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Fibrinogen and coronary heart disease: test of causality by 'Mendelian randomization'

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Background Blood concentrations of fibrinogen have been associated with coronary heart disease risk in epidemiological studies, but it is uncertain whether this association is causal or reflects residual confounding by other risk factors. We investigated the relationship between the single nucleotide polymorphism at position −148 in the beta-fibrinogen gene promoter (β − 148C/T), blood fibrinogen levels, and risk of myocardial infarction (MI) in sufficiently large numbers of coronary disease cases to reliably address this question.

Methods Genotyping and measurement of blood fibrinogen concentration were carried out in 4685 cases of confirmed MI and 3460 controls with no history of coronary disease. A meta-analysis of ISIS and 19 other studies of beta-fibrinogen genotypes involving a total of 12 220 coronary disease cases and 18 716 controls was conducted.

Results Among the ISIS controls, mean plasma fibrinogen concentrations with the C/C, C/T and T/T genotypes were 3.34 (SE 0.015), 3.48 (0.022), and 3.60 (0.064) g/l, respectively, corresponding to an increase of 0.14 (0.024) g/l per T allele (trend $P < 0.0001$). In the case–control comparison, 0.14 g/l higher usual plasma fibrinogen concentration was associated with an age-adjusted and sex-adjusted risk ratio for MI of 1.17 [95% confidence interval (95% CI) 1.14–1.19; $P < 0.0001$]. But, after further adjustment for smoking, body mass index, and plasma apolipoprotein B/A_1 ratio, this risk ratio fell to 1.03 (95% CI 1.00–1.05; $P = 0.05$). Moreover, fibrinogen genotype was not significantly associated with MI incidence: risk ratio of 1.06 (95% CI 0.96–1.16) per higher-fibrinogen allele in ISIS alone and of 1.00 (95% CI 0.95–1.04) per allele in the meta-analysis.

Conclusions Genotypes that produce lifelong differences in fibrinogen concentrations do not materially influence coronary disease incidence. As these genotype-dependent differences in fibrinogen were allocated randomly at conception (Mendelian randomization), this association is not likely to be confounded by other factors. Consequently, these genetic results provide strong evidence that long-term differences in fibrinogen concentrations are not a major determinant of coronary disease risk.

Keywords Genetics, coronary heart disease, fibrinogen, meta-analysis, Mendelian randomization

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Rapid improvements in analytic technology are offering the opportunity to investigate associations between the blood levels of many different factors and the risks of various common conditions, such as cardiovascular disease or cancer, in large epidemiological studies.[1,2] This approach is likely to result in the identification of many definite differences in the usual blood levels of such factors between disease cases and healthy individuals. But, these differences may be a result rather than a cause of disease or may merely reflect confounding by other risk factors. Statistical adjustment for such confounding tends to be incomplete in most studies owing to the failure to measure all importantly relevant confounders and measurement error in such confounders.[3] On the other hand, adjustment for other factors that actually lie in the causal pathway between the putative risk factor and disease may inadvertently lead to its relevance being under-estimated. In some circumstances, randomized controlled trials of interventions that influence the levels of such risk factors may help to assess the causal nature of the association with disease (e.g. lowering LDL cholesterol with statin drugs reduces coronary disease).[4,5] But, where such evidence is lacking, genetic information may provide an alternative approach to assessing causality unbiasedly.

In particular, if a genetic polymorphism (e.g. in the promoter region of a gene) selectively affects the levels, but not the function, of a putative risk factor then lifelong differences in the levels of the factor due to genotype will effectively have been allocated randomly at conception. Such 'Mendelian randomization' should ensure that the relationship between these genotype-determined differences in the factor and disease is not materially affected either by the subsequent development of disease or by confounding.[6–9] Hence, demonstration of significant associations between these genotypes and disease risk would generally provide strong support for a causal association between the risk factor and disease. By contrast, clear demonstration of a lack of such gene–disease associations in the presence of significant gene-factor associations would provide strong evidence against a causal relationship. Typically, the contribution of a common polymorphism to the level of a particular factor is small (e.g. a few per cent of the total population variability), so the contribution of any individual polymorphism to disease risk is also likely to be small.[10,11] Consequently, for this approach to produce statistically reliable evidence, studies may well need to involve many thousands of cases, which has not hitherto been usual.[12]

Blood concentrations of fibrinogen have been found to be associated with coronary disease in many epidemiological studies,[13] but they are also strongly correlated with some established risk factors (such as smoking, obesity, and plasma lipid profile) that confound the association of fibrinogen with disease risk. Fibrinogen is made up of three polypeptide chains (α, β, and γ), and polymorphisms in the regulatory region of the gene encoding the β chain are associated with differences of a few per cent in plasma concentrations of fibrinogen.[11,14,15] We genotyped one of these polymorphisms, and measured plasma fibrinogen concentration, in several thousand cases with recent myocardial infraction (MI) and controls without evidence of coronary disease. These results were considered in the context of a meta-analysis of all available studies of such beta-fibrinogen genotypes and coronary disease, involving a total of ~30 000 cases and controls.

Methods

Study population

The design of the ISIS genetic study has been described elsewhere.[16] Blood was collected from patients living in the UK within 24 h of the onset of suspected acute MI (mean 6 h).[17] A few months after hospital discharge, information was sought from surviving cases about various aspects of their lifestyle. A similar questionnaire was then sent to their siblings and children aged >30, and to spouses of such relatives, with blood sought from those first-degree relatives and spouses who completed the questionnaire.[16,18] Cases to be genotyped in this study were males aged 30–54 and females aged 30–64 with MI confirmed by cardiac enzyme or electrocardiographic criteria. Controls to be genotyped were aged 30–64 with no history of MI, angina, or other definite heart disease. Among the 6002 potential controls, all 1923 women and 1537 of the men were related only by marriage to first-degree relatives of MI cases in the ISIS study, while a further 2542 male controls were siblings or adult sons of such cases. (Blood was not analysed from all these potential cases and controls, although it was from most: see Results.) Ethnicity was not recorded, but the family-based recruitment strategy should have yielded similar distributions among the cases and controls (and, based on other studies in these UK hospitals, >95% would be expected to be Caucasian[5]).

Laboratory methods

DNA was extracted from frozen buffy-coat samples.[19] The −148C/T polymorphism of the beta-fibrinogen promoter was genotyped by PCR amplification of genomic DNA, followed by restriction enzyme digestion and agarose gel electrophoresis (as previously described).[19] DNA extraction and genotyping were carried out without knowledge of disease status, with samples from cases and controls distributed within each 96-well genotyping plate. Internal controls were used to check the consistency of genotyping between plates, with an estimated genotyping error rate <1%. An automated Behring (Marburg, Germany) Analyser II nephelometer measured plasma fibrinogen concentrations (using Behring reagents) according to the manufacturer's protocols. Beckman (Brea, CA) autoanalysers measured plasma concentrations of apolipoproteins A_1 and B [using Immuno (Vienna, Austria) reagents], with an initial blank reading subtracted from the final reading to correct for any discolouration from haemolysis.[20] Samples from a large plasma pool were included in each analytical run, yielding coefficients of variation of 4% for fibrinogen and for both apolipoproteins.

Statistical analysis

The association between plasma fibrinogen concentration and beta-fibrinogen genotype in the ISIS controls was calculated by linear regression on the number of T alleles. The age-adjusted and sex-adjusted risk ratio for MI associated with a unit increase in the measured concentration of plasma fibrinogen

was calculated from the case–control comparisons using logistic regression fitted by unconditional maximum likelihood, as was the impact of further adjustment for recorded values of smoking, body mass index, and the ratio of plasma apolipoprotein B to apolipoprotein A_1. Repeat blood samples were obtained from 1044 controls at 2–3 years after the first sample, and the self-correlation ($R = 0.59$) between the measured values of plasma fibrinogen in the original and the repeat samples was used to calculate the effects on MI risk of long-term 'usual' differences in plasma fibrinogen concentrations (by multiplying the regression coefficient by $1/R$).[3,21] Plasma fibrinogen was found to increase by 0.4% per hour from pain onset in the cases; so the measured values were corrected to time zero by subtracting this percentage change per hour. The age-adjusted and sex-adjusted risk ratios for MI between genotypes were calculated from the case–control comparisons by logistic regression. Floated absolute risks and their confidence intervals (CIs) were used in order to share the variances of the log risk ratios appropriately between different groups.[22] The analyses in the present report included all 1923 female controls but only those 1537 male controls who were unrelated to an MI case except by marriage and are given with 95% CIs. Subsidiary analyses involving both unrelated and related controls found similar associations, albeit with slightly smaller standard errors (SEs) due to the larger numbers (available on request). Among the MI cases, the logrank test was used to test for differences between genotypes in 6 month survival after admission to hospital (chiefly to exclude the possibility that case fatality rates might differ sufficiently by genotype to bias case–control comparisons in this retrospective study).

Meta-analysis of beta-fibrinogen polymorphisms and coronary disease risk

Based on data from over 1200 chromosomes in Caucasians, the −148C/T polymorphism of the beta-fibrinogen gene appears to be in complete linkage disequilibrium with the nearby −455G/A polymorphism (i.e. knowledge of the genotype at one locus predicts, with certainty, the genotype at the other locus).[19,23,24] Studies of coronary disease that had typed either polymorphism were, therefore, sought for a meta-analysis using computer-assisted searches involving various combinations of key words (myocardial, coronary, vascular, isch[a]emic, heart, fibrinogen, gene, polymorphism), supplemented by reviews of reference lists, handsearching of relevant journals and correspondence with authors. Studies were included if genotype proportions could be obtained for controls and for cases of non-fatal myocardial infarction or coronary death. Two investigators independently reviewed study eligibility and extracted data according to a fixed protocol, with any discrepancies resolved by discussion. Standard statistical methods were used to obtain inverse-variance-weighted combined estimates of the overall risk ratio for coronary disease with respect to these genotypes.

Results

The 4685 cases of confirmed MI in the ISIS study had an average age of 50.5 years (Table 1), with 3052 men aged 30–54 and 1633 women aged 30–64 at presentation. The 3460 unrelated controls had an average age of 46.2 years, with 1537 men and 1923 women aged 30–64. The distribution among these cases and controls of a variety of cardiovascular risk factors is shown in Table 1. Plasma fibrinogen concentration was higher in older individuals and, within each stratum of age, was higher in females than in males (Table 2). It was positively correlated with apolipoprotein B concentration, body mass index, and smoking and negatively correlated with apolipoprotein A_1 concentration, albumin concentration, and alcohol consumption (Table 3).

Table 1 Characteristics of confirmed MI cases and controls

Characteristic	Cases (n = 4685)	Controls (n = 3460)
Age (years)	50.5 (0.12)	46.2 (0.14)
Male (%)	65.1 (0.70)	44.4 (0.84)
Plasma biochemistries[a]		
Fibrinogen (g/l)	3.80 (0.0142)	3.45 (0.0137)
Apolipoprotein A_1 (g/l)	1.12 (0.0034)	1.24 (0.0032)
Apolipoprotein B (g/l)	1.22 (0.0049)	1.05 (0.0046)
Apolipoprotein B/A_1 ratio	1.11 (0.0049)	0.87 (0.0046)
Albumin (g/l)	37.7 (0.0631)	40.8 (0.0600)
Questionnaire data[a]		
Body mass index (kg/m^2)	26.6 (0.09)	24.9 (0.07)
Current cigarette smoking (%)	55.8 (0.87)	17.8 (0.67)
Hypertension (%)	24.0 (0.74)	11.2 (0.53)
Diabetes mellitus (%)	6.5 (0.43)	1.5 (0.21)

[a] Adjusted for age and sex. Plasma measures were available for 3961–3967 of the 4685 cases and 3091–3162 of the 3460 controls; and questionnaire data were available for 3276–3293 cases and 3453–3460 controls. Values in brackets are standard errors.

Table 2 Plasma fibrinogen by age and sex in controls

Sex	Age group (years)	Number of controls[a]	Mean (SE) plasma fibrinogen (g/l)
Female	30–44	874	3.25 (0.02)
	45–54	459	3.52 (0.03)
	55–64	406	3.79 (0.03)
Male	30–44	633	3.11 (0.02)
	45–54	377	3.39 (0.03)
	55–64	342	3.59 (0.04)

[a] Plasma fibrinogen was measured in 3091 (89%) of the 3460 controls.

Association between genotype and plasma fibrinogen concentration

Genotyping the −148C/T polymorphism of the beta-fibrinogen gene was successful in 4490 (95.8%) of the 4685 cases and 3290 (95.1%) of the 3460 controls. The allele frequencies in the controls were 0.815 for the C allele and 0.185 for the T allele, and genotype frequencies among both cases and controls satisfied Hardy–Weinberg equilibrium. Among the controls, the age-adjusted and sex-adjusted mean plasma fibrinogen concentrations with the C/C, C/T, and T/T genotypes were 3.34 (SE 0.015), 3.48 (0.022), and 3.60

Table 3 Age-adjusted and sex-adjusted mean (SE) values of various risk factors by fifths of plasma fibrinogen in controls

	Age-adjusted and sex-adjusted fifths of plasma fibrinogen (g/l)					
Characteristic[a]	<2.89 (n = 586)	⩾2.89 < 3.18 (n = 587)	⩾3.18 < 3.46 (n = 586)	⩾3.46 < 3.84 (n = 587)	⩾3.84 (n = 586)	**Trend *P*-value**
Fibrinogen (g/l)	2.61 (0.011)	3.04 (0.011)	3.32 (0.011)	3.63 (0.011)	4.36 (0.011)	
Apolipoprotein A_1 (g/l)	1.27 (0.007)	1.26 (0.007)	1.25 (0.007)	1.23 (0.007)	1.22 (0.007)	<0.0001
Apolipoprotein B (g/l)	0.92 (0.009)	0.96 (0.009)	0.99 (0.009)	1.03 (0.009)	1.06 (0.009)	<0.0001
ApolipoproteinB/A_1 ratio	0.74 (0.009)	0.77 (0.009)	0.80 (0.009)	0.86 (0.009)	0.90 (0.009)	<0.0001
Albumin (g/l)	40.90 (0.121)	41.03 (0.121)	40.81 (0.121)	40.74 (0.121)	39.76 (0.121)	<0.0001
Body mass index (kg/m^2)	23.58 (0.147)	23.77 (0.146)	24.44 (0.146)	25.01 (0.146)	26.11 (0.146)	<0.0001
Units of alcohol/week	10.70 (0.542)	8.72 (0.540)	9.90 (0.540)	9.58 (0.542)	7.55 (0.541)	0.0015
Current cigarette smoker (%)	12.65 (1.388)	13.80 (1.452)	16.86 (1.566)	21.92 (1.723)	26.77 (1.840)	<0.0001

[a] All analyses in Tables 3–5 are restricted to the 2932 (85%) of 3460 controls with data on plasma fibrinogen, beta-fibrinogen genotype, and all of these risk factors.

Table 4 Association between genotype and age-adjusted and sex-adjusted risk factors for MI in controls

	Beta-fibrinogen −148C/T genotype			
Characteristic[a]	C/C (n = 1943)	C/T (n = 888)	T/T (n = 101)	**Trend *P*-value**
Fibrinogen (g/l)	3.34 (0.015)	3.48 (0.022)	3.60 (0.064)	<0.0001
Apolipoprotein B/A_1 ratio	0.81 (0.005)	0.82 (0.007)	0.86 (0.022)	0.01
Albumin (g/l)	40.68 (0.068)	40.61 (0.100)	40.41 (0.294)	0.36
Body mass index (kg/m^2)	24.51 (0.083)	24.66 (0.123)	25.25 (0.363)	0.06
Units of alcohol/week	9.25 (0.300)	9.55 (0.441)	7.92 (1.303)	0.90
Diabetes (%)	1.34 (0.261)	1.56 (0.418)	1.97 (1.386)	0.53
Hypertension (%)	10.46 (0.694)	10.46 (1.028)	12.64 (3.332)	0.70
Current cigarette smoker (%)	18.48 (0.880)	19.92 (1.341)	18.66 (3.889)	0.48

[a]See footnote to Table 3.

(0.064) g/l, respectively, corresponding to a difference of 0.14 (0.024) g/l per T allele (trend $P < 0.0001$; Table 4). The strength of the association between genotype and plasma fibrinogen concentration did not differ significantly between men and women, smokers and non-smokers, older and younger people, or cases and controls (data available on request). Fibrinogen genotype was not significantly associated with other characteristics measured in these unrelated controls, with the exception of the plasma apolipoprotein B/A_1 ratio, which appeared to be somewhat higher with the T allele ($P = 0.01$; Table 4). In view of the multiple comparisons made, however, this significance level is not particularly extreme. Moreover, this apparent association was not significant either in the cases or when both unrelated and related controls were considered together, so it may well largely or wholly reflect the play of chance. (Any genotype risk ratios would be altered only slightly by adjustment for the observed association of adverse lipid profiles with the T-allele among the unrelated controls: see below.)

Association between plasma fibrinogen concentration and MI risk

The case–control comparison yielded a risk ratio for MI of 2.99 (95% CI 2.55–3.30) per 1 g/l higher usual plasma fibrinogen concentration, which is equivalent to a risk ratio of 1.17 (95% CI 1.14–1.19; $\chi^2_1 = 203.5$) per 0.14 g/l higher plasma fibrinogen, after adjustment for age and sex (Table 5). There was no evidence that the strength of this association differed between individuals with different β − 148C/T genotypes (heterogeneity $\chi^2_2 = 0.2; P = 0.9$) or between smokers and non-smokers (heterogeneity $\chi^2_1 = 1.7; P = 0.2$). Adjustment for smoking status reduced the overall risk ratio to 1.11 (95% CI 1.08–1.13) per 0.14 g/l higher usual plasma fibrinogen and more than halved the chi-squared value for the apparent strength of the association ($\chi^2_1 = 74.4$). The estimated strength of the association was reduced still further by adjustment for body mass index (1.08; 95% CI 1.05–1.11; $\chi^2_1 = 41.9$) and, after additional adjustment for a single measure of the plasma apolipoprotein B/A_1 ratio, it was no longer clearly significant (1.03; 95% CI 1.00–1.05; $\chi^2_1 = 3.8$; $P = 0.05$).

Association between genotype and MI risk

As there was a stepwise 0.14 g/l increase in plasma fibrinogen concentration with the addition of each T allele, a co-dominant genetic model was used in the case–control comparison of beta-fibrinogen genotype and MI risk (Table 6). By comparison with the C/C genotype, which was associated with the lowest plasma fibrinogen concentration, the age-adjusted and sex-adjusted risk ratios for MI were 1.12 (95% CI 1.02–1.23) with

Table 5 Effects of adjustment for various factors on the association between 0.14 g/l higher than usual plasma fibrinogen concentration, and MI risk

Adjustment factors[a]	Risk ratio (95% CI)	$\chi_1 2$
Age and sex	1.17 (1.14–1.19)	203.5
Age and sex plus smoking status	1.11 (1.08–1.13)	74.4
All above plus body mass index	1.08 (1.05–1.11)	41.9
All above plus plasma apolipoprotein B/A_1 ratio	1.03 (1.00–1.05)	3.8

* Analyses are restricted to the 2685 (57%) of 4685 cases and 2932 (85%) of 3460 potential controls with data on plasma fibrinogen, beta-fibrinogen genotype and all of these risk factors.

Table 6 Age- and sex-adjusted association of beta-fibrinogen genotype with non-fatal MI risk

Genotype[a]	Case	Control	Risk ratio (95% CI)
C/C	2919 (65.0%)	2186 (66.4%)	1.00 (0.94–1.07)
C/T	1422 (31.7%)	989 (30.1%)	1.12 (1.02–1.23)
T/T	149 (3.3%)	115 (3.5%)	0.91 (0.69–1.21)
Per allele change			1.06 (0.96–1.16)
Trend *P*-value			0.3

[a] The beta-fibrinogen −148C/T genotype was measured successfully in 4490 (95.8%) of 4685 cases and 3290 (95.1%) of 3460 controls.

the C/T genotype and 0.91 (95% CI 0.69–1.16) with the T/T genotype. This corresponds to a risk ratio per T allele of 1.06 (95% CI 0.96–1.16), which is not statistically significant (trend *P*-value = 0.3; Table 6). Inclusion of the 2397 additional male controls on whom genotype data were available who were siblings or adult sons of cases in these analyses provided very similar results (available as Supplementary Data at *IJE* Online). In analyses restricted to the 2685 cases and 2932 controls in Table 5 (i.e. with data on plasma fibrinogen, fibrinogen genotype, and other risk factors), the age-adjusted and sex-adjusted risk ratio per T allele was 1.04 (95% CI 0.92–1.17) and, after additional adjustment for smoking and apolipoprotein B/A_1 ratio, it was reduced to 1.00 (95% CI 0.88–1.13). Inclusion of all 5687 unrelated and related controls that were genotyped yielded a similar, non-significant, risk ratio of 1.02 (95% CI 0.92–1.13) per T allele. In principle, large differences between genotypes in case-fatality rates following MI could bias such retrospective case–control comparisons, but 6 month follow-up of the cases did not indicate any such difference in mortality following admission to hospital ($\chi_2^2 = 0.6$; $P = 0.7$). Nor were genotype frequencies different between patients who were admitted soon after the onset of MI and those who were admitted during later time intervals after symptom onset, which might have been expected if early case fatality rates had differed substantially ($\chi_6^2 = 6.0$; $P = 0.4$).

The absence of any significant association in the ISIS study between genetically determined differences in plasma fibrinogen concentration and MI risk corroborates the absence of any significant association between plasma fibrinogen (independent of genotype) and MI risk after correction for multiple other factors. But, because these genetically determined differences in fibrinogen concentration are only moderate, even larger numbers of disease cases than in ISIS alone are needed to assess the association of genotype with coronary disease sufficiently reliably. There were 19 previously reported genetic-association studies of coronary disease in which the −148C/T or −455G/A polymorphisms of the beta-fibrinogen promoter had been genotyped.[11,15,23,25–40] The differences in plasma fibrinogen concentration between genotypes in the control populations of these other studies were generally concordant with those observed in the ISIS study, with no evidence of heterogeneity among the 20 available studies [$\chi_{19}^2 = 15.20$; $P = 0.77$; $I^2 = 0\%$ (95% CI 0–47%)]. In aggregate, those case–control comparisons involved another 7730 coronary disease cases and 15 426 controls, and combining their results with those of ISIS yielded a weighted risk ratio per allele of 1.00 with a 95% CI of 0.95–1.04 (Figure 1), which excludes any material association.

Discussion

The present report demonstrates the potential for large-scale genetic epidemiology to help assess the nature of relationships between putative risk factors and disease. It had not previously been possible to determine with certainty whether the observed association between blood fibrinogen concentrations and coronary risk was causal or due to residual biases from incomplete adjustment for established risk factors. In the present study, 0.14 g/l higher than usual plasma fibrinogen was associated with an age-adjusted and sex-adjusted risk ratio for MI of 1.17 (95% CI 1.14–1.19), which is consistent with the results of the Fibrinogen Studies Collaboration's meta-analysis of individual participant data from 154 000 participants in 31 prospective studies.[41] But, adjustment for the baseline levels of just a few other factors measured in this study greatly reduced the strength of the apparent association. Indeed, after adjustment for smoking, body mass index, and apolipoprotein B/A_1 ratio, no definite association remained (risk ratio 1.03; 95% CI 1.00–1.05), indicating that long-term differences in plasma fibrinogen concentrations are not an independent determinant of MI risk. 'Mendelian randomization' provides an independent test of this conclusion because—based on the beta-fibrinogen genotype inherited—individuals should effectively be 'randomized' at conception to lifelong differences in plasma fibrinogen concentrations.[6–9] Consequently, any association between genotype and disease should not be confounded by other causative factors or by the presence of pre-existing disease.

Rare mutations may cause large and informative differences in the levels of putative risk factors (as, for example, with some types of familial hypercholesterolaemia) that can be investigated by relatively small studies of high-risk families. But, the effects of common genetic polymorphisms on physiologically important factors are typically quite small: for example, the beta-fibrinogen polymorphisms studied are responsible for only a few per cent of the total inter-individual variance of fibrinogen in these populations. Hence, very large numbers of cases and controls may be required to determine reliably whether the association of such a genotype with disease risk indicates a causal role for the risk factor under investigation. Based on 4490 MI cases and similar numbers of controls, the present study indicates that a genetically determined 0.14 g/l

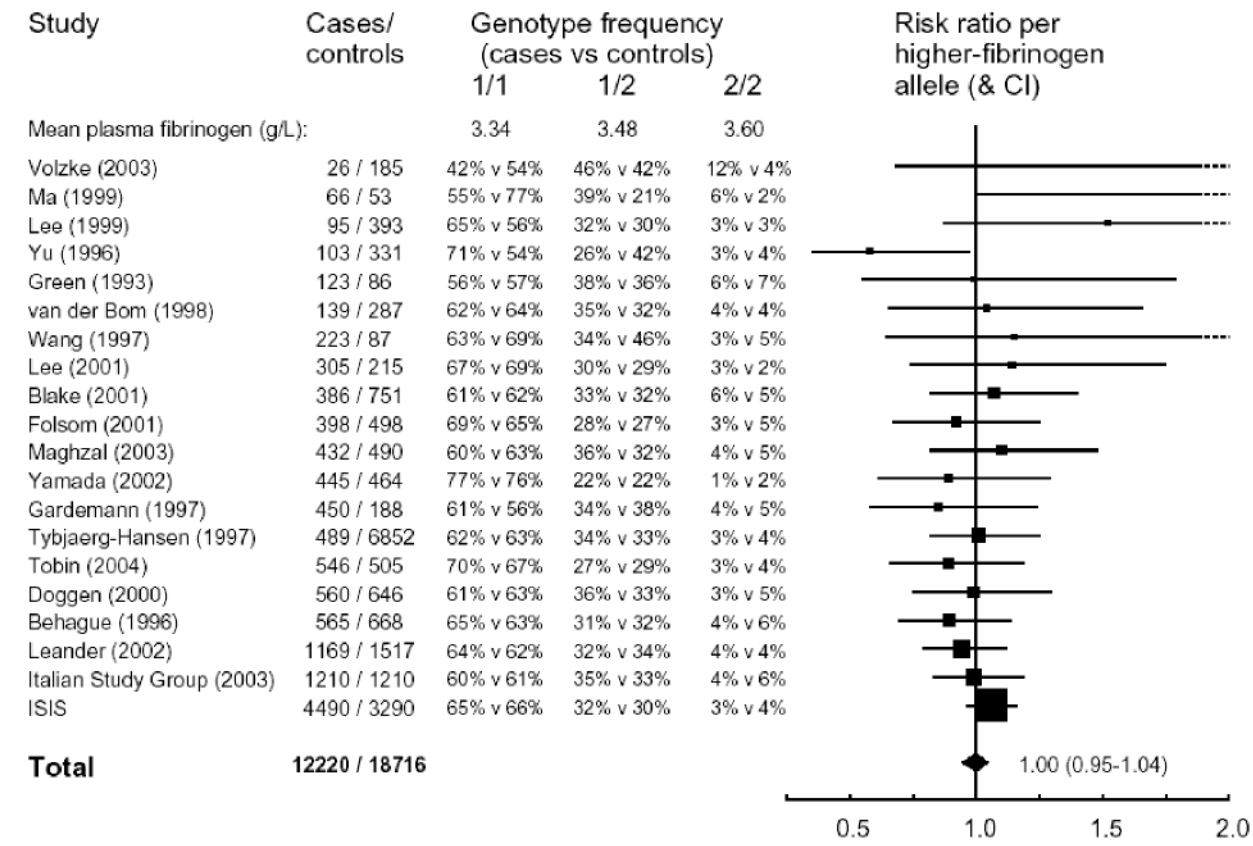

Figure 1 Meta-analysis of studies of coronary disease and −148C/T or −455G/A polymorphisms in the beta-fibrinogen gene. These two polymorphisms are in complete linkage disequilibrium, so knowledge of genotype at one locus predicts genotype at the other locus with certainty. For each study, the risk ratio for coronary disease per higher-fibrinogen allele is represented by a square (area proportional to the information content of the study), with a horizontal line denoting the 99% CI. The overall risk ratio and 95% CI is represented by a diamond, with values alongside. Some groups provided additional data on case and control numbers, which were used in this analysis in preference to the published data (for details see Web Table 2 at *IJE* Online)

higher plasma fibrinogen concentration is not associated with a significant increase in the incidence of MI, but the upper 95% CI is 1.16. That null result was, however, strongly reinforced by an updated meta-analysis involving a total of 12 220 coronary disease cases in studies of such beta-fibrinogen polymorphisms, which yielded a risk ratio for coronary disease per higher-fibrinogen allele of 1.00 with a much narrower 95% CI of 0.95–1.04. Hence, this meta-analysis strongly supports the conclusion that unadjusted associations between plasma fibrinogen concentration and coronary disease risk are due chiefly, if not wholly, to confounding by other risk factors. During the preparation of the present report, others published a meta-analysis of 19 studies of fibrinogen involving 12 393 cases.[42] That meta-analysis incorporated preliminary data from the present study, which included both related and unrelated control subjects in the calculation of the genotype–disease association.[6] Although theoretically this could bias the result towards the null, there was, as we show here (Web Table 1 at *IJE* Online), no significant difference between the disease risk ratios in ISIS when related controls were or were not included. Despite minor differences between the meta-analyses as a result of additional information obtained from authors (see Web Table 2 at *IJE* Online), the results of the two meta-analyses are consistent, indicating no association between fibrinogen genotypes and coronary heart disease.

In principle, various issues could complicate the use of such genetic-epidemiological approaches to the assessment of causality. First, a particular genetic polymorphism could influence risk not only through its effects on plasma concentrations of a particular protein but also through effects on the physiological function of the protein.[43] With regard to the present findings, functional studies in individuals of different genotype at the beta-fibrinogen promoter −455G/A and coding Arg448Lys single nucleotide polymorphisms (SNPs), which are both in very tight linkage disequilibrium with the −148C/T SNP typed in the present study, have previously shown no association of fibrin clot structure with genotype.[34,44,45] This supports the view that the principal difference in plasma fibrinogen between people of different genotypes at the −148C/T SNP is in concentration rather than function. Second, early adaptation during development to genetically determined differences in plasma fibrinogen concentration might attenuate the impact of such differences on the later risk of disease. While this possibility cannot be absolutely excluded, it seems unlikely that there would be substantial developmental adaptation to the small differences in plasma fibrinogen between the

genotypes studied (amounting to ~5% per allele). Third, if a typed polymorphism also affected potential confounding factors then proper 'randomization' might not be achieved. Among the potential confounders measured in the present ISIS study, the β−148C/T polymorphism was associated with a conventionally significant difference in plasma apolipoprotein B/A_1 ratio among the 3460 unrelated controls. But that association was no longer significant after adjustment for multiple testing, and no significant association was found between beta-fibrinogen genotypes and plasma apolipoprotein concentrations among the 2685 cases, or among all 4987 related and unrelated controls in ISIS, or, indeed, among the 9000 participants in the largest previous study.[15] Although one previous study has reported shown that HepG2 cells in culture, which overexpress fibrinogen 4-fold owing to transfection with fibrinogen chains, also have increased secretion of apolipoprotein B, there is no *in vivo* data supporting this as an important mechanism of apolipoprotein B regulation.[46] It seems most likely, therefore, that the apparent association between beta-fibrinogen genotype and apolipoprotein B/A_1 ratio among unrelated controls in the present study merely reflects the play of chance. Moreover, adjustment for smoking and the slightly higher apolipoprotein B/A_1 ratio among unrelated controls with the T allele in the ISIS study only changes the genotype-associated risk ratio for MI from 1.04 to 1.00, reinforcing the null result.

The present study involved cases who had survived a sufficient duration from the onset of MI to be admitted to hospital. As MI carries a significant early mortality, there is the potential for survivor bias in the case ascertainment for such a retrospective study. The β − 148C/T genotype has at most a very small effect on the incidence of non-fatal MI, but it would need to have a large effect on early mortality to produce any material bias in these case–control comparisons. There were no significant differences between the genotype frequencies of patients who were admitted within just a few hours of symptom onset and those who were admitted later, and the mortality rates during the 6 months following admission to hospital did not differ significantly between genotypes. Consequently, it does not seem likely that the present findings have been materially influenced by genotype-associated differences in case-fatality rates. A prospective study would avoid the potential for such survivor bias, but it would need to involve hundreds of thousands of healthy individuals followed for several years in order to yield similar number of cases of premature MI (and, hence, similar statistical power).

It is clear that much of the endemic non-replication of genetic associations with complex diseases hitherto has resulted from inadequate sample sizes. The present study alone has adequate power to rule out a materially significant association between the β − 148C/T polymorphism and CHD. However, because the influence of the polymorphism on fibrinogen levels is small, even the present large study was not of sufficient size alone to address the issue of whether differences in plasma fibrinogen cause CHD. Even the meta-analysis data cannot entirely rule out some causal role for fibrinogen, although this would have to be relatively small. Future 'Mendelian randomization' studies of other potential biomarkers may be similarly hampered by the relative paucity of regulatory polymorphisms thus far identified, which have sufficiently large effects on such biomarkers that discordance between genetic and observational risk estimates could be robustly identified in cohorts of tractable size. With respect to fibrinogen, however, it seems unlikely that further genotyping in CHD cases and controls, even if polymorphisms were discovered that had larger effects on plasma levels, would materially alter the conclusions of the present study.

Many putative risk factors for coronary disease have been proposed in recent years, including inflammatory proteins (notably C-reactive protein),[47] lipoprotein (a),[48] homocysteine,[49,50] and various components of the coagulation pathway, and new technologies (such as proteomics) for measuring very large numbers of compounds in blood will increase further the number of factors associated with disease. But, incomplete information on potential confounding factors, and random error in their measurement, will continue to complicate the assessment of causality by standard epidemiological methods. 'Mendelian randomization' has the potential to avoid some of these difficulties of interpretation, provided that regulatory genetic polymorphisms (such as those in the beta-fibrinogen promoter region) can be identified that influence the levels of the factor of interest without influencing its function and without any material effects on other risk factors.[7,9,51] Novel high-throughput genomic technologies should speed the discovery of such polymorphisms,[52,53] and genetic-epidemiological studies that involve appropriately large numbers of disease cases would then allow reliable confirmation or (as in the present study of fibrinogen) refutation of importantly causal relationships.

Supplementary Data

Supplementary data are available at *IJE* Online.

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KEY MESSAGES

- Observational epidemiology may not alone be able to determine whether associations between putative risk factors and coronary heart disease are causal.
- Genetic epidemiology provides a potential method ('Mendelian randomization') to address such questions.
- This large 'Mendelian randomization' study of plasma fibrinogen levels suggests that differences in plasma fibrinogen level do not cause coronary heart disease.
- This method may have wider applications in complex diseases.

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