RESEARCH ARTICLE

Genotype–phenotype relationship of *F7* R353Q polymorphism and plasma factor VII coagulant activity in Asian Indian families predisposed to coronary artery disease

JAYASHREE SHANKER[1]*, GANAPATHY PERUMAL[1], ARINDAM MAITRA[1], VEENA S. RAO[2], B. K. NATESHA[2], SHIBU JOHN[3], SRIDHAR HEBBAGODI[3] and VIJAY V. KAKKAR[1,4]

[1]*Mary and Garry Weston Functional Genomics Unit,* [2]*Tata Proteomics and Coagulation Unit,*
[3]*Elizabeth and Emmanuel Kaye Bioinformatics and Statistics Unit, Thrombosis Research Institute, 258/A, Bommasandra Industrial Area, Bangalore 560 099, India*
[4]*Thrombosis Research Institute-London, Emmanuel Kaye Building, Manresa Road, Chelsea SW3 6LR, London*

Abstract

Elevated factor VII (FVII) level is a risk factor for coronary artery disease (CAD). We investigated the role of R353Q polymorphism in the *F7* gene in 139 Indian families with CAD, comprising of 222 affected subjects, 105 unaffected subjects and 126 affected sibling pairs. Plasma per cent FVIIc activity (FVII.c activity) differed significantly across R353Q genotype ($P < 0.0001$). Frequency of subjects with RR and QQ genotypes were higher in 4th quartile and 1st quartile of FVII.c activity, respectively ($P < 0.0001$). *F7* R353Q SNP was able to explain up to 7% of variation in FVII.c activity by regression analysis and an additive genetic component of variance of 28.04% by heritability analysis. Quantitative trait loci analysis showed suggestive linkage evidence of *F7* SNP with per cent FVII.c activity (LOD score −1.82; $P = 0.002$). Individuals with RR and RQ genotypes carried an OR of 2.071 (95% c.i. = 1.506–2.850) and 2.472 (95% c.i. = 1.679–3.641), respectively, towards CAD risk. There was significant correlation of FVII.c activity with lipid markers, particularly among those with RR and RQ genotype after covariate adjustment. In conclusion, the *F7* R353Q SNP appears to moderately influence plasma FVII.c activity and risk of CAD in Indians.

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Introduction

Factor VII (FVII.) is a key component in the extrinsic coagulation pathway (Rao and Rapaport 1988) Both genetic and environmental factors influence plasma FVII levels. Age, obesity and food habits, as well as use of oral contraceptives and onset of menopause among females are some of the commonly reported non-genetic factors (Balleisen *et al*. 1985; Meade *et al*. 1986; Scarabin *et al*. 1996). Levels of plasma FVII show inter-individual differences as well as variation within populations (Meade *et al*. 1977; Thompson *et al*. 1987).

Studies on large families have assisted in determining the genetic contribution towards variation in FVII levels. The non-synonymous R353Q single nucleotide polymorphism (SNP) in exon 8 in the *F7* gene has been shown to influence FVII levels (Green *et al*. 1991). The R353Q SNP, which involves the replacement of the amino acid arginine (R) by glutamine (Q) due to the substitution of guanine with adenine at the codon 353 position in the *F7* gene, was shown to account for over 20% of the variance in FVII levels (Green *et al*. 1991). Strong linkage disequilibrium (LD) has been observed between the R353Q SNP and a10-bp ins/del polymorphism (Liu *et al*. 2002). Molecular dissection of a 15-kb-genomic region around the *F7* gene was able to identify 49 SNPs, of which four to seven functional variants were

*For correspondence. E-mail: jayashreeshanker@triindia.org.in.

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shown to exert a regulatory control on circulating FVII levels (Soria *et al*. 2005). While there is consensus on the genotype specific effect of the *F7* gene variants on FVII levels, their role in CAD is debatable (Doggen *et al*. 1998; Feng *et al*. 2000; Batalla *et al*. 2001). The aim of the present study was to look at the association and linkage between the R353Q polymorphism in the *F7* gene and plasma factor VII. coagulant activity (FVII.c activity) and CAD status in Asian Indian families predisposed to CAD. In addition, the relationship of the F7 SNP with other atherothrombotic blood phenotypes was assessed.

Materials and methods

A total of 139 families consisting of 327 participants, 222 affected and 105 unaffected members, were recruited in the ongoing Indian Atherosclerosis Research Study (IARS), which investigates the molecular basis of atherothrombosis in Indian families with strong family history of CAD. Families were enrolled through the proband who had angiographically proven CAD and diagnosed as stable angina/unstable angina or had an episode of myocardial infarction based on cardiological diagnosis. The age at onset of CAD was $\leq$ 60 years for males and $\leq$ 65 years for females. There were 194 males (87.4%) and 28 females (12.6%) in the CAD affected group and 49 males (46.7%) and 56 females (53.3%) in the unaffected group, respectively.

Around 126 affected sibling pairs (ASPs) were selected from 87 IARS families for linkage analysis. All study participants provided fasting blood and urine samples as well as clinical and relevant personal information by informed, signed, voluntary consent. The institutional ethics committee, which follows the Indian Council of Medical Research guidelines on conduct of research on human subjects, approved the IARS protocol.

Laboratory investigations

Genomic DNA was extracted using the salting-out procedure (Miller *et al*. 1988). Genotyping of the *F7* R353Q SNP (rs6046) was performed according to the procedure reported by Green *et al*. (1991). In short, the genomic region encompassing the polymorphic site in exon 8 of the *F7* gene was PCR amplified, digested with *Msp*1 restriction enzyme and resolved on a 2% agarose gel. Genotyping was confirmed by bi-directional sequencing of the amplified 312-bp product in five samples using Big Dye Terminator 3.1 sequencing kit and 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA). Figure 1a depicts a gel picture of the three genotypes, RR, RQ and QQ, whereas figure 1b shows the sequence variation in a heterozygote.

Various atherothrombotic blood phenotypes were assayed using plasma samples. Coagulation parameters, namely FVII.c activity (expressed as per cent activity), fibrinogen (g/L) and prothrombin time (PT expressed in seconds) were analysed by clotting assays on the ACL 300 automated coagulation analyzer (IL Systems, Milano, Italy). Lipid markers (TC, TG, HDL-C (mg/dL), lp(a) (mg/L), Apo AI, ApoB (g/L)) and hsCRP (μg/dL) were estimated on a Clinical Chemistry analyzer (Hoffman La Roche, Switzerland) while PAI-1 (ng/mL) and IL6 (pg/mL) were assayed by ELISA method.

Linkage analysis

ASP analysis was used to test for linkage between CAD and the *F7* R353Q genotype (Blackwelder and Elston 1985). Appropriate input files were created using the MEGA2 program (Mukhopadhyay *et al*. 2005). Single-point-linkage analysis was performed using the SAGE version 5.3.1 package (Elston and Gray-McGuire 2004). Significant presence of linkage was assigned based on the criteria of Kruglyak *et al*. (1996). Mean proportion of allele sharing (*pi*), identity by descent (IBD) between the ASPs, was calculated using the GENIBD program in SAGE and was considered to be significant if the estimated value was greater than 0.50. Quantitative trait analysis (QTL) of the *F7* SNP and FVII.c activity was performed using MERLIN program (Abecasis *et al*. 2002). Age, gender and body mass index (BMI) were used as covariates.

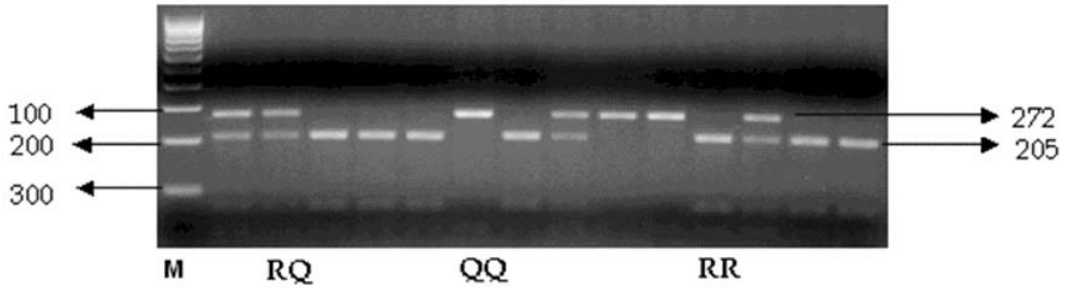

Figure 1a. FV11 R353Q genotypes resolved on 2% agarose gel. M, 100-bp DNA ladder; genotypes are resolved based on the presence or absence of *Msp*1 restriction enzyme cutting site. 'R' allele has two cutting sites and 'Q' allele has no cutting site. RR genotype (205 bp), RQ genotype (272 and 205 bp), QQ genotype (272 bp).

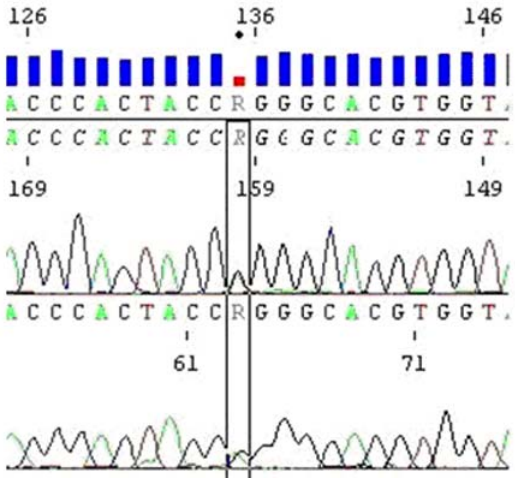

Figure 1b. Sequence of FVII R353Q region showing the RQ (AG) heterozygote at base position 135.

Association studies

The CAD affected participants (n = 222) and their unaffected siblings (n = 105) were included for the association studies. All quantitative traits were expressed as mean ± s.e. Conformity to Hardy–Weinberg equilibrium was determined by the χ^2 test. Student's t-test or one-way ANOVA was employed to calculate mean differences for the continuous variables, while Pearson's χ^2 test was used to estimate the differences in proportions for discrete variables including the R353Q genotypes. Estimation of correlation between FVII.c activity and other atherothrombotic phenotypes were performed by partial two-tailed correlation test after adjusting for age and gender. Variance component analysis was used to estimate the extent of genetic contribution of the *F7* SNP to the variance in per cent FVII.c activity. Multivariate analysis was performed for all quantitative variables across the R353Q genotypes and gender. Factors such as lipids, coagulation markers, inflammatory markers, PAI-1, BMI and waist: hip ratio were analysed with FVII.c activity in each of these groups, using age as covariate to understand the relationship of the various atherothrombotic variables to plasma FVII.c activity. Discriminant analysis was performed across the *F7* genotype for males and females separately. Both simple and multiple linear regression analyses were used to explain the contributory variables that influenced FV11 levels. The extent of association of *F7* SNP with CAD status was estimated using the logistic regression model with QQ genotype as the reference. The Q–Q plot was used to test for normality of distribution for the quantitative variables. Values with skewed distribution were log-transformed prior to analysis. The number of diseased coronary vessels, mean age at onset and event score (ischemic heart disease with symptoms of chest pain −1, stable angina −2 and unstable angina / MI −3) defined CAD severity scoring. A nominal P value of 0.05 or less was considered statistically significant. SPSS version 13 for Windows (SPSS, Chicago, USA) was used for the analyses.

Results

The distribution of the various characteristics and phenotypic markers in the CAD affected and unaffected subjects is provided in table 1. Mean values of most biomarkers; namely lipids and pro-coagulant factors, were significantly higher in the unaffected than the affected family members and this trend remained when separately tested for subjects who were not on statins (n = 172) (data not shown). Mean FVII.c activity were lower in CAD subjects (104.45 ± 1.59%) than the unaffected group (116.87 ± 2.3%) ($P < 0.001$). Frequency of diabetes, hypertension and smoking was higher among the affected (51.8%, 45.9%, 47.3%) than the unaffected subjects (19.0%, 25.7%, 18.1%) ($P < 0.0001$).

The mean age at onset of CAD was 50.28 (±0.65) years for males and 52.69 (±1.83) for the female cases. Two or more vessel disease was present in 78% of the CAD patients with over 64% suffering from unstable angina or myocardial infarction event (event score = 3). The proportion of affected subjects who were taking medication was significantly higher than the unaffected subjects: lipid-lowering drugs (statins: 65% vs 3%), anti-hypertensive agents (ACE inhibitor/beta blocker: 41%/30% vs 11%/13%) and hypoglycemic agents (38% vs 13%), respectively.

Linkage studies

There was no evidence of linkage between the R353Q SNP and CAD using 126 ASPs in the IARS cohort (LOD score 0.1376). There was no significant deviation in the proportion of mean allele sharing (pi = 0.51) between the ASPs. Suggestive evidence of linkage was obtained between the R353Q SNP and FVII.c activity (LOD score 1.82; P = 0.002) by QTL analysis.

F7 genotype–phenotype analysis

The *F7* R353Q SNP was in Hardy–Weinberg equilibrium ($P > 0.05$) in the whole data as well as in the affected and unaffected groups. The three genotypes were confirmed by sequencing of the region of interest in five samples. Allele and genotype frequencies showed similar distribution in the affected and unaffected subjects and across gender with a minor allele frequency of 0.28–0.29. The frequency of QQ genotype was higher in the unaffected subjects (12.4%; n = 13) as compared to the affected group (7.7%; n = 7). However, this difference was not statistically significant.

R353Q genotype and plasma FVII.c activity

There was significant difference in the mean plasma FVII.c levels across RR, RQ and QQ genotypes in both affected

Table 1. Distribution of clinical and biomarker data between CAD affected and unaffected family members (mean ± s.e.).

Characteristic	CAD	Non CAD	*P* value*
Number	222	105	–
Age, y	55.2 ± 0.6	49.4 ± 1.0	**< 0.001**
Sex, % male	80	20	**< 0.0001**
BMI, kg/m^2	25.71 ± 0.28	26.21 ± 0.39	0.31
WHR	0.95 ± 0.003	0.91 ± 0.005	**< 0.0001**
Diabetes, %	85	15	**< 0.0001**
Hypertension	79	21	**< 0.0001**
Smoking, % (males)	85	15	**< 0.0001**
Alcohol, %	18.1	15.2	0.32
SBP, mm Hg	127.9 ± 1.13	130.8 ± 1.7	0.16
DBP, mm Hg	82.7 ± 0.59	83.6 ± 0.91	0.37
TC (mg/dl)	151.63 ± 2.6	185.86 ± 3.9	**< 0.0001**
TG (mg/dl)	151.08 ± 4.6	171.1 ± 11.0	**0.049**
HDL-c (mg/dl)	34.5 ± 0.6	36.1 ± 0.8	0.084
LDL-c (mg/dl)	86.84 ± 2.3	117.7 ± 3.68	**< 0.0001**
ApoA1 (g/l)	1.12 ± 0.02	1.25 ± 0.03	**< 0.0001**
ApoB (g/l)	0.93 ± 0.02	1.18 ± 0.04	**< 0.0001**
Lp (a)	28.13 ± 2.13	21.33 ± 1.95	**0.047**
LVII.c activity, %	104.45 ± 1.59	116.87 ± 2.3	**< 0.0001**
PT, sec	12.5 ± 0.07	12.02 ± 0.13	**< 0.0001**
Fibrinogen	4.15 ± 0.08	4.23 ± 0.09	0.54
IL6	6.9 ± 1.6	4.8 ± 0.8	0.39
hsCRP	3.39 ± 0.35	4.12 ± 0.52	0.32
PAI-1	52.51 ± 2.81	63.98 ± 5.35	**0.04**

ApoA1, apolipoprotein A1; Apo B, apolipoprotein B; BMI, body mass index; FVII.c, factor VII coagulant activity; HDL-c, high density lipoprotein-cholesterol; Lp (a), lipoprotein (a), LDL, low density lipoprotein; PT, prothrombin time, WHR, waist–hip ratio.
*Figures in bold indicate significant *P* value.

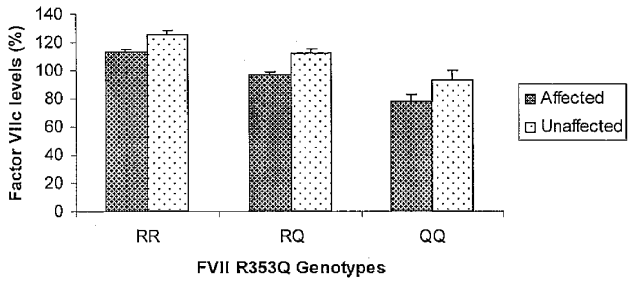

Figure 2. Mean FVII.c levels (%) across the R353Q genotypes among the CAD affected and unaffected subjects.

(113.53 ± 23.47%, 97.57 ± 18.16% and 78.47 ± 23.66%) and unaffected (125.38 ± 21.47%, 112.19 ± 18.80% and 93.15 ± 26.01%) subjects, respectively ($P < 0.0001$) (figure 2). Individuals with one or two R alleles showed an average of 20%–30% higher FVII.c activity when compared to those harbouring the Q allele. Due to the difference between the two homozygotes, RR and QQ, the additive genetic component was manually estimated to be 28.04% using variance component analysis.

Simple linear regression analysis showed that the *F7* R353Q genotype was able to independently explain up to 18.7% of variation in FVII.c activity ($P < 0.0001$). When the association of the *F7* SNP was analysed across the four quartiles of plasma factor VII activity in the whole dataset, over 36% of subjects with RR genotype belonged to the 4th quartile and only 10.4% were in the 1st quartile. Conversely, around 63.3% of individuals with QQ genotype were in the 1st quartile, while only 1.3% was in the 4th quartile (χ^2 with 6 $df = 66.63$; $P < 0.0001$) (table 2). This significant trend was retained when affected and unaffected subjects were analysed independently (data not shown).

R353Q genotype and CAD status

Individuals with RR genotype showed an OR of 2.07 (95% c.i. = 1.51–2.85) when tested against the QQ genotype for CAD risk while those with the RQ genotype showed an OR of 2.47 (95% c.i. = 1.68–3.64) employing logistic regression analysis (table 3). No significant association was observed between the R353Q genotypes and the mean age at onset, number of diseased vessels or event score.

Table 2. Distribution of *F7* R353Q genotypes across the FVII.c quartiles.

	Quartile distribution of FVII.c activity*			
	1	2	3	4
RR Genotype N (%)	18 (10.5)	42 (24.4)	50 (29.1)	**62 (36.0)**
RQ Genotype N (%)	45 (36.0)	37 (29.6)	29 (23.2)	14 (11.2)
QQ Genotype N (%)	**19 (63.3)**	5 (16.7)	1 (3.3)	

$\chi^2 = 66.63$ (6 d.f.); $P < 0.0001$. *The figures in bold indicate maximum frequency of individuals with RR genotype in the 4th quartile and minimum frequency of individuals with QQ genotype in the 1st quartile of FVII.c activity.

Table 3. Association of *F7* R353Q SNP with CAD affected status.

Genotype	*P* value*	Odds ratio (95% c.i.)
QQ genotype (reference genotype)	–	–
RR genotype	**< 0.0001**	2.07 (1.51–2.85)
RQ genotype	**< 0.0001**	2.47 (1.68–3.67)

*Figures in bold indicate significant *P* values.

Genotype–phenotype relationship of F7 R353Q genotype, plasma FVII.c activity and atherothrombotic phenotypes

Correlation estimation of FVII.c activity and other atherothrombotic biomarkers across the *F7*genotypes showed that there was significant correlation for some markers only in the group with RR genotype and to a certain extent, the RQ genotype, while there was no correlation with any of the biomarkers within the QQ genotype group (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/).

Gender showed significant influence on plasma FVII.c activity by multiple linear regression analysis with the *F7* SNP contributing to over 7% of variation. PT (45%), LDL-c (5%), TC (3%) and PAI-1 (2%) were the other significant contributors in this dataset. Multivariate analysis of variance showed a significant effect of gender and genotype on FVII.c activity. HDL-c, BMI and WHR, and PT (see table 2 in electronic supplementary material). There was significant association in the pattern of distribution of TC and LDL-c across the quartiles of FVII.c activity ($P < 0.01$) (see table 3 in electronic supplementary material). Discriminate analysis across the *F7* genotypes and gender showed that 80% of the variability was extracted by the first function ($P < 0.0001$) defined by FVII.c activity and PT with standardized discrimination coefficient values of $r = 0.691$ and 0.393, respectively.

Discussion

Polymorphisms within the *F7* gene undoubtedly contribute to plasma FVII.c activity, although there is considerable difference in the relative extent of their influence on the phenotype across populations. We have observed a high heritability (76%) and a low spouse pair correlation ($r = 0.038$) for plasma FVII.c activity in the IARS cohort (508 families and 2305 individuals) indicating the significant contribution of genetic factors towards FVII.c activity. The additive genetic variance of the *F7* SNP was around 28%, suggesting that this SNP may be an important variant that influences FVII.c activity.

The R353Q SNP accounts for over 20% of the variation in FVII activity among Caucasians (Green *et al*. 1991; Lane *et al*. 1996) and around 5%–11% among Asians (Saha *et al*. 1994) and this association is dependent on gender. While the *F7* R353Q SNP independently accounted for over 18% of the variability in the FVII.c activity in our cohort, inclusion of other correlating factors such as lipids and coagulation factors reduced the contribution of the *F7* SNP to 7%. This polymorphism was the strongest predictor of FVII antigen levels and explained up to 7.7% of the total variance in the Framingham study (Feng *et al*. 2000). A higher proportion of subjects with RR and QQ genotypes in the 4th and 1st quartiles of per cent FVII.c activity, respectively, provide evidence of influence of the R allele on plasma FVII.c activity. However, the magnitude of effect is low, given the fact that the overall contribution by this SNP is only 7% in the present study.

QTL analysis on ASPs showed suggestive evidence of linkage of the R353Q locus to FVII.c activity but not with CAD. This may be attributable to the small numbers of ASPs analysed in the study and/or due to the presence of other SNPs in close proximity to the R353Q locus that may impart a greater effect on FVII.c activity in circulation. This warrants further investigation.

We observed a minor allele (Q) frequency of 0.28, which is comparable to the prevailing frequency in the Caucasian population (Bernardi *et al*. 1997). The RR and the RQ genotype were significantly associated with CAD status in comparison to the QQ genotype based on the estimated odds ratio which indicates the relative influence of this *F7* SNP on CAD risk. Also, the frequency of the QQ genotype was higher in the unaffected group than the affected group, though the difference was not statistically significant. As the present comparison has been from a family-based cohort of related samples, it would be worthwhile to confirm these preliminary results, independently, on a larger study based on population bases, case–control analysis.

Genotype-specific association of plasma FVII levels with lipids, mainly triglycerides, has been reported among Caucasian in non-fasting states (Humphries *et al*. 1994; Lane *et al*. 1996) and among Gujarati–Indian population in fasting state (Saha *et al*. 1994) particularly in those with RR or RQ genotypes. We observed a similar trend wherein, following covariate adjustment, there was significant correlation of per cent FVII.c activity with lipids and other prothrombotic factors only among those with the RR and to some extent the RQ genotypes but not for the QQ genotype (see table 1 in electronic supplementary material).

Lipids and procoagulant factors including FVII.c were higher among the unaffected members and in the female cohort in our study. Several factors may have contributed to this observation. Firstly, our cohort has been selected from a family-based study where strong genetic relationship and shared environmental background between siblings could act as potential confounders. Females have an inherent tendency for higher biomarker levels than males; however, the incidence of CAD is kept in check among young women in their reproductive age, probably due to the protective influence of oestrogen against heart diseases that slowly diminishes in post-menopausal women (Scarabin *et al*. 1996). In the present study, out of total 222 CAD affected subjects, only 13% ($n = 28$) were females, whereas more than half of the 105 unaffected members constituted females (53%; $n = 56$). The mean BMI levels and other proatherogenic and coagulation markers were elevated in females than in males in our cohort. Whether this increase in biomarker levels can be attributed to the influence of the higher BMI levels observed in females is a matter that warrants further investigation but may to a certain extent explain the predominance of biomarker levels in the unaffected subjects who have a greater proportion of females in their midst.

Although, over 63% of the affected cohort was on lipid-lowering therapy as compared to 3% of the unaffected subjects, statins did not appear to influence the biomarker levels as evinced by the independent analysis conducted on those subjects who were not on lipid-lowering therapy.

Factor VII.c activity has been considered as a risk factor for coronary heart disease in the presence of smoking, fibrinogen and lipids in the PROCAM study (Junker *et al*.

1997) while the risk of MI that has been associated with the haplotypes in the *F7* gene through their intermediary influence on BMI, low HDL-c etc. (Reiner *et al*. 2007). Our present family-based study suggests a similar trend wherein high coagulant activity of FVII, in the presence of other atherothrombotic traits, particularly the lipids and traditional risk factors may exert a combined influence on CAD status with each factor contributing in a small way to enhance the overall risk of CAD in the Asian Indian population.

In conclusion, both association and linkage studies indicate the significant influence of *F7* R353Q genotype on plasma FVII.c activity while its association with CAD may be indirectly influenced by the presence of other intermediary risk phenotypes.

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