Published in final edited form as:

Thromb Res. 2012 August ; 130(2): 221–225. doi:10.1016/j.thromres.2011.08.029.

Relationships of plasma factor VIIa-antithrombin complexes to manifest and future cardiovascular disease

Angela Silveira^{1,*}, Daniela Scanavini¹, Susanna Boquist², Carl-Göran Ericsson³, Mai-Lis Hellénus⁴, Karin Leander⁵, Ulf de Faire^{4,5}, John Öhrvik¹, Barry Woodhams⁶, James H. Morrissey⁷, and Anders Hamsten¹

¹Cardiovascular Genetics Group, Atherosclerosis Research Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden

²Department of Cardiology, Karolinska University Hospital, Stockholm, Sweden

³Cardiology Unit, Department of Clinical Sciences, Danderyd Hospital, Stockholm, Sweden

⁴Cardiology Unit, Department of Medicine, Karolinska Institutet, Stockholm, Sweden

⁵Cardiovascular Epidemiology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

⁶Diagnostica Stago, Gennevilliers, France

⁷Department of Biochemistry, College of Medicine, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Abstract

Background—Low levels of free activated coagulation factor VII (VIIa) are normally present in plasma to prime the coagulation of blood in normal hemostasis and during thrombus formation. VIIa also circulates in inactive form, in complex with antithrombin (VIIaAT) formed when VIIa is bound to tissue factor (TF). This study evaluated VIIaAT in relation to cardiovascular disease (CVD).

Methods—We determined the plasma VIIaAT concentration in samples from the Stockholm Coronary Atherosclerosis Risk Factor (SCARF) study, a population-based case-control study of myocardial infarction (MI) and in samples from the Stockholm study of 60-years-old individuals, a prospective study of CVD. VIIaAT was measured with a sandwich ELISA that captures the complex between a monoclonal antibody to VIIa and a polyclonal antibody to AT.

Results—In the SCARF study (200 post-MI cases, 340 controls), VIIaAT was statistically significantly associated with patient status [odds ratio (95% confidence interval (CI)) 1.51 (1.09–2.08), $p=0.0126$]. The case-control differences were however small, with VIIaAT values that

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*Corresponding author: Angela Silveira, Centre for Molecular Medicine (L8:02), Karolinska University Hospital Solna ; S-171 76 - Stockholm Sweden; Tel +46 8 517 732 24; Fax +46 8 31 12 98; Angela.Silveira@ki.se.

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Part of this work was presented at the XXIst International Congress of the International Society of Thrombosis and Haemostasis, Genève, Switzerland. 2007.

Conflict of Interest

B.W. is a full time employee of Diagnostica Stago; J.H.M. receives patent royalties on assay technologies used in this study.

largely overlap between the two groups. When a nested case-control design (211 incident CVD cases and 633 matched controls) was applied on 5- to 7-year follow-up results of the Stockholm prospective study of 60-year-olds, plasma VIIaAT concentration was not associated with incident CVD (odds ratio (95% CI) 1.001 (0.997–1.005), $p=0.5447$).

Conclusions—Plasma VIIaAT concentration had no predictive value for future CVD in our study population. Slightly increased plasma VIIaAT concentrations observed after MI may reflect processes that occur in connection with the acute event when TF and VIIa availability is increased.

Keywords

VIIaAT complexes; activated factor VII; factor VII; antithrombin; cardiovascular disease

Low levels of activated factor VII (VIIa) are normally present in human plasma and serve to prime the blood coagulation process (1), which is triggered when circulating VIIa forms a complex with tissue factor (TF) that is exposed to blood upon perturbation of the endothelium. The VIIa-TF complex converts the zymogen forms of factors X and IX into active proteases, thus initiating the reactions leading to thrombin generation, fibrin formation and fibrin deposition at the site of endothelial injury (2). Abundantly expressed in the core of atherosclerotic plaques, TF is also exposed to blood and to circulating VIIa upon physical disruption of the plaque (3,4). The subsequent formation of the VIIa-TF complex initiates formation of the fibrin network and the thrombus, the size of which might be partially controlled by the amount of TF exposed and the amount of VIIa (and other coagulation factors) available in the surroundings (4). Thus, VIIa is involved in initiating blood clotting in normal hemostasis as well as in thrombosis.

The amount of free active VIIa in plasma is small, less than 1% of the total factor VII mass, with a mean (range) of 3.6 ng/ml (0.5–8.4 ng/ml) in normal adults (1). VIIa also circulates in plasma in complex with antithrombin (VIIaAT), this complex containing up to 3% of the total factor VII mass (5). Regarding the complex formation, *in vitro* studies have demonstrated that factor VIIa alone reacts very poorly with AT, presence of TF and heparin being required for optimal binding (6–8). Once the VIIa-TF complex is formed, the binding and transfer of VIIa to AT is facilitated and VIIa activity is inhibited. This process suggests that a relationship may exist between the plasma VIIaAT concentration and the degree of intravascular exposure of TF, which might have clinical relevance. To address this question, Spiezia et al have recently explored the plasma VIIaAT concentration in relation to arterial and venous thrombosis (9). They observed that individuals with a previous thrombotic event tended to have higher plasma VIIa-AT levels than patients with either acute arterial or venous thrombosis, or healthy controls, and VII/VIIa was found to be the main determinant of VIIaAT (9).

In the present study, we extend the observations of Spiezia et al by exploring the potential usefulness of plasma VIIaAT concentration to discriminate between patients and controls in a larger study of myocardial infarction (MI). In addition, we studied whether VIIaAT concentration has any value to predict future events in a prospective study of incident cardiovascular disease (CVD), using subjects from the same population.

Subjects and Methods

Subjects

For the study of VIIaAT after MI, the Stockholm Coronary Atherosclerosis Risk Factor (SCARF) database and biobank were used (10,11). VIIaAT analyses were performed on a total of 200 survivors of a first MI before the age of 60 years and 340 age- and sex-matched

control subjects who were not treated with lipid lowering drugs or anticoagulants at the time of blood sampling and from whom citrate plasma samples were available. The original cohort, with participants recruited during 1996–2000, comprised a total of 387 consecutive, unselected survivors of a first MI aged less than 60 years and age- and sex-matched controls recruited in parallel from the general population of the same catchment area. Recruitment strategies, inclusion/exclusion criteria and protocol features have been published (10,11).

To assess the predictive value of VIIaAT in relation to future CVD events, a nested case-control design was applied to a total of 4232 subjects, recruited through a health screening of the population of the County of Stockholm, who participated in a follow-up study of incident CVD events. Between July 1997 and June 1998, every third man and woman reaching the age of 60 years and living in the area were invited to participate (response rate 78%). Information on demographic details, life-style, and medical history was obtained by a self-administered questionnaire. Physical examination was performed and blood samples collected. All details of the screening procedure have been published (12). Incident cases of first CVD were recorded by regular examinations of the national cause of death registry (fatal events until December 31, 2003) and the national in-hospital registry (non-fatal events until December 31, 2005). Through these surveys, a total of 211 incident cases of CVD were recorded (77 MI, 85 angina pectoris and 49 ischemic strokes). Care was taken to enroll subjects without a history of CVD prior to recruitment to guarantee registration of first CVD events. For each incident case, three controls were randomly selected amongst the screenees who remained free of CVD, matched for follow-up time ($\pm$ 60 days) and sex (13).

The studies were approved by the Ethics Committee of the Karolinska Institutet, and all participants gave their informed consent.

Blood sampling

The patients who participated in the SCARF study were sampled 3–6 months after the acute event, in parallel with sampling of the controls. Participants of the prospective study of 60-year-old individuals were sampled at inclusion. Venous blood was drawn into plastic tubes containing sodium citrate for analysis of hemostatic factors, and into tubes containing potassium EDTA for analysis of inflammatory markers, lipids and lipoproteins and for DNA preparation, and plasma was prepared (10–12), aliquoted and stored at -80°C . Samples for determination of VIIaAT, VIIa, factor VII antigen (VIIag) and antithrombin (AT) were not thawed until used for the present report.

Assays

VIIaAT concentration was determined in citrate plasma samples by a sandwich ELISA that captures the complex between a monoclonal antibody to VIIa (coating) and a polyclonal antibody to AT (probing). In the study of 60-year old individuals, kits and protocols supplied by STAGO R&D (Gennevillieres Cedex, France) were used. The STAGO assay represents an optimized version of the in-house assay used for the samples of the SCARF study. The plasma VIIa concentration was measured in an ACL-9000 coagulometer (Instrumentation Laboratory Spa, Milan, Italy) by the clotting assay using soluble recombinant truncated tissue factor (sTF), as described (1), with samples diluted in factor VII deficient plasma (Instrumentation Laboratory) and activated with rabbit brain cephalin (Haemachem Inc., St. Louis, MO, USA), before the addition of calcium and sTF. The First International Standard VIIa Concentrate (National Institute for Biological Standards and Control, Hertfordshire, UK) was used as calibrator. Total factor VII in plasma was determined as VIIag, in a sandwich ELISA with antibodies from Affinity Biologicals Inc. (Ancaster, Canada). To render direct comparisons between the factor VII-related variables (VIIaAT, VIIa and VIIag) easier, all results are expressed in pmol/l. Fibrinogen and AT

were measured by assays from Instrumentation Laboratory Co and C-reactive protein (CRP, high sensitivity assay) by particle-enhanced immunonephelometry in the BN system, Dade Behring (Liederbach, Germany). Cholesterol and triglycerides were determined with kits from ABX Diagnostics (Montpellier, France) and Roche Diagnostics (Indianapolis, IN, USA), respectively, in the SCARF study, and with kits from Bayer Diagnostics (Tarrytown, NY, USA) in the study of the 60-year olds.

Each case sample was analyzed together with the sample of the matched control(s). For analyses performed in duplicate, samples with intra-assay coefficient of variation (CV) higher than 10% were re-analyzed. Coagulation Reference Plasma (Baxter AG, Wien, Austria) and NKP 160 (Global Hemostasis Institute, Linköping, Sweden) were used for determination of the following inter-assay CVs: 14% for VIIaAT using the in-house assay (n=22), 10% for VIIaAT using the STAGO kit (n=22), 10% for VIIa (n=20), 9% for VIIag (n=20), 4% for AT (n=40) and 4% for fibrinogen (n=24).

Genotyping for the factor VII gene (*F7*) R353Q polymorphism (rs6046) was performed in DNA samples of the SCARF participants by restriction fragment length polymorphism method, as described (14).

Statistical analysis

Continuous variables are summarized as mean±SD or median (interquartile range). Variables which presented skewed distribution were logarithmically transformed before any analyses. Group comparisons were performed by two-sample t-test if the variables were approximately normally distributed, otherwise the Wilcoxon-Mann-Whitney rank sum test was used, or by analysis of variance (ANOVA) when more than two groups were compared. Categorical variables are reported as numbers and percentage of group, and between-group comparisons were performed by Fisher's exact test or a chi-square test. Associations of VIIaAT to other biochemical variables were assessed by computation of Spearman rank correlation coefficients. Determinants of VIIaAT were identified by multiple stepwise regression analysis. Partial correlation coefficients were calculated between biochemical or clinical variables and VIIaAT, and the variable with highest partial correlation was entered at each step until no variable remained with an F-to-enter of 4 or more ($p < 0.05$). Multiple stepwise logistic regression analysis was used to determine the set of independent variables discriminating between post-infarction patients and controls. For the nested case-control data, matched logistic regression was used to assess the crude and adjusted prospective associations between VIIaAT and CVD.

Results

Study groups

Overall, the cardiovascular risk factor burden was higher in cases than in controls included in both study groups (Table 1), as reported for the complete SCARF cohort (10,11) and already published for the 60-year old individuals (12,13).

VIIaAT and MI/CVD

In the SCARF study, the plasma VIIaAT concentration was statistically significantly higher in post-infarction patients than in controls, although the differences were small (Table 2) with VIIaAT values that largely overlap between the two groups (Fig. 1). No significant differences were observed for VIIa and VIIag, although patients tended to have lower VIIag. Consequently, compared with the controls, post-infarction cases showed a significantly higher proportion of VIIaAT complex relative to the total VII mass (VIIaAT/VIIag,%), as well as higher proportion of total VIIa [(VIIa+VIIaAT)/VIIag, %], a difference that was not

seen for the proportion of free VIIa (VIIa/VIIag, %). In univariable logistic regression analysis VIIaAT emerged as the only factor VII-related measure significantly ($p=0.0126$) associated with post-infarction patient status [odds ratio (95% confidence interval (CI)) 1.51 (1.09–2.08)]. In stepwise multiple logistic regression analysis, including variables that significantly differed between cases and controls in univariable analysis, VIIaAT appeared as being independently associated with MI along with HDL cholesterol, AT, LDL triglycerides, smoking habits, CRP and alcohol intake.

Correlation coefficients were calculated separately in post-infarction patients and controls of the SCARF study between VIIaAT and hemostatic, metabolic, anthropometric and clinical variables. A strong, positive and consistent relationship was observed only with VIIa. Using multiple linear regression analysis, including values of VIIag, AT, age, body-mass index (BMI), triglycerides, LDL cholesterol and glucose for all subjects, plasma VIIa concentration was found to be the strongest predictor of plasma VIIaAT concentration, accounting for about 10% of its variation, whereas glucose and BMI each added about 1.5% to the variation in VIIaAT. The *F7R353Q* polymorphism (rs6046), which strongly influenced the plasma VIIa concentration in the SCARF controls (mean $\pm$ SD: 96 ± 39 , 66 ± 36 and 40 pmol/l in RR ($n=290$), RQ ($n=45$) and QQ ($n=1$), respectively), showed accordingly a tendency to influence VIIaAT (159 ± 91 , 144 ± 70 and 56 pmol/l, results for comparisons between carriers vs non-carriers of the minor Q allele in Table 2).

In the study of 60-year-old individuals, plasma concentrations of VIIaAT and VIIag were similar in the baseline samples of the subjects who later suffered a CVD event (cases) compared with samples from the subjects who remained free of disease (controls) (Table 2). Of note, no significant differences were observed for VIIaAT among cases who attained different CVD end-points (MI, angina or stroke, $p=0.7134$, ANOVA) in the study of the 60-year-olds. The predictive capacity of plasma VIIaAT concentration for future occurrence of CVD was further assessed by the best subset logistic regression analysis, forcing VIIaAT into the calculations. In the final model, plasma VIIaAT concentration neither contributed to the risk of future CVD by itself [odds ratio (95% CI) 1.001 (0.997–1.005), $p=0.5447$], nor after adjustment (1.000 (0.996–1.004), $p=0.886$) for the confounding variables (apolipoprotein B, CRP, diabetes (fasting plasma glucose ≥ 7.0 and/or use of anti-diabetes medication) and current smoking, which, all proved to be significant predictors of CVD).

Discussion

This study shows that complexes between VIIa and AT were slightly more abundant in plasma from patients who have suffered a MI compared with matched healthy individuals. Depending on the slightly lower VIIag in the patients, the calculated VIIaAT/VIIag ratio (or proportion of total factor VII mass represented by the complex) was a better patient-control discriminator. Although these case-control differences may be considered subtle, they constitute novel observations, which extend the findings of Spezia et al (9) associating elevated plasma VIIaAT with previous occurrence of arterial thrombosis in a smaller group of 45 CVD cases, of whom 26 had suffered MI, since the present results were generated in a different experimental setting and comprised a total of 200 post-infarction patients and 340 controls. The question then arose of whether determination of VIIaAT in individuals free of symptoms and signs of CVD can predict future cardiovascular events. On advancing the study of VIIaAT to a prospective setting (with 211 incident CVD cases and 633 controls), we found, however, that the plasma VIIaAT concentration in samples taken at baseline did not differ between subjects who in the course of 5–7 years suffered a first CVD event (MI, angina or stroke) and subjects who remained free of disease during follow-up. Overall, these findings suggest that an increase in VIIaAT seems to occur in connection with MI and that

VIIaAT does not reflect processes that later result in CVD. Whether the rise in VIIaAT precedes or parallels the acute event cannot be elucidated from this study.

Early *in vitro* observations on the mechanisms of VIIaAT formation provide some points for consideration in relation to the present results. VIIa alone reacts poorly with AT; TF and heparin are necessary for efficient binding (6–8). Once the VIIa-TF complex is formed, the binding and transfer of VIIa to AT is facilitated and VIIa activity inhibited. These mechanisms elucidated *in vitro* are in line with the finding that VIIaAT is elevated in plasma of patients with a previous thrombotic event (Spiezia et al (9) and the present study), because the presence of TF protein and TF synthesizing cells have been demonstrated in atherosclerotic plaques, protein expression and cell number being higher in specimens from patients with previous MI and unstable angina as compared to specimens obtained from patients with stable angina (15,16). Also, elevated levels of circulating TF have been reported in patients who have presented with acute coronary syndrome (17) and in patients with unstable angina (18). In the event of plaque rupture or erosion, TF contained within plaques or underneath their endothelial coverage is exposed and binds to circulating VIIa, initiating the process which in its initial phase leads to bulk conversion of VII to VIIa. These conditions would provide the increased availability of TF and VIIa for VIIaAT formation. In this respect, lack of data regarding plasma concentration of TF in the present study is a limitation that precludes us to confirm this association. However, we observed that plasma VIIa in the range found in the post-infarction patients was, indeed, the strongest determinant of the plasma VIIaAT concentration, and none of the biochemical and anthropometric variables tested significantly contributed to the variation of VIIaAT plasma levels.

After VIIaAT formation, intravascular TF is left exposed and this situation may have adverse clinical implications, as pointed out by Spiezia et al (9) and as indicated in a study of MI (19), in which the plasma concentrations of TF and factor VII (VIIag) at admission were reported to be independent predictors of mortality and reinfarction, the highest risk being observed in patients with simultaneous elevation of both TF and factor VII. It should therefore be interesting to study VIIaAT as a marker of such states, because here we cannot conclude whether the subtle differences observed between VIIaAT plasma concentrations in post-infarction patients and controls of the SCARF study can be directly translated to a clinically relevant risk of recurrences.

VIIaAT complex formation (in the presence of TF) results in inactivation of VIIa, which no longer can be measurable in a clotting assay. That could explain why VIIa results obtained in the present and other studies (20–22) were not significantly different between patients with manifest coronary artery disease and controls. In particular, increased thrombin generation and activity have been observed during the acute and chronic phases of MI and unstable angina, unaccompanied, however, by any detectable change in plasma VIIa concentration (22).

As judged from the evidence obtained in the present study, plasma VIIaAT concentration is not a useful biomarker for risk of future CVD, but further clinical studies need to be conducted, focusing for example on assessment of the prognostic capacity in individuals presenting with acute coronary syndrome. Clinical studies also need to be complemented by studies in animal models of atherosclerosis, vascular thrombosis and MI. Furthermore, important roles of VIIaAT complex formation remain to be explored. Firstly, AT present in large excess (3–5 μ M) in relation to VIIa (less than 0.5 nM) should be considered as an important regulator/inactivator of VIIa, when the latter is bound to TF and possesses full catalytic capacity. Secondly, VIIaAT should constitute a global marker of factor VII turnover on TF exposure, because the sum of VIIaAT+VIIa encompasses total VIIa (the active and inactive forms of VIIa) generated *in vivo*. Finally, evidence is emerging for

VIIaAT formation as an important mechanism for the elimination of factor VII(VIIa) from the circulation (23).

Acknowledgments

Funding

This study was supported by grants from the Swedish Research Council (8691), the Swedish Heart-Lung Foundation, the Knut and Alice Wallenberg Foundation, the Foundation for Strategic Research, Fondation Leducq, the European Commission (LSHM- CT- 2007- 037273), the Torsten and Ragnar Söderberg Foundation, the Strategic Cardiovascular Programme of KI, Stockholm County Council (560283), the Foundation for Old Servants and NIH grant HL47014.

Abbreviations

VIIa	activated coagulation factor VII
AT	antithrombin
VIIaAT	VIIa in complex with AT
TF	tissue factor
SCARF	Stockholm Coronary Atherosclerosis Risk Factor
MI	myocardial infarction
CVD	cardiovascular disease
ELISA	enzyme immune sorbent assay
VIIag	factor VII antigen
sTF	soluble recombinant truncated TF
F7	Factor VII gene
R	Arginine
R	Glutamine
ANOVA	Analysis of variance
BMI	body mass index
BP	blood pressure
LDL	low density lipoprotein
HDL	high density lipoprotein
CRP	C-reactive protein
PAI-1	plasminogen activator inhibitor-1

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Fig. 1. Plasma VIIaAT concentration in post-infarction cases (N=200) and controls (N=340) enrolled in the SCARF study.

Table 1

Characteristics of participants

	SCARF			Stockholm study of 60-year-old individuals		
	Post-infarction cases	Controls	P-value	Incident CVD cases	Controls	P-value
N	200	340		211	633	
Sex M/F, n (% of Females)	160/40 (20%)	279/61 (18%)		140/71 (33.6%)	420/213 (33.6%)	
Age, years	52.5±5.4	52.8±5.0		60	60	
BMI, kg/m ²	27.3±3.9	25.7±3.1	<0.0001	27.7±4.6	26.7±3.8	0.0031
Alcohol intake, g/week	57 (15–118)	90 (45–156)	0.0016			
Smoking habits, n (%)			0.0023			0.0050
present smoker	106 (53.0)	129 (38.2)		67 (32.2)	122 (19.7)	
former smoker	40 (20.0)	75 (22.3)		77 (37.0)	244 (39.3)	
never smoked	54 (27.0)	133 (39.5)		64 (30.8)	255 (41.0)	
Systolic BP, mmHg	133±20	129±17	0.0250	148±22	139±21	<0.0001
Diastolic BP, mmHg	81±10	82±10	0.7718	89±11	85±10	<0.0001
Total cholesterol, mmol/l	5.4±1.0	5.4±1.0	0.5563	6.1±1.0	6.0±1.2	0.1366
LDL cholesterol, mmol/l	3.5±0.9	3.5±1.0	0.6725	3.9±1.2	3.8±1.1	0.4490
HDL cholesterol, mmol/l	1.0±0.3	1.4±0.4	<0.0001	1.3±0.4	1.4±0.4	0.0005
Triglycerides, mmol/l	1.6 (1.2–2.2)	1.2 (0.8–1.6)	<0.0001	1.4 (1.0–1.9)	1.1 (0.8–1.6)	0.0005
Glucose, mmol/l	5.4 (5.0–5.9)	4.8 (4.6–5.2)	<0.0001	5.4 (4.9–6.0)	5.3 (4.9–5.7)	0.0004
CRP, mg/l	1.4 (0.8–3.4)	1.0 (0.5–1.8)	<0.0001	2.4 (1.3–4.7)	1.70 (0.9–3.2)	<0.0001
Fibrinogen, g/l	3.8±1.0	3.6±0.8	0.0087	3.2±0.9	2.9±0.8	0.0015
PAI-1, IU/ml	12.4 (4.5–23.1)	7.4 (3.1–17.3)	0.0004	6.6 (2.8–16.3)	4.8 (1.5–14.4)	0.0043
AT, % Standard	104 (97–113)	111 (105–120)	<0.0001	ND	ND	
Diabetes, n (%)	24 (12.0)	0 (0)	<0.0001	32 (15.2)	50 (7.9)	0.0020

Diabetes, fasting plasma glucose 7.0 mmol/l or use of anti-diabetes medication; ND, not determined. Values are number of individuals in the group (%), mean±SD or median (interquartile range); P values are from unpaired t test (continuous variables) or chi-square test (categorical variables).

Table 2

Factor VII variables

According to study groups		Stockholm study of 60-year-old individuals			
SCARF		Post-infarction cases	Controls	P-value	P-value
N	200	145 (104–209)	137 (102–190)	0.0129	0.7818
VIIaAT, pmol/l	79 (67–97)	80 (62–106)	ND	0.8956	-
VIIa, pmol/l	11 900 (10 200–13 810)	12 160 (10 700–14 050)	9 190 (7 870–10 110)	0.0870	0.8132
VIIaAT/VIIa, %	1.15 (0.82–2.03)	1.12 (0.82–1.55)	1.48 (1.26–1.77)	0.0027	0.8813
VIIa/VIIa, %	0.684 (0.558–0.816)	0.650 (0.513–0.835)	-	0.3520	-
(VIIa+VIIaAT)/VIIa, %	2.55 (1.83–3.90)	2.4 0 (1.83–3.10)	-	0.0023	-
According to F7 R353Q (rs6046) genotypes (in SCARF Controls)					
Non carriers (RR)		Carriers (RQ+QQ)	P-value		
N	290	46			
VIIaAT, pmol/l	138 (102–191)	131 (83–181)	0.0843		
VIIa, pmol/l	86 (67–110)	55 (47–65)	<0.0001		

ND, not determined.

Values are median (interquartile range) and P-values are from unpaired t test of logarithmically transformed variables.