 6 except in Barcelona where it was around 7). The median fibrinogen level in the overall study population was 3.5 g/l; however, there was a significant difference in fibrinogen levels between patients from different geographical areas (lowest in Rome [median: 3.0 g/l] and highest in Barcelona [median: 4.1 g/l]). City of residence was the strongest predictor of fibrinogen levels in our population, despite the fact that 99.7% of the study participants were Caucasians.

Table 1 Characteristics of the Study Population*

Number of participants, n	
All cities	895
Augsburg	200
Barcelona	169
Helsinki	195
Rome	134
Stockholm	197
Number of visits, mean [range]	
All cities	6.2 [2–8]
Augsburg	5.9 [2–7]
Barcelona	7.0 [2–8]
Helsinki	6.0 [2–6]
Rome	5.9 [2–8]
Stockholm	6.0 [2–8]
Male gender, n (%)	694 (77.5)
Diabetes, n (%)†	175 (19.6)
Respiratory disease, n (%)†‡	101 (11.3)
Family history of MI, n (%)	129 (14.4)
Hypertension, n (%)†	452 (50.5)
Heart failure, n (%)	98 (11.0)
Ever smokers, n (%)§	603 (67.4)
Age (yrs)	63.1 ± 9.0
Body mass index (kg/m^2)	28.3 ± 4.2
Smoking (pack-yrs)	18.6 ± 23.7
Glycosylated hemoglobin (%)‖	5.4 ± 1.0
Serum cholesterol (mg/dl)‖	183.3 ± 39.0
Alcohol intake per day (g)	14.4 ± 19.9
Systolic blood pressure (mm Hg)‖	134.1 ± 21.5
Inflammatory markers (median [range] in 5,327 person-visits)	
Fibrinogen (g/l)	3.49 [3.0–4.1]
C-reactive protein (mg/l)	1.27 [0.7–2.8]
Interleukin-6 (pg/ml)	2.23 [1.6–3.3]
Hour of visit¶	12 [7–19]
Trend#	240 [1–423]

*Values are expressed as mean ± standard deviation, unless stated otherwise. †Self-reported through the baseline questionnaire and defined as ever diagnosed by a physician. ‡Includes asthma, chronic bronchitis, and emphysema. §Includes ex-smokers, occasional smokers, and active smokers. ‖Includes treatment. ¶Hour of visit makes reference to the hour of the day when the blood was withdrawn. #Trend makes reference to the number of days elapsed between the date of the first visit of the full study (May 19, 2003, in Augsburg) and the date of the corresponding visit for any subject in any city.
MI = myocardial infarction.

Genotype analysis. Analysis of LD for the 21 SNPs in the 3 fibrinogen genes (FGA, FGB, and FGG on chromosome

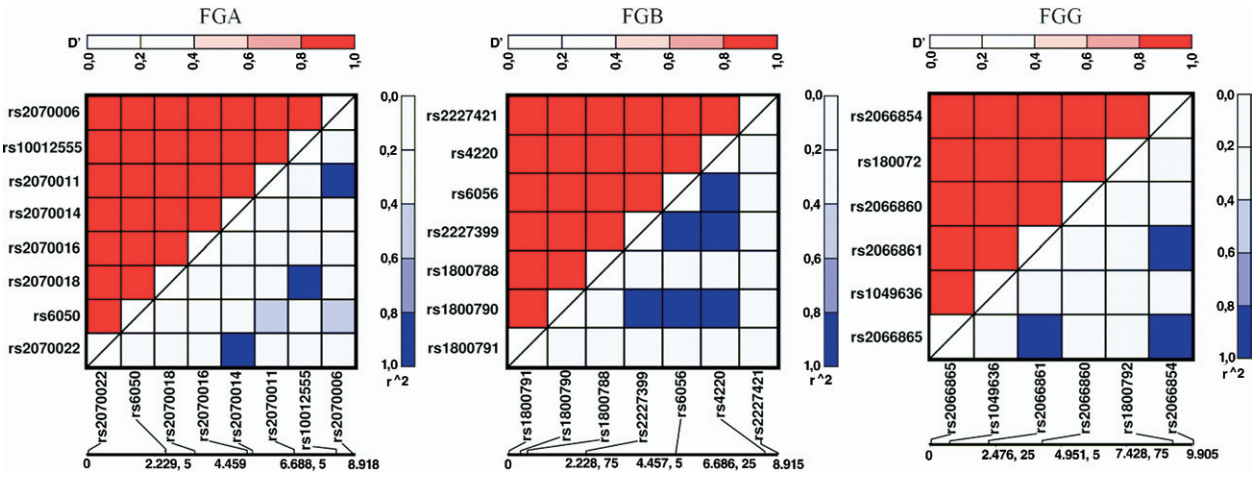

Figure 1 **LD Maps for FGA, FGB, and FGG**

Linkage disequilibrium (LD) maps for the 3 fibrinogen chain genes: fibrinogen chain alpha (FGA), beta (FGB), and gamma (FGG). D' **(red)** and r^2 **(blue)** measures of LD are shown.

4) indicated that for each gene all SNPs are in 1 haplotype block (Fig. 1). In addition, high correlation (r^2 >0.8) was observed for 3 separate pairs of SNPs in FGA, a group of 6 SNPs in FGB, and a group of 3 SNPs in FGG. The frequencies of these SNPs and the respective haplotypes in the study population are presented in Tables 2 and 3, respectively. The allele frequencies and genotype distribution were similar to those found in a general population from Augsburg (data not shown).

There was no significant effect of any of the examined SNPs and haplotypes on baseline clinical status, as evaluated by the presence of angina pectoris, arrhythmias, or heart failure (p = NS for all associations after taking into account the p value adjusted for multiple comparisons).

Associations between SNPs and fibrinogen levels. Eight SNPs (out of 21 included in the study) were significantly associated with plasma fibrinogen levels in the analyses that included 1 SNP at a time (Table 2): 1) for FGA: rs2070011, rs2070006, rs2070022, rs2070014; and 2) for FGB: rs1800790, rs2227399, rs6056, rs4220.

Four of these SNPs held the calculated significance level corrected for multiple testing (alpha = 0.0026): 1) for FGA: rs2070011; and 2) for FGB: rs1800790, rs2227399, rs6056. Two SNPs (FGA rs2070011 and FGB rs1800790s) remained in the mutually adjusted model. FGB rs1800790s explained 1.9% of the variability in the fibrinogen concentration and FGA rs2070011 explained 1.2%, in the crude bivariate model. When both SNPs were included in the same model, 3.4% of the variability in the fibrinogen concentration was explained by the genetic variants, and 31.37% of the variability was explained by the nongenetic covariates.

None of the tested SNPs in the FGG, IL-6, or CRP genes showed a significant association with fibrinogen levels (data not shown).

Associations between haplotypes and fibrinogen levels. We found that 1 haplotype of the FGA gene, TTTAACG (defined by the minor allele in rs6050 as described in Table 3), showed significant association with fibrinogen levels. In addition, interestingly, 1 haplotype of FGA (CTCGATG), defined by SNP rs2070016, was also associated with fibrinogen levels, although this SNP was not significantly associated in the SNP analyses.

We also observed that 3 haplotypes in the FGB gene were associated with plasma fibrinogen levels. Two were defined only by SNPs associated with fibrinogen levels (Table 2): GCGTA was the only haplotype with the minor allele in rs6056, and GCGCA was defined by the allelic combination in both SNPs (rs2227399 and rs6056). The third haplotype (GTTCA) associated with fibrinogen levels was mainly defined by 1 SNP not associated with fibrinogen levels in single SNP analyses (rs1800788). The distinction between this haplotype and the rare variant ATTCA (frequency 0.1%) can be made in rs1800791.

None of the haplotypes in the FGG gene showed a significant association with fibrinogen levels as in the SNPs analyses.

Effect of SNPs and haplotypes on the response to low-grade inflammation. Individual means of plasma fibrinogen levels in the overall population were significantly correlated with both IL-6 (r = 0.40, 95% confidence interval [CI]: 0.38 to 0.42) and CRP mean levels (r = 0.53, 95% CI: 0.51 to 0.55). To test whether the examined SNPs/haplotypes modify the response of fibrinogen genes

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Table 2 Association Between SNPs in the FGA and FGB Genes and Plasma Fibrinogen Levels

		Crude (Adjusted Only for City)		Adjusted for Identified Confounders*	
	Frequency, %	% Change (95% CI)	p Value	% Change (95% CI)	p Value
fgb_rs1800790					
AA	4.0	13.5 (5.83 to 21.81)	**0.0004**	11.5 (4.44 to 19.10)	**0.0010**
GA	31.9	3.5 (0.67 to 6.37)	0.0149	3.8 (1.23 to 6.46)	0.0040
GG	64.1	0 (ref.)	***0.0003***	0 (ref.)	***<0.0001***
fgb_rs2227399					
GG	4.1	12.7 (4.87 to 21.05)	**0.0011**	10.8 (3.60 to 18.44)	0.0030
GT	32.2	3.6 (0.79 to 6.50)	0.0117	3.9 (1.34 to 6.56)	0.0030
TT	63.7	0 (ref.)	***0.0005***	0 (ref.)	***<0.0001***
fgb_rs6056					
TT	3.0	13.6 (4.83 to 23.13)	**0.0019**	10.8 (2.98 to 19.32)	0.0060
CT	30.6	3.1 (0.28 to 6.05)	0.0311	3.6 (0.96 to 6.27)	0.0070
CC	66.4	0 (ref.)	***0.0015***	0 (ref.)	***0.0010***
fgb_rs4220					
AA	2.9	13.0 (4.04 to 22.75)	0.0038	10.8 (2.73 to 19.49)	0.0080
GA	30.7	3.2 (0.38 to 6.15)	0.0262	3.6 (1.00 to 6.31)	0.0070
GG	66.4	0 (ref.)	***0.0023***	0 (ref.)	***0.0010***
fga_rs2070011					
TT	15.9	5.3 (1.35 to 9.31)	0.0080	5.9 (2.23 to 9.60)	**0.0010**
CT	46.2	0.3 (−2.44 to 3.14)	0.8274	0.4 (−2.16 to 2.94)	0.7820
CC	37.9	0 (ref.)	*0.0194*	0 (ref.)	*0.0030*
fga_rs2070006					
TT	15.7	4.6 (0.77 to 8.63)	0.0184	5.4 (1.83 to 9.12)	0.0030
CT	47.2	0.3 (−2.43 to 3.18)	0.8162	0.4 (−2.12 to 3.01)	0.7530
CC	37.1	0 (ref.)	*0.0451*	0 (ref.)	*0.0070*
fga_rs2070022					
AA	3.2	−4.9 (−11.36 to 2.09)	0.1660	−6.0 (−11.88 to 0.22)	0.0590
GA	28.0	−3.7 (−6.40 to −0.85)	0.0111	−3.7 (−6.24 to −1.19)	0.0040
GG	68.8	0 (ref.)	*0.0221*	0 (ref.)	*0.0050*
fga_rs2070014					
TT	3.2	−4.8 (−11.33 to 2.13)	0.1693	−6.0 (−11.86 to 0.25)	0.0600
CT	27.3	−3.6 (−6.31 to −0.74)	0.0137	−3.7 (−6.20 to −1.13)	0.0050
CC	69.6	0 (ref.)	*0.0266*	0 (ref.)	*0.0060*
fga_rs2070016					
GG	2.0	12.3 (2.58 to 23.04)	0.0121	8.7 (0.06 to 18.16)	0.0485
AG	25.6	0.5 (−2.36 to 3.53)	0.7192	1.7 (−0.97 to 4.50)	0.2113
AA	72.4	0 (ref.)	*0.0424*	0 (ref.)	*0.0777*
fgg_rs2066860					
TT	0.1	−11.4 (−37.25 to 25.24)	0.4944	−5.2 (−30.76 to 29.75)	0.7382
CT	7.1	−2.2 (−6.79 to 2.57)	0.3571	−3.0 (−7.17 to 1.32)	0.1701
CC	92.7	0 (ref.)	*0.5225*	0 (ref.)	*0.3713*
fgg_rs2066854					
AA	6.3	5.2 (−0.15 to 10.89)	0.0568	6.0 (0.93 to 11.23)	0.0196
AT	36.6	1.2 (−1.50 to 4.02)	0.3835	1.4 (−1.11 to 3.95)	0.2788
TT	57.1	0 (ref.)	*0.1444*	0 (ref.)	*0.0539*
fgb_rs1800791					
AA	2.8	−3.6 (−10.78 to 4.05)	0.3430	−4.2 (−10.66 to 2.74)	0.2295
GA	25.5	−3.4 (−6.23 to −0.53)	0.0206	−4.1 (−6.61 to −1.46)	0.0025
GG	71.7	0 (ref.)	*0.0534*	0 (ref.)	*0.0069*
fgb_rs2227421					
CC	9.0	−1.6 (−6.14 to 3.06)	0.4860	−2.2 (−6.33 to 2.01)	0.2963
AC	45.2	−1.8 (−4.45 to 0.85)	0.1788	−1.5 (−3.89 to 0.98)	0.2354
AA	45.9	0 (ref.)	*0.3886*	0 (ref.)	*0.3846*

Continued on next page

Table 2 Continued

		Crude (Adjusted Only for City)		Adjusted for Identified Confounders*	
	Frequency, %	% Change (95% CI)	p Value	% Change (95% CI)	p Value
fgg_rs2066861					
TT	6.1	4.9 (−0.54 to 10.54)	0.0789	5.9 (0.81 to 11.21)	0.0225
CT	36.4	1.2 (−1.51 to 4.02)	0.3847	1.4 (−1.08 to 3.98)	0.2679
CC	57.6	0 (ref.)	*0.1860*	0 (ref.)	*0.0589*
fgg_rs2066865					
AA	6.2	4.8 (−0.61 to 10.48)	0.0831	5.8 (0.76 to 11.15)	0.0238
GA	36.4	1.1 (−1.67 to 3.85)	0.4529	1.3 (−1.22 to 3.83)	0.3188
GG	57.4	0 (ref.)	*0.2048*	0 (ref.)	*0.0662*
fga_rs6050					
CC	7.7	3.5 (−1.42 to 8.59)	0.1670	4.5 (−0.07 to 9.27)	0.0538
TC	37.3	2.1 (−0.64 to 4.92)	0.1342	2.0 (−0.48 to 4.61)	0.1132
TT	55.0	0 (ref.)	*0.1850*	0 (ref.)	*0.0779*
fgb_rs1800788					
TT	5.2	3.8 (−2.05 to 9.92)	0.2092	5.5 (0.09 to 11.29)	0.0462
CT	32.6	0.0 (−2.78 to 2.77)	0.9763	−0.3 (−2.83 to 2.24)	0.7993
CC	62.2	0 (ref.)	*0.4396*	0 (ref.)	*0.1134*
fga_rs2070018					
GG	2.1	1.8 (−6.75 to 11.17)	0.6877	−0.7 (−8.39 to 7.59)	0.8598
AG	21.1	1.3 (−1.85 to 4.45)	0.4326	2.1 (−0.73 to 5.09)	0.1458
AA	76.8	0 (ref.)	*0.6928*	0 (ref.)	*0.3328*
fga_rs10012555					
CC	1.8	2.2 (−6.62 to 11.94)	0.6323	−0.9 (−8.83 to 7.62)	0.8224
TC	21.6	1.1 (−1.99 to 4.33)	0.4838	2.1 (−0.79 to 5.06)	0.1562
TT	76.6	0 (ref.)	*0.7143*	0 (ref.)	*0.3462*
fgg_rs1049636					
GG	9.9	−2.9 (−7.10 to 1.54)	0.1983	−4.2 (−7.98 to −0.19)	0.0402
AG	41.4	−2.0 (−4.61 to 0.66)	0.1390	−1.4 (−3.78 to 1.09)	0.2720
AA	48.7	0 (ref.)	*0.2214*	0 (ref.)	*0.1054*
fgg_rs1800792					
CC	20.3	0.5 (−3.09 to 4.21)	0.7896	0.3 (−2.95 to 3.75)	0.8388
TC	48.6	−0.4 (−3.28 to 2.57)	0.7897	0.1 (−2.58 to 2.81)	0.9551
TT	31.2	0 (ref.)	*0.8694*	0 (ref.)	*0.9783*

Of the 21 tested single nucleotide polymorphisms (SNPs) in fibrinogen chains alpha (FGA), beta (FGB), and gamma (FGG), only the 8 with an Akaike information criterion lower than the null model (without any SNP), are shown. % change: percentage of change of fibrinogen geometric mean from the reference category. The p value is given for each genotype relative to the common homozygote reference. The *italic* p value in the reference row refers to the overall p value for both genotypes of the respective SNP. **Bold** p values remain statistically significant when considering the corrected significance level of alpha = 0.0026 for multiple comparisons. *Adjusted for city, gender, age, body mass index, glycosylated hemoglobin HbA1c (%), and smoothed trend.
CI = confidence interval.

to proinflammatory stimuli, we first examined whether plasma levels of IL-6 and CRP had any impact on the relationship between SNPs and fibrinogen levels (Table 4). Indeed, there was a significant interaction between IL-6 levels and the association of rs2070011 in FGA with fibrinogen levels and a borderline interaction between CRP levels and the association of the same SNP with fibrinogen levels. Interestingly, the haplotype effect patterns of all 3 fibrinogen genes showed significant heterogeneity across quartiles of IL-6 levels, indicating effect modification at the haplotype level. The IL-6 levels appeared particularly to significantly modify the association between fibrinogen levels and 1 haplotype of FGA, 2 haplotypes of FGB (1 significantly and 1 borderline), and 2 haplotypes of FGG (both borderline) (Table 5), none of which showed significant overall association with fibrinogen levels (Table 3).

Effect of SNPs and haplotypes on intraindividual variability of plasma fibrinogen. As fibrinogen levels appear to have high intraindividual variability across time, we then examined whether any of the examined SNPs and haplotypes have an effect on this intraindividual variability of plasma fibrinogen, in serial measurements from the same subjects across a 6- to 8-month period. The homozygote AA variant of SNP rs2227421 in the FGB gene was positively associated with the intraindividual standard deviation of logarithmically transformed fibrinogen levels (coefficient: 0.015, 95% CI: 0.002 to 0.029), and the SNP rs2070006 in FGA showed a borderline negative association, for both variants (heterozygote: coefficient: −0.008, 95% CI: −0.016 to 0.001; rare homozygote variant GG: coefficient: −0.023, 95% CI: −0.049 to 0.003). One FGA haplotype, CTCGATG (which was associated

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Table 3 Association Between FGA, FGB, and FGG Haplotypes and Plasma Fibrinogen Levels*

	Haplotype Frequency (%)	% Change (95% CI) of Fibrinogen Levels Versus Reference	p Value
FGA†			
CTCAATG	29.3	0 (ref.)	*<0.001*
TTTAACG	25.8	2.9 (0.7 to 5.0)	0.008
CTCAATA	17.0	−1.7 (−4.1 to 0.7)	0.168
CTCGATG	14.5	3.3 (0.7 to 6.0)	0.014
TCTAGTG	12.1	2.5 (−0.2 to 5.2)	0.068
Rare		3.0 (−4.0 to 10.4)	0.418
FGB‡			
GCTCC	31.5	0 (ref.)	*<0.001*
GTTCA	21.5	2.2 (0.1 to 4.4)	0.039
GCGTA	18.4	4.1 (1.9 to 6.4)	<0.001
ACTCA	15.5	−1.3 (−3.5 to 1.0)	0.270
GCTCA	11.2	0.4 (−2.1 to 2.9)	0.780
GCGCA	1.9	7.9 (1.9 to 14.2)	0.010
ATTCA	0.1	15.4 (−14.8 to 56.3)	0.355
FGG§			
TCCA	40.4	0 (ref.)	*0.072*
TTCG	30.0	−1.0 (−2.7 to 0.8)	0.298
ATCA	24.4	1.4 (−0.6 to 3.4)	0.171
TCTA	3.6	−3.0 (−7.0 to 1.1)	0.148
Rare		−3.6 (−9.2 to 2.4)	0.237

Frequency: haplotype frequency in person-visits. % change: Percentage of change of fibrinogen geometric mean from the reference category. The *italic* p values in the reference row come from a likelihood ratio test of the model with all the haplotypes versus the null model (without any haplotype). *Adjusted for city, gender, age, body mass index, glycosylated hemoglobin HbA1c (%), and smoothed trend. †FGA: rs2070006, rs10012555, rs2070011, rs2070016, rs2070018, rs6050, rs2070022. ‡FGB: rs1800791, rs1800788, rs2227399, rs6056, rs2227421. §FGG: rs2066854, rs1800792, rs2066860, rs1049636.

Abbreviations as in Table 2.

Table 4 Association Between Mean Plasma Fibrinogen Levels and SNPs rs2070011 in FGA and rs1800790 in FGB, Stratified by Quartiles of Plasma IL-6 and CRP Levels*

	Q1		Q2		Q3		Q4		
	% Change	95% CI	% Change	95% CI	% Change	95% CI	% Change	95% CI	p Inter
IL-6									
rs1800790									
AA	12.3	(4.3 to 20.8)	11.8	(5.1 to 18.9)	10.0	(1.8 to 18.8)	10.1	(0.4 to 20.7)	0.805
GA	3.2	(0.5 to 6.1)	3.9	(1.1 to 6.8)	2.8	(−0.2 to 5.9)	3.0	(−0.8 to 6.9)	0.960
GG	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.950*
rs2070011									
TT	5.3	(1.4 to 9.5)	3.1	(−0.7 to 7.0)	5.3	(1.2 to 9.5)	6.4	(1.1 to 12.1)	0.079
CT	0.1	(−2.6 to 2.8)	0.3	(−2.4 to 3.2)	0.9	(−2.1 to 4.0)	1.7	(−2.0 to 5.6)	0.046
CC	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.003*
CRP									
rs1800790									
AA	11.8	(2.4 to 21.9)	9.4	(0.9 to 18.6)	8.1	(1.1 to 15.6)	4.6	(−2.8 to 12.6)	0.114
GA	3.0	(−0.0 to 6.1)	3.1	(0.3 to 6.0)	3.8	(0.9 to 6.9)	3.3	(−0.2 to 7.0)	0.940
GG	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.287*
rs2070011									
TT	3.6	(−0.6 to 8.0)	5.1	(1.3 to 9.2)	4.7	(0.7 to 8.9)	5.8	(0.9 to 10.9)	0.098
CT	0.2	(−2.7 to 3.2)	1.6	(−1.3 to 4.5)	0.2	(−2.8 to 3.2)	0.1	(−3.3 to 3.7)	0.442
CC	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.058*

% change: percentage of change of fibrinogen geometric mean from the reference category. p Inter: p value for interaction (from a model with the continuous variable for interleukin [IL]-6 or C-reactive protein [CRP] plasma level and the SNP). The *italic* p value in reference row comes from the likelihood ratio test between the model containing the interaction term versus the one without it. Q1 contains the values less than the first quartile, Q2 contains the values between the first and second quartile, and so on. *Adjusted for city, gender, age, body mass index, glycosylated hemoglobin HbA1c (%), and smoothed trend.

Abbreviations as in Table 2.

Table 5 Association Between Mean Plasma Fibrinogen Levels and FGA, FGB, and FGG Haplotypes, Stratified by Quartiles of Plasma IL-6 Levels*

	Q1 IL-6		Q2 IL-6		Q3 IL-6		Q4 IL-6		
	% Change	95% CI	% Change	95% CI	% Change	95% CI	% Change	95% CI	p Inter
FGA†									
CTCAATG	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.013*
TTTAACG	1.6	(−0.9 to 4.2)	2.3	(−0.4 to 4.9)	3.2	(0.5 to 6.0)	1.9	(−1.5 to 5.3)	0.180
CTCAATA	−1.0	(−3.8 to 1.8)	−0.6	(−3.6 to 2.5)	−0.7	(−3.8 to 2.6)	−3.0	(−6.7 to 0.8)	0.002
CTCGATG	3.4	(0.3 to 6.6)	2.7	(−0.4 to 5.8)	2.2	(−1.2 to 5.8)	2.8	(−1.5 to 7.3)	0.142
TCTAGTG	3.6	(0.3 to 7.0)	0.7	(−2.5 to 4.0)	1.0	(−2.3 to 4.4)	1.7	(−2.7 to 6.2)	0.778
Rare	7.7	(−1.3 to 17.5)	−3.3	(−11.1 to 5.1)	−1.9	(−9.7 to 6.7)	0.9	(−9.9 to 13.0)	0.762
FGB‡									
GCTCC	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.001*
GTTCA	2.0	(−0.6 to 4.6)	1.0	(−1.6 to 3.6)	2.4	(−0.3 to 5.1)	2.3	(−1.1 to 5.8)	0.654
GCGTA	4.5	(1.8 to 7.3)	3.7	(1.1 to 6.5)	2.6	(−0.4 to 5.7)	3.1	(−0.5 to 6.9)	0.218
ACTCA	−0.1	(−2.8 to 2.7)	−1.0	(−3.8 to 1.9)	−1.2	(−4.1 to 1.8)	−2.8	(−6.2 to 0.8)	0.001
GCTCA	3.1	(−0.1 to 6.3)	−1.8	(−4.8 to 1.3)	−1.0	(−4.0 to 2.2)	−0.2	(−4.3 to 4.0)	0.055
GCGCA	6.4	(−0.9 to 14.1)	7.3	(0.7 to 14.5)	8.9	(1.6 to 16.7)	6.0	(−3.1 to 16.0)	0.100
ATTCA	—		—		23.3	(−8.4 to 65.9)	9.5	(−22.7 to 55.0)	0.952
FGG§									
TCCA	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	0.0	(ref.)	*0.033*
TTCG	−0.8	(−3.0 to 1.5)	−0.9	(−3.1 to 1.3)	−1.2	(−3.5 to 1.1)	−2.3	(−5.0 to 0.5)	0.072
ATCA	0.3	(−2.1 to 2.8)	0.5	(−1.9 to 2.9)	1.4	(−1.1 to 3.9)	0.4	(−2.6 to 3.5)	0.960
TCTA	−0.6	(−5.2 to 4.2)	0.0	(−5.3 to 5.5)	−2.7	(−7.9 to 2.7)	−8.0	(−14.0 to −1.5)	0.084
Rare	−5.5	(−13.5 to 3.2)	−9.7	(−16.8 to −2.0)	−3.8	(−11.0 to 4.0)	−2.0	(−10.1 to 6.9)	0.011

%change: percentage of change of fibrinogen geometric mean from the reference. p Inter: p value for interaction (from a model with the continuous variable for IL-6 and the haplotypes). The italic p value in reference row comes from the likelihood ratio test between the model containing the interaction term versus the model without it. Q1 contains the values less than the first quartile, Q2 contains the values between the first and second quartile, and so on. *Adjusted for city, gender, age, body mass index, glycosylated hemoglobin HbA1c (%), and smoothed trend. †FGA: rs2070006, rs10012555, rs2070011, rs2070016, rs2070018, rs6050, rs2070022. ‡FGB: rs1800791, rs1800788, rs2227399, rs6056, rs2227421. §FGG: rs2066854, rs1800792, rs2066860, rs1049636.
Abbreviations as in Tables 2 and 4.

with plasma fibrinogen levels) (Table 3), was also negatively associated with the intraindividual standard deviation of logarithmically transformed fibrinogen levels (coefficient: −0.003, 95% CI: −0.020 to −0.002), and similarly 1 FGB haplotype, the GCGTA (also associated with fibrinogen levels) (Table 3), showed a negative association with the standard deviation of the logarithmically transformed fibrinogen level (coefficient: −0.007, 95% CI: −0.016 to 0.001).

Discussion

In the present study, we found that 8 SNPs in the FGA and FGB genes had significant associations with plasma fibrinogen levels when evaluated one by one. Furthermore, we show that specific haplotypes of FGA, FGB, and FGG appear to modify the expression of fibrinogen in response to proinflammatory stimuli such as IL-6, because their effects on fibrinogen levels became stronger in the presence of high IL-6 levels. We also provide evidence that SNPs and haplotypes on both FGA and FGB genes may be implicated in the normally observed intraindividual variability of fibrinogen levels.

It is now widely accepted that systemic low-grade inflammation is a key feature in atherogenesis (23), and evidence suggests that increased plasma levels of inflammatory biomarkers such as fibrinogen are associated with increased cardiovascular risk (1,23). It is therefore of importance to determine the genetic variables that affect the expression of fibrinogen cluster genes and their response to the underlying proinflammatory stimuli.

Genetic determinants of fibrinogen levels. The FGB chain synthesis is considered the rate-limiting factor for the production of fibrinogen (24), and SNPs on the FGB gene have been associated with fibrinogen levels (10,13,25), with G455A (FGB rs18000790) and C148T (FGB rs1800787), most widely studied (8,26). The FGB rs18000790 SNP is probably the most physiologically relevant, because the genetic variants defined by this SNP have distinct nuclear protein-binding properties (26). However, conflicting data (8,27) suggest that complex gene-environment interactions and especially the underlying inflammatory status or disease state could be important modifiers of this relationship (8).

In this study, we found that 4 SNPs in high mutual LD on the FGB gene affect fibrinogen levels, with FGB rs18000790 giving the strongest association. Indeed, the rs1800790 SNP is located in the promoter region of the FGB gene (11) and it seems to be a functional polymorphism, affecting the expression of FGB and hence the synthesis of fibrinogen in experimental models (12,23). On the other hand, we also found similar but slightly weaker

associations between fibrinogen levels and the other 3 FGB SNPs in this LD group: rs6056 (C4651T), rs4220 (C7589A), and rs2227399 (G1683T). Although 1 of these could also represent a true relationship, their association with fibrinogen levels may well be indirect through their high LD with the functional FGB rs18000790 SNP in our population.

We also observed that FGA SNPs rs2070011 (C58T, located in the promoter region) and the correlated rs2070006 (C2027T, located in the promoter region) as well as rs2070022 (A6892G, located in the untranslated region) and the correlated rs2070014 (C1374T located in an intronic area) were each individually associated with fibrinogen levels, with rs2070011 giving the strongest association.

More importantly, this is the first study demonstrating that specific haplotypes on fibrinogen cluster genes regulate plasma fibrinogen levels in MI survivors. Indeed, we made the novel observation that 3 haplotypes (1 haplotype in FGA and 2 in FGB) are strong determinants of fibrinogen levels, not entirely as expected from the SNP analyses, suggesting that haplotype analyses could provide an additional future option in evaluating the effects of genetic background on fibrinogen levels.

Other determinants of fibrinogen levels: gene-environment interactions. It is now widely known that fibrinogen levels vary across different ethnic groups, as a result of genetic differences (28), dietary modifiers and the overall lifestyle (29), with season of the year being an additional important modifier (6). In the present study, we observed that apart from some demographic characteristics known to influence plasma fibrinogen levels (e.g., age, gender, and body mass index) (30), city of residence was the strongest predictor. As 99.7% of study participants were Caucasians, it seems that "city" captures differences beyond ethnicity. In our study, city but not ethnicity was a strong determinant of both fibrinogen levels and the distribution of the examined SNPs/haplotypes. This finding suggests that factors beyond ethnicity (i.e., cultural and dietary habits, weather variations, pollution and many others) may affect fibrinogen levels and genetic differences of European populations are best described by geographical, linguistic, and cultural influences, which are best described by "city of stay" rather than "ethnic group," as previously reported (31).

By performing serial monthly measurements of fibrinogen levels in the same subjects, we were able: 1) to calculate more stable estimates of average individual fibrinogen levels, which is an advantage over most previous studies as it reduces measurement random errors; and 2) to examine the effect of genotype on the intraindividual variability of fibrinogen levels over time. We demonstrate for the first time that the intraindividual variability of plasma fibrinogen is associated with SNPs on the FGB (rs2227421) and possibly on FGA (rs2070016) genes, and more importantly with haplotypes of both FGA and FGB genes. These novel findings imply that the genetic background may be partly responsible for the high intraindividual variability of fibrinogen levels normally observed in patients with atherosclerosis (6,32).

Effects of proinflammatory stimuli on the association between SNPs/haplotypes and fibrinogen. Interleukin 6 is the main mediator of acute phase-induced fibrinogen synthesis, mainly because it acts on the promoter regions of the genes encoding all 3 fibrinogen chains (12). The SNPs in FGB (12) or FGA (27) may modify the response of these genes to IL-6 in vitro, but at a clinical level, preliminary data suggested that these SNPs affect plasma fibrinogen levels only in subgroups with higher IL-6/CRP levels such as healthy smokers (13,14). However, the possible effect of the haplotypes of FGA, FGB, and FGG on IL-6–induced fibrinogen expression has not previously been investigated.

In this study, we were able to show that SNP rs2070011 in FGA modified the association between IL-6 and fibrinogen levels. Somewhat surprisingly, there was no clear association between the rs18000790 SNP in FGB and the IL-6–induced fibrinogen expression.

In addition to the finding that several SNPs in different areas of the FGA, FGB, and FGG genes affect the response of these genes to IL-6, we also made the novel observation that haplotypes on these genes may have an even stronger effect. Indeed, we observed an effect modification of the IL-6 impact on fibrinogen levels, which is related mainly to 1 haplotype on FGA, 2 haplotypes on FGB, and 2 haplotypes on FGG. These novel findings suggest that the haplotype-based approach may be more accurate than individual SNPs to define complex associations between fibrinogen genes and variations of its plasma levels in response to proinflammatory stimuli.

Genetics of fibrinogen chains: clinical perspectives. As plasma fibrinogen is considered to be a risk factor for atherosclerosis (23), it is still unclear whether therapeutic strategies targeting fibrinogen levels may have the potential to modify cardiovascular risk. Treatments currently used against atherosclerosis (such as statins or angiotensin-converting enzyme inhibitors) improve clinical outcome in primary and secondary prevention (33), and they also decrease fibrinogen levels (23,34). However, it is unknown whether plasma fibrinogen is a reliable marker to follow the response to these treatments or whether it could ever be used as a therapeutic target itself. As fibrinogen appears to have seasonal variability, only serial measurements can provide useful information regarding the progression of the underlying proinflammatory status. A novel finding of the present study is the suggestion that haplotypes affect the response of fibrinogen genes to low-grade inflammation observed in MI survivors. These findings imply that multiple genetic variants in these genes are of importance and that the described haplotypes could modify the response of fibrinogen levels to anti-inflammatory treatments, such as statins or angiotensin-converting enzyme inhibitors. The present study was not designed to test any pharmacogenomic value

of these haplotypes, and prospective clinical trials should be designed to test such a hypothesis.

Study limitations. A limitation of the present study is the absence of any prospective data examining the effect of these SNPs/haplotypes on long-term clinical outcome. As the study was designed to test the effect of these genotypes on fibrinogen levels and variations throughout a period of 6 months, it was underpowered to detect any impact on clinical events during the follow-up period. In addition, the borderline, nonsignificant effects of these genotypes on clinical status of these patients at baseline suggests that further large-scale prospective clinical studies are required to demonstrate the effect of these SNPs and haplotypes on hard clinical end points.

Conclusions

In the present study, we demonstrate that genetic background, as defined by specific SNPs and haplotypes of the FGA, FGB, and FGG genes, represents a major determinant of the expression of fibrinogen in post-MI patients, modifies the response to proinflammatory stimuli, and possibly also modifies the intraindividual variability of fibrinogen levels. These findings may have important implications on risk stratification of patients with advanced atherosclerosis. Although it is still premature to suggest an immediate clinical application of these findings, the results of the present study may guide the design of genetically based approaches for secondary prevention in these patients in the future.

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APPENDIX

For a list of the AIRGENE study group partners, please see the online version of this article.