

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol 2011, 31:2345-2352: originally published online July 14, 2011

doi: 10.1161/ATVBAHA.111.232710

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75214

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Assessment of Genetic Determinants of the Association of γ' Fibrinogen in Relation to Cardiovascular Disease

Rehana S. Lovely, Qiong Yang, Joseph M. Massaro, Jing Wang, Ralph B. D'Agostino, Sr, Christopher J. O'Donnell, Jackilen Shannon, David H. Farrell

Objective— γ' fibrinogen is a newly emerging biomarker that is associated with cardiovascular disease (CVD). However, the genetic determinants of γ' fibrinogen levels are unknown. We therefore conducted a genome-wide association study on 3042 participants from the Framingham Heart Study Offspring Cohort.

Methods and Results—A genome-wide association study with 2.5 million single-nucleotide polymorphisms (SNPs) was carried out for γ' fibrinogen levels from the cycle 7 examination. Fifty-four SNPs in or near the fibrinogen gene locus demonstrated genome-wide significance ($P < 5.0 \times 10^{-8}$) for association with γ' fibrinogen levels. The top-signal SNP was rs7681423 ($P = 9.97 \times 10^{-110}$) in the fibrinogen gene locus near *FGG*, which encodes the γ chain. Conditional on the top SNP, the only other SNP that remained genome-wide significant was rs1049636. Associations between SNPs, γ' fibrinogen levels, and prevalent CVD events were examined using multiple logistic regression. γ' fibrinogen levels were associated with prevalent CVD ($P = 0.02$), although the top 2 SNPs associated with γ' fibrinogen levels were not associated with CVD. These findings contrast those for total fibrinogen levels, which are associated with different genetic loci, particularly *FGB*, which encodes the $B\beta$ chain.

Conclusion— γ' fibrinogen is associated with prevalent CVD and with SNPs exclusively in and near the fibrinogen gene locus. (*Arterioscler Thromb Vasc Biol.* 2011;31:2345-2352.)

Key Words: coronary heart disease ■ epidemiology ■ fibrin ■ gene mutations ■ risk factors

γ' fibrinogen is an alternatively spliced form of the clotting factor fibrinogen (Figure 1) that has shown an association with arterial thrombosis and venous thrombosis in case-control studies.¹⁻⁵ Total fibrinogen is itself a well-validated risk factor for cardiovascular disease (CVD).⁶ Fibrinogen is a disulfide-bonded dimer, with each half of the dimer containing 1 $A\alpha$ chain, 1 $B\beta$ chain, and 1 γ chain, which can be either the more common γA chain or a γ' chain. γ' fibrinogen consists of approximately 90% heterodimers containing 1 γ' chain and 1 γA chain, and $\approx 10\%$ homodimers containing 2 γ' chains. γ' fibrinogen constitutes $\approx 7\%$ of total fibrinogen levels,^{2,3,7} although the amount varies considerably among individuals, particularly under pathological conditions.^{8,9} Unlike many CVD risk markers, γ' fibrinogen has biochemical properties that have the potential to actually contribute to the etiology of CVD. In particular, compared with total fibrinogen, γ' fibrinogen forms fibrin blood clots that show differences in clot architecture,^{10,11} are mechanically stiffer,¹² and are resistant to fibrinolysis.¹²⁻¹⁴ These properties have been hypothesized to contribute in a causative way to thrombosis,¹⁵ although this is an area of ongoing controversy.¹⁶

In previous case-control studies, elevated γ' fibrinogen levels have been associated with arterial thrombosis, including myocardial infarction,^{1,3} coronary artery disease,² and stroke.^{4,5} But paradoxically, γ' fibrinogen levels have been inversely associated with venous thrombosis¹⁷ and thrombotic microangiopathy.¹⁸ The reasons behind these differences are still unclear and may be the result of genetic variation or the design of previous studies. The genetic determinants of γ' fibrinogen levels have not been fully investigated, which provided the impetus for the present studies. In addition, previous epidemiological studies on γ' fibrinogen have used a case-control design, which is more susceptible to various forms of bias.¹⁹ The association between γ' fibrinogen and CVD has not been investigated in a large community-based cohort to date. The present study was therefore undertaken to investigate the association between γ' fibrinogen, genetic determinants, and CVD in a well-characterized cohort, the Framingham Offspring Study.

Methods

Study Population

We conducted an analysis of γ' fibrinogen concentrations in stored blood specimens from subjects enrolled in the Framingham Off-

Received on: June 9, 2011; final version accepted on: June 25, 2011.

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Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.111.232710

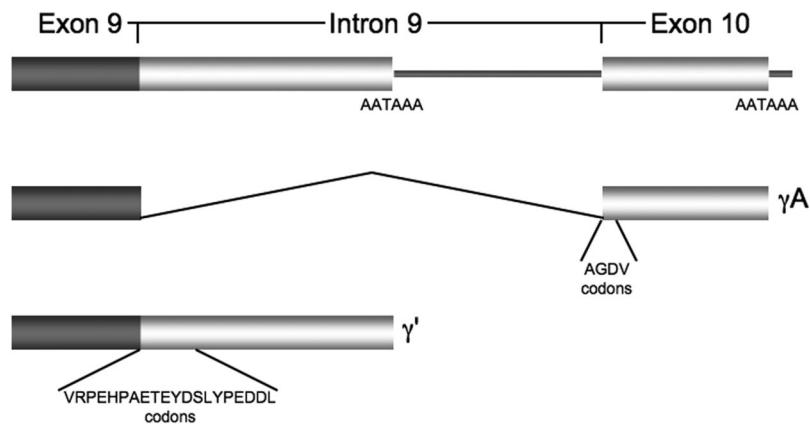


Figure 1. Alternative splicing of the γ -chain gene *FGG*. The current hypothesis is that competition between spliceosome cleavage of intron 9, which removes the intron and generates the γ A mRNA, vs polyadenylation within intron 9 at the AATAAAA site that cleaves off the 3' end of the pre-mRNA to generate the γ' mRNA, regulates the ratio of γ A to γ' mRNA.

spring Study. The design and methodology of this study for the long-term evaluation of risk factors for CVD have been described previously.²⁰ Briefly, 5124 offspring and the spouses of the offspring of the original Framingham cohort members, 5 to 70 years of age at entry, were enrolled and provided baseline data.²¹ Participants had completed 7 examinations, including a blood draw, over intervals of 4 to 6 years and were followed for CVD morbidity and mortality. Plasma samples from 3300 individuals available from the seventh examination cycle (1998 to 2001) were obtained for measuring γ' fibrinogen levels. Total fibrinogen levels had been measured in a previous study.²² The subjects gave informed consent, and the study was approved by the relevant institutional review boards. Aliquots were frozen at -20°C after the initial phlebotomy at the time of the baseline examination.

γ' Fibrinogen Assay

γ' fibrinogen was assayed using an ELISA protocol described previously.⁷ The precision and variability of this assay have been validated.⁷ The ELISA coefficient of variability was 9.3% at the mean γ' fibrinogen level.

Genotyping and Samples for Genome-Wide Association Study

DNA samples from 9274 Framingham Heart Study 3-generation participants were genotyped using the Affymetrix 500K mapping array and the Affymetrix 50K supplemental array. Of those, 8481 samples were genotyped successfully (sample call rate $\geq 97\%$) and had person heterozygosity within ± 5 standard deviations from the mean. Of these, 3042 had complete data on γ' fibrinogen values and other key covariates and were included in the subsequent genome-wide association analyses. A total of 549 781 single-nucleotide polymorphisms (SNPs) were genotyped. Of those, 503 551 were genotyped successfully with a call rate $< 95\%$ or Hardy-Weinberg equilibrium probability value of $< 10^{-6}$. Imputation of 2.5 million autosomal SNPs in HapMap based on successfully genotyped SNPs from the chips and phased chromosomes for 60 HapMap CEU founders was conducted using a Hidden Markov Model algorithm implemented in the MACH software (<http://www.sph.umich.edu/csg/abecasis/MACH/index.html>).

Genome-Wide Association Analyses

To adjust for potential population stratification, principal components of the genotypes of directly genotyped SNPs were computed using the Eigenstrat software.²³ The first 10 principal components were included as covariates in addition to sex, age, body mass index (BMI), fasting blood glucose, systolic blood pressure, diabetes, smoking status, total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides in the association analyses with each of the 2.5 million imputed SNPs. We used, in the association analyses,

a linear mixed-effects model with a fixed additive genotype effect and subject specific random effects that are correlated within family with correlation proportional to kinship coefficients between family members.²⁴

Determination of Risk Factors and Prevalent CVD

All visits preceding and including the seventh examination cycle included assessment of the prevalence of CVD and evaluation of CVD risk factors. Cases of myocardial infarction and stroke, as well as related end points, such as angina, coronary insufficiency, and transient ischemic attack, were adjudicated by a 3-physician end point review committee. Prevalent "hard CVD" events that occurred before the seventh examination consisted of the prior occurrence of myocardial infarction or coronary insufficiency. Prevalent "total CVD" consisted of the prior occurrence of either one of the CVD events listed above or angina pectoris, transient ischemic attack, stroke, or intermittent claudication. Specific criteria for the clinical and laboratory methods and the CVD event adjudication have been published previously.^{21,25}

During the seventh clinical examination cycle, information was obtained on cigarette smoking during the past year and use of medications. Blood pressure after sitting for 5 minutes was measured using standardized methods. Phlebotomy took place under fasting conditions. Lipid determinations were made at the time of the seventh examination cycle in the Framingham Heart Study laboratory. Plasma cholesterol was measured according to the Lipid Research Clinics Program Protocol, and HDL cholesterol was determined after precipitation of non-HDL lipoproteins with heparin-manganese.²⁶

Relating γ' Fibrinogen Levels and Identified SNPs to Prevalent Cardiovascular Events

The difference in the distribution of each of prevalent CVD and myocardial infarction across γ' fibrinogen tertiles was assessed using logistic regression adjusting for sex, age, BMI, systolic blood pressure, diabetes mellitus, smoking, total cholesterol, HDL cholesterol, and triglycerides. The association between γ' fibrinogen and risk of prevalent CVD and myocardial infarction were estimated using unconditional logistic regression. All variables independently associated with CVD or with γ' fibrinogen at $P < 0.05$ were considered as potential confounders in the final model.

Results

Study Sample Characteristics

Descriptive statistics (mean \pm standard deviation for continuous risk factors, count, and percentage of prevalence for

Table 1. Characteristics of the Study Participants*

Variable	Value
Age, y	61 (± 10)
Female, %, n	53.5, 1629
Body mass index, kg/m ²	28.1 (± 5.3)
Cigarette smoking, %, n	13.4, 408
Diabetes mellitus, %, n	13.1, 399
Fasting blood glucose, mmol/L	5.80 (± 1.54)
Systolic blood pressure, mm Hg	127 (± 18.6)
Total cholesterol, mmol/L	5.18 (± 0.95)
High-density lipoprotein cholesterol, mmol/L	1.39 (± 0.44)
Triglycerides, mmol/L	1.56 (± 1.02)
γ' fibrinogen, g/L	0.26 (± 0.12)
Total fibrinogen, g/L	3.80 (± 0.75)
Prevalent CVD, %, n	12.8, 388
Prevalent hard CVD, %, n	5.6, 172
Prevalent myocardial infarction, %, n	4.3, 132
Prevalent stroke, %, n	1.5, 47

CVD indicates cardiovascular disease.
*(n=3,042).

dichotomous risk factors and γ' and total fibrinogen) are presented in Table 1.

Genome-Wide Association Study

To identify genetic loci related to γ' fibrinogen levels, we conducted a genome-wide association study (GWAS).

Quantile-quantile plots of the observed versus expected probability values showed little evidence of potential inflation in the results, with a genomic control λ value of 1.01. A total of 54 SNPs exceeded the threshold for genome-wide significance ($P < 5.0 \times 10^{-8}$) and clustered exclusively in or near the fibrinogen gene locus on chromosome 4 (Figure 2). No other loci on other chromosomes reached the threshold for significance. The strongest statistical evidence for an association with γ' fibrinogen levels was with rs7681423 (minor allele frequency [MAF]: 0.227, $P = 9.97 \times 10^{-110}$, variance explained: 13%), which is 8.35 kb upstream of the γ chain gene *FGG* (Table 2; Figure 3). Interestingly, the A>G variant in *FGB* with the strongest statistical association with total fibrinogen levels, rs1800789,²⁷ was not significantly associated with γ' fibrinogen levels (MAF: 0.209, $P = 4.34 \times 10^{-4}$, variance explained: 0.4%).

Several SNPs in *FGG* were identified by genome-wide association that have previously been associated with γ' fibrinogen levels, particularly rs1049636,³ the 9340C>T variant within intron 9 (MAF: 0.319, $P = 3.84 \times 10^{-50}$, $R^2 = 0.13$ with rs7681423). In addition, rs2066861, the 7874G>A variant in intron 8 (MAF: 0.227, $P = 1.90 \times 10^{-109}$, $R^2 = 1.0$ with rs7681423); rs2066864, the 9615C>T variant in intron 9 (MAF: 0.227, $P = 5.38 \times 10^{-109}$, $R^2 = 1.0$ with rs7681423); and rs2066865, the 10 034C>T variant in the 3' untranslated region (MAF: 0.225, $P = 7.13 \times 10^{-109}$, $R^2 = 1.0$ with rs7681423), which have previously been shown to be in linkage disequilibrium,¹⁷ were associated with γ' fibrinogen levels. We performed additional GWAS, sequentially adjust-

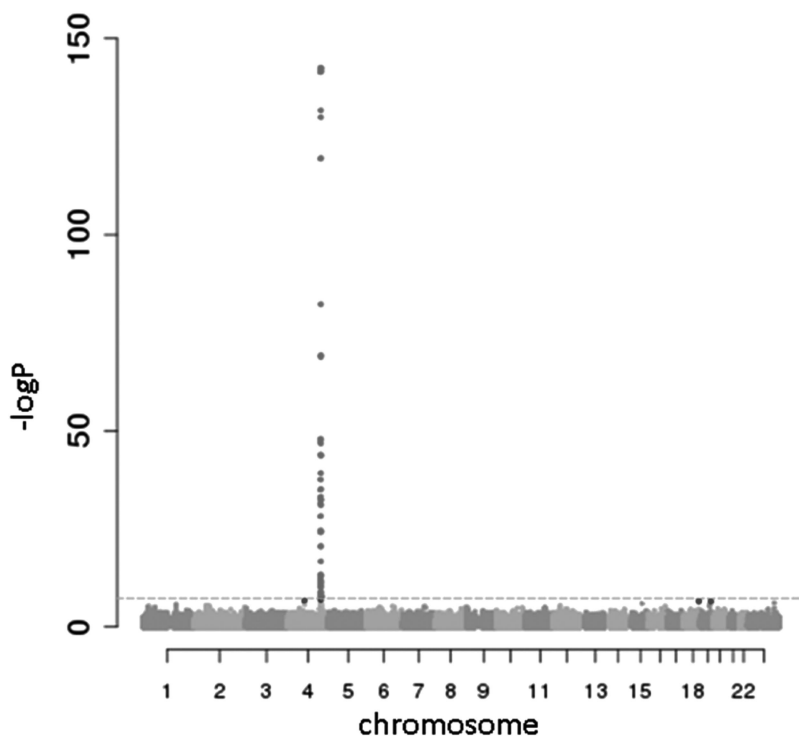


Figure 2. Genome-wide association analysis for γ' fibrinogen levels. -Log probability values are shown across 22 autosomal and sex chromosomes. Multivariate-adjusted natural log-transformed γ' fibrinogen levels included the covariates sex, age, body mass index, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, high-density lipoprotein cholesterol, and triglycerides, plus principal components that accounted for potential population admixture. Separate chromosomes are indicated by alternating shading. The dashed gray horizontal line corresponds to the probability value threshold of 5.0×10^{-8} . Black dots represent findings with a probability value $\leq 5 \times 10^{-8}$, and gray dots represent findings with a probability value $\leq 4 \times 10^{-7}$ but $> 5 \times 10^{-8}$.

Table 2. Top SNPs Having Significant Associations With γ' Fibrinogen Levels

rsID	Location	Physical Position	Minor (Major) Allele	MAF*	R^2 †	β ‡	SE	P Value	Imputation Quality
rs7681423	5' of <i>FGG</i>	155761698	T(C)	0.23	0.130	-0.294	0.013	9.97×10^{-110}	0.99
rs7654093	5' of <i>FGG</i>	155764522	T(A)	0.23	0.130	-0.295	0.013	1.05×10^{-109}	0.97
rs12644950	5' of <i>FGG</i>	155756771	A(G)	0.23	0.130	-0.292	0.013	1.15×10^{-109}	0.99
rs2066861	<i>FGG</i> intron 8	155746886	T(C)	0.23	0.129	-0.291	0.013	1.90×10^{-109}	1.00
rs2066864	<i>FGG</i> intron 9	155745145	A(G)	0.23	0.129	-0.291	0.013	5.38×10^{-109}	1.00
rs2066865	3' of <i>FGG</i>	155744726	A(G)	0.23	0.129	-0.291	0.013	7.13×10^{-109}	0.99
rs7659024	Between <i>FGG</i> and <i>FGA</i>	155740380	A(G)	0.23	0.129	-0.291	0.013	1.15×10^{-108}	0.99
rs13130318	5' of <i>FGG</i>	155757920	G(T)	0.22	0.119	-0.301	0.014	5.50×10^{-100}	0.89
rs13109457	Between <i>FGG</i> and <i>FGA</i>	155734329	A(G)	0.24	0.119	-0.279	0.013	1.05×10^{-99}	0.96
rs6050	<i>FGA</i> exon 5 (Thr312Thr)	155727040	C(T)	0.24	0.108	-0.264	0.013	8.90×10^{-90}	0.97
rs6825454	Between <i>FGA</i> and <i>FGB</i>	155720638	C(T)	0.24	0.108	-0.265	0.013	1.12×10^{-89}	0.96
rs6536024	5' of <i>FGG</i>	155762819	T(C)	0.44	0.085	0.221	0.013	1.16×10^{-68}	0.81
rs1049636	<i>FGG</i> intron 9	155745420	G(A)	0.32	0.063	0.182	0.012	3.84×10^{-50}	0.99
rs1118823	Between <i>FGG</i> and <i>FGA</i>	155743296	A(T)	0.32	0.063	0.182	0.012	4.31×10^{-50}	0.99
rs12648395	5' of <i>FGG</i>	155760739	C(T)	0.32	0.063	0.180	0.012	7.92×10^{-50}	1.00
rs1800788	5' of <i>FGB</i>	155703364	T(C)	0.20	0.050	-0.189	0.014	1.37×10^{-39}	0.97
rs12648258	5' of <i>FGB</i>	155696822	A(T)	0.20	0.050	-0.189	0.014	2.51×10^{-39}	0.97
rs12642469	5' of <i>FGB</i>	155693672	A(G)	0.19	0.049	-0.187	0.014	1.22×10^{-38}	0.98
rs12511469	3' of <i>PLRG1</i>	155671209	A(T)	0.19	0.046	-0.180	0.014	2.46×10^{-36}	1.00
rs10008078	3' of <i>PLRG1</i>	155668003	A(G)	0.19	0.046	-0.180	0.014	2.85×10^{-36}	0.99
rs13147579	3' of <i>PLRG1</i>	155668498	T(C)	0.19	0.046	-0.180	0.014	2.85×10^{-36}	0.99
rs7662567	3' of <i>PLRG1</i>	155668598	C(T)	0.19	0.046	-0.180	0.014	2.88×10^{-36}	1.00
rs13435101	3' of <i>PLRG1</i>	155669282	C(A)	0.47	0.044	0.145	0.012	6.38×10^{-35}	0.97
rs12642770	<i>PLRG1</i> intron 11	155679909	C(T)	0.21	0.040	-0.164	0.014	1.15×10^{-31}	0.99
rs7659613	3' of <i>PLRG1</i>	155734866	C(G)	0.37	0.038	-0.138	0.012	3.45×10^{-30}	0.97
rs2070006	Between <i>FGG</i> and <i>FGA</i>	155733316	T(C)	0.37	0.038	-0.137	0.012	4.60×10^{-30}	0.97
rs13435192	3' of <i>PLRG1</i>	155669608	C(T)	0.47	0.038	0.131	0.012	8.92×10^{-30}	1.01
rs7689945	3' of <i>PLRG1</i>	155669079	C(T)	0.47	0.038	0.131	0.012	9.09×10^{-30}	1.01
rs13123551	3' of <i>PLRG1</i>	155668514	T(A)	0.47	0.038	0.131	0.012	9.17×10^{-30}	1.01
rs4463047	Between <i>FGA</i> and <i>FGB</i>	155714983	C(T)	0.08	0.034	-0.313	0.029	1.09×10^{-26}	0.55
rs2070011	<i>FGA</i> promoter	155731347	T(C)	0.37	0.033	-0.126	0.012	2.52×10^{-26}	1.00
rs4642230	3' of <i>PLRG1</i>	155664208	A(G)	0.18	0.033	-0.158	0.015	3.88×10^{-26}	0.98
rs4235247	3' of <i>PLRG1</i>	155657789	A(G)	0.17	0.030	-0.151	0.015	6.03×10^{-24}	1.00
rs4550901	Between <i>FGA</i> and <i>FGB</i>	155720983	A(C)	0.13	0.023	0.156	0.018	7.72×10^{-19}	0.94
rs4308349	Between <i>FGA</i> and <i>FGB</i>	155721563	G(A)	0.13	0.023	0.156	0.018	7.73×10^{-19}	0.94
rs2070018	<i>FGA</i> intron 4	155728077	G(A)	0.13	0.023	0.156	0.018	7.75×10^{-19}	0.94
rs12642646	3' of <i>PLRG1</i>	155665902	A(G)	0.46	0.022	-0.107	0.012	4.90×10^{-18}	0.89
rs2070022	<i>FGA</i> exon 6 (3' UTR)	155724398	A(G)	0.17	0.022	0.135	0.016	5.85×10^{-18}	0.96
rs2227412	<i>FGB</i> intron 4	155708545	G(A)	0.16	0.019	0.129	0.016	2.99×10^{-15}	0.93
rs9997519	5' of <i>PLRG1</i>	155692620	T(C)	0.16	0.019	0.129	0.016	3.25×10^{-15}	0.92
rs12651106	<i>DCHS2</i> intron 2	155520509	A(C)	0.17	0.015	-0.136	0.019	1.62×10^{-12}	0.64
rs4323084	3' of <i>PLRG1</i>	155643681	T(C)	0.23	0.013	-0.092	0.014	3.05×10^{-11}	0.95
rs11737226	3' of <i>PLRG1</i>	155661696	G(A)	0.32	0.013	-0.081	0.012	6.19×10^{-11}	0.99
rs17373860	<i>DCHS2</i> exon 1 (Pro209Ser)	155631333	A(G)	0.11	0.011	-0.143	0.023	7.30×10^{-10}	0.62
rs4622984	3' of <i>PLRG1</i>	155671683	T(C)	0.34	0.011	-0.073	0.012	2.35×10^{-09}	1.01
rs6819508	3' of <i>PLRG1</i>	155670637	A(G)	0.14	0.011	0.099	0.017	2.57×10^{-09}	1.00
rs12645631	<i>PLRG1</i> intron 11	155680219	A(G)	0.14	0.011	0.098	0.017	2.77×10^{-09}	1.00
rs7698829	<i>PLRG1</i> intron 10	155680917	C(T)	0.14	0.011	0.098	0.017	2.81×10^{-09}	1.00
rs1873369	<i>DCHS2</i> intron 1	155627928	A(C)	0.24	0.010	-0.079	0.014	5.71×10^{-09}	0.99
rs13122184	3' of <i>PLRG1</i>	155675439	T(C)	0.06	0.010	0.159	0.028	8.01×10^{-09}	0.72
rs11731813	<i>DCHS2</i> intron 1	155599333	G(A)	0.25	0.010	-0.080	0.014	9.30×10^{-09}	0.90
rs1490683	<i>DCHS2</i> intron 1	155582326	C(T)	0.37	0.009	-0.073	0.013	2.31×10^{-08}	0.84
rs4482740	3' of <i>PLRG1</i>	155665755	A(G)	0.37	0.009	0.071	0.013	2.54×10^{-08}	0.90
rs12504201	<i>DCHS2</i> intron 1	155578137	A(C)	0.37	0.009	-0.068	0.012	3.13×10^{-08}	0.95

*MAF indicates frequency of the minor (less frequent) allele.

†Variance of natural log-transformed γ' fibrinogen explained by the additive coding of the single-nucleotide polymorphism genotype.‡The increase in natural log γ' fibrinogen per copy increment of the minor allele.

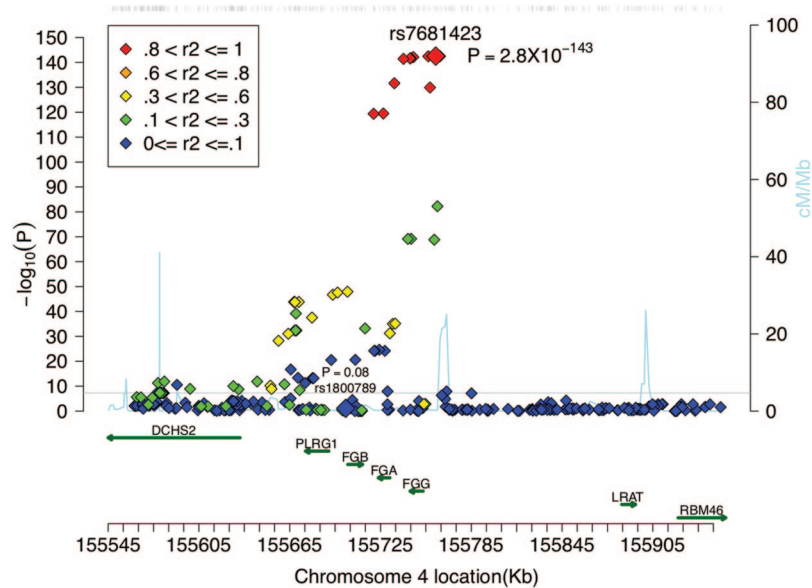


Figure 3. Regional plots of loci associated with γ' and total fibrinogen. The associated probability values (natural log-transformed) for single-nucleotide polymorphisms (SNPs) in each of the loci are plotted vs their chromosome positions. Each diamond represents a SNP, with the color indicating the linkage disequilibrium between the SNP and the top associated SNP, which is indicated by the largest red diamond. The gray horizontal line corresponds to the probability value threshold of 5.0×10^{-8} . This figure shows that the top SNP in the fibrinogen gene cluster for γ' fibrinogen levels (rs7681423) does not correlate with the top SNP (rs1800789, the largest blue diamond) that was reported in association with total fibrinogen levels. The tick marks at the top of graph indicate the density of SNPs.

ing for top signal SNPs from the previous GWAS. After adjusting for the top signal SNP rs7681423 from the initial GWAS, rs1049636 became the top signal SNP ($P=3.4 \times 10^{-17}$, variance explained: 1.8%), and all aforementioned SNPs were no longer genome-wide significant. After additionally adjusting for rs1049636, no genome-wide significant signal remained (Supplemental Table I, available online at <http://atvb.ahajournals.org>).

Perhaps the most intriguing association with SNPs near the fibrinogen gene locus was in the *PLRG1* gene near the fibrinogen gene locus; rs12642770 (MAF: 0.205, $P=8.69 \times 10^{-30}$, 81 kb from the top SNP rs7681423, $R^2=0.35$ with rs7681423), rs12645631 (MAF: 0.147, $P=2.77 \times 10^{-9}$, $R^2=0.09$ with rs7681423), and rs7698829 (MAF: 0.147, $P=2.81 \times 10^{-9}$, $R^2=0.06$ with rs7681423) in *PLRG1* all showed genome-wide significance. *PLRG1* encodes pleiotropic regulator 1, which plays a direct role in mRNA splicing.²⁸ No SNPs in *PLRG1* were found previously in association with total fibrinogen levels.²⁷ Because the γ' chain arises from an alternative mRNA splicing event,^{29,30} this raises the intriguing possibility that SNPs in *PLRG1* may play a functional role in modulating γ' fibrinogen levels. After adjusting for the top signal SNP rs7681423 in *FGG*, however, these SNPs were no longer genome-wide significant (P ranged from 0.01 to 0.19); after additional adjusting for rs1049636, the associations of these SNPs were further weakened (P ranged from 0.17 to 0.27), which suggests that association signals observed in *PLRG1* may be attributable to linkage disequilibrium (Supplemental Table I).

Association Between SNPs and CVD

The 2 independent top signal SNPs, rs7681423 and rs1049636, near and in *FGG* were not associated with any of

the cardiovascular events examined in this study (Table 3), but the effects were directionally consistent with their association with γ' fibrinogen. The previously identified top signal SNP for total fibrinogen, rs1800789, was not associated with any of the cardiovascular events either,²⁷ even though total fibrinogen levels are well known to be associated with CVD.⁶

Association Between γ' Fibrinogen Levels and CVD

γ' fibrinogen has previously been shown to be significantly associated (all $P < 0.05$) with the cardiovascular risk factors of age, sex, BMI, smoking, diabetes, blood glucose, and triglycerides and inversely associated with HDL cholesterol in this cohort.⁷ Individuals with prevalent CVD had significantly higher risk-factor adjusted mean (\pm standard error) γ' fibrinogen concentrations than those without CVD (0.278 ± 0.006 mg/mL versus 0.258 ± 0.002 mg/mL; $P=0.002$). Results were similar for men and women separately (sex-by-prevalent-CVD probability value = 0.220). In a cross-sectional assessment of the relationship between γ' fibrinogen and CVD, the odds ratio (OR) of event per 0.1 mg/mL increase in γ' fibrinogen was 1.12 (1.03 to 1.21) after adjustment for sex, age, BMI, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, HDL cholesterol, and triglycerides. In a univariate model, the age-adjusted OR (95% confidence interval) comparing the prevalence of CVD in the highest versus lowest γ' fibrinogen tertile was 1.84 (1.15 to 2.93) for women, 1.70 (1.19 to 2.43) for men, and 1.76 (1.33 to 2.34) for men and women combined. This association (Table 4) remained significant after multivariable adjustment for sex, age, BMI, smoking, diabetes, fasting blood glucose, systolic blood pressure, total cholesterol, HDL

Table 3. Association Between Cardiovascular Events and Single-Nucleotide Polymorphisms That Associate With γ' Fibrinogen or Total Fibrinogen, Respectively*

SNP	Minor (Major) Allele and MAF	Phenotype	β †	SE	P Value	Odds Ratio	CI, Lower	CI, Upper
rs7681423	T (C), 0.23	CVD	-0.049	0.10	0.62	0.95	0.78	1.16
		Hard CVD	-0.074	0.15	0.61	0.93	0.70	1.24
		MI	-0.028	0.17	0.87	0.97	0.70	1.35
		Stroke	-0.119	0.25	0.64	0.89	0.54	1.45
rs1800789	A (G), 0.21	CVD	-0.076	0.11	0.48	0.93	0.75	1.14
		Hard CVD	0.066	0.14	0.64	1.07	0.81	1.41
		MI	0.025	0.17	0.88	1.03	0.74	1.42
		Stroke	0.109	0.25	0.67	1.11	0.68	1.83
rs1049636	G (A), 0.32	CVD	0.032	0.10	0.74	1.03	0.85	1.25
		Hard CVD	0.170	0.14	0.21	1.18	0.91	1.55
		MI	0.069	0.16	0.66	1.07	0.79	1.46
		Stroke	0.334	0.22	0.13	1.40	0.91	2.15

*All analyses were multivariable adjusted in a model with the covariates sex, age, body mass index, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, high-density lipoprotein cholesterol, and triglycerides. MAF indicates minor allele frequency; CI, confidence interval; CVD, cardiovascular disease; MI, myocardial infarction.

†Log of odds ratio per 1 copy increment of the minor allele, ie the less frequent allele.

cholesterol, and triglycerides for men and women combined (highest versus lowest tertile, OR=1.53, 95% CI [1.14 to 2.05]). Similarly elevated magnitudes of risk were noted in sex-specific analyses for women (highest versus lowest tertile, OR=1.66, 95% CI [1.04 to 2.68]) and for men (highest versus lowest tertile, OR=1.44, 95% CI [0.99 to 2.11]), although the relationship was borderline significant in men.

Similar significant associations were found between γ' fibrinogen and hard CVD (multivariable adjusted OR 1.61 [1.05 to 2.47] for both sexes combined) and myocardial infarction (multivariable adjusted OR 1.76 [1.06 to 2.92]). The association between γ' fibrinogen and stroke (multivariable adjusted OR 1.42 [0.68 to 2.95]) did not reach statistical significance ($P=0.36$), although there was a nonsignificant trend toward higher γ' levels with stroke. For all CVD outcomes, there was no significant γ' fibrinogen-by-sex

interaction on associations with prevalent CVD ($P=0.082$ for hard CVD, $P=0.270$ for myocardial infarction, $P=0.335$ for stroke).

Further adjustment for total fibrinogen rendered nonsignificant associations of γ' fibrinogen with CVD ($P>0.05$ for men and women combined). This is in part due to the correlation between the 2 fibrinogen measurements (age- and sex-adjusted Pearson correlation coefficient of 0.44; $P<0.001$). However, in multivariable-adjusted models (Table 4), the OR for the highest versus lowest tertile of γ' fibrinogen alone for CVD was 1.53 (1.14 to 2.05) and for the highest versus lowest tertile of total fibrinogen alone, it was 1.54 (1.14 to 2.07), but the OR for the highest tertile of both total fibrinogen and γ' fibrinogen compared with the lowest tertile of both was 2.17 (1.42 to 3.32). The OR for association between fibrinogen and myocardial infarction was also increased when considering both γ' fibrinogen and total fibrinogen simultaneously than when considering either type of fibrinogen alone. In multivariable-adjusted models, the OR for highest versus lowest tertile of γ' fibrinogen alone was 1.76 (1.06 to 2.92), and for the highest versus lowest tertile of total fibrinogen alone, it was 1.99 (1.21 to 3.28), but the OR for the highest tertile versus the lowest tertile of both total fibrinogen and γ' fibrinogen was 3.08 (1.41 to 6.72). These results suggest that γ' and total fibrinogen are not simply surrogate markers for one another but have different associations with CVD. In addition, adjusting for the top signal SNPs rs7681423 and rs1049636 of γ' fibrinogen and rs1800789 of total fibrinogen levels resulted in minimal changes to these results (Supplemental Tables II and III).

Taken together, these results suggest that genetics may not play a major role in the association between γ' fibrinogen and CVD. Because γ' fibrinogen is an acute phase reactant that increases in response to inflammation,^{8,9} environmental factors may play a greater role than genetic factors in its association with CVD.

Table 4. Association Between Fibrinogen Types and Prevalent Cardiovascular Disease*

Disease	Adjusted Odds Ratio (95% Confidence Interval)		
	γ' Fibrinogen, Tertile 3 vs 1	Total Fibrinogen, Tertile 3 vs 1	γ' and Total Fibrinogen, Tertiles 3 vs 1
Total CVD	1.53 (1.14 to 2.05)	1.54 (1.14 to 2.07)	2.17 (1.42 to 3.32)
Hard CVD	1.61 (1.05 to 2.47)	1.79 (1.16 to 2.74)	2.67 (1.38 to 5.15)
Myocardial infarction	1.76 (1.06 to 2.92)	1.99 (1.21 to 3.28)	3.08 (1.41 to 6.72)
Stroke	1.42 (0.68 to 2.95)	1.25 (0.62 to 2.53)	2.03 (0.66 to 6.30)

*Adjusted for sex, age, body mass index, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, high-density lipoprotein cholesterol, and triglycerides. CVD indicates cardiovascular disease.

Discussion

The genetic loci associated with total fibrinogen levels²⁷ are different from those associated with γ' fibrinogen levels in the current study. For total fibrinogen, 4 loci are marked by 1 or more SNPs with genome-wide significance ($P < 5.0 \times 10^{-8}$); *FBG*, the fibrinogen B β chain gene; *IRF1*, the interferon regulatory factor 1 gene; *PCCB*, the propionyl coenzyme A carboxylase gene; and *NLRP3*, the NLR family pyrin domain containing 3 isoforms gene. In contrast, the loci we identified that are significantly associated with γ' fibrinogen levels are all located in or near the fibrinogen gene locus, including the *PLRG1* gene.

Some of the SNPs in the γ gene *FGG* have been previously associated with γ' fibrinogen levels. In particular, rs1049636, the 9340C>T variant within intron 9 has been shown to be significantly associated with γ' fibrinogen levels.³ Individuals in the Stockholm Coronary Artery Risk Factor study homozygous for the 9340T variant had a lower mean γ' fibrinogen level (0.25 mg/mL for cases, 0.21 mg/mL for controls) compared with those homozygous for the 9340C variant (0.38 mg/mL for cases, 0.31 mg/mL for controls), whereas heterozygotes displayed intermediate levels (0.29 mg/mL for cases, 0.27 mg/mL for controls). In addition, rs2066861, the 7874G>A variant in intron 8, rs2066864, the 9615C>T variant in intron 9, and rs2066865, the 10 034C>T variant in the 3' untranslated region, which are in linkage disequilibrium and are haplotype-tagging SNPs, have been associated with γ' fibrinogen levels.¹⁷

Perhaps the most intriguing association with SNPs near the fibrinogen gene locus is in the *PLRG1* gene next to the fibrinogen gene locus; rs12642770, rs12645631, and rs7698829 in *PLRG1* all showed genome-wide significance. None of these SNPs are significantly associated with total fibrinogen levels.²⁷ *PLRG1* encodes pleiotropic regulator 1, which plays a direct role in mRNA splicing.²⁸ This raises the possibility that *PLRG1* may be mechanistically involved in modulating the splicing events that give rise to the γ' and γ A splice variants.^{29,30} However, it is also possible that the *PLRG1* SNPs are simply in linkage disequilibrium with other functional SNPs within the fibrinogen locus that constitute a specific haplotype. However, the minor allele frequencies of rs12645631 and rs7698829 in the *PLRG1* locus, 0.14, are different from the MAF of rs1049636 in *FGG* intron 9 (0.32); the estimated R^2 with the 2 *PLRG1* SNPs was 0.25 and different from the minor allele frequencies of 0.224 to 0.225 for the H2H2 haplotype-tagging SNPs rs2066861, rs2066864, and rs2066865 (estimated R^2 between each of the tagging SNPs and each of the 2 *PLRG1* loci was only 0.04) that have been associated with γ' fibrinogen levels previously.¹⁷ On the other hand, the MAF of rs12642770 in the *PLRG1* locus, 0.21, is similar to those of the H2H2 haplotype-tagging SNPs (estimated R^2 between each of the tagging SNPs and rs12642770 was 0.36). Additional studies will be required to determine whether the *PLRG1* locus plays a functional role in γ' fibrinogen regulation.

Of interest, the top SNP in the fibrinogen gene cluster that was reported in association with total fibrinogen levels was not associated with γ' fibrinogen levels. The strongest statistical evidence for an association with total fibrinogen levels

was with rs1800789,²⁷ which is located in exon 7 of the fibrinogen B β chain gene *FBG*; the association of this SNP with γ' fibrinogen levels did not even reach statistical significance ($P = 4.34 \times 10^{-4}$). Rather, the top SNP in association with γ' fibrinogen levels was rs7681423, 8.35 kb upstream of the γ chain gene *FGG*. These findings suggest that γ' fibrinogen and total fibrinogen levels are under differential genetic control, consistent with the partially additive ORs for CVD observed in Table 4.

The results of the present study show a significant increased multivariable-adjusted association of plasma γ' fibrinogen with prevalent total CVD, as well as prevalent myocardial infarction. The association was not as robust as was seen in our previous case-control study of acute coronary artery disease, which showed an OR of 7.16 comparing the top and bottom γ' fibrinogen quartiles.² However, although the Framingham Offspring Study participants in the present study had no documented history of CVD, it is likely that some of the participants may have had underlying subclinical CVD that had not yet manifested itself as an acute event. In addition, the time since the onset of CVD varied widely among the Framingham Offspring cohort, whereas our previous case-control study examined acute coronary artery disease at the time of diagnosis.

In addition, there was a higher order interaction between γ' fibrinogen and total plasma fibrinogen that manifested as a further increased association with myocardial infarction (Table 4). However, further adjustment for total fibrinogen attenuated the statistical significance of the association between γ' fibrinogen and CVD. This is to be expected, because γ' fibrinogen is a subset of total fibrinogen. By analogy, low-density lipoprotein cholesterol levels, a subset of total cholesterol, lose significant association with CVD if adjusted for total cholesterol levels. However, in addition, variables that affect total fibrinogen expression may also affect γ' fibrinogen expression. As an example, the *FGG* T9430C polymorphism in the fibrinogen γ gene promoter, rs1049636, which increases total fibrinogen concentration, is also associated with increased plasma γ' fibrinogen concentration.³ Thus, although there is significant correlation between total fibrinogen and γ' fibrinogen, there is also considerable interindividual variation between these 2 biomarkers.^{2,7,9} The partially additive effect of γ' and total fibrinogen on the ORs for CVD indicates that γ' and total fibrinogen are not simply surrogates for one another. Another finding suggesting that γ' and total fibrinogen are not surrogate markers comes from the GWAS, in which different SNPs are associated with γ' and total fibrinogen levels.

It remains to be seen whether or not γ' fibrinogen is simply a marker of CVD or a prospectively defined risk factor for CVD. However, the data presented here provide a compelling rationale for further investigation into the association between γ' fibrinogen and CVD. Future prospective studies are therefore warranted to examine the ability of γ' fibrinogen to predict CVD.

Sources of Funding

This work was supported by the National Heart, Lung, and Blood Institute's Framingham Heart Study (Contract No. N01-HC-25195);

American Heart Association Grant 0865486G (to R.S.L.); National Heart, Lung, and Blood Institute, National Institutes of Health, Grants R21-HL-75006 and R21-HL-097298 (to D.H.F.); and Office of Naval Research Grant N000140610411 (to D.H.F.).

Disclosures

Dr Farrell is founder and chief scientific officer of Gamma Therapeutics, Inc.

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Supplementary Table 3s. Association (Odds ratio and 95% confidence interval) of fibrinogen tertiles with CVD events with and without adjusting for SNPs associated with fibrinogen measures.

Disease	N Disease	Adjustment	Separate Models		Joint Models		γ' and Total Fibrinogen Tertiles 3 vs. 1
			γ' Fibrinogen Tertile 3 vs. 1	Total Fibrinogen Tertile 3 vs. 1	γ' Fibrinogen Tertile 3 vs. 1	Total Fibrinogen Tertile 3 vs. 1	
Total CVD	388	MV	1.47(1.01- 2.02)	1.45(1.06-1.96)	1.33 (0.95-1.86)	1.32 (0.96- 1.83)	1.83 (1.14-2.94)
		MV and SNPs	1.53(1.09 - 2.16)	1.46(1.07 - 1.99)	1.36 (0.94 - 1.96)	1.33 (0.96 - 1.85)	2.03 (1.22 - 3.38)
Hard CVD	172	MV	1.49(0.95- 2.34)	1.74 (1.09- 2.77)	1.26 (0.78- 2.04)	1.64 (1.00-2.69)	2.09 (1.05-4.19)
		MV and SNPs	1.48(0.91 - 2.41)	1.73(1.08 - 2.77)	1.22 (0.72 - 2.07)	1.66 (1.00 - 2.76)	2.00 (0.98 - 4.10)
Myocardial infarction	132	MV	1.59 (0.94- 2.71)	2.04 (1.17-3.55)	1.29 (0.74-2.25)	1.94 (1.09-3.45)	2.53 (1.13-5.69)
		MV and SNPs	1.68(0.96 - 2.94)	2.04(1.17 - 3.56)	1.32 (0.73 - 2.39)	1.93 (1.07 - 3.48)	2.73 (1.21 - 6.19)
Stroke	47	MV	1.29(0.57-2.90)	0.98 (0.47-2.06)	1.31 (0.53- 3.22)	0.90 (0.39-2.09)	1.20 (0.36-3.93)
		MV and SNPs	1.18(0.47 - 2.94)	0.95(0.45 - 1.99)	1.20 (0.42 - 3.43)	0.90 (0.38 - 2.17)	0.91 (0.28 - 3.01)

All analyses were restricted to the sample of 3,042 individuals having both fibrinogen measures, all covariates in MV analyses and SNP genotypes.

MV: Multivariable adjusted model that has both γ' fibrinogen and total fibrinogen as predictors, adjusted for

sex, age, BMI, systolic blood pressure, fasting blood glucose, diabetes mellitus, smoking, total cholesterol, HDL cholesterol, and triglycerides.

MV and SNP: Same as the MV model but also adjusted for rs7681423,rs1049636 and rs1800789, SNPs that associated with γ' fibrinogen and total fibrinogen, respectively.