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Genetic variation in fibrinogen; its relation to fibrinogen levels and the risk of myocardial infarction and ischaemic stroke

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Abstract

Introduction Confounding by common causes and reverse causation have been proposed as explanations of the association of high fibrinogen levels and cardiovascular disease. Genetic variants, such as plasma levels, can alter fibrinogen characteristics and are not subject to these problems.

This study aims to determine the fibrinogen plasma levels for genotypic variants in fibrinogen-alpha (FGA Thr312Ala, rs6050) and fibrinogen-beta (FGB -455G/A, rs1800790) and whether these variants are associated with myocardial infarction and ischaemic stroke in young women.

Methods Fibrinogen genotypes were determined in a population-based case-control study including women aged 18-50 years; 218 cases with myocardial infarction, 190 cases with ischaemic stroke and 767 healthy controls. Fibrinogen levels were determined in the control population. Blood samples were available for 205 cases with myocardial infarction, 175 ischaemic stroke cases and 638 patients.

Results The FGB -455G/A variant increased plasma fibrinogen levels, whereas the FGA Thr312Ala variant lowered plasma fibrinogen levels, albeit to a modest extent. The risk of ischaemic stroke was altered when the homozygote minor allele was compared with the homozygote major allele. The FGA Thr312Ala single-nucleotide polymorphism (SNP) was associated with a decrease in risk [odds ratio (OR) 0.43; 95% confidence interval (CI) 0.21 - 0.87], whereas the FGB -455G/A SNP might have increased the risk (OR 1.76; 95% CI 0.7 - 4.03). The risk of myocardial infarction was not altered for either SNP (FGA Thr312Ala, OR 0.98, 95% CI 0.40 - 2.40; FGB -455G/A, OR 0.98, 95% CI 0.40 - 2.40).

Discussion With the genetic variations as markers of plasma fibrinogen levels alterations, thereby ruling out confounding and reverse causation, our results suggest that plasma fibrinogen levels play a more pronounced role as risk factor for ischaemic stroke than for myocardial infarction.

Introduction

Fibrinogen is an acute phase coagulation factor produced by hepatocytes and is activated by thrombin to form fibrin monomers and polymers. It is covalently cross-linked by coagulation Factor XIIIa to form a fibrin mesh of which is the main constituent of blood clots. Fibrinogen consists of three pairs of polypeptide chains, denoted (Aα / Bβ / γ), which are linked in the central domain. All three genes for these polypeptide chains are located in the fibrinogen module located on the short arm of chromosome 4.[1,2]

High fibrinogen levels have been associated with cardiovascular diseases in large studies such as the Framingham Study and Northwick Park Heart study and further in meta analyses such as the one by the Fibrinogen Studies Collaboration.[3–5] Results from the RATIO study also showed high fibrinogen to be associated with myocardial infarction (3.5-4.5 g/L vs. <2.5 g/L OR 2.1; 95% confidence interval 1.2 to 3.6).[6]

However, the acute phase property of fibrinogen together with the associations with environmental risk factors of cardiovascular disease (e.g. smoking / oral contraceptive (OC) use) make it difficult to determine whether fibrinogen is either a cause or a mere marker of increased.[7–9] This question can be addressed by means of Mendelian randomisation, a process which uses genetic variation a as marker for lifelong exposure to altered protein levels, in this case an increase of fibrinogen.[10,11] With these genetic variants as markers for fibrinogen levels confounding by extraneous factors and reverse causation are virtually excluded as possible explanations of the association. The genetic marker might also be in linkage disequilibrium with another variation causing variation in the plasma fibrinogen level. Common genetic variants in the fibrinogen module have been associated with increased fibrinogen plasma level and therefore can be used as markers of lifelong increased levels of plasma fibrinogen.[12–14] This current study uses the fibrinogen-Aα (FGA) Thr312Ala (rs6050) single- nucleotide polymorphism (SNP) as a marker of decreased plasma fibrinogen levels, and fibrinogen-Bβ (FGB -455G/A) (rs1800790) as a marker for an increase in level.

In spite of the associations between FGA Thr312 as well as FGB -455A and increased levels of plasma fibrinogen, no association was found for these SNPs or associated haplotypes with myocardial infarction and ischaemic stroke in the Cardiovascular Health Study.[14] Similar results were obtained earlier in the Copenhagen City Study, the SMILE study, the Rotterdam study and a cross sectional analysis of the CARDIA cohort; the genetic variations

are associated with increased plasma fibrinogen level, but not with cardiovascular disease.[12,13,15,16] Another study, however, found an increase in myocardial infarction risk for carriers of the Bcl I β-chain fibrinogen polymorphism (rs209502) which is also a marker of increased plasma fibrinogen levels.[17] This SNP is almost completely in linkage disequilibrium with the FGB - 455G/A SNP (|D'| = 0.96 in the ECTIM study).[18]

The FGA Thr312Ala SNP has also been associated with a decreased risk of pulmonary embolism as well with a lower risk of post-stroke mortality in patients with atrial fibrillation but not of deep venous thrombosis. These decreases in risk are not likely to be caused by increases in plasma fibrinogen level, but through the changes in the mechanical properties of the clot by the genetic variant. The FGA Thr312Ala variant alters clot stability by affecting FXIII cross-linking making carriers of the FGA Thr312 less prone to embolisation.[19,20] This study assessed the risk implications of genetic variations in the fibrinogen module on myocardial infarction and ischaemic stroke in young women. We estimated the risks for carriers of the genetic variants FGA Thr312Ala and FGB - 455G/A as markers for altered fibrinogen levels for myocardial infarction and ischaemic stroke.

Methods

Study design & participants The Risk of Arterial Thrombosis In relation to Oral Contraceptives (RATIO) study is a multicenter population-based case-control study. It consists of three sub-studies which included cases with myocardial infarction, ischaemic stroke and peripheral arterial disease and frequency-matched controls. The study was initiated to evaluate the risk of myocardial infarction and ischaemic stroke due to oral contraceptives of different generations (1990 - 1995).[21–23] Blood and buccal swabs were collected during the second phase of the study in order to study prothrombotic conditions of the coagulation system (1998 - 2002). Informed consent was obtained from all participants and the study was approved by the medical ethics committees of the participating hospitals. For the current study we used data from the myocardial infarction and ischaemic stroke sub-studies.

Patient selection has been described in detail previously. [21–24] In summary, we included women aged 18 to 50 years old who presented with myocardial infarction or ischaemic stroke to one of the sixteen participating hospitals in the Netherlands between 1990 and 1995. Myocardial infarction was diagnosed by the presence of clinical symptoms, elevated cardiac enzyme levels and electrocardiographic changes. Clinical symptoms of ischaemic

stroke were confirmed by either computed tomography (CT) or magnetic resonance imaging (MRI). Controls were approached by random digit dialing, and matched on age, area of residence and year of event. A standardised questionnaire on (familial) medical history, use of oral contraceptives, smoking habits and participant characteristics was filled in by all participants. Some of these questions were targeted at the year prior to the event for cases. Controls were asked to answer these same questions for a particular year (index year) to ensure comparability. Blood or buccal swaps were collected for DNA analysis from 218 myocardial infarction cases, 190 ischaemic stroke cases and 767 controls.

Measurements and definitions DNA was isolated from 10 ml EDTA-augmented blood or buccal swabs and amplified by polymerase chain reaction. Two SNPs in fibrinogen Aα (Thr312Ala, rs6050) and fibrinogen Bβ (-455G/A, rs1800790) were genotyped with the 5' nuclease / Taqman Assay.[25] Polymerase chain reactions based on fluorescent allele specific oligonucleotide probes (assay-by-design / assay-on-demand; Applied Biosystems, Foster city, USA) were performed on a PE9700 thermal cycler (Biozym, Hessisch Oldendorf,

Table 1. Characteristics of RATIO participants

	Myocardial infarction N=218	Ischaemic stroke N=190	Control N=767
Mean Age (SD)	43 (6)	39 (8)	39 (8)
Caucasian ethnicity	207 (95%)	182 (96%)	732 (94%)
History of *			
Hypertension	55 (25%)	55 (29%)	47 (6%)
Diabetes	11 (5%)	8 (4%)	10 (1%)
Hypercholesterolaemia	23 (11%)	15 (8%)	22 (33%)
Oral contraceptives use *	86 (39%)	98 (52%)	272 (35%)
Smoking *	181 (83%)	115 (61%)	319 (42%)
alcohol use			
never	83 (38%)	89 (47%)	227 (30%)
0-15 glasses per week	128 (59%)	84 (44%)	500 (65%)
>15 glasses per week	7 (3%)	2 (1%)	32 (4%)

All data are with respect to the year of event (cases) or index date (controls). Data are missing on ethnicity for one ischaemic stroke (IS) case and four controls, on history of hypertension for four controls, on history of diabetes for one myocardial infarction (MI) case and three controls, on hyperlipidaemia for one MI case and five controls, on oral contraceptive use for six controls, on smoking for six controls, and on alcohol use for one IS case and six controls. SD, standard deviation.

Germany) and fluorescence endpoint reading for allelic discrimination was performed on a ABI 7900 HT (Applied Biosystems). Primers and probe sequence are available upon request. During genotyping, technicians were unaware of outcome status. Fibrinogen in the control population was determined according to Von Clauss using the reagents of Behringwerke AG (Marburg, Germany).

Statistical analyses Logistic regression was used to obtain odds ratios as measures of rate ratios and accompanying 95% confidence intervals (95% CI) for the relation between genetic variants in the fibrinogen gene and myocardial infarction ischaemic stroke. Odds ratios were adjusted for the three stratification variables age (as a continuous variable), area of residence and index event. Differences in fibrinogen plasma levels and corresponding 95% CI were calculated per genotype. Expected relative risks corresponding the observed fibrinogen differences were based on hazard ratios from a meta analysis.[5] For all analyses, the homozygote carriers of the major allele were used as the reference group. Women with missing data were excluded from analysis when appropriate.

Results

Study participants Table 1 shows the baseline characteristics of the study participants. Known risk factors, such as hypertension, diabetes and hyperlipidaemia, were more prevalent in both case groups than in controls. A larger proportion of patients than of controls were smokers. The overall call rate of the genotyping was 97% for the two SNPs, and the success rate was irrespective of case - control status. The allele frequencies of the variants were 0.31 for FGA Thr312Ala and 0.19 for FGB - 455G/A. The likelihood of departure from Hardy - Weinberg equilibrium (HWE) was determined in the control population, and was P = 0.043 for FGA Thr312Ala and P = 0.84 for FGB - 455G/A.

Genetic variants and fibrinogen plasma levels Plasma fibrinogen levels were available for 602 control women. The mean fibrinogen level in this population was 3.20 g/L. The plasma fibrinogen levels per genotype as well as the associated expected relative risks are shown in Table 2. L Levels were lower in carriers of the FGA - 455G/A variant (3.15 g/L for carriers vs 3.24 g/L for non-carriers, 95% CI of difference -0.20 to 0.02 g/L). Levels were higher for carriers of the FGB Thr312Ala variant (3.32 g/L for carriers vs 3.13 g/L for non-carriers, 95% CI of difference 0.07 to 0.30 g /L).

Table 2. Mean fibrinogen plasma levels per genotype

	N	fibrinogen level	difference	95% CI of difference	expected RR	
FGA Thr312Ala SNP (rs6050)					MI	IS
Thr / Thr	319	3.24 g/L	ref	-	-	-
Thr / Ala	237	3.14 g/L	-0.06	-0.21 to 0.01 g/L	0.90 (0.80-1.01)	0.90 (0.81-1.01)
Ala / Ala	46	3.20 g/L	-0.04	-0.25 to 0.17 g/L	0.96 (0.76-1.20)	0.96 (0.77-1.19)
FGB -455G/A SNP (rs1800790)						
G / G	397	3.13 g/L	ref	-		
A / G	184	3.30 g/L	0.17 g/L	0.05 to 0.28 g/L	1.20 (1.01-1.35)	1.19 (1.05-1.33)
A / A	21	3.50 g/L	0.37 g/L	0.06 to 0.67 g/L	1.49 (1.06-2.05)	1.46 (1.04-1.98)

Fibrinogen was measured in 602 control women. The expected relative risk (RR) was calculated on the basis of the hazard ratio (HR) per increase of fibrinogen plasma level of 1 g L)1 for (non)-fatal coronary heart disease [i.e. HR 2.93; 95% confidence interval (CI) 2.59 - 3.31] and (non)-fatal stroke (i.e. HR 2.77; 95% CI 2.17 - 3.53) for ages 40 - 59 years reported by the Fibrinogen Studies Collaboration.[5] MI, myocardial infarction; IS, ischemic stroke; RR, relative risk; OR, odds ratio; Ref, reference; FGA, fibrinogen-Aα; FGB, fibrinogen-Bβ.

Genotype analyses The genotype distribution among case groups and controls, aswell as the risk per genotype with the homozygote major allele genotype as reference, are shown in Table 3. After adjustment for the stratification variables, no effect on myocardial risk was observed for the FGA Thr312Ala variant (OR 0.82; 95% CI 0.49 - 1.39) but a decreased risk was found for ischaemic stroke (OR 0.43; 95% CI 0.21 - 0.87) when the homozygote minor allele genotype was compared with the homozygote major allele genotype. The homozygote carriers of the FGB -455G/A variant also showed no altered risk of myocardial infarction (OR 0.98; 95% CI 0.40 - 2.40). If anything, the risk of ischaemic stroke was increased (OR 1.76; 95% CI 0.77 - 4.03). No substantial effect on myocardial infarction or ischaemic stroke was observed for heterozygotes when compared with those with the homozygote major alleles for the FGA Thr312Ala SNP or the FGB -455G/A SNP.

The results of this per-genotype analysis are consistent with the risks when one assumes a recessive inheritance pattern: homozygous minor allele genotype of the FGA Thr312Ala SNP, when compared with the homozygous major allele genotype combined with the heterozygote genotype, lowered the risk of ischaemic stroke (OR 0.43; 95% CI 0.21 - 0.85), but not of myocardial infarction (OR 0.91; 95% CI 0.55 - 1.53). The homozygous minor allele genotype of the FGB -455G/A SNP tended to elevate the risk of ischaemic stroke (OR 1.84; 95% CI 0.81 - 4.19) but not of myocardial infarction (OR 0.94; 95% CI 0.39 - 2.29) when compared with the homozygous major allele and heterozygote genotype combined. A dominant inheritance pattern diluted all effects; no substantial effect of the FGA Thr312Ala

Table 3. Genotype distribution and the relative risk of MI and IS

		Genotype distribution						OR (95%CI)			
FGA Thr312Ala SNP (rs6050)		**MI**		**IS**		**Control**		**MI**		**IS**	
Homozygote major allele	Thr/Thr	121	56%	97	53%	370	49%	1	[ref]	1	[ref]
Heterozygote	Thr/Ala	81	33%	74	40%	295	39%	0.76	0.54 - 1.07	1.00	0.69 - 1.46
Homozygote minor allele	Ala/Ala	24	11%	12	7%	89	12%	0.82	0.49 - 1.39	0.43	0.21 - 0.87
FGB -455G/A SNP (rs1800790)											
Homozygote major allele	G/G	135	64%	122	67%	483	66	1	[ref]	1	[ref]
Heterozygote	A/G	70	33%	60	27%	221	30%	1.15	0.80 - 1.62	0.85	0.57 - 1.27
Homozygote minor allele	A/A	7	3%	10	5%	29	4%	0.98	0.40 - 2.40	1.76	0.77 - 4.03

Percentages might not add up to 100%, because of rounding. Single-nucleotide polymorphism (SNP) determination was either unsuccessful or ambiguous in seven control women, one myocardial infarction (MI) case and seven ischaemic stroke (IS) cases for the fibrinogen-Aα (FGA) Thr312Ala SNP. The fibrinogen-Bβ (FGB) -455G/A SNP determination did not succeed in 34 controls, six MI cases and eight IS cases. ORs were calculated per genotype, with the homozygote major allele genotype as reference, and are adjusted for matching factors (i.e. age, area of residence, and year of event).

SNP was observed on myocardial infarction (OR 0.77; 95% CI 0.56 - 1.07) or on ischaemic stroke (OR 0.86; 95% CI 0.60 - 1.22). The FGB -455G/A SNP also showed no effect on either myocardial infarction (OR 1.13; 95% CI 1.13 - 1.58) or ischaemic stroke (OR 0.94; 95% CI 0.64 - 1.37).

Smoking and oral contraceptive use OC use in the year of the event increased the crude risk of myocardial infarction and ischaemic stroke two-fold to three-fold (OR 2.3, 95% CI1.6 - 3.3, for myocardial infarction; OR 2.6, 95% CI 1.7 - 3.9, for ischaemic stroke). Smoking in the year prior to the event also increased arterial thrombotic risk (OR 7.1, 95% CI 4.7 - 10.7, for myocardial infarction; OR 2.2, 95% CI 1.6 - 3.3, for ischaemic stroke). These effects showed no marked interaction with the effects of the SNPs (not shown).

Discussion

We used two genetic variants in the fibrinogen module as markers of decreased (FGA Thr312Ala) and increased (FGB -455G/A) plasma fibrinogen levels. We found that the effects of these SNPs on fibrinogen levels in our control population were according to the literature, albeit to a modest extent. Both genetic variant SNPs as markers of altered fibrinogen levels also altered the risk the risk of ischaemic stroke in the expected direction; FGA Thr312Ala decreased the risk twofold (OR 0.43; 95% CI 0.21 - 0.87), and FGB -455G/A possibly increased this risk (OR 1.76; 95% CI 0.77 - 4.03). Neither SNP affected the risk of myocardial infarction. These altered risks were greatest in the per-genotype analysis, with similar results being obtained, assuming a recessive inheritance pattern. Smoking and the use of OCs, both established risk factors for arterial thrombosis and related to increased

fibrinogen, increased the risk of both myocardial infarction and ischaemic stroke, but showed no marked interaction with the FGA Thr312Ala and FGB -455G/A SNPs, possibly because of lack of power.

We used a Mendelian randomisation line of thought to rule out any confounding by environmental factors and to ascertain whether the role of fibrinogen is a cause or a consequence. For this process, it is necessary to establish the relationship between genetic variants and plasma fibrinogen level, either from the literature or in the control population; both the FGA Thr312Ala and FGB -455G/A SNPs have been found to alter fibrinogen plasma levels. [12,13,15,19,26,27] We showed similar effects of the genetic variants in our control population, albeit to a modest extent. The expected increase in risk based on the increase in fibrinogen levels associated with these genetic variants is 4 - 49%, which is lower than the increase in risk that we established. These calculations are based on the hazard ratios from the meta-analysis by the Fibrinogen Studies Collaboration, focusing on coronary heart disease and stroke in the age group 40 - 59 years.[5] This meta-analysis also includes studies with participants aged 18 - 90 years, with an emphasis on the elderly. The effect of fibrinogen on the risk of arterial thrombosis is larger in the young, which might explain why the effect was larger in the young women in our study.

This fair-sized population-based case - control study for young women with myocardial infarction or ischaemic stroke provides a unique opportunity to determine (genetic) risk factors for this specific group that may be indicative of risk factors in older age categories. Nonetheless, our subgroup analyses (i.e. smoking and OC use analyses) had limited numbers for some subgroups. For this reason, we chose to use a dominant inheritance pattern. The dilution of effect that is observed in the risk analysis with a dominant inheritance pattern could therefore also affect these subgroup analyses. However, numbers were limited and strong conclusions cannot be drawn. We can therefore only conclude that there is no evidence for a strong interaction between the environmental factors and genetic variants.

The control population can be considered not to be in HWE because a P-value of 0.043 was calculated for the FGA Thr312Ala SNP. As our controls were sampled from the entire Netherlands through random digit dialing, we consider this slight deviation of the HWE to be probably due to chance. We chose to study two SNPs in the fibrinogen module that are known to be associated with altered fibrinogen levels. It is possible that these SNPs are

causal for this change in plasma level, but they could also be in linkage with other genetic traits that influence fibrinogen levels (e.g. Bcl I β-chain fibrinogen polymorphism). These other traits could potentially affect the plasma fibrinogen levels, still leaving our SNPs as proper markers of lifelong exposure to altered fibrinogen levels. The used SNPs could also be in linkage with genetic variations that result in functionally different fibrinogen and alter the risk via a mechanism other then altered plasma fibrinogen levels. This might be the case for the FGA Thr312Ala SNP. It has been negatively associated with venous thromboembolism, pulmonary embolism and poststroke mortality in patients with atrial fibrillation and ischaemic stroke.[19,20,27,28] This increase in risk might be caused by the changed characteristics of the fibrin fibers, which make the clot more prone to embolisation.[20] This increase in risk is in contrast with our results. But as the stroke cases included in our study are primarily of non-cardiac origin, we feel that the FGA Thr312Ala variation is a marker not of decreased disposition to embolisation but primarily of lower fibrinogen plasma levels. Nevertheless, conclusions based on the FGA Thr312 results should be drawn cautiously. The large individual-participant meta-analysis by the Fibrinogen Studies Collaboration showed a similar increase in risk for all major cardiovascular diseases, including non-fatal ischaemic stroke and myocardial infarction. The difference between our results (different effects of fibrinogen on the risks of myocardial infarction and ischaemic stroke) and those of the Fibrinogen Studies Collaboration (similar effects of fibrinogen on the risks of those diseases) might be due to confounding and reverse causation, problems that are resolved by the use of Mendelian randomisation in our study. Earlier studies with the same approach also showed an association of the FGB -455G/A SNP and increased plasma fibrinogen levels, but failed to show an association with myocardial infarction or ischaemic stroke.[13–15,27,29] These studies mainly included middle-aged to old subjects, in contrast to our young female population: the older age, and hence more developed stage of atherosclerosis, of the previously studied populations might explain why no direct relationship between the genetic variants and arterial thrombosis were found. If it is present at all, we hypothesize that our cases have minimal late stage atherosclerosis, because of their young age; only 11% of the myocardial infarction cases and 8% of the ischaemic stroke cases were reported to be diagnosed with hyperlipidaemia at the time of the event. Fibrinogen levels and other coagulation factors might therefore play a more pronounced role in arterial thrombosis in the young than in the old, either as a direct causal factor for thrombosis or a strong

cofactor in thrombosis initiated by subclinical atherosclerosis. This may be even more the case for the development of ischaemic stroke than for myocardial infarction.

Because of the use of a detailed questionnaire in the first phase of this case-control study, we were able to include only survivors of myocardial infarction and ischaemic stroke; fatal events could not be included, possibly leading to a survivor bias. Even if there is a difference between the aetiologies of fatal and non-fatal arterial thrombosis, we hypothesize that the effect of fibrinogen, or any other risk factor, is more pronounced in the fatal events. Therefore, if our study is affected by this survivor bias, this bias is likely to have led to an underestimation of the true effect of fibrinogen levels.

Conclusion In conclusion, this study showed an association between the FGA Thr312Ala SNP and a decreased risk of ischaemic stroke. If anything, the FGB -455G/A SNP was associated with an increase in risk. The direction of the effects is in accordance with the effects of the two SNPs on plasma fibrinogen levels. Neither SNP affected the risk of myocardial infarction. The FGA Thr312Ala SNP could also be a marker of altered clot characteristics, and the effect of the FGB -455G/A SNP has not been established firmly, which makes the interpretation of the increased risk associated with this variant difficult. Taking survivor bias into account, as well as the non-cardiac origin of the ischaemic stroke included in this study and the fact that the SNPs used in this study are proper markers of altered fibrinogen levels, we conclude that fibrinogen probably plays a causal role in the development of ischaemic stroke in young women.

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