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Fibrinogen and *IL6* Gene Variants and IL-6 Levels in Relation to Plasma Fibrinogen Concentration and Cardiovascular Disease Risk in the Cardiovascular Health Study

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Summary

Background: The inflammatory cytokine interleukin-6 (IL-6) is a main regulator of fibrinogen synthesis, though its interaction with fibrinogen genes (*FGA*, *FGB*, *FGG*) in relation to CVD risk is not well-studied in humans.

Methods and Results: We investigated joint associations of common fibrinogen and *IL6* tagSNPs with fibrinogen level, carotid intima-media thickness (IMT) and risk of myocardial infarction (MI) or ischemic stroke in 3900 European-American participants of the Cardiovascular Health Study. To identify combinations of genetic main effects and interactions associated with each outcome, we used logic regression. We also evaluated whether the relationship between fibrinogen SNPs and fibrinogen level varied by IL-6 level using linear regression models with multiplicative interaction terms. Combinations of fibrinogen and *IL6* SNPs were associated with fibrinogen level ($p<0.005$), but not with IMT ($p>0.30$), MI ($p=0.73$) or stroke ($p=0.21$). Fibrinogen levels were higher in higher in individuals having *FGB*1437 (rs1800790) minor alleles and lacking *FGA*6534 (rs6050) minor alleles; these SNPs interacted with IL6 rs1800796 to influence fibrinogen level. Marginally significant ($p=0.03$) interactions between IL-6 level and SNPs located in promoter regions of *FGA* and *FGG* associated with fibrinogen levels were detected.

Conclusion: We identified potential gene-gene interactions influencing fibrinogen levels. Although IL-6 responsive binding sites are present in fibrinogen gene promoter regions, we did not find strong evidence of interaction between fibrinogen SNPs and IL6 SNPs or levels influencing CVD risk.

Background

Fibrinogen is a key component of the inflammation and clotting pathways and an established risk factor for cardiovascular disease (CVD).(Danesh *et al.*, 2005) Fibrinogen production is upregulated in response to cytokines released during inflammation, infection, neoplasia or tissue damage.(Ernst, 1993)

Three genes (*FGA*, *FGB* and *FGG*) on human chromosome 4 code for the two sets of α, β, and γ–chains that constitute fibrinogen. Although each gene is separately transcribed and translated, transcription is well coordinated so that when expression of one gene is elevated,

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the other genes are also upregulated.(Duan & Simpson-Haidaris, 2003, Fuller & Zhang, 2001) Interleukin-6 (IL-6) is the key regulator, though other mediators, such as glucocorticoids, IL-1β and TNF-α also modulate acute phase fibrinogen synthesis.(Fuller & Zhang, 2001) Acting via an intracellular pathway, IL-6 binds to its receptor and activates STAT proteins. In particular, STAT3, also known as acute phase response factor, is the major transcription factor mediating regulation of IL-6-responsive genes.(Duan & Simpson-Haidaris, 2006) STAT3 relays signals from the IL-6 receptor to the nucleus where it binds to response elements in the promoter regions of the fibrinogen genes. Human fibrinogen genes are highly responsive to the IL-6 cytokine; several IL-6 responsive binding sites in the promoter regions of the fibrinogen genes have been identified.(Duan & Simpson-Haidaris, 2003, Heinrich *et al.*, 1990)

Though IL-6 is a key regulator of fibrinogen synthesis, few studies have investigated whether having specific combinations of variants in the *IL6* and fibrinogen genes predisposes to CVD. In one such study, no interaction between an *IL6* SNP and three fibrinogen SNPs influencing risk of MI was detected, though the study was small and included few variants.(Mannila *et al.*, 2006)

Given the role of IL-6 in fibrinogen regulation, we hypothesized that genetic variation in the fibrinogen locus could affect binding to IL-6 response elements and potentially the regulation of fibrinogen levels in humans. Furthermore, the potential synergism between these genes on the fibrinogen phenotype may have implications for subclinical and clinical CVD development. In this study, we assessed the potential interaction of both common *IL6* SNPs and the gene product, IL-6 cytokine levels, with fibrinogen SNPs in influencing fibrinogen levels. Fibrinogen and *IL6* gene-gene interactions associated with markers of subclinical CVD, internal and common carotid artery intima-medial thickness (IMT), and risk of future ischemic stroke or myocardial infarction (MI) were also evaluated.

Methods

Study Population

The Cardiovascular Health Study is a population-based cohort of mainly white (European-descent) adults aged 65 years and older recruited from 4 field centers in North Carolina, California, Maryland and Pennsylvania.(Fried *et al.*, 1991) Demographic, lifestyle and medical history information and fasting blood samples were collected on CHS participants at baseline. Blood collection procedures and laboratory methods have been reported previously.(Cushman *et al.*, 1995) Hypertension was defined as systolic BP>=140mmHG or diastolic BP >=90mmHG or having a history of hypertension with concurrent use of anti-hypertensive medication. Diabetes status was defined as 'established/new' for participants using insulin or oral hypoglycemics and for participants with fasting glucose ≥ 126mg/dL, or as 'normal.' We restricted the current study to individuals of European descent, 'whites,' for issues of power; our genetic analyses precluded missing data and thus pooling of racial groups with different genotyped tagSNPs was not feasible without loss of gene coverage. Additionally, adequate power for interaction analyses (of moderate effect size) is a problem in the smaller minority cohort. Of the 4925 individuals whose self-identified race was 'white,' we excluded individuals who did not provide informed consent for participation in DNA studies, or for whom DNA was not available (n=211). Additionally, participants with a history of MI or stroke at baseline (n=610), those for whom baseline measures of fibrinogen were unavailable (n=44) and those with incomplete genotype data (n=160) were also excluded for a final sample of 3900. An additional 299 individuals without baseline measurements were dropped from analyses involving IL-6 levels for a total of 3621 participants.

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Using baseline samples, plasma fibrinogen levels were measured using the Clauss assay which measures the functional clotting activity of fibrinogen. Both IL-6 and C-reactive protein (CRP) were measured in stored frozen serum samples using a commercial assay (Quantikine HS Human IL-6 Immunoassay, R&D Systems, Minneapolis, MN, USA) and a validated in-house high-sensitivity enzyme-linked-immunosorbent-assay (ELISA), respectively.(Macy *et al.*, 1997) Analytic coefficients of variation were 9.7%, 6.3% and 5.1% for the assays, respectively. Maximum carotid artery intima-media thickness (IMT) in mm was determined at the baseline examination by high-resolution B-mode ultrasonography.(O'Leary *et al.*, 1991) IMT measures were calculated for the common and internal carotid arteries by averaging maximum wall thicknesses obtained from scans of the near and far walls on the left and right sides.

Participants were followed through June 2003 (maximal follow-up of 14 years) with median follow-up of 13 years. Censoring date was defined by death, loss to follow-up, study drop out or event date. Incident, non-procedure-related (not occurring during surgery or re-vascularization) MI or ischemic stroke events were analyzed. MI was defined using standard CHS criteria: history of chest pain, cardiac enzyme levels, and characteristic changes on serial electrocardiograms. Stroke was validated based on criteria that included onset of symptoms, duration of deficits, and findings on computed tomography or magnetic resonance imaging. Participants with multiple incident events were censored on the date of the first event. All events were adjudicated by CHS committee as reported previously.(Ives *et al.*, 1995)

SNP Selection Strategy and Genotyping

TagSNPs in the fibrinogen (n=16) and *IL6* (n=10) genes were identified by the SNP discovery resource, SeattleSNPs, among European descent individuals (whites) using the LD select algorithm.(Carlson *et al.*, 2004) Of these, 12 fibrinogen and 6 *IL6* tagSNPs were common (minor allele frequency (MAF) ≥ 5%) in CHS whites. Genotyping was performed at the Laboratory for Clinical Biochemistry Research (University of Vermont) with the ABI TaqMan platform using an ABI 7900 real time thermal cycler under standard conditions (Applied Biosystems, Foster City, CA). Deviations from Hardy Weinberg equilibrium were tested with the Chi^2-test; no severe deviations were apparent, but it was determined that a few of the tagSNPs were highly correlated ($r^2 \geq 0.60$). We excluded the following correlated SNPs with slightly lower MAF; specifically, *FGA*2224 (rs2070011), *FGA*9205 (rs2070022) and *FGG*10034 (rs2066865) and one *IL6* tagSNP (rs1800795), leaving 9 fibrinogen and 5 *IL6* tagSNPs for analysis. [Supplemental table and Figure]

Statistical Analysis

To investigate complex interaction between *IL6* SNPs and fibrinogen SNPs, and simple interaction between IL-6 concentration and fibrinogen SNPs associated with CVD outcomes, we applied two separate statistical methods. Combinations of SNPs in fibrinogen and *IL6* associated with fibrinogen level, carotid IMT or CVD events were evaluated by a data-driven approach to model building using logic regression.(Ruczinski *et al.*, 2003) Briefly, logic regression builds potential models by searching for a variable (called a leaf) and/or Boolean expression (called a tree) that best predicts outcome (i.e. minimizes the scoring function); this process is repeated until additional variables do not improve model scores. It is adaptable to different forms of regression-based methods including linear regression and Cox proportional hazards methods. To simplify its interpretation and for discovery of combinations of predictors affecting relatively larger groups of individuals, we limited final model size *a priori*, by setting the maximum tree size to 2 and the number of leaves (i.e. variables) to 8, as suggested by Kooperberg *et al.*(Kooperberg *et al.*, 2001) We used linear regression to analyze interactions between fibrinogen SNPs and *IL6* SNPs

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associated with the continuous outcomes of baseline fibrinogen level and carotid IMT. MI and ischemic stroke events were analyzed using Cox proportional hazards models. Since logic regression is adaptive, appropriate model selection is necessary to prevent 'over-fitting' of the data. The logic regression software includes model selection tools such as cross-validation and conditional permutation tests which are used for interpretation of results. These tests account for multiple testing and thus reduce the chance of type I error. Once potential models have been created, a null model permutation test is run (n=200). Pseudo p-values derived from this test represent the proportion of times the 'best fit model' scores better than the 'null model,' suggesting evidence against the null model. Provided this null test indicated that the predictors had some discriminatory power, we chose a threshold of $p<0.20$, we pursued additional model selection tests using cross-validation (10-fold) and conditional permutation (n=200) tests conditioning on successive models of increasing size; a successive model was only chosen if it fit data better (had a smaller score) than the prior smaller model. Gene-gene interaction models were minimally adjusted for age, sex and recruitment site since acquired factors associated with fibrinogen level or risk of CVD events are unlikely to confound the genotypes. Both dominant and recessive genetic models were tested using this approach. For logic regression analyses, we used R software and the logic regression package (v.1.0.4) on the Linux platform; *STATA*/SE software v.8.2 (StataCorp, College Station, TX) was used for all other analyses.

To evaluate potential interactions between IL-6 levels and genetic variants in the fibrinogen genes with fibrinogen level, the significance of interaction terms on a multiplicative scale (fibrinogen SNPs in an additive genetic model multiplied by IL-6 concentration) was assessed using Wald's test. Only two-way interactions were tested. In sensitivity analyses, genotype was modeled using categorical variables for each genotype (common homozygote as reference) and the interaction effects were tested using a likelihood ratio test statistic, with two degrees of freedom. Results differed little from additive models, therefore for brevity, only results from additive genetic models results are presented. For the IL-6 level and fibrinogen SNP interactions, confounding of the IL-6 interaction term was an important consideration. For all models involving IL-6 level in the interaction term, we adjusted for age, sex and recruitment site *a priori* and investigated the following baseline variables for potential confounding of the IL-6 term*:* body mass index (BMI), current smoking, systolic blood pressure, diastolic blood pressure, medication use (anti-hypertensive or lipid-lowering agents and aspirin), diabetes or hypertension status and serum lipid levels (LDL-cholesterol, HDL-cholesterol, triglycerides and total cholesterol).

Results

Descriptives

In this subset of the CHS population without a prior history of MI or stroke, the majority of participants were female (60%).[Table 1] Diagnosis of hypertension was common in this older population (mean age at study entry= 73 years), though the prevalence of other CVD risk factors such as current smoking or diabetes was relatively low.

Mean fibrinogen level was 317± 63 mg/dL and was approximately normally distributed, but due to the highly skewed distribution of IL-6 (median=1.6 pg/ml, IQR: 1.1-2.5), it was ln-transformed for analyses, mean ln(IL-6)= 0.6 ± 0.6. While several fibrinogen SNPs are strongly associated with fibrinogen levels in this population, *IL6* SNPs did not appear to be strongly associated with plasma fibrinogen in univariate analyses. (data not shown)

Mean common carotid IMT was 0.99 ± 0.20 mm whereas internal carotid IMT was slightly skewed, median=1.32mm (IQR: 0.90-1.88), and was ln-tranformed for analysis. A total of

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486 incident non-procedure related MIs and 419 ischemic strokes were observed; median follow-up times were approximately 13.1 years for both events.

Evaluation of gene-gene interactions in relation to fibrinogen levels, carotid IMT and CVD events

Using logic regression and modeling fibrinogen level as the outcome in linear regression models, the p-value for the null randomization test was significant, p<0.005.[Table 2] This finding suggests evidence of an association between fibrinogen level and at least one SNP or combination of SNPs so we pursued model selection to identify the minimal set of variables that best modeled the fibrinogen level outcome.

Of the top five parsimonious models evaluated using conditional permutation [Table 3] and/or cross-validation tests [Figure 1], the two best models included combinations of functional variants in *FGA*6534 (rs6050) and *FGB*1437 (rs1800790), both with dominant effects. Specifically, cross validation identified two models for the best fit: 1) 1 tree with 2 leaves and 2) 2 trees and 2 leaves.[Figure 2] The p-values estimated from conditional randomization testing of these models were p=0.055 and 0.180, respectively. The first model with '1 tree and 2 leaves' includes one gene-gene interaction term and no main effect terms. It may be interpreted as individuals with no *FGA*6534 (rs6050) minor alleles and at least 1 minor allele of *FGB*1437 (rs1800790) have 18.5 mg/dL higher fibrinogen level on average than individuals who do not have that combination of genotypes, holding site, age and sex constant. The second model (2 trees and 2 leaves) includes main effect terms for the same *FGA* and *FGB* SNPs, but no interaction term. It suggests that after adjustment for site, age, sex and *FGA*6534 genotype, individuals with at least one *FGB*1437 (rs1800790) minor allele have 13.3 mg/dL higher levels of fibrinogen than individuals with no minor alleles. In addition, after adjustment for site, age, sex and *FGB*1437 (rs1800790) genotype, individuals with no *FGA*6534 (rs6050) minor alleles have 10.2 mg/dL higher fibrinogen level on average than individuals who have at least one minor allele. The pairwise correlation for these *FGA* and *FGB* SNPs was low, r^2=0.03 though minor allele frequencies were similar, 25% and 22% respectively. These same SNPs appeared in the remaining top five models, along with *IL6*-1111 (rs1800796) (dominant genetic model) and *FGA*251 (rs2070006) (recessive genetic model). Specifically, permutation tests identified a model supporting interaction between *FGA*6534 (rs6050) and *IL6*-1111 (rs1800796), but because this model also included an additional term involving *FGB*1437 (rs1800790), it was not the most parsimonious compared with the two models listed above. We did not find strong evidence of interaction between fibrinogen SNPs and *IL6* SNPs in relation to carotid IMT, MI or ischemic stroke; all global test p-values > 0.20.

Effect of interaction between fibrinogen SNPs and IL-6 levels on fibrinogen levels

Baseline measures of plasma fibrinogen and IL-6 were strongly associated, for each 1 natural log unit increase in IL-6, mean fibrinogen was 41 mg/dL (95%CI: 37-45; p<0.0001) higher. Marginally significant interactions were found between IL-6 levels and two fibrinogen SNPs *FGA*251 (rs2070006), p=0.029, and *FGG*902 (rs1800792), p=0.033, associated with fibrinogen level.[Table 4] For interpretation of these findings, we plotted the relationship between fibrinogen level and ln(IL-6), by fibrinogen genotype. The slope describing the relationship between ln(IL-6) and fibrinogen is not as steep among carriers of two *FGG*902 alleles compared with other genotypes.[Figure 3] Conversely, the slope describing the association between ln(IL- 6) and fibrinogen is lower among individuals homozygous for the rare *FGA*251 allele (rs2070006).[Figure 4]

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Interaction terms were slightly attenuated by adjustment for potential confounders of IL-6 including BMI, current smoking and diabetes status[Table 4], but given their imprecision, we are cautious in our interpretation of adjusted vs. unadjusted models.

Sensitivity Analyses

The minor alleles of *IL6* SNPs rs1554606 and rs1800795 have been previously associated with elevated IL-6 plasma levels in CHS Caucasians.(Walston *et al.*, 2007) We found that these tagSNPs were highly correlated (r^2=0.87) and chose to exclude rs1800795, which has a slightly lower MAF of 0.41 than rs1554606, MAF= 0.44. When we included rs1800795 instead of rs1554606 in logic regression models, results did not change. Similarly, the fibrinogen SNPs *FGA*6534 (rs6050) and *FGG*10034 (rs2066865) which also tag a common regional haplotype, were highly correlated in this population (r^2=0.86). We excluded *FGG*10034, but when substituted for *FGA*6534 in the logic regression models with fibrinogen level as an outcome, it performed similarly to *FGA*6534 in that it was present in the three top models along with *FGB*1437, and had a comparable impact on fibrinogen levels; however the model supporting its interaction with *IL6*1111 was ranked fourth with a non-significant p-value of 0.250. Interaction by genotype-sex was tested and not detected in any of the logic regression models.

Discussion

Elevated levels of fibrinogen(Tzoulaki *et al.*, 2007, Kannel, 2005, Danesh *et al.*, 2005) and IL- 6(Zakai *et al.*, 2007, Tzoulaki *et al.*, 2007) have been moderately associated with increased risks of CVD but whether they interact to influence risk has not been extensively studied in humans. In this study, we used two methods to assess interaction between these biologically related proteins associated with risk of CVD. Using logic regression to investigate combinations of variants in the fibrinogen and *IL6* genes associated with CVD outcomes, our exploratory results support potential interaction between two fibrinogen SNPs, *FGB*1437 (rs1800790) and *FGA*6534 (rs6050) in determining fibrinogen levels, but no associations with subclinical or clinical CVD. These SNPs appeared in one model which included their main effects only and a second model including only their interaction term. It is unclear which model is best, since their scores were of similar magnitude and as demonstrated in Fig.2, their implications are somewhat similar. The main effect findings for these SNPs which tag different common haplotypes have been previously reported in CHS, (Carty *et al.*, 2008) as has the haplotype describing the particular combination of SNPs identified in the current study. However, the current analysis is novel in that it includes additional predictors and genetic models, an assessment of complex interaction in the absence of main effects and conditional permutation tests which account for multiple tests performed. Though not statistically significant after correction for multiple testing, a model supporting interaction between *FGA*6534 and *IL6*-1111 (rs1800796) associated with fibrinogen level may be worthy of additional investigation.

In our standard analysis of interaction on a multiplicative scale between fibrinogen SNPs and baseline IL-6 level, we found marginally significant interactions in minimally adjusted models (unadjusted for multiple testing) between IL-6 level and fibrinogen SNPs located in the promoter regions of *FGA* and *FGG* so that in individuals having the homozygous rare *FGG*902 (rs1800792) genotype or lacking the rare *FGA*251 (rs2070006) allele, the slope describing the association between fibrinogen level and ln(IL-6) level was not as steep as with the other genotypes. These results are consistent with a recessive effect for *FGG*902 whereas the similar slopes for individuals with either 1 or 2 minor alleles are consistent with a dominant gene effect for *FGA*251, though the overlap of the confidence intervals should be noted. As reported previously(Carty *et al.*, 2008), in single SNP analyses without interaction terms, each *FGG*902 allele was significantly associated with higher fibrinogen

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and each *FGA*251 minor allele was associated with significantly lower fibrinogen levels. Consideration of these findings in context with the smaller slope describing the relationship between IL-6 and fibrinogen levels among *FGG*902 rare homozygotes, may suggest that *FGG*902, located in the promoter region of the *FGG* gene, potentially interferes with IL-6 mediated binding. At a cutoff of $r^2>0.64$, it is not in LD with any validated *FGG* SNPs, though it is in LD with *FGA* and *FGB* SNPs. However, these interaction results should be viewed as exploratory as they would not be significant following correction for multiple testing using the Bonferroni method.

Our fibrinogen level findings are generally consistent with results from laboratory studies identifying IL-6 response elements in the promoter regions of human *FGA*(Liu & Fuller, 1995), *FGG*(Zhang *et al.*, 1995, Duan & Simpson-Haidaris, 2003, Mizuguchi *et al.*, 1995) and to a lesser extent, *FGB.*(Huber *et al.*, 1990) While the promoter SNPs studied are not located in the putative IL-6 response element sites, it is possible that they could indirectly modulate the IL-6 response by interacting with factors bound to the response element. Specifically, the *FGG*902 variant (rs1800792) is located at approximately −251 base pairs (bp), close to an IL-6 response element reported at −306 to −301 bp in the 5′ flanking region of human *FGG*. (Mizuguchi *et al.*, 1995) Similarly the *FGA*251 variant, located at −201 bp, is within a 5′ flanking *FGA* region (from −217 to +1bp) reported to have significant promoter activity and be inducible by IL-6, (Hu *et al.*, 1995) whereas the two FGB promoter SNPs rs1800791 and rs1800790, located at approximately −301 bp and −485 bp are not especially close to the putative IL-6 response region reported at −142 to −137 bp.(Dalmon *et al.*, 1993) For *FGB*, we also did not detect evidence supporting *in vitro* findings indicating that the A allele of *FGB*1437 (rs1800790) is associated with higher IL-6- induced expression of fibrinogen than the G allele(van't Hooft *et al.*, 1999, Brown & Fuller, 1998) or that a variant at *FGB* -148 (which is in complete LD with *FGB*1437) is associated with lower IL-6-induced levels of the fibrinogen-β protein.(Verschuur *et al.*, 2005) The contrasts between our findings and *in vitro* studies may be related to issues of power or biological complexity of the *in vivo* regulation of fibrinogen synthesis as detailed below.

The heritability of fibrinogen level is reported as 20-50%,(de Lange *et al.*, 2001, de Maat *et al.*, 2004, Yang *et al.*, 2003, Reed *et al.*, 1994, Bladbjerg *et al.*, 2006) yet several recent population-based association studies report that common variants in three fibrinogen genes explain less than 5% of the variance in fibrinogen level.(Reiner *et al.*, 2006, Carty *et al.*, 2008) One explanation for this discrepancy is that fibrinogen levels are affected by combinations of other inherited genes such as those involved in its regulation and break-down. Even in healthy individuals, fibrinogen level is heterogeneous due to variable rates of formation (or transcription) and removal (processing, stability and degradation) of the fibrinogen protein.(de Maat & Verschuur, 2005) Furthermore, the various molecular forms of fibrinogen resulting from splice variants, post-translational modification and proteolytic degradation have functional consequences with respect to fibrinolysis, cellular interactions, clotting time, and coagulation characteristics.(de Maat & Verschuur, 2005) Other environmental factors not accounted for in these analyses could also affect fibrinogen levels, such as glucocorticoids (GCs), which act synergistically with IL-6 to up-regulate fibrinogen gene expression.(Otto *et al.*, 1987, Simpson Haidaris, 1997)

Similarly, *IL6* SNPs alone explain little of the variance in IL-6 levels which presumably are reflective of other genetic and environmental factors. Laboratory studies suggest that an *IL6* polymorphism (rs1800795) is associated with inter-individual differences in the degree of IL-6 response to stressful stimuli.(Fishman *et al.*, 1998) This SNP was previously found to be significantly associated with higher fibrinogen levels (Jenny *et al.*, 2002), IL-6 and CRP levels in CHS.(Walston *et al.*, 2007) However, a previous study tested, but was unable to detect interaction between rs1800795 and three fibrinogen SNPs associated with risk of MI,

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though the study was small and included few variants.(Mannila *et al.*, 2006) Rs1800795, excluded from our main analyses due to its high correlation with rs1554606 ($r^2 = 0.87$), was also associated with higher IL-6 and CRP levels.(Walston *et al.*, 2007) We did not identify any interactions involving rs1554606 or rs1800795 (in sensitivity analyses), but rather identified a potential interaction between another *IL6*promoter SNP, rs1800796 and an *FGA* SNP. This *IL6*SNP also has been associated with higher fibrinogen and CRP levels in a Chinese population.(Wong *et al.*, 2007) Although this SNP is not in strong LD with either of the aforementioned *IL6*SNPs, our finding is consistent with the *IL6*promoter region being potentially important for the downstream expression of acute phase reactants.

In our gene-gene interaction analyses, we chose to use logic regression since it is particularly useful for binary variables such as those in genetic analyses.(Kooperberg *et al.*, 2007) In addition, it can be used to detect within and between gene effects and easily incorporates testing of both dominant and recessive genetic models. It also can identify interactions among three or more variables and provides tools for use during model selection that account for multiple testing. A limitation of the logic regression approach is that it requires that continuous predictors such as IL-6 level be categorized into binary (or a series of binary) variables for modeling. This categorization may be more susceptible to misclassification error or a loss of power due to the increased number of degrees of freedom. For this reason, we analyzed interactions involving IL-6 levels using a different approach.

Because elevated fibrinogen levels are associated with increased risks of CVD and stroke, elucidation of the molecular mechanisms that regulate fibrinogen expression may be relevant for control of diseases associated with high levels of fibrinogen.(Humphries *et al.*, 1999) Thus, in addition to furthering our understanding of how fibrinogen is up-regulated in response to inflammatory stimuli, the mechanisms that down-regulate fibrinogen gene expression could be important for normalization of elevated plasma fibrinogen levels in individuals at increased risk for cardiovascular disease.(Duan & Simpson-Haidaris, 2003) We hypothesized that IL-6 variants could potentially influence fibrinogen levels and CVD through their effects on IL-6 levels or IL-6 structure/binding and subsequent regulation of IL-6 induced genes. For our gene-gene interactions, we estimated that we had 98% power to estimate fibrinogen betas of approximately 11mg/dL for the interaction term; however, power dropped as low as approximately 58% for combinations of SNPs with the lowest minor allele frequencies. Similarly, we were well powered for fibrinogen SNP-IL6 level interactions, with at least 80% power to detect interaction terms accounting for 0.2% of the variance in fibrinogen levels, though power decreased for low frequency SNPs. In spite of the *in vitro* evidence of a biological interaction, we did not find strong evidence of interaction in risk of subclinical or clinical disease in this older population. Previous studies of *IL6* and fibrinogen SNPs also did not reveal strong main effect associations between variants and complex disease in CHS,(Walston *et al.*, 2007, Carty *et al.*, 2008) though significant findings have been reported in other populations.(Mannila *et al.*, 2006) It is likely that the interactions of many genetic variants, some of which may not encode proteins and thus can be difficult to identify, contribute to development of complex diseases such as CVD.(Freimer & Sabatti, 2007) Lacking blood samples drawn during an acute inflammatory event, when both IL-6 and fibrinogen would be upregulated, we relied on single baseline measures of these proteins. Future research may be better directed at larger populations for better power to detect the likely small effects of combinations of genes on risk of clinical CVD or on individuals having combinations of certain genotypes that make them more responsive to acute stimuli.(Humphries *et al.*, 1999) Given the exploratory nature of our findings, validation of our results in an independent data set and using other statistical methods for determining interaction is necessary.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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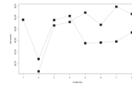

Figure 1. Logic regression cross-validation testing results for models of fibrinogen and IL6 SNPs associated with fibrinogen level

Model size (x-axis scale) indicates the number of leaves; shaded boxes indicate the number of trees in the models. The y-axis scale, 'test score' reflects the residual variance scores of each model. Lower test scores are indicative of better fitting models. In this figure, both 1 tree and 2 tree models with 2 leaves have the best scores (< 62.75) in 10-fold cross-validation testing.

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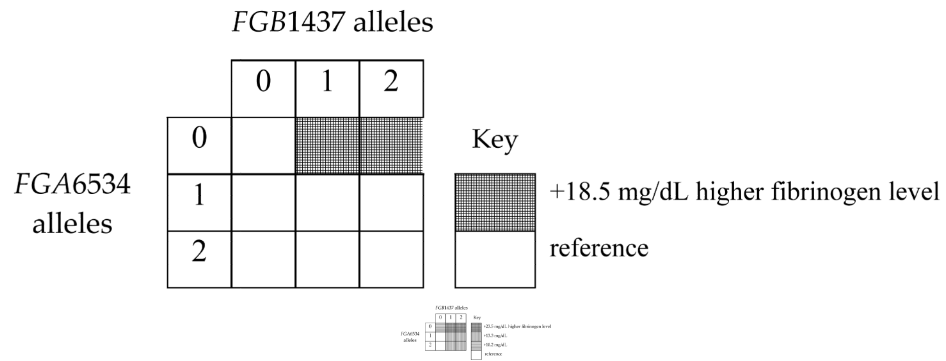

Figure 2. Interaction between fibrinogen and IL6 SNPs in relation to fibrinogen level: best models identified by logic regression

A. 1 tree with 2 leaves: score is 62.12

E(fibrinogen) = 243.35 + 18.50 ((no *FGA*6534 minor alleles) and ≥1 minor allele at *FGB1*437) + 1.22(site A) +17.67(site B) + 0.82(site C) + 0.91(age) − 4.25(male)

As shown below, this equation includes one gene-gene interaction term and no main effects. It suggests that holding site, age and sex constant, individuals with no *FGA*6534 minor alleles and at least 1 minor allele of *FGB*1437 have 18.5 mg/dL higher fibrinogen level on average than individuals who do not have that combination of genotypes.

B. 2 trees with 2 leaves: score is 62.03

E(fibrinogen) = 252.72 − 13.29(no *FGB*1437 minor alleles) + 10.21(no *FGA*6534 minor alleles) + 1.16(site A) +17.31(site B) + 0.47(site C) + 0.88(age) − 4.07(male)

As shown below, this equation includes a main effect for the same *FGA* and *FGB* SNPs, but no interaction term. It suggests that after adjustment for site, age, sex and *FGA*6534 genotype, individuals with no *FGB*1437 minor alleles have 13.3 mg/dL lower levels of fibrinogen than individuals with at least one *FGB*1437 minor allele. After adjustment for site, age, sex and *FGB*1437 genotype, individuals with no *FGA*6534 minor alleles have 10.2 mg/dL higher fibrinogen level on average than individuals who have at least one minor allele.

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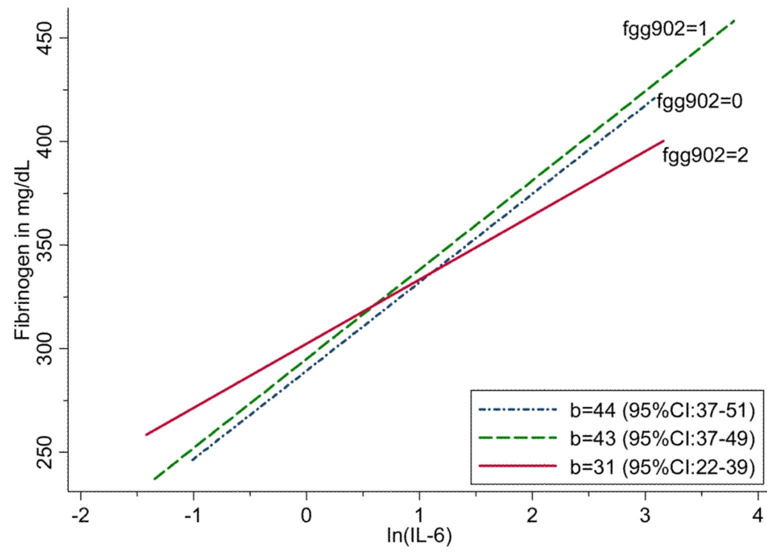

Figure 3. Association between fibrinogen level and ln(IL-6), by FGG902 (rs1800792) genotype

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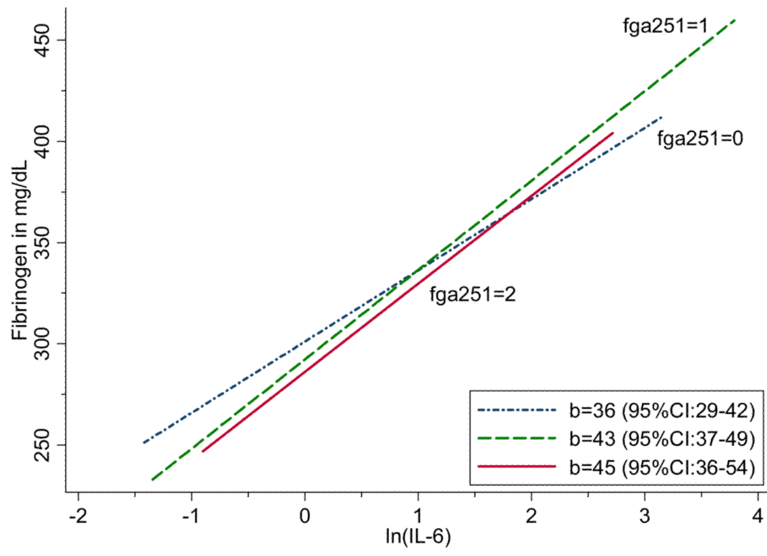

Figure 4. Association between fibrinogen level and ln(IL-6), by FGA251 (rs2070006) genotype

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Table 1

Baseline Characteristics of the Study Population

Characteristic[1]	**N=3900**
Female	2322 (59.5)
Age in years	72.6 ± 5.5
BMI in kg/m^2	26.3 ± 4.5
Current Smoker	428 (11.0)
Diabetes (established/new)	508 (13.0)
Hypertension	2129 (54.8)
Hypertension Medication Use	1584 (40.7)
Lipid-Lowering Medication Use	189 (4.9)
Aspirin Use (>2 days in prior 2 weeks)	1210 (31.1)
Total Cholesterol in mg/dL	212.3 ± 39.0
LDL Cholesterol in mg/dL	130.1 ± 35.7
HDL Cholesterol in mg/dL	54.6 ± 15.8
Triglycerides in mg/dL	141.4 ± 74.8
ln(CRP) in mg/L	0.6 ± 1.0

[1]Data are presented as number (%), or mean ± standard deviation.

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Table 2

Logic Regression Results for the Within Gene and Gene-Gene Interactions between Fibrinogen SNPs and IL6 SNPs in Relation to Tested Outcomes

Continuous Outcome	Signal Test p-value
Fibrinogen level in mg/dL	<0.005
Ln(internal carotid IMT in mm)	0.31
Common carotid IMT in mm	0.61
Incident MI	0.73
Incident ischemic stroke	0.21

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Table 3

Top Five Models from Conditional Permutation Tests

Size	Model	p-value
1 tree, 1 leaf	14.8 * (≥1 *FGB*1437 minor allele)	0.010
1 tree, 2 leaves	18.5 * ((no *FGA*6534 minor alleles) & ≥1 *FGB*1437minor allele)	0.055
1 tree, 3 leaves	−18.6*((no *FGB*1437 minor alleles) or ((no *IL6*_1111 minor alleles) & ≥1 *FGA*6534 minor allele))	0.105
1 tree, 4 leaves	−19.6 * ((≥1 *FGA*6534 minor allele & (no *IL6*_1111 minor alleles)) or (2 *FGA*251 minor alleles or (no *FGB*1437 minor alleles)))	0.150
2 trees, 2 leaves	−13.3 * (no *FGB*1437 minor alleles) + 10.2 * (no *FGA*6534 minor alleles)	0.180

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Table 4

Interactions between Fibrinogen SNPs and IL-6 Plasma Concentration on Fibrinogen Plasma Concentration

Interaction Term	β (95%CI)[1] *p-value*	β (95%CI)[2] *p-value*
*FGA*251 × lnIL6	5.8 (0.6, 10.9) ***0.029***	5.0 (−0.1, 10.2) *0.057*
*FGA*3807 × lnIL6	−5.4 (−12.3, 1.4) *0.119*	−5.2 (−12.0, 1.6) *0.137*
*FGA*5498 × lnIL6	6.5 (−0.6, 13.6) *0.074*	5.9 (−1.2, 12.9) *0.103*
*FGA*6534 × lnIL6	3.4 (−2.7, 9.5) *0.270*	2.9 (−3.2, 8.9) *0.349*
*FGB*1038 × lnIL6	−1.0 (−8.2, 6.3) *0.797*	−0.9 (−8.1, 6.3) *0.801*
*FGB*1437 × lnIL6	0.3 (−6.2, 6.9) *0.920*	0.5 (−6.1, 7.0) *0.884*
*FGB*9952 × lnIL6	−3.6 (−9.2, 2.2) *0.222*	−3.3 (−9.0, 2.4) *0.255*
*FGG*902 × lnIL6	−5.6 (−10.8, −0.5) ***0.033***	−5.0 (−10.2, 0.2) *0.058*
*FGG*9340 × lnIL6	4.6 (−1.2, 10.5) *0.121*	4.4 (−1.5, 10.3) *0.140*

[1] Model adjusted for age, sex, site and ln(IL6).

[2] Model adjusted for age, sex, site, ln(IL6), BMI, current smoking and diabetes.