Blood Coagulation, Fibrinolysis and Cellular Haemostasis

The factor VII activating protease G511E (Marburg) variant and cardiovascular risk

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Summary

A previous study had shown a strong relationship between a variant in factor VII activating protease (FSAP G511E) and advanced carotid atheroma. *In-vitro*, the variant has reduced fibrinolytic but normal pro-coagulant activity, which may constitute a prothrombotic state. The current study has addressed risk for coronary heart disease in a prospective study of cardio-vascular disorders (Northwick Park Heart Study II). An interactive effect upon risk was found between the 511E allele and elevated levels of cholesterol and triglyceride. Fibrinogen could substitute for triglyceride levels in this risk-interaction analysis. The findings support the proposal that the FSAP 511E allele exacerbates atherosclerosis or its clinical sequelae.

Introduction

In 1996, Choi-Muira et al. described a hitherto unrecognised serine protease (1). Following isolation of this glycoprotein (2), its ability to activate factor VII (FVII) in the absence of tissue factor was reported (3). Soon after, this FVII activating protease (FSAP) was shown to be a potent activator of single-chain plasminogen activators e.g., pro-urokinase (scuPA) (4). Thus, FSAP appeared to be both a potent pro-coagulant and pro-fibrinolytic agent. More recently FSAP has also been reported to inhibit platelet-derived growth factor BB-mediated proliferation and migration of vascular smooth muscle cells (5).

In plasma, FSAP circulates at a concentration of about 12 μg/mL in single- chain form, though interaction with biomolecular surfaces, such as provided by acidic glycosaminoglycans, stimulates autoactivation to the two-chain form and degradation (6). Of the many haemostatic and fibrinolytic pro-teases, only pro-urokinase/urokinase (uPA) activates FSAP (6). The main inhibitors of FSAP appear to be C1-inhibitor and antiplasmin (4).

Romisch et al (7) reported that, in 9% of healthy blood donors, the ability of plasma to activate scuPA was impaired, despite normal FSAP antigen levels and a normal FVII activating ability (8). Analysis of genomic DNA revealed a polymorphism of FSAP at nucleotide 1601, with substitution of adenine (A) for guanine (G), and resulting in the substitution of glycine (G) by glutamic acid (E) at position 511 (1601G>A; G511E) (9). This variant was named FVII Marburg I by Romisch et al (9). Willeit et al speculated that the reduced scuPA activation but normal activation of FVII associated with the G511E polymorphism might constitute a prothrombotic state (10). They therefore investigated the effect of this polymorphism on the evolution of carotid stenosis by prospective surveillance of an Italian population aged 40 to 79 years. Over a 5-year follow-

up, no association was observed between the polymorphism and the development of early atherosclerosis, but a strong relation was found with advanced atheroma (lumen narrowed by > 40%). These findings (10) prompted us to explore the relationship between Marburg I polymorphism and coronary heart disease (CHD) in the prospective Second Northwick Park Heart Study (NPHS II) (11).

Materials and methods

Study cohort

NPHS II is a study of men aged 50-64 years at baseline, who were clinically free of cardiovascular disease at that time. The

Table 1: Baseline characteristics and measured coagulation factor traits in NPHS men within each G511E genotype group.

	GG N=1900	GE/EE N=176	P value
Age (years)	56.2 (3.4)	56.1 (3.4)	0.84
BMI (kg/m^2)	26.3 (3.3)	26.4 (3.3)	0.84
Smokers, % (N)	28.3 (537)	33.5 (59)	0.14
Systolic BP (mm Hg)	138.7 (19.6)	138.3 (17.8)	0.78
Triglyceride (mmol/L)*	1.77 (0.93)	1.70 (0.88)	0.32
Cholesterol (mmol/L)	5.73 (0.98)	5.53 (0.99)	0.01
FVIIc (% standard)*	106.6 (28.0)	107.2 (28.4)	0.75
FVIIa (ng/mL)*	2.22 (1.20)	2.28 (1.15)	0.81
FVIIag (% standard)*	126.7 (35.0)	127.1 (37.8)	0.90
Fibrinogen (mg/dL)* -smoking adjusted	2.65 (0.50)	2.69 (0.51)	0.22
FPA (nM)*	1.19 (0.60)	1.14 (0.57)	0.37
F1.2 (nM)*	0.70 (0.27)	0.70 (0.28)	0.97
FIX pep (pM)*	197.7 (57.6)	219.4 (60.3)	0.05
FX pep (pM)*	80.2 (28.0)	84.3 (26.6)	0.48

*Variables are log-normal. Means are geometric and standard deviations approximate.
p values for comparing EEs with GE/GG are: cholesterol p=0.20, FVIIc p=0.06, FVIIag p=0.31, F1.2 p=0.01, FIX p=0.17

study commenced in 1989, and is based within 9 general medical practices in England and Scotland. Of 4600 men, 4141 were eligible for study and 3052 were recruited. Blood was taken in the non-fasting state for haemostatic factors (11). DNA was obtained from 2775 men. For the current analysis non-caucasian subjects were excluded, leaving 2735. Body mass index (BMI), serum lipids, smoking habit and blood pressure (BP) were recorded as described elsewhere (11). All subjects had results for FVII coagulant activity (FVIIc), while fibrinopeptide A (FPA) and prothrombin fragment 1+2 ($F_{1.2}$) were recorded in 1871 and 1906, respectively. Factor IX activation peptide (FIX pep, n=401) and factor X activation peptide (FX pep, n=347) were analysed in cases and matched with others from the cohort for age, practice and the year of sampling. All coagulation parameters were measured in base-line samples. The Office for National Statistics, hospitals, coroners and general practices supplied details of morbidity and mortality. CHD was defined as those who had a myocardial infarction (silent, determined by ECG, or clinical), or those who had coronary intervention procedures. Stable angina was not included as an end-point when the prospective study was initiated, although ascertained by questionnaire. For the current analysis patients with angina (n=58) were included in the non-CHD group, as angina is considered a subjective end-point. Patients with diabetes (n=51) were excluded in the current analysis, but including diabetics did not change the results. All men gave written informed consent. Of the genotyped, non-diabetic men, 158 had a CHD event over the period of follow up, and based on the observed allele frequency, the study had 80% power at the 5% level of significance to detect a relative risk of 1.92 for carriers of the 511E allele as compared to the 511GG men.

Genetic analysis

Genotype was determined by an allele specific amplification method. Oligonucleotide primers reported previously (9) resulted in a 292bp fragment, and were used as "outer" primers, and allele specific primers were used (Invitrogen Life Technologies) to amplify the A (511E) allele 5'CTGGGGC-CTGGAGTGTGAG3' and the G (511G allele) 5'CTGGGGC-CTGGAGTGTGGG3' (i.e. differing only in the penultimate 3' base). These primers produced a 178bp fragment. PCRs were performed using an MJ Research PTC-225 Peltier Thermal Cycler, in a 20μl reaction volume using $1 \times$ KCl buffer containing 50mM KCL, 10mM Tris (pH 8.3), 0.001% gelatin and 0.2mM each dNTP. PCR reactions included 5ng of DNA, 5pmol of the two outer and 5pmol of either the "A" or "G" allele specific primer, 1.5 mmol MgCl and 10% DMSO. Amplification conditions were 95^0 for 2mins, followed by 40 cycles of 95^0 for 45secs, 61^0 for 1min, and 72^0 for 1min, with a final extension period of 72^0 for 5mins. The fragments were resolved by electrophoresis on a 1% agarose gel for 1 hr at 75 volts. The 292bp fragment was used as an amplification control within

Table 2: Stepwise model to determine factors having a risk-interaction with FSAP genotype (GE+EE).

	HR for continuous variables	Interaction with genotype
Smoking	1.66 (1.27 – 2.18) p=0.001	p=0.20
BMI	1.36 (1.04 – 1.77) p=0.02	p=0.13
SBP	1.39 (1.07 – 1.82) p=0.01	p=0.15
Cholesterol	1.77 (1.34 – 2.33) p<0.0001	p=0.02
Triglyceride	1.49 (1.13 – 1.96) p=0.004	p=0.05
Fibrinogen	1.92 (1.46 – 2.33) p<0.0001	p=0.04
Genotype	p=0.63	-

Triglyceride and fibrinogen levels were ln-transformed.
*Interaction p values use levels above and below the mean (median).

each reaction. The accuracy of the method was confirmed using DNA from 10 subjects whose genotype had been obtained by sequencing, and each PCR run included a control for each of the three genotypes. Genotype was determined for 2127 individuals (78% of those with DNA). Poor quality DNA prevented amplification of all samples. None of the baseline characteristics of those genotyped, differed significantly from the whole sample (not shown).

Statistical analysis

Statistical analysis was performed using STATA version 7.0 (STATA Corporation, Texas, USA). Deviations from Hardy-Weinberg equilibrium were considered using the chi-squared test. Log transformations were for variables, which were not normally distributed. Differences in baseline characteristics were tested by analysis of variance. Differences in smoking status were tested by chi-squared test. Associations with CHD risk were examined using Cox proportional hazards models (Hazard Ratio, HR). Age was included as a covariate in the models and differences in the baseline hazard by practice were permitted (using the strata option in STATA). Interactions were considered as deviations from multiplicative effects. Ten-year risk of CHD was estimated using the covariates from the Cox model and the baseline hazard obtained from the Kaplan-Meier survival function. The effect of raised cholesterol, triglyceride, fibrinogen and genotype on haemostatic variables were assessed by analysis-of-variance models. Confidence Intervals (CI) quoted at the 95% level).

Results

Genotype associations

The genotype distribution was as expected for Hardy-Weinberg proportions and the frequency of the 511E allele was 0.043 (CI 0.038 to 0.050), which is similar to that reported previously (9, 10). The number of individuals homozygous for the rare allele was non-significantly higher in the current study, compared to the Bruneck Study cohort (5/2127, 0.24%, including 1 diabetic, compared to 1/800, 0.125%, p=0.48) and may be due to sampling differences. In non-Caucasians (not included in analysis) no estimate of frequency could be made for the 511E allele, as

G511E	Chol<=5.7			Chol>5.7		
	N	No of events (%)	HR (95% CI)*	N	No of events (%)	HR (95% CI)*
GG	975	62 (6.4%)	1.00	909	81 (8.9%)	1.37 (0.98 – 1.91)
GE/EE	113	4 (3.5%)	0.53 (0.19 – 1.47)	62	11 (17.7%)	2.99 (1.57 – 5.70)
	TG<=1.73			**TG>1.73**		
	N	No of events (%)	HR (95% CI)*	N	No of events (%)	HR (95% CI)*
GG	958	60 (6.3%)	1.00	927	83 (9.0%)	1.43 (1.02 – 1.99)
GE/EE	97	3 (3.1%)	0.48 (0.15 – 1.53)	79	12 (15.2%)	2.56 (1.37 – 4.77)
	Fibrinogen<=2.7			**Fibrinogen>2.7**		
	N	No of events (%)	HR (95% CI)*	N	No of events (%)	HR (95% CI)*
GG	945	62 (6.6)	1.00	945	81 (8.6)	1.38 (0.99 – 1.93)
GE/EE	78	1 (1.3)	0.19 (0.03 – 1.36)	98	14 (14.3)	2.40 (1.34 – 4.30)

Mean used as cut-off for cholesterol, median used as cut-off for triglyceride and fibrinogen. *age and practice adjusted; Genotype by cholesterol interaction p=0.03; Interaction persists after adjustment for age, practice, smoking, triglyceride, BMI, SBP, fibrinogen and VIIc (p=0.03). Genotype by triglyceride interaction p=0.06; Interaction persists after adjustment for age, practice, smoking, BMI, cholesterol, SBP, fibrinogen and VIIc (p=0.06). Genotype by fibrinogen interaction p=0.03; Interaction persists after adjustment for age, practice, smoking, triglyceride, BMI, cholesterol, SBP and VIIc (p=0.03).

Table 3: CHD risk in those with cholesterol, triglyceride or fibrinogen levels below or above the mean (median), setting common homozygotes with levels below the mean as HR 1.0.

Table 4: CHD risk for FSAP 511E allele for 1SD increase in cholesterol, triglyceride (ln-transformed) or fibrinogen level (ln-transformed).

G511E	HR* (95% CI)	P value
	Cholesterol	
GG	1.25 (1.06 – 1.48)	0.01
GE/EE	3.04 (1.76 – 5.25)	<0.0001
interaction	p=0.002	
	Triglyceride	
GG	1.34 (1.14 – 1.57)	<0.0001
GE/EE	1.76 (1.08 – 2.86)	0.02
interaction	p=0.29	
	Fibrinogen	
GG	1.19 (1.01 – 1.39)	0.03
GE/EE	1.63 (1.05 – 2.53)	0.03
interaction	p=0.19	

*Adjusted for age and practice. The cholesterol by genotype interaction persisted after adjustment for age, practice, triglyceride, BMI, SBP, smoking, fibrinogen and VIIc (p=0.004).

all carried the common allele (n=40). Selected baseline characteristics by genotype are shown in Table 1. There were no significant differences between levels for age, BMI, systolic blood pressure (SBP), or triglyceride levels, in those with the common compared with those with the rare allele. However, cholesterol levels were significantly lower in those with the rare allele (heterozygotes and homozygotes combined) compared to those homozygous for the common allele, Table 1, and were lower, although not significantly, in the four, non-diabetic, individuals who were homozygous for the rare allele (GG mean 5.73mM, SD 0.98; GE mean 5.53mM, SD 1.00; EE mean 5.30mM, SD 0.32). No significant differences were observed between heterozygotes and rare homozygotes, for age, BMI, SBP, or triglyceride levels. Further analysis was done using the combined group of heterozygotes and rare homozygotes.

Risk-interaction analysis

Initially, this analysis was done with diabetics included. The finding of a risk-interaction between genotype and factors present in 'metabolic syndrome' led us to repeat the analysis, excluding diabetics. Essentially, the results were the same as presented, when diabetics were included. No significant overall risk for CHD was determined for the 511E allele (HR1.14; CI 0.67-1.94, p=0.64). Risk-interaction was assessed between FSAP genotype and established clinical and biochemical risk factors for CHD. No significant risk interactions were seen with BMI, SBP, or smoking, but significant interactions were detected for the 511E allele (heterozygotes and homozygotes combined) with cholesterol levels (p=0.02, Table 2) and fibrinogen levels (p=0.04, Table 2), as well as a borderline significant interaction with triglyceride levels (p=0.05, Table 2). The HR suggested no increased risk for the variant in those with low cholesterol, tryglceride or fibrinogen levels (rather, the tendency was towards a protective effect) but a significant risk for the 511E allele in those with cholesterol, triglyceride or fibrinogen levels above the mean/median, Table 3. When cholesterol, triglyceride and fibrinogen levels were analysed as continuous variables, in their interaction with FSAP genotype, only cholesterol exhibited a statistical interaction (Fig 1 shows estimated 10-year risk for cholesterol, triglyceride and fibrinogen levels). The HR for 1 SD increase in these three variables, for both the common and rare FSAP genotype, is shown in Table 4.

Since cholesterol, triglyceride and fibrinogen, exhibited an interaction with FSAP genotype, these factors were further studied using Cox regression analysis: In a model including genotype, triglyceride and cholesterol levels a borderline interaction was found between genotype and cholesterol levels (p=0.05) but did not reach significance between genotype and triglyceride levels (p=0.11). Fibrinogen could substitute for triglyceride in the model (with a borderline interaction, p=0.057) but including it in a model with cholesterol and triglyceride, removed the significance of all factors.

Triglyceride is primarily a component of chylomicrons, very low (VLDL) and intermediate density (IDL) lipoprotein, while cholesterol is primarily a component of IDL and LDL. Apolipoprotein B (ApoB) circulates as an integral component of LDL and lower-density lipoproteins (one molecule per particle) but is not present on HDL particles. ApoB had been measured in NPHSII, and we therefore determined, in a model including cholesterol, tryglyceride and Apo B, which factor(s) persisted in

No. of risk factors raised	G511E	N	No. of events (%)	HR (95% CI)*
0/1	GG	969	62 (6.4)	1.00
	GE/EE	106	2 (1.9)	0.29 (0.07 – 1.18)
2	GG	609	46 (7.6)	1.19 (0.81 – 1.75)
	GE/EE	38	4 (10.5)	1.57 (0.57 – 4.33)
3	GG	297	35 (11.8)	1.85 (1.22 – 2.80)
	GE/EE	31	9 (29.0)	5.64 (2.79 – 11.40)

*Number of risk factors by genotype interaction p=0.004

Table 5: Risk associated with 0/1 risk factors raised above the mean (cholesterol), or median (triglyceride, fibrinogen) compared to 2 or 3 risk factors raised, for FSAP G511 compared to 511E allele.
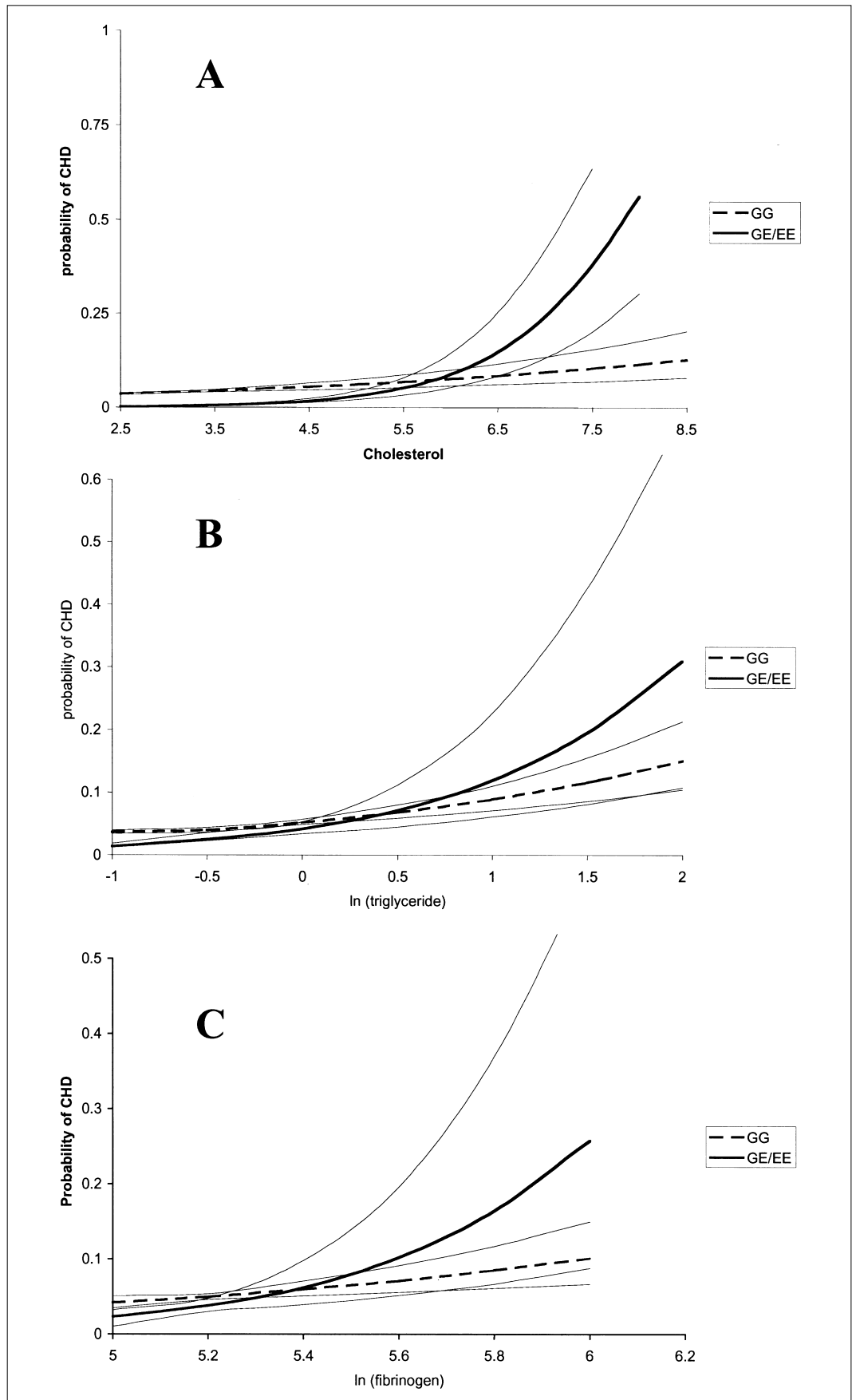
Figure 1: Ten year risk of CHD for cholesterol (A), triglyceride (B) and fibrinogen (C) for FSAP 511GG compared to GE/EE (calculated using the Kaplan Meier survival function and estimates from the Cox model). Risk is adjusted to the mean age 56 years. Solid lines represent GE/EE, large dashed line represents common allele, GG. Small dashed lines represent standard error for each probability curve.

an interaction with FSAP genotype. The triglyceride interaction was now borderline (p=0.05), but neither cholesterol nor Apo B exhibited a significant interaction after adjustment for the other. These results suggest that cholesterol and ApoB levels are interchangeable as risk factors. Furthermore, these data would suggest that cholesterol-risk interaction of FSAP 511E is acting through LDL and IDL, and that triglyceride contributes to the genotype risk interaction independently of cholesterol.

We then analysed CHD risk-interaction with FSAP genotype in those who had either two or three plasma factors elevated above the mean (for either fibrinogen, cholesterol or triglyceride). ApoB was not included in this analysis as it is likely that ApoB risk is synonymous with cholesterol risk (correlation of ApoB with cholesterol, r=0.62, p=<0.0001). Fibrinogen was included with triglyceride, because while it substituted for triglyceride in a risk model, there was no correlation of fibrinogen with triglyceride levels (r=0.02, p=0.31). Setting risk in those with the common FSAP genotype, and with zero or one elevated plasma marker, as HR 1.0, a significant genotype interaction was identified in those with three elevated factors and the FSAP 511E genotype (heterozygotes and homozygotes combined), reaching HR 5.64 (CI 2.79-11.40; p=0.003), Table 5.

Haemostatic markers by genotype

Haemostatic parameters in those homozygous for the common allele, G511, were compared to those with GE and EE combined, Table 1. Only FIX peptide exhibited a borderline significant difference between the two groups, those with the E allele having 11% higher levels (p=0.05).

Interaction analysis to determine the effect of either one, two or three of the factors (cholesterol, triglyceride or fibrinogen) on levels of haemostatic markers revealed no significant interaction with FSAP 511E genotype and levels of FVIIc, FVIIag, FVIIa, FPA, F1.2, FIX or FX (p>0.26).

Discussion

Overall, the 511E allele did not associate with increased risk of MI. It was shown previously, in the Bruneck study cohort, that FSAP 511E did not relate to the development of early carotid atherosclerosis, but was found to be an independent risk for development of advanced atheroma (10). There are differences between the two study designs that may have led to the different findings in terms of an independent risk for atherosclerosis conferred by the 511E allele in the Bruneck, but not the current study. Firstly, the Bruneck study assessed carotid atherosclerosis and not heart disease. While some relationship exists between them, they are not synonymous pathologies. Secondly, in the Bruneck study, factors used in the logistic regression model to assess risk, were those previously shown to interfere with coagulation or fibrinolysis, while the factors tested in the current study were classical risk factors for CHD. The factors

used in the Bruneck study included fibrinogen but not cholesterol or triglyceride levels. It is possible that the individuals with advanced atheroma in the Bruneck study had self-selected for increased cholesterol and triglyceride levels. The current, prospective study of CHD would support the finding in the Bruneck study if the development of an initial atherosclerotic plaque is associated with high levels of cholesterol and triglyceride (and/or fibrinogen), and the progression of atheroma (leading to a clinical event, monitored in the current study) is exacerbated by FSAP 511E.

In these healthy NPHS men, the genotype alone was not sufficient to increase risk significantly, but in association with elevated cholesterol and triglyceride, a genotype -associated risk became apparent. Fibrinogen levels could be substituted for triglyceride to produce a similar effect upon risk in association with FSAP genotype. Individuals who had at least one 511E allele, and levels of cholesterol, triglyceride and fibrinogen levels above the mean/median, were at 5-fold increased risk of a CHD event compared to those with the common allele with none or one factor above their mean/median level. By comparison, individuals with the common allele and high cholesterol, triglyceride and fibrinogen levels were only 2-fold more at risk of a CHD event than those with levels below the mean/median.

FSAP has been associated with a number of activities, in both the coagulation and fibrinolytic pathways. Which of these, if any, is the prime function of the protein, remains to be determined. However, a previous finding of reduced fibrinolytic activity of FSAP 511E but normal ability to activate Factor VII, may be one of the mechanisms by which this variant contributes to atherosclerosis and its clinical sequelae. In the current study, no measurement of fibrinolytic activity had been made, but several markers of coagulation activation had been measured previously. FIX peptide and FX peptide are formed during activation of factors IX and X respectively. Prothrombin fragment F1.2 is cleaved from prothrombin as thrombin is generated, while FPA is cleaved from fibrinogen by the action of thrombin to form fibrin, and is a measure of thrombin activity. FVIIa is the activated form of FVII, an activation, at least in-vitro, that can be achieved by FSAP (3). While we would not have expected levels of FVIIc or FVIIag to differ by FSAP genotype, the enzyme, as its name suggests, was originally identified by its FVII activating activity. However, no effect of the FSAP G511E variant on FVII activating activity was determined previously (9). The current study suggests that 511E has no effect upon FVIIa levels, either considering genotype only or genotype in combination with increased cholesterol, triglyceride, or fibrinogen levels. However, apart from an effect of fatty meals (12) the determinants of plasma FVIIa levels are largely unknown. An independent effect of FSAP genotype on FVIIa levels may be difficult to isolate from other contributors. We could show no difference in either thrombin generation (F1.2) or activity (FPA), for FSAP G511E, either overall or in combi-

nation with increased cholesterol, triglyceride, or fibrinogen levels. FIXa levels were higher in those with FSAP 511E, but this finding was of borderline statistical significance and requires confirmation. Surprisingly, cholesterol levels were different by FSAP genotype, being significantly lower in those with the rare allele. This may have occurred by chance and requires confirmation in a second cohort.

While the pathophysiological mechanism involving the Marburg variant cannot be elucidated from the current studies, the findings suggest an exacerbation of atherosclerosis or clinical event in those already compromised by established cardiovascular risk factors. A recent study has shown that FSAP inhibits growth factor mediated cell proliferation and migration of vascular smooth muscle cells (5). Although the effect of the Marburg variant on this property of FSAP was not studied, the possibility is raised that pathophysiological mechanisms associated with the variant in atherosclerosis may lie outside the coagulation and fibrinolytic pathways.

There was a non-significant protective effect of the 511E allele (borderline significance in Cox regression analysis) in the absence of raised cholesterol, triglyceride or fibrinogen levels.

The reason for this is not clear and may have occurred by chance. It appears that when cholesterol, triglyceride (or fibrinogen) levels increase, risk associated with the 511E allele becomes apparent and when all three are raised a 5-fold elevation in risk ensues. This analysis highlights the importance of sub-group analysis in large cohorts, as factors contributing to heart disease may otherwise be overlooked. Furthermore, sub-group analysis may direct research into novel patho-physiological mechanisms. However, because of the post-hoc nature of the sub-group analysis carried out we cannot rule out the possibility that the statistical findings here result by chance. Further analysis of other large prospective studies is required to confirm these findings.

Acknowledgements

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