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ORIGINAL ARTICLE

Elevated plasma fibrinogen γ′ concentration is associated with myocardial infarction: effects of variation in fibrinogen genes and environmental factors

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Summary. *Background:* Fibrinogen γ′, a fibrinogen γ-chain variant generated via alternative mRNA processing, has been associated with susceptibility to thrombotic disease. *Objective:* The present case–control study searched for potential determinants of the plasma fibrinogen γ′ concentration and examined the relationship between this variant and risk of myocardial infarction (MI). *Patients and methods:* The Stockholm Coronary Artery Risk Factor study, comprising 387 postinfarction patients and 387 healthy individuals, was employed. The fibrinogen gamma (*FGG*) 9340T > C [rs1049636], fibrinogen alpha (*FGA*) 2224G > A [rs2070011] and fibrinogen beta (*FGB*) 1038G > A [rs1800791] polymorphisms were determined. The plasma fibrinogen γ′ concentration was measured by enzyme-linked immunosorbent assay. The multifactor dimensionality reduction method was used for interaction analyses on risk of MI. *Results:* The *FGG* 9340T > C and *FGA* 2224G > A polymorphisms, total plasma concentrations of fibrinogen, insulin and high-density lipoprotein, and gender appeared to be independent determinants of plasma fibrinogen γ′ concentration in patients, and the corresponding determinants in controls included *FGG* 9340T > C and *FGA* 2224G > A polymorphisms and plasma fibrinogen concentration. An elevated plasma fibrinogen γ′ concentration proved to be an independent predictor of MI [adjusted odds ratio (OR) (95% CI): 1.24 (1.01, 1.52)]. The plasma fibrinogen γ′ concentration was involved in a high-order interaction with total plasma fibrinogen and the *FGG* 9340T > C and *FGA* 2224G > A polymorphisms, associated with a further increased risk of MI [OR (95% CI): 3.22 (2.35, 4.39)]. *Conclusions:* Plasma fibrinogen γ′ concentration influences the risk of MI, and this relationship seems to be strengthened by the presence of an elevated total plasma fibrinogen concentration and the *FGG* 9340T and *FGA* 2224G alleles.

Keywords: fibrinogen γ′, fibrinogen polymorphisms, interaction, myocardial infarction.

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Introduction

Fibrinogen is a 340-kDa glycoprotein synthesized predominantly in hepatocytes. It circulates in plasma as a dimer, composed of three pairs of polypeptide chains denoted Aα, Bβ and γ. These chains are encoded by the fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*) and fibrinogen gamma (*FGG*) genes clustered on chromosome 4q31 (UCSC Genome Browser, URL: http://genome.ucsc.edu/). Two distinct fractions of fibrinogen can be separated by using ion exchange chromatography [1]. The major fraction, containing the most abundant γ-chain variant (γA), amounts to approximately 84% of the plasma fibrinogen and is a homodimer with the stoichiometry $(A\alpha B\beta\gamma A)_2$, referred to as γA/γA [1]. The minor fraction, containing the less abundant γ-chain variant (γ′), is either a heterodimeric molecule (AαBβγA) (AαBβγ′) referred to as γA/γ′, amounting to approximately 15% of the plasma fibrinogen, or a homodimer (AαBβγ′) (AαBβγ′), referred to as γ′/γ′, which constitutes around 1% of the fibrinogen contained in plasma [1,2]. Fibrinogen γ′ is generated by alternative mRNA processing [3,4], resulting in replacement of the last four residues of the γA-chain with an anionic 20-amino-acid sequence [2].

There is a close relationship between the structure of the fibrinogen molecule and its various biological functions, some of which are disclosed upon fibrin clot formation. The structure

of the fibrin clot seems to be clinically relevant, as tightly packed and rigid clots with thinner fibers and low porosity are less susceptible to the action of thrombolytic agents [5]. The fibrin clot structure is determined both by quantitative phenotypes (e.g. the plasma fibrinogen concentration) [6] and by qualitative phenotypes (e.g. the fibrinogen γ′-chain variant [7] and the *FGA* Thr312Ala polymorphism [8]). Fibrin clots formed in the presence of the fibrinogen γ′-chain variant are resistant to fibrinolysis, although the mechanisms are a matter of debate [9–11]. Clinically, an abnormal clot structure has been associated with the extent and severity of coronary artery disease (CAD) [12] and with an increased risk of venous thromboembolism [5] and myocardial infarction (MI) [13]. It is of note that the ratio of fibrinogen γ′ (γA/γ′ plus γ′/γ′) to total plasma fibrinogen concentration has been associated with atherothrombotic disease [14], and a raised fibrinogen γ′ concentration has been associated with an increased risk of CAD, independently of the plasma fibrinogen concentration [15]. On the other hand, an *FGG* haplotype, which has been associated with a decrease in plasma fibrinogen γ′ concentration, seems to confer a higher risk of deep vein thrombosis [16]. In addition, a recent report has concluded that *FGG* haplotypes do not affect the risk of MI [17], in contrast to a previous report [18]. Thus, the impact of plasma fibrinogen γ′ concentration on thrombotic diseases remains elusive and warrants further evaluation.

The present study was undertaken to identify potential genetic and environmental determinants of the plasma fibrinogen γ′ concentration and to explore the impact of this fibrinogen variant on the risk of MI. For these purposes, we employed the database and biobank of the Stockholm Coronary Artery Risk Factor (SCARF) study, designed to form a basis for studies of genetic and biochemical factors predisposing to precocious MI.

Materials and methods

Subjects

The SCARF study, comprising 387 survivors of a first MI under the age of 60 years and 387 population-based sex and age-matched controls, has been described elsewhere [18]. All patients included at two of the recruiting hospitals ($n = 269$) were offered routine coronary angiography, of whom 243 (90%) agreed to be included in the coronary angiography substudy. Angiograms were analyzed by quantitative coronary angiography (Medis QCA-CMS system, Leiden, The Netherlands).

Biochemical analyses

Fibrinogen γ′ and total fibrinogen concentrations were measured in plasma containing trisodium citrate (0.13 mol L^{-1}). Fibrinogen γ′ antigen concentration was measured by enzyme-linked immunosorbent assay (ELISA) using a modification of the assay described by Lovely *et al.* [15]. In brief, fibrinogen γ′ in the samples and calibrators was captured by the monoclonal antibody 2.G2.H9 (purified from a preparation generated by Scheuchenzuber at the Pennsylvania State University Biotechnology Institute, University Park, PA, USA) on the wells of the plates, and probed with a horseradish peroxidase (HRP)-conjugated sheep antihuman fibrinogen (Innovative Research Inc., Southfield, MI, USA). The amount of HRP antibody bound was detected with 3,3′-5,5′-tetramethylbenzidine (TMB, Super Sensitive 1 Component HRP microwell substrate; BioFX Laboratories, Owings Mills, MD, USA), in a reaction terminated with 50 μL of stop solution (Liquid Stop Solution for TMB microwell; BioFX Laboratories), and the absorbance was read at 450 nm. Heat-defibrinated plasma reconstituted with purified fibrinogen γA/γ′ was used as standard. Interassay coefficients of variation (CV) calculated for two plasma samples containing fibrinogen γ′ concentrations of 0.17 and 0.45 g L^{-1} were 23% and 11%, respectively. To compare our method with another described assay, we also measured fibrinogen γ′ antigen concentration according to de Willige *et al.* [16]. This ELISA is also based on the ability of the 2.G2.H9 antibody to capture fibrinogen γ′ antigen but works with 100-μL volumes, different buffers, other sample/reagent volume ratios, and a different commercially available probing HRP antibody. Coagulation Reference Plasma (Technoclone GmbH, Vienna, Austria), standardized against a similar material as above, was used as calibrator. The interassay CV for the control plasma (0.32 g L^{-1}) was 11%. The mean difference (95% CI) between the methods was 0.00065 (−0.010, 0.011) g L^{-1}, i.e. close to 0, suggesting lack of an overall bias [19]. Statistical analyses were based on the measurements performed with the first method described.

Plasma fibrinogen concentration was determined by the Clauss method [20], using reagents from Instrumentation Laboratory Spa, Milano, Italy (range of measurement: 1.0–5.5 g L^{-1}). Plasma lipoproteins were determined by a combination of preparative ultracentrifugation and precipitation of apolipoprotein B-containing lipoproteins followed by lipid analysis [21]. Insulin and interleukin-6 (IL-6) concentrations were assayed in plasma containing Na_2EDTA (0.34 mol L^{-1}) using ELISAs (Dako Ltd, Cambridgeshire, UK, and high-sensitivity IL-6 from R&D Systems Inc., Minneapolis, MN, USA, respectively).

Genetic analyses

Three haplotype-tagging single-nucleotide polymorphisms (SNPs), *FGG* 9340T > C [rs1049636], *FGA* 2224G > A [rs2070011] and *FGB* 1038G > A [rs1800791], were selected for the present study. The selection of the *FGG* 9340T > C SNP was based on the hypothesis that it might interfere with the splicing process in intron 9 generating the fibrinogen γ′-chain (Fig. 1). Genotyping was performed in 377 patients (DNA was unavailable from 10 patients) and 387 controls, using Taqman technology (Applied Biosystems, Stockholm, Sweden) [18].

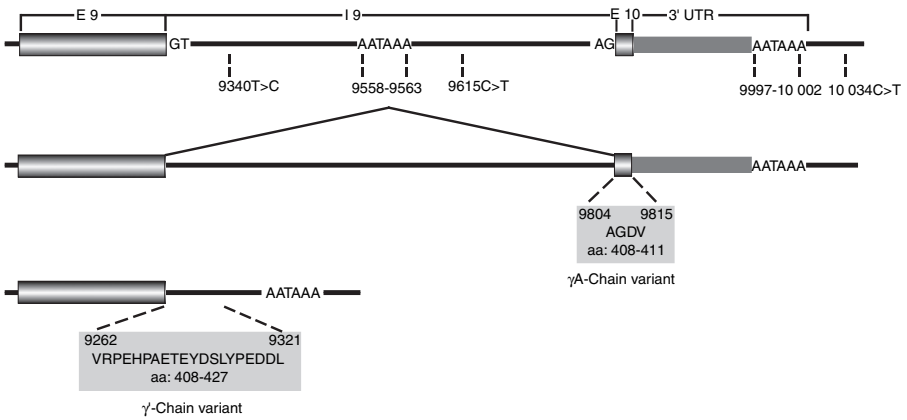

Fig. 1. Alternative mRNA processing of the *FGG* gene. The location of the *FGG* 9340T > C single-nucleotide polymorphism (SNP) and two other common SNPs (*FGG* 9615C > T and *FGG* 10 034C > T), the AATAAA polyadenylation sites and the 3′-untranslated region are illustrated. The γA-chain containing 411 amino acid (aa) residues arises when all introns of the *FGG* pre-mRNA are removed and the polyadenylation occurs downstream of exon 10 (E 10). The γ′-chain containing 427 aa residues arises because of partial retention of intron 9 (I 9), the polyadenylation occurring downstream of exon 9 (E 9).

Fibrin gel structure analyses

A total of 60 controls from the SCARF study population were selected on the basis of homozygosity for either the minor C allele ($n = 30$) or the major T allele ($n = 30$) of the *FGG* 9340T > C SNP for determination of the structure of fibrin clot formed *in vitro* from plasma samples [permeability coefficient (K_s, cm^2) reflecting fibrin clot porosity, and the fiber mass/length ratio (μ, Da cm^{-1})] [13]. The interassay CV for K_s was 11.3%.

Statistical analyses

Statistical analyses were conducted using the STATVIEW package (version 5.0.1 for Windows, SAS Institute, Inc, Cary, NC, USA). Variables that did not adhere to a normal distribution were normalized by logarithmic transformation prior to statistical analyses. Factorial analysis of variance (ANOVA) was used in order to estimate both main and interaction effects on plasma fibrinogen γ′ concentration. Differences between groups were estimated by using the unpaired *t*-test and one-way ANOVA using the Bonferroni test for *post hoc* analysis. Odds ratios (ORs) and standardized odds ratios (SORs) for a 1 SD increase in plasma fibrinogen γ′ concentration were calculated using logistic regression analysis. Multiple stepwise regression analysis was performed in order to identify determinants of plasma fibrinogen γ′ concentration. The proportion of variation accounted for by individual variables was derived by calculation of adjusted R^2.

The multifactor dimensionality reduction (MDR) software was used to study interactions in relation to risk of MI [22]. The MDR method was specifically developed in order to improve the power to detect gene–gene and gene–environment interactions in relatively small samples. The plasma fibrinogen and fibrinogen γ′ concentrations were dichotomized using the 75th percentile as cut-off values before they were included in the MDR analyses along with the *FGG* 9340T > C, *FGA* 2224G > A and *FGB* 1038G > A SNPs. The data were analyzed 10 times with different random seeds, each time using tenfold cross-validation intervals in order to ensure that the analysis was not influenced by chance division of the data. The average prediction error (PE) across all runs is presented in the final model. Statistical significance was determined by the permutation tests implemented in the MDR software.

Ethical considerations

The Ethics Committee of the Karolinska University Hospital approved the study. All participants gave their informed consent.

Results

Plasma fibrinogen γ′ concentration

The plasma fibrinogen γ′ concentration was significantly higher in patients than in controls (0.28 ± 0.12, $n = 367$ vs. 0.25 ± 0.11 g L^{-1}, $n = 381$, $P = 0.001$). There were no significant interactions between case–control status and gender, smoking, body mass index (BMI), fibrinogen, insulin and high-density lipoprotein (HDL)-cholesterol on plasma fibrinogen γ′ concentration (data not shown). Taken as a group, women had significantly higher plasma fibrinogen γ′ concentrations than men (0.28 ± 0.13, $n = 127$ vs. 0.26 ± 0.11 g L^{-1}, $n = 621$, $P = 0.03$), as did individuals with fibrinogen concentrations in the top quartile compared to those with lower fibrinogen concentrations [0.29 ± 0.12, $n = 265$ (4th quartile) vs. 0.26 ± 0.11, $n = 180$ (3rd quartile), 0.24 ± 0.09, $n = 145$ (2nd quartile) and 0.23 ± 0.11 g L^{-1}, $n = 150$ (1st quartile), $P < 0.004$ for all comparisons]. A weak correlation between the plasma fibrinogen γ′ concentration and total plasma

fibrinogen concentration was noted in both patients and controls ($r = 0.29$, $P < 0.001$, and $r = 0.18$, $P < 0.001$, respectively). There was no significant difference in the fibrinogen γ′/total fibrinogen concentration ratio between patients and controls ($P = 0.65$).

Influence of fibrinogen SNPs on plasma fibrinogen γ′ concentration

All three polymorphisms were in Hardy–Weinberg equilibrium. The *FGG* 9340T > C, *FGA* 2224G > A and *FGB* 1038G > A SNPs appeared to influence the plasma fibrinogen γ′ concentration (Table 1). The effect of the *FGG* 9340T > C and *FGA* 2224G > A SNPs was independent of case–control status ($P = 0.12$ and $P = 0.31$ for interaction, respectively). On the other hand, a significant interaction between the *FGB* 1038G > A SNP and case–control status on plasma fibrinogen γ′ concentration was detected ($P = 0.02$). However, this interaction, as well as the higher plasma fibrinogen γ′ concentration among heterozygotes in the control group (Table 1), should be interpreted with caution, given the modest number of homozygotes for the minor allele of this SNP. The plasma fibrinogen γ′ concentration increased with increasing number of the minor *FGG* 9340C allele or the major *FGA* 2224G allele, this pattern being consistent in both patients and controls (Table 1).

Gene–gene interaction analyses on the plasma fibrinogen γ′ concentration were then performed, from which the *FGB* 1038G > A SNP was excluded, because of its linkage disequilibrium with the other two SNPs [18] and to the low number of homozygotes for the minor allele. The *FGG* 9340T > C and *FGA* 2224G > A SNPs appeared to interact on the plasma fibrinogen γ′ concentration ($P < 0.001$). This interaction was independent of case–control status ($P = 0.53$) and was found in both patients and controls (Fig. 2).

Table 1 Plasma fibrinogen γ′ concentration according to fibrinogen single-nucleotide polymorphisms (SNPs) in cases and controls

	Fibrinogen γ′ (g L^{-1})			
SNP	Case	*n*	Control	*n*
FGG 9340T > C				
TT	0.25 (0.12)	179	0.21 (0.10)	166
TC	0.29 (0.11)	156	0.27 (0.11)	169
CC	0.38 (0.14)	30	0.31 (0.10)	46
P-value	< 0.001*		< 0.001*	
FGA 2224G > A				
GG	0.30 (0.12)	148	0.26 (0.10)	149
GA	0.27 (0.12)	172	0.25 (0.10)	178
AA	0.22 (0.13)	45	0.21 (0.12)	54
P-value	< 0.001*		0.02	
FGB 1038G > A				
GG	0.27 (0.12)	251	0.24 (0.10)	263
GA	0.28 (0.12)	106	0.28 (0.11)	105
AA	0.37 (0.07)	8	0.24 (0.07)	13
P-value	0.06		< 0.001*	

Values are mean (SD) and number (*n*) of subjects in each group.
*These comparisons remain statistically significant after Bonferroni correction for multiple testing ($n = 6$).

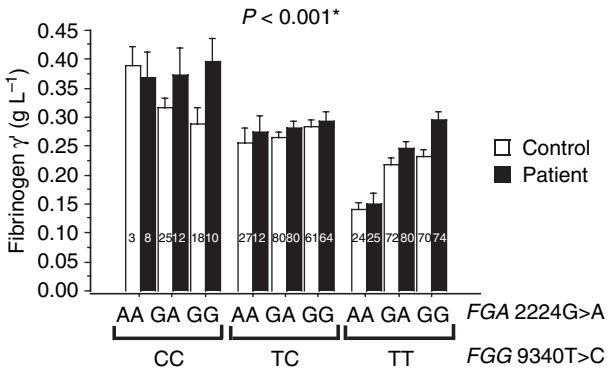

Fig. 2. Epistatic effects between the *FGG* 9340T > C and *FGA* 2224G > A single-nucleotide polymorphisms on plasma fibrinogen γ′ concentration. The numbers in boxes represent the number of individuals in each group. **P*-value for interaction estimated in the entire study population.

Determinants of plasma fibrinogen γ′ concentration

Multiple stepwise regression analysis was performed separately in patients and controls in order to assess the potential contributions of age, gender, BMI, smoking, alcohol consumption, fibrinogen, triglycerides, low-density lipoprotein-cholesterol, HDL-cholesterol, glucose, insulin, and the *FGG* 9340T > C and *FGA* 2224G > A SNPs, to plasma fibrinogen γ′ concentration. In postinfarction patients, fibrinogen (9.2%), *FGG* 9340T > C (5.9%), *FGA* 2224G > A (3.9%), HDL-cholesterol (1.4%), insulin (1.1%) and gender (0.9%) emerged as independent determinants, together accounting for 22.4% of the variation in plasma fibrinogen γ′ concentration. In controls, *FGG* 9340T > C (10.4%), fibrinogen (3.4%) and *FGA* 2224G > A (2.2%) emerged as independent predictors, together accounting for 16.0% of the variation in plasma fibrinogen γ′ concentration.

Plasma fibrinogen γ′ concentration in relation to CAD severity, fibrin gel structure and risk of MI

No statistically significant associations between total plasma fibrinogen and fibrinogen γ′ concentrations and CAD severity scores were detected (Table 2). The plasma fibrinogen γ′ concentration was not associated with fibrin clot structure (K_s and μ), whereas an elevated total plasma fibrinogen concentration was associated with lowered K_s (Table 2).

The fibrinogen γ′ concentration appeared to be an independent discriminator between patients and controls [unadjusted SOR (95% CI): 1.25 (1.09, 1.44), $P = 0.001$]. The risk of MI associated with an increased fibrinogen γ′ concentration remained significant after controlling for the *FGG* 9340T > C and *FGA* 2224G > A SNPs [SOR (95% CI): 1.34 (1.16, 1.55), $P < 0.001$]. Controlling for the effects of age, gender, smoking, alcohol consumption, BMI, fibrinogen, IL-6, insulin,

Table 2 Association of plasma fibrinogen γ′ and total plasma fibrinogen concentration with coronary artery disease (CAD) severity scores and fibrin gel structure

Variable	Fibrinogen γ′			Fibrinogen		
	< 75th percentile	≥ 75th percentile	*P*-value	< 75th percentile	≥ 75th percentile	*P*-value
CAD						
n	139	89		143	88	
MLD (mm)	2.23 (0.45)	2.21 (0.45)	0.78	2.18 (0.46)	2.28 (0.45)	0.10
MSD (mm)	3.01 (0.41)	3.05 (0.49)	0.49	2.97 (0.42)	3.12 (0.51)	0.02
Mean % stenosis	31.38 (9.58)	32.70 (8.64)	0.29	31.93 (9.99)	32.19 (7.96)	0.84
Plaque area mm^{-1}	0.22 (0.07)	0.23 (0.09)	0.22	0.22 (0.08)	0.23 (0.08)	0.41
Fibrin gel structure						
n	43	17		44	16	
Permeability coefficient (cm^2 10^{-9})	7.72 (2.38)	7.16 (2.13)	0.40	8.14 (2.27)	5.96 (1.56)	< 0.001*
Fiber mass/length ratio (Da cm^{-1} 10^{-12})	112.26 (41.14)	121.64 (43.17)	0.44	116.91 (43.89)	109.44 (35.11)	0.54

MLD, minimum lumen diameter; MSD, mean segment diameter.
Values are mean (SD) and number (*n*) of subjects in each group.
*The comparison remains statistically significant after Bonferroni correction for multiple testing ($n = 12$).

triglycerides and HDL-cholesterol together with the *FGG* 9340T > C and *FGA* 2224G > A SNPs did not abolish the discriminative power of fibrinogen γ′ [SOR (95% CI): 1.24 (1.01, 1.52), $P = 0.04$]. In contrast, the fibrinogen γ′/total fibrinogen concentration ratio was not an independent discriminator between patients and controls (data not shown).

Potential interactions on MI risk were searched for. All three fibrinogen SNPs were included in these analyses, along with the plasma fibrinogen γ′ and total fibrinogen concentrations, the latter two being dichotomized using the 75th percentile as cut-off values. A significant four-factor interaction on the risk of MI ($P = 0.02$ from permutation test) with an average PE of 38% was detected between the fibrinogen γ′ concentration, the total fibrinogen concentration, and the *FGG* 9340T > C and *FGA* 2224G > A SNPs. The best models corresponding to two-factor (fibrinogen, *FGA* 2224G > A), three-factor (fibrinogen, *FGA* 2224G > A, *FGG* 9340T > C) and five-factor (fibrinogen, *FGA* 2224G > A, *FGG* 9340T > C, *FGB* 1038G > A, fibrinogen γ′) combinations offered a higher PE (> 40%). The ORs in the testing dataset (used as part of the internal validation implemented in the MDR method) were significant for the four-factor and five-factor combinations [OR (95%): 3.16 (1.16, 8.64) and 2.67 (1.02, 7.00), respectively] but not for the two-factor and three-factor combinations [OR (95%): 2.78 (0.97, 7.97) and 2.61 (0.97, 7.05), respectively]. Although the five-factor model is significant, the four-factor model is better, as it offers a lower PE and is more parsimonious. The best predictive model [OR (95% CI): 3.22 (2.35, 4.39), $P < 0.001$] was the result of a marked shift in genotype frequency distributions among individuals with total fibrinogen and/or fibrinogen γ′ concentrations above the 75th percentile (Fig. 3). There was no difference in risk of MI in carriers of the major *FGG* 9340T and *FGA* 2224G alleles (i.e. the TC/GA plus TC/GG plus TT/GA plus TT/GG genotypes) who had a plasma fibrinogen γ′ concentration but not a total plasma fibrinogen concentration above the 75th percentile as compared to non-carriers of these alleles who had both total fibrinogen and fibrinogen γ′ below the 75th percentile (used as the reference category) [OR (95% CI): 1.58 (0.84, 2.99), $P = 0.16$]. In contrast, the presence of the *FGG* 9340T allele together with the *FGA* 2224G allele was more common among patients who had a total plasma fibrinogen concentration above the 75th percentile ($P < 0.001$) as compared to those individuals having fibrinogen γ′ and total fibrinogen concentrations below the 75th percentile (Fig. 3). The risk of MI for these individuals was markedly increased [OR (95% CI): 2.79 (1.53, 5.08), $P < 0.001$; Fig. 3]. Individuals who had both total plasma fibrinogen and fibrinogen γ′ concentrations above the 75th percentile had a further increase in the risk of MI [OR (95% CI): 3.33 (1.73, 6.40), $P < 0.001$; Fig. 3].

Discussion

We here report that a raised plasma fibrinogen γ′ concentration is associated with increased risk of MI, independently of the plasma fibrinogen concentration and established cardiovascular risk factors. These results are in agreement with the previously reported association between fibrinogen γ′ concentration and CAD [15]. Second, the risk of MI is markedly increased in individuals with raised plasma fibrinogen γ′ and/or total fibrinogen concentrations who are also carriers of the *FGG* 9340T and *FGA* 2224G alleles.

Fibrinogen γ′ arises because of alternative processing of the *FGG* mRNA. This process is conserved across several different species [23], suggesting that it is of physiologic importance. It involves tightly linked reactions of capping, splicing and polyadenylation in which a host of factors (e.g. small nuclear RNAs, multisubunit protein complexes) interact and affect each others' specificity and efficiency [24]. Differences in the mechanisms regulating mRNA processing, to which genetic variation in the *FGG* gene and in the splicing machinery is likely to contribute, partly account for the variation in the concentration of alternatively spliced transcripts [25]. Consequently, the rather weak relationship between the plasma fibrinogen γ′ and total fibrinogen concentration noted in the present study, which is in accordance with data from a previous report [15],

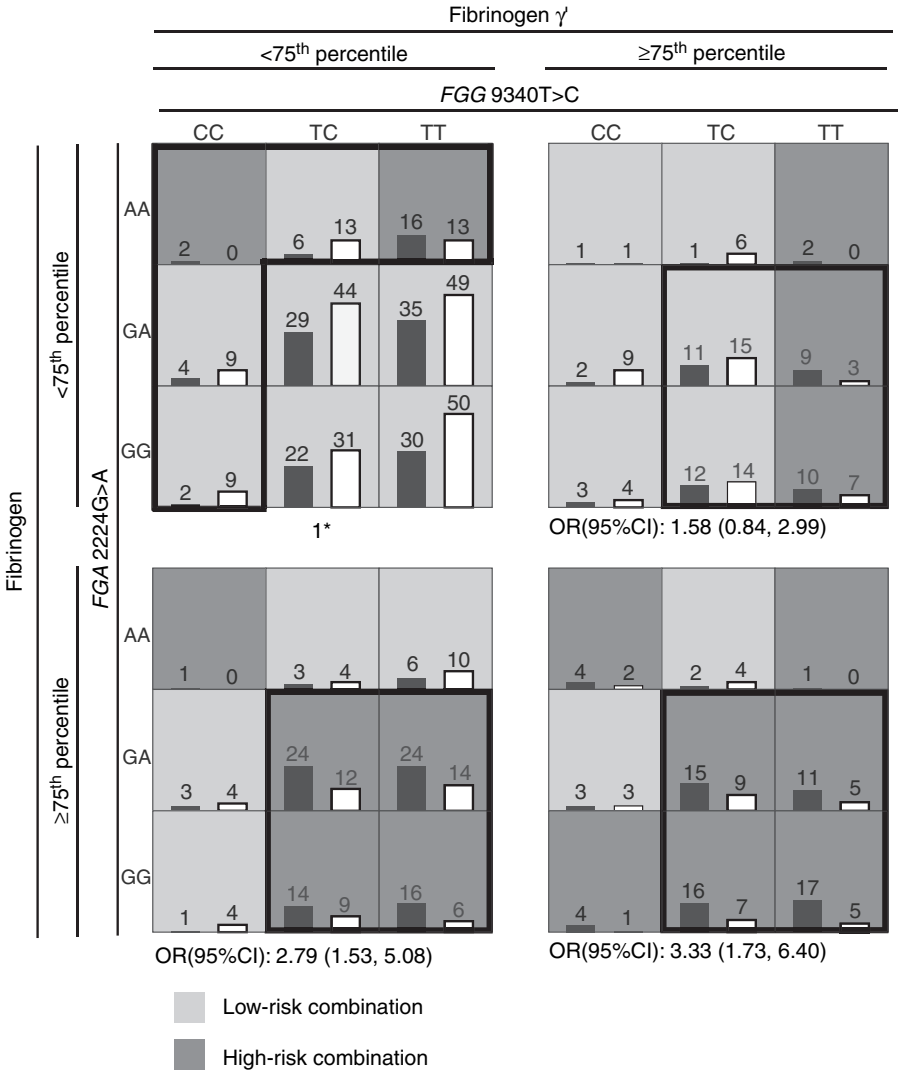

Fig. 3. Interaction model associated with risk of myocardial infarction ($P = 0.02$). The corresponding distribution of cases (dark bars in boxes) and controls (white bars in boxes) is depicted for each multifactor dimension. In each multifactor dimension, the ratio of the number of cases to the number of controls is calculated. If the ratio exceeds a threshold (e.g. > 1), the high-risk label (dark gray) is attributed to that multifactor dimension, whereas the low-risk label (light gray) is attributed when the opposite occurs. The shift in pattern of high-risk and low-risk combinations across the different multifactor dimensions indicates the presence of interaction. ORs with corresponding 95% CI estimated for the distribution of variables within the boxes demarcated with bold lines and using box 1* (demarcated with bold lines) as the reference category are presented.

probably reflects the involvement of several complex levels of regulation in *FGG* mRNA processing. It may also explain the lack of association between the fibrinogen γ′/total fibrinogen concentration ratio and MI. In contrast, the fibrinogen γ′/total fibrinogen concentration ratio has been found to be associated with deep vein thrombosis [16]. The fact that it was unrelated to MI in the present study may at least partly be due to the greater imprecision of a ratio compared to individual variables. Also, it should be emphasized that the pathogenesis of MI is not identical to that of deep vein thrombosis. Thus, the relationship between plasma fibrinogen γ′ concentration and MI cannot be considered to be merely a reflection of the well-established association between plasma fibrinogen concentration and MI.

Among the patients participating in the present study, fibrinogen, the *FGG* 9340T > C and *FGA* 2224G > A SNPs, insulin, HDL-cholesterol and gender emerged as independent determinants of plasma fibrinogen γ′ concentration, together accounting for 22.4% of the variation. In controls, approximately 16% of the variation in plasma fibrinogen γ′ concentration was accounted for by the *FGG* 9340T > C and the *FGA* 2224G > A SNPs and fibrinogen. An influence on mRNA stability and/or interference with the splicing machinery may explain the relationship between the *FGG* 9340T > C SNP and plasma fibrinogen γ′ concentration. It is of note that many regulatory SNPs that influence gene transcription (by altering mRNA stability, altering the efficiency of mRNA processing, or causing epigenetic changes) have been detected either inside or outside of promoter regions [26]. As the fibrinogen genes contain similar regulatory *cis*-elements [27] and are transcribed in a tightly coordinated manner [28],

genetic variants located in regulatory regions (e.g. promoters) in the fibrinogen gene cluster might have an impact on the expression of any of the three fibrinogen genes, which could explain the association between the *FGA* 2224G > A SNP and the plasma fibrinogen γ′ concentration. Alternatively, as the *FGG* 9340T > C and *FGA* 2224G > A SNPs are haplotype-tagging, they could be proxies for functional SNPs located elsewhere in the fibrinogen genes.

Interestingly, a significant interaction between the *FGG* 9340T > C and *FGA* 2224G > A SNPs on plasma fibrinogen γ′ concentration was detected. As a consequence of this interaction, it appears that in homozygotes for the major *FGG* 9340T allele, the plasma fibrinogen γ′ concentration increases with increasing number of the major *FGA* 2224G allele. It could be speculated that carriers of the *FGG* 9340T and *FGA* 2224G alleles might exhibit a more pronounced increase in plasma fibrinogen γ′ concentration in response to a disease-related stimulus. This finding may therefore at least partly explain the previously reported 1.5-fold increased risk of MI seen in carriers of the FGG-FGA*1 (TG) haplotype [18]. It is of note that in the present study, a further increase (up to 3-fold) in the risk of MI was seen in carriers of the *FGG* 9340T and *FGA* 2224G alleles in whom the total plasma fibrinogen and/or fibrinogen γ′ concentration was in the top quartile.

A plausible explanation for the association between fibrinogen γ′ concentration and risk of MI could be an unfavorable influence on fibrin clot structure. However, in the present study, no association was detected between plasma fibrinogen γ′ concentration and fibrin clot porosity, these findings being consistent with those of a previous study using recombinant γ′/γ′ [10]. Despite a similar structure, the fibrin clots formed using recombinant γ′/γ′ were about three times stiffer and showed a tenfold lower fibrinolysis rate than clots formed from fibrinogen γA/γA, but only in the presence of coagulation factor XIII, which stabilizes the clot through cross-linking reactions [10]. Notably, Collet *et al*. [29] recently reported that fibrin clot stiffness remained the only independent correlate with premature CAD in a multivariate model including several well-established risk factors.

As discussed earlier, results from a previous report indicate that a reduced plasma fibrinogen γ′ concentration is associated with an increased risk of deep vein thrombosis, and inhibition of thrombin by its binding to this fibrinogen variant has been suggested to explain this finding [16]. However, inhibition of thrombin is also known to occur through well-established anticoagulant mechanisms, e.g. antithrombin and the activated protein C pathway. On the other hand, the presence of fibrinogen γ′ has consistently been associated with resistance to fibrinolysis [9–11], which may explain why a raised plasma fibrinogen γ′ concentration is associated with CAD [15] and MI, as indicated by the data reported here. It is of note that in the present study, two ELISAs, which generated closely similar results, were used to measure the plasma fibrinogen γ′ concentration on samples collected 3 months after the MI, when the inflammatory response most likely had subsided.

One limitation of the present study is the exclusion of *FGG* SNPs that might affect the plasma fibrinogen γ′ concentration. As the 5′-capping process is coupled with pre-mRNA splicing and 3′-polyadenylation [24], it cannot be excluded that *FGG* promoter SNPs are relevant in this context. However, the inclusion of the *FGG* 9340T > C, *FGA* 2224G > A and *FGB* 1038G > A SNPs was based on our previous findings suggesting a relationship between fibrinogen haplotypes and risk of MI [18]. Moreover, the *FGG* 9340T > C and *FGA* 2224G > A SNPs are independent of each other [18] and are therefore appropriate candidates for interaction analyses on intermediate phenotypes. Second, the retrospective design of this study might have conferred a selection bias, as it cannot be excluded that patients who died during the acute phase of MI had a different genetic predisposition and/or different plasma fibrinogen γ′ and total fibrinogen concentrations as compared with the survivors.

In conclusion, plasma fibrinogen γ′ concentration is independently related to MI, and this association is strengthened by the presence of an elevated plasma fibrinogen concentration and the *FGG* 9340T and *FGA* 2224G alleles.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Mosesson MW, Finlayson JS, Umfleet RA. Human fibrinogen heterogeneities: III. Identification of γ chain variants. *J Biol Chem* 1972; **247**: 5223–7.
2 Wolfenstein-Todel C, Mosesson MW. Human plasma fibrinogen heterogeneity: evidence for an extended carboxyl-terminal sequence in a normal γ chain variant (γ′). *Proc Natl Acad Sci USA* 1980; **77**: 5069–73.
3 Fornace Jr AJ, Cummings DE, Comeau CM, Kant JA, Crabtree GR. Structure of the human γ-fibrinogen gene. Alternate mRNA splicing near the 3′ end of the gene produces γA and γB forms of γ-fibrinogen. *J Biol Chem* 1984; **259**: 12826–30.
4 Chung DW, Davie EW. γ and γ′ chains of human fibrinogen are produced by alternative mRNA processing. *Biochemistry* 1984; **23**: 4232–6.
5 Collet JP, Soria J, Mirshahi M, Hirsch M, Dagonnet FB, Caen J, Soria C. Dusart syndrome: a new concept of the relationship between fibrin clot architecture and fibrin clot degradability: hypofibrinolysis related to an abnormal clot structure. *Blood* 1993; **82**: 2462–9.
6 Blomback B, Carlsson K, Fatah K, Hessel B, Procyk R. Fibrin in human plasma: gel architectures governed by rate and nature of fibrinogen activation. *Thromb Res* 1994; **75**: 521–38.

7 Cooper AV, Standeven KF, Ariens RA. Fibrinogen gamma-chain splice variant γ′ alters fibrin formation and structure. *Blood* 2003; **102**: 535–40.

8 Lim BC, Ariens RA, Carter AM, Weisel JW, Grant PJ. Genetic regulation of fibrin structure and function: complex gene–environment interactions may modulate vascular risk. *Lancet* 2003; **361**: 1424–31.

9 Falls LA, Farrell DH. Resistance of γA/γ′ fibrin clots to fibrinolysis. *J Biol Chem* 1997; **272**: 14251–6.

10 Collet JP, Nagaswami C, Farrell DH, Montalescot G, Weisel JW. Influence of γ′ fibrinogen splice variant on fibrin physical properties and fibrinolysis rate. *Arterioscler Thromb Vasc Biol* 2004; **24**: 382–6.

11 Siebenlist KR, Mosesson MW, Hernandez I, Bush LA, Di Cera E, Shainoff JR, Di Orio JP, Stojanovic L. Studies on the basis for the properties of fibrin produced from fibrinogen-containing γ′ chains. *Blood* 2005; **106**: 2730–6.

12 Fatah K, Hamsten A, Blombäck B, Blombäck M. Fibrin gel network characteristics and coronary heart disease: relations to plasma fibrinogen concentration, acute phase protein, serum lipoproteins and coronary atherosclerosis. *Thromb Haemost* 1992; **68**: 130–5.

13 Fatah K, Silveira A, Tornvall P, Karpe F, Blomback M, Hamsten A. Proneness to formation of tight and rigid fibrin gel structures in men with myocardial infarction at a young age. *Thromb Haemost* 1996; **76**: 535–40.

14 Drouet L, Paolucci F, Pasqualini N, Laprade M, Ripoll L, Mazoyer E, Bal dit Sollier C, Vanhove N. Plasma γ′/γ fibrinogen ratio, a marker of arterial thrombotic activity: a new potential cardiovascular risk factor? *Blood Coagul Fibrinolysis* 1999; **10**(Suppl. 1): 35–9.

15 Lovely RS, Falls LA, Al-Mondhiry HA, Chambers CE, Sexton GJ, Ni H, Farrell DH. Association of γA/γ′ fibrinogen levels and coronary artery disease. *Thromb Haemost* 2002; **88**: 26–31.

16 Uitte de Willige S, de Visser MC, Houwing-Duistermaat JJ, Rosendaal FR, Vos HL, Bertina RM. Genetic variation in the fibrinogen gamma gene increases the risk of deep venous thrombosis by reducing plasma fibrinogen γ′ levels. *Blood* 2005; **106**: 4176–83.

17 Uitte de Willige S, Doggen CJ, de Visser MC, Bertina RM, Rosendaal FR. Haplotypes of the fibrinogen gamma gene do not affect the risk of myocardial infarction. *J Thromb Haemost* 2006; **4**: 474–6.

18 Mannila MN, Eriksson P, Lundman P, Samnegard A, Boquist S, Ericsson CG, Tornvall P, Hamsten A, Silveira A. Contribution of haplotypes across the fibrinogen gene cluster to variation in risk of myocardial infarction. *Thromb Haemost* 2005; **93**: 570–7.

19 Bland M. Comparing two methods of measurement. In: Bland M, ed. *An Introduction to Medical Statistics*, 3rd edn. Oxford: Oxford University Press, 2000: 272–5.

20 Clauss A. Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta Haematol* 1957; **17**: 237–46.

21 Carlson K. Lipoprotein fractionation. *J Clin Pathol Suppl* 1973; **5**: 32–7.

22 Hahn LW, Ritchie MD, Moore JH. Multifactor dimensionality reduction software for detecting gene–gene and gene–environment interactions. *Bioinformatics* 2003; **19**: 376–82.

23 Crabtree GR, Kant JA. Organization of the rat γ-fibrinogen gene: alternative mRNA splice patterns produce the γA and γB (γ′) chains of fibrinogen. *Cell* 1982; **31**: 159–66.

24 Proudfoot NJ, Furger A, Dye MJ. Integrating mRNA processing with transcription. *Cell* 2002; **108**: 501–12.

25 Bai Y, Lee D, Yu T, Chasin LA. Control of 3′ splice site choice in vivo by ASF/SF2 and hnRNP A1. *Nucleic Acids Res* 1999; **27**: 1126–34.

26 Pastinen T, Hudson TJ. Cis-acting regulatory variation in the human genome. *Science* 2004; **306**: 647–50.

27 Mizuguchi J, Hu CH, Cao Z, Loeb KR, Chung DW, Davie EW. Characterization of the 5′-flanking region of the gene for the γ chain of human fibrinogen. *J Biol Chem* 1995; **270**: 28350–6.

28 Otto JM, Grenett HE, Fuller GM. The coordinated regulation of fibrinogen gene transcription by hepatocyte-stimulating factor and dexamethasone. *J Cell Biol* 1987; **105**: 1067–72.

29 Collet JP, Allali Y, Lesty C, Tanguy ML, Silvain J, Ankri A, Blanchet B, Dumaine R, Gianetti J, Payot L, Weisel JW, Montalescot G. Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb Vasc Biol* 2006; **26**: 2567–73.