Journal of Thrombosis and Haemostasis, **14**: 655–666 DOI: 10.1111/jth.13274

ORIGINAL ARTICLE

Activated factor VII–antithrombin complex predicts mortality in patients with stable coronary artery disease: a cohort study

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To cite this article: Martinelli N, Girelli D, Baroni M, Guarini P, Sandri M, Lunghi B, Tosi F, Branchini A , Sartori F, Woodhams B, Bernardi F, Olivieri O. Activated factor VII–antithrombin complex predicts mortality in patients with stable coronary artery disease: a cohort study. *J Thromb Haemost* 2016; **14**: 655–66.

Essentials

- Activated factor VII–antithrombin complex (FVIIa-AT) in plasma may reflect tissue factor exposure.
- FVIIa-AT levels were assessed in an angiographically controlled coronary artery disease (CAD) cohort.
- High FVIIa-AT levels correlated with an increased thrombin generation.
- High FVIIa-AT levels were associated with a greater risk of mortality in patients with stable CAD.

Summary. *Background:* Plasma concentration of activated factor VII (FVIIa)–antithrombin (AT) complex has been proposed as an indicator of intravascular exposure of tissue factor. *Objectives:* The aims of this observational study were to evaluate (i) FVIIa-AT plasma concentration in subjects with or without coronary artery disease (CAD) and (ii) its association with mortality in a prospective cohort of patients with CAD. *Methods:* FVIIa-AT levels were measured by ELISA in 686 subjects with (n = 546) or without (n = 140) angiographically proven CAD. Subjects with acute coronary syndromes and those taking anticoagulant drugs at the time of enrollment were excluded. CAD patients were followed for total and cardiovascular mortality. *Results:* There was no difference in FVIIa-AT levels between CAD (84.8 with 95% confidence interval [CI] 80.6–88.2 pmol L^{-1}) and CAD-free subjects (83.9 with 95% CI 76.7–92.8 pmol L^{-1}). Within the CAD population, during a 64-month median follow-up, patients with FVIIa-AT levels higher than the median value at baseline (≥ 79 pmol L^{-1}) had a two-fold greater risk of both total and cardiovascular mortality. Results were confirmed after adjustment for sex, age, the other predictors of mortality (hazard ratio for total mortality: 2.05 with 95% CI 1.22–3.45, hazard ratio for cardiovascular mortality 1.94 with 95% CI 1.01–3.73, with a slight improvement of C-statistic over traditional risk factors), FVIIa levels, drug therapy at discharge, and even patients using all the usual medications for CAD treatment. High FVIIa-AT levels also correlated with increased thrombin generation. *Conclusions:* This preliminary study suggests that plasma concentration of FVIIa-AT is a thrombophilic marker of total and cardiovascular mortality risk in patients with clinically stable CAD.

Keywords: coronary artery disease; hypercoagulability; laboratory marker; secondary prevention; tissue factor.

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Received 10 August 2015
Manuscript handled by: I. Pabinger
Final decision: F. R. Rosendaal, 12 January 2016

Introduction

Despite significant therapeutic advances, coronary artery disease (CAD) remains one of the leading causes of death and morbidity worldwide [1,2]. Coagulation plays a crucial role in CAD development and complications (e.g. myocardial infarction [MI]) [3]. Transmembrane protein tissue factor (TF) is one of the key initiators of the coagulation cascade through the binding and activation of coagulation factor VII (FVII). TF is constitutively expressed by vascular smooth muscle cells and fibroblasts leading to rapid initiation of coagulation when a blood vessel is damaged [4]. Endothelial cells at rest do not express TF, but they can be induced to TF expression by several triggers, including inflammatory cytokines, oxidized lipoprotein, and thrombin [5,6]. Similarly, monocytes and macrophages show very little or no basal expression of TF, but it can be induced by inflammatory stimuli [5]. Although controversial, there is

some indication that TF can be transferred to platelets or that they can express TF from residual TF mRNA [7,8]. TF exists on the membrane surface both in an inactive (encrypted) and an active (decrypted) form, mostly in the inactive form. The mechanism of TF activation still remains incompletely understood [9]. TF in the active form can be detected in plasma associated with microparticles [10,11]. Antigenic assays measure all forms of TF without giving functional information, while basal TF activity, due to its very low levels in plasma, is hardly measurable. It has been reported that circulating levels of active TF do not exceed 20 fmol L^{-1} in plasma, with higher concentrations likely being incompatible with life [12]. Nonetheless, due to the crucial role of full-length TF in thrombosis on the disruption of atherosclerotic plaque [4,13], the direct or surrogate measurement of active TF could be clinically relevant.

Contextually, until now the plasma levels of activated FVII (FVIIa), the active enzyme initiating the coagulation cascade when bound to TF, have been associated with CAD only with controversial results [14–18].

The serine protease inhibitor antithrombin (AT), along with tissue factor pathway inhibitor (TFPI), acts as an inhibitor of the FVIIa–TF pathway. Both AT and TFPI form stable complexes with TF-bound FVIIa, but only the FVIIa-AT complex is released and accumulates in the plasma, which offers an opportunity for an affordable measure (Fig. S1). TF expression contributes to FVIIa-AT complex formation in a mouse model [19], and plasma levels of FVIIa-AT have been reported to directly correlate with TF mRNA expression [20,21]. The assay of this complex could indirectly reflect TF–FVIIa interaction and TF exposure. Thus, the plasma concentration of FVIIa-AT has been proposed as a potential biomarker of a prothrombotic diathesis. [22,23].

The clinical significance of plasma concentrations of FVIIa-AT in patients with CAD is unknown. The aims of the present study were (i) to evaluate FVIIa-AT in subjects with or without angiographically demonstrated CAD; (ii) to assess correlations with several clinical and laboratory parameters, including thrombin generation assay; and (iii) to evaluate plasma levels of FVIIa-AT as a risk predictor of total and cardiovascular mortality in a cohort of CAD patients.

Materials and methods

Study population

This observational study was performed within the framework of the Verona Heart Study (VHS), a regional survey that assessed new CAD risk factors in subjects with angiographic documentation of the state of their coronary vessels [24,25]. The study design is summarized in Fig. 1. All the subjects in the VHS are required to have no history of any acute illness in the month preceding the enrollment. CAD patients with acute coronary syndromes were excluded from this study. Subjects with severe renal failure (estimated glomerular filtration rate [GFR] < 30 mL min^{-1}) and those with severe hepatic impairment (clinically defined diagnosis of liver cirrhosis) were also excluded from this study. A total of 686 adult subjects of both sexes who were not taking any anticoagulant drugs and for whom frozen citrate plasma samples for FVIIa-AT assay were available, were included in the present study. One hundred forty subjects with completely normal coronary arteries, undergoing coronary angiography for reasons other than CAD (mainly valvular heart disease), were used as controls (CAD-free group). These subjects had no history or clinical or instrumental evidence of atherosclerosis outside the coronary bed, and none were taking any antiplatelet drugs. Five hundred forty-six subjects had angiographically-proven CAD (CAD group), with at least one of the major epicardial coronary arteries (left anterior descending coronary, circumflex, and right) affected with one or more significant stenoses ($\geq$ 50% lumen reduction). All the CAD patients were newly diagnosed at time of enrollment (i.e. at the time of coronary angiography) and were included as incident cases. The angiograms were assessed in a blinded manner by two cardiologists who were unaware that the patients were to be included in this study.

All participants came from the same geographical area of northern Italy. At the time of blood sampling, a complete clinical history was collected, as well as data about drug therapies. The study complies with the Declaration of Helsinki and was approved by the ethics committee of our institution (Azienda Ospedaliera Universitaria Integrata, Verona). A written informed consent was obtained from all the participants.

Assessment of outcome in follow-up analysis

Subjects were followed until death or until June 30, 2012. The mortality status at June 30, 2012, was incomplete for patients who changed their domicile during the follow-up ($n = 36$). Study subjects' status was determined by searching in the National Population Register and by an ambulatory or telephone survey. Certification and date of death were obtained from the National Population Register. The causes of death were obtained from death certificates kept at the Italian Institute of Statistics. Death from cardiovascular causes was defined as death caused by ischemic heart disease, heart failure, peripheral vascular disease, or cerebrovascular disease.

Biochemical analysis and thrombin generation assay

Samples of venous blood were drawn from each subject, after an overnight fast, at the time of enrollment before coronary angiography. Serum lipids, as well as other CAD risk factors, including high-sensitivity C-reactive

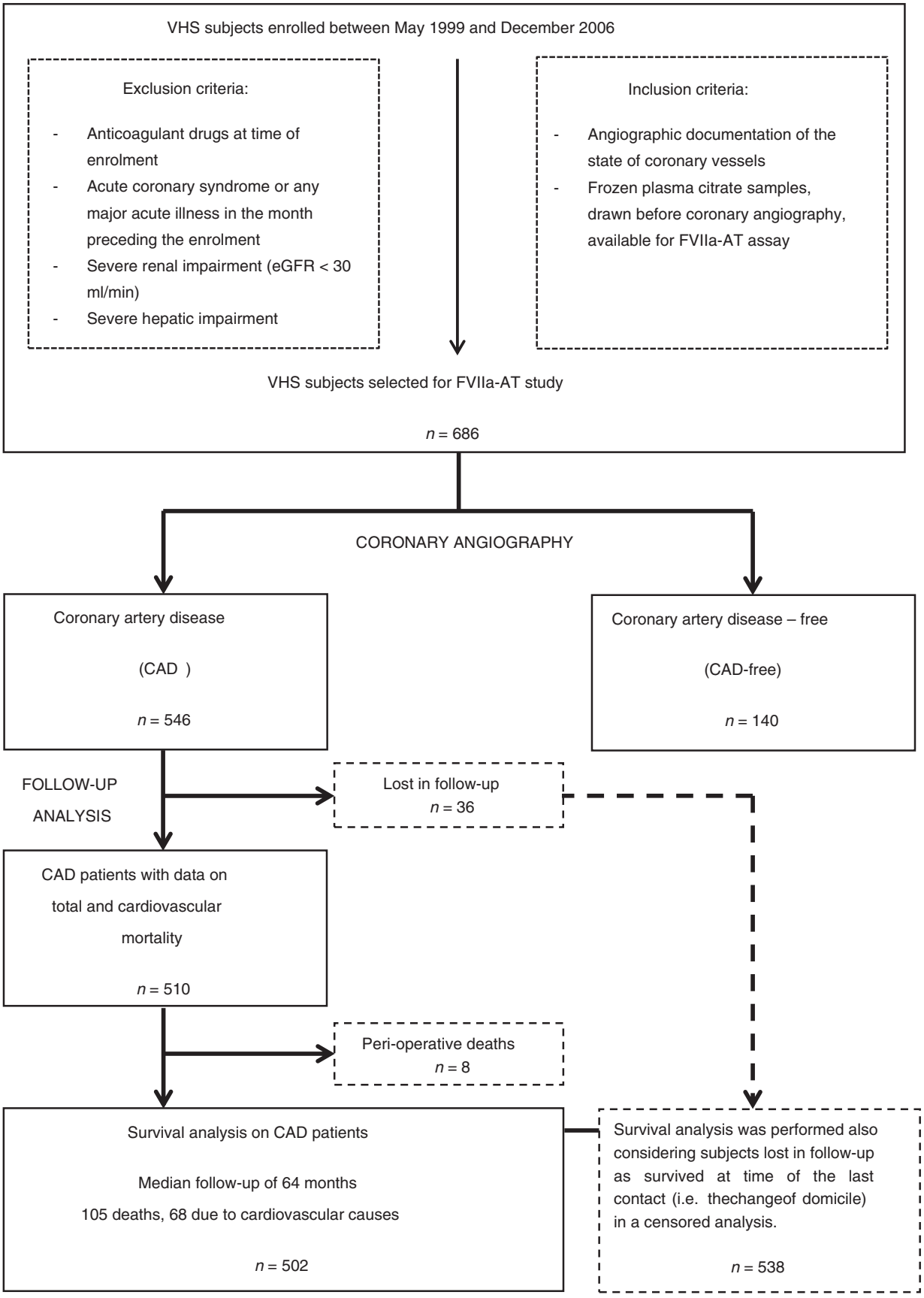


Fig. 1. Verona Heart Study (VHS) design for the analysis of activated factor VII–antithrombin complex (FVIIa-AT) levels.

protein (hsCRP), were determined as previously described [24,25]. The four-variable version of the Modification of Diet in Renal Disease (MDRD) equation was used to estimate the glomerular filtration rate (GFR) from serum creatinine levels [26].

Thrombin generation in plasma samples was evaluated by the addition of a thrombin specific fluorogenic substrate (Thrombin Substrate III; Calbiochem, EDM Millipore, MA, USA), as previously described [27]. The generation of thrombin in diluted plasma samples (1:5) was initiated by the addition of a volume mixture of $CaCl_2$ (2.5 mmol L^{-1}), thrombin fluorogenic substrate (250 μmol L^{-1}), and Innovin (Dade® Innovin®, Siemens Healthcare, Marburg, Germany). The estimated TF concentration was 85 pmol L^{-1} in our assay. Fluorescence (excitation 355 nm–emission 460 nm) was measured overtime in a fluorimeter (Fluoroskan Ascent BioMed, Thermo Fisher Scientific, Helsinki, Finland) and the amount of the generated thrombin was calculated using a normal pooled human plasma (Hyphen BioMed, Neuville-sur-Oise, France) as a standard. Specific parameters of thrombin generation (lag time, peak, time to peak, and endogenous thrombin potential (ETP) estimated by area under the curve) were calculated using the statistic software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) [28]. All the experiments were performed in duplicate. The between-run coefficients of variation were 1.31% (lag time), 2.24% (time to peak), 1.48% (peak), and 1.53% (ETP), respectively.

FVIIa-AT assay

The concentration of FVIIa-AT was measured by ELISA (Asserachrom VIIa-AT; Diagnostica Stago, Asnieres, France) on frozen citrate plasma samples that had never been thawed before this study. Venous blood samples collected at the time of enrolment were centrifuged, stored in 0.5-mL aliquots, and frozen at −80°C within 1 h of sample collection. Plasma samples were thawed in a water bath at 37°C for 5 min before FVIIa-AT assay. All testing was performed in duplicate. The intra-assay and interassay coefficients of variations were < 5%.

Statistics

All calculations were performed using the IBM SPSS 20.0 (IBM Inc., Armonk, NY, USA), Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. StataCorp LP, College Station, TX, USA), and R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria, 2015. https://www.R-project.org/, accessed at November 2015) statistical packages.

Distributions of continuous variables in groups were expressed as mean ± standard deviations. Skewed variables, including FVIIa-AT, FVIIa, triglyceride, hsCRP, and fibrinogen, were logarithmically transformed, and geometric means with 95% confidence intervals (CIs) were reported. Quantitative data were assessed using the Student *t*-test or by ANOVA, with polynomial contrast for linear trend when indicated. Qualitative data were analyzed with use of the χ^2 test and with χ^2 for linear trend analysis when indicated.

Significant associations between FVIIa-AT plasma concentration and other continuous or categorical variables were evaluated by linear regression models estimating R^2 and standardized β-coefficients in the whole study population, as well as in either CAD or CAD-free subgroups. Categorical variables were used after transformation into binary variables. To assess the independent predictors of FVIIa-AT levels, all the variables showing an association with the FVIIa-AT complex at univariate analysis were included in an adjusted regression model with backward stepwise selection of variables (removal if $P > 0.10$). Taking into account the low number of subjects for whom FVIIa levels were available ($n = 223$), data of FVIIa were added separately in a second adjusted regression model.

Survival was assessed by using the Kaplan–Meier method (log-rank statistic) and Cox regression. Kaplan–Meier curves were used for survival plots stratifying the CAD population on the basis of either quartile distribution or median value. Multivariate Cox proportional hazards for both total and cardiovascular mortality were performed considering the FVIIa-AT median value as threshold and including in the different models of sex, age, all the predictors of mortality at univariate analyses at baseline, drug therapy at discharge, and other variables that showed an association with FVIIa-AT at baseline (e.g. plasma lipids and classic coagulation times). Final models were obtained with backward stepwise logistic regression models, with $P > 0.10$ as the critical value for excluding variables in the model. Hazard ratios (HRs) and 95% CIs are reported with two-tailed probability values. All the survival analyses were performed also by censoring follow-up times for subjects lost in follow-up ($n = 36$) at date of change of domicile (i.e. their last documented certification of being alive). A value of $P < 0.05$ was considered statistically significant. Statistical power was calculated by means of Altman's nomogram [29].

Subjects with missing data were at first excluded from multivariate analysis in Cox regression models. Then, missing data were handled using multiple imputation by chained equations [30,31], a statistical technique that operates under the missing at random assumption (i.e. the probability that a value is missing depends only on observed values and not on unobserved values). The *mi impute chained* command of Stata 13 (StataCorp. 2013. Stata Statistical Software: Release 13. StataCorp LP) was used to generate 100 complete data sets with imputed missing values. The event indicator and the Nelson–Aalen estimator of the cumulative hazard were included in the imputation model [32]. For each imputed data set, a Cox model was fitted and the 100 estimates of the HRs were

then combined into an overall estimate and variance–covariance matrix using Rubin's rules [30]. HRs estimation using multiple imputations was performed within Stata using the *mi estimate* command with the *stcox* estimation command.

C-statistics and net reclassification improvement (NRI) were used to evaluate risk predictions from models containing the other predictors of mortality in our study population (i.e. sex, age, body mass index [BMI], history of MI, degree of CAD, type of therapeutic approach, hypertension, diabetes, renal function, fibrinogen, and hsCRP as explanatory variables) and models containing the above-mentioned predictors with the addition of FVIIa-AT data. To evaluate the improvement in risk discrimination performance gained by adding FVIIa-AT, the C-statistic for survival data proposed by Uno *et al*. [33] was estimated for the reference Cox model containing the other predictors of mortality and the FVIIa-AT–implemented Cox model (i.e. reference model plus FVIIa-AT). The difference between the two C-statistics was estimated for total and cardiovascular mortality at different truncation times (i.e. at 24, 48, 72, and 96 months). The *P*-values of the Wald test of the Cox regression coefficient for FVIIa-AT were reported because a comparison of C-statistics is less powerful than Wald test [34]. Calculation of the C-statistics was performed using the SurvC1 package [35] of R version 3.2.2 [36]. The ability of FVIIa-AT plasma level to change risk stratification was also assessed by means of reclassification analysis [37]. Continuous NRI (cNRI) for survival data was estimated according to Pencina *et al*. [38]. cNRI was defined by the sum of two subcomponents, that is, the amount of correct reclassification among events and among non-events. The SurvIDINRI package of R allows the estimation of cNRI with CIs at specified truncation times [39].

Results

FVIIa-AT plasma levels in subjects with or without CAD

The clinical and laboratory characteristics of the study population, divided into CAD and CAD-free subgroups, are summarized in Table 1. As expected, the traditional cardiovascular risk factors were mostly expressed in CAD subjects. No difference in mean plasma concentration of FVIIa-AT was seen between CAD and CAD-free subjects (Table 1), and no difference was found between the distributions of CAD and CAD-free subjects according to FVII-AT deciles (Table S1).

Associations of FVIIa-AT plasma levels with clinical and laboratory characteristics

The associations between FVIIa-AT plasma concentration and other clinical and laboratory variables were evaluated by linear regression models (Table S2).

Table 1 Clinical and laboratory characteristics of the study population, divided in subgroups with or without coronary artery disease (CAD)

	CAD free (n = 140)	CAD (n = 546)	P*
Age (y)	60.3 ± 12.1	61.4 ± 10.7	NS
Male sex (%)	68.6	76.3	NS
BMI (kg m^{-2})	25.8 ± 3.7	26.7 ± 3.8	0.010
Smoker (%)	42.2	68.0	< 0.001
Hypertension (%)	46.0	69.0	< 0.001
Diabetes (%)	8.30	21.3	0.001
eGFR (mL min^{-1})†	79.1 ± 22.2	78.0 ± 20.9	NS
Total cholesterol (mmol L^{-1})	5.20 ± 1.05	5.18 ± 1.12	NS
LDL cholesterol (mmol L^{-1})	3.34 ± 0.96	3.42 ± 0.94	NS
HDL cholesterol (mmol L^{-1})	1.39 ± 0.41	1.16 ± 0.29	< 0.001
Triglycerides (mmol L^{-1})	1.27 (1.18–1.36)	1.67 (1.60–1.72)	< 0.001
hsCRP (mg L^{-1})	2.36 (1.86–2.97)	5.37 (4.76–6.05)	< 0.001
Fibrinogen (g L^{-1})	3.35 (3.16–3.56)	3.94 (3.82–4.01)	< 0.001
PT (ratio)	1.01 ± 0.07	0.99 ± 0.07	0.016
APTT (ratio)	1.01 ± 0.10	1.02 ± 0.13	NS
FVIIa-AT complex (pmol L^{-1})	83.9 (76.7–92.8)	84.8 (80.6–88.2)	NS
F VIIa (mU mL^{-1})‡	42.5 (36.2–49.9)	46.1 (42.9–49.9)	NS
Drug therapy at discharge (%)§			
Antiplatelet therapy	–	97.6	
Warfarin	–	3.7	
β-Blockers	–	55.8	
Statins	–	56.5	

*By *t*-test or χ2-test. †Estimated glomerular filtration rate (eGFR) by the abbreviated Modification of Diet in Renal Disease (MDRD) equation. ‡Data were available for 305 subjects (82 CAD-free and 223 CAD). §Data of drug therapy at discharge were available only for CAD patients. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; PT, prothrombin time; APTT, activated partial thromboplastin time; NS, not significant.

Table 2 Thrombin generation parameters according to activated factor VII–antithrombin complex (FVIIa-AT) levels*

	FVIIa-AT < 79 pmol L^{-1} (n = 122)	FVIIa-AT ≥ 79 pmol L^{-1} (n = 150)	P†
Lag time (s)	56.3 (54.1–58.0)	57.4 (55.7–59.2)	NS
Time to peak (s)	142.6 (139.8–146.9)	141.2 (138.4–144.0)	NS
Peak (Rfu/s)	6.36 (6.17–6.55)	6.82 (6.55–7.03)	0.009
ETP (Rfu)	854.1 (837.2–871.3)	906.9 (880.1–925.2)	0.001

The median level (79 pmol L–1) was used as threshold value. *Thrombin generation data were available for 272 subjects (198 coronary artery disease [CAD] and 74 CAD free). †By *t*-test. ETP, endogenous thrombin potential; Rfu, relative fluorescence units; NS, not significant.

In an adjusted linear regression model (not including FVIIa levels, which were available only in a limited number of subjects), estimated GFR level, high-density lipoprotein cholesterol (HDL-C), and triglyceride plasma concentration were predictors of FVIIa-AT variability (Table S3A). When FVIIa was included in the regression model, HDL-C and triglyceride plasma concentrations and FVIIa levels remained significant predictors of FVIIa-AT variability (Table S3B).

FVIIa-AT complex levels and thrombin generation parameters

Citrate plasma samples for thrombin generation assay were available for 272 subjects (198 CAD and 74 CAD-free). FVIIa-AT plasma concentration correlated directly with peak and ETP levels (Table S4). Subjects with FVIIa-AT levels higher than the median value had increased peak and ETP levels (Table 2), and high FVIIa-AT plasma concentration remained a significant predictor of peak (β-coefficient = 0.168; $P = 0.006$) and ETP levels (β-coefficient = 0.200; $P < 0.001$) also after adjustment for sex, age, and CAD diagnosis.

Prospective study in CAD patients

Eight of the 510 CAD patients in the follow-up study died perioperatively and were excluded from subsequent survival analysis. Of the remaining 502 subjects, 105 (20.9%) subjects died, with 68 (13.5%) events due to

Table 3 Clinical and laboratory characteristics of CAD patients at baseline according to quartiles of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration

	FVIIa-AT plasma concentration				
	< 58 pmol L^{-1} (n = 120)	58–78.9 pmol L^{-1} (n = 127)	79–119 pmol L^{-1} (n = 128)	> 119 pmol L^{-1} (n = 127)	P*
Age (y)	60.2 ± 10.3	63.5 ± 9.1	61.7 ± 11.1	61.5 ± 11.3	NS
Male sex (%)	86.7	78.7	75.0	66.9	< 0.001
BMI (kg m^{-2})	28.2 ± 4.0	26.7 ± 3.6	26.2 ± 4.0	26.4 ± 3.9	0.001
Smoker (%)	68.4	70.6	69.3	63.4	NS
Hypertension (%)	64.4	72.4	71.1	68.0	NS
Diabetes (%)	19.1	16.0	31.7	22.6	NS
History of MI (%)	65.5	54.3	60.6	52.0	NS
eGFR (mL min^{-1})†	79.5 ± 17.2	81.9 ± 18.1	77.5 ± 20.8	73.8 ± 23.8	0.007
Total cholesterol (mmol L^{-1})	5.02 ± 1.00	5.21 ± 1.10	5.12 ± 1.03	5.21 ± 1.25	NS
LDL cholesterol (mmol L^{-1})	3.44 ± 0.86	3.47 ± 0.99	3.41 ± 0.86	3.43 ± 1.05	NS
HDL cholesterol (mmol L^{-1})	1.13 ± 0.33	1.14 ± 0.25	1.16 ± 0.29	1.19 ± 0.30	NS
Triglycerides (mmol L^{-1})	1.58 (1.48–1.70)	1.65 (1.54–1.77)	1.68 (1.55–1.80)	1.77 (1.57–1.86)	NS
hsCRP (mg L^{-1})	5.42 (4.26–6.96)	6.42 (5.05–8.17)	5.58 (4.48–7.03)	4.22 (3.25–5.23)	NS
Fibrinogen (g L^{-1})	4.01 (3.78–4.26)	3.94 (3.74–4.14)	3.86 (3.63–4.06)	3.90 (3.67–4.14)	NS
PT (ratio)	1.02 ± 0.08	0.99 ± 0.06	0.98 ± 0.08	0.98 ± 0.07	< 0.001
APTT (ratio)	1.04 ± 0.15	1.02 ± 0.12	1.00 ± 0.11	1.00 ± 0.12	0.006
FVIIa (mU mL^{-1})‡	32.4 (27.1–39.3)	49.4 (40.5–60.3)	41.2 (35.5–48.4)	66.7 (60.9–73.0)	< 0.001
LVEF (%)¶					
Preserved	64.1	66.0	58.7	67.6	NS
Moderately reduced	32.0	27.0	33.7	24.8	
Severely reduced	3.9	7.0	7.6	7.6	
Degree of CAD (%)					
1 vessel	29.6	21.4	25.3	18.4	0.026
2 vessels	28.0	32.5	31.0	24.0	
3 vessels	42.4	46.2	43.7	57.6	
Therapeutic approach (%)					
Only medical therapy	22.5	11.0	21.1	16.5	0.039
PTCA ± stent	45.0	57.5	42.2	35.5	
CABG	32.5	31.5	36.7	48.0	
Drug therapy at discharge (%)					
Antiplatelet therapy	95.0	100.0	99.2	96.1	NS
Warfarin	6.7	0.8	2.3	5.5	NS
β-Blockers	65.8	56.9	54.4	47.8	0.007
Statins	59.2	67.7	53.9	46.5	0.008

Subjects who died perioperatively (n = 8) or were lost in follow-up (n = 36) were excluded from this analysis. *By ANOVA with polynomial contrasts for linear trend or by $\chi 2$ for linear trend, when appropriate. †Estimated glomerular filtration rate (GFR) by the abbreviated Modification of Diet in Renal Disease (MDRD) equation. ‡Data of FVIIa levels were available for 212 subjects (41.6%). ¶Data of left ventricular ejection fraction (LVEF) levels were available for 412 subjects (80.8%). MI, myocardial infarction; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; PT, prothrombin time; APTT, activated partial thromboplastin time; PTCA, percutaneous transluminal coronary angiography; CABG, coronary artery bypass graft surgery; NS, not significant.

cardiovascular causes during the follow-up with a median period of 64 months. Baseline FVIIa-AT plasma concentrations were higher in subjects who died (95.6 with 95% CI 86.5–106.7 pmol L^{-1}; $P = 0.002$) and in those who died from cardiovascular causes (92.8 with 95% CI 81.9–105.6 pmol L^{-1}; $P = 0.038$) than in survivors (81.5 with 95% CI 77.5–85.6 pmol L^{-1}). Clinical and laboratory characteristics at baseline according to FVIIa-AT quartiles are reported in Table 3.

CAD patients within the highest FVIIa-AT quartile had a higher total and cardiovascular mortality rate than did those within the lowest FVIIa-AT quartile (Fig. 2).

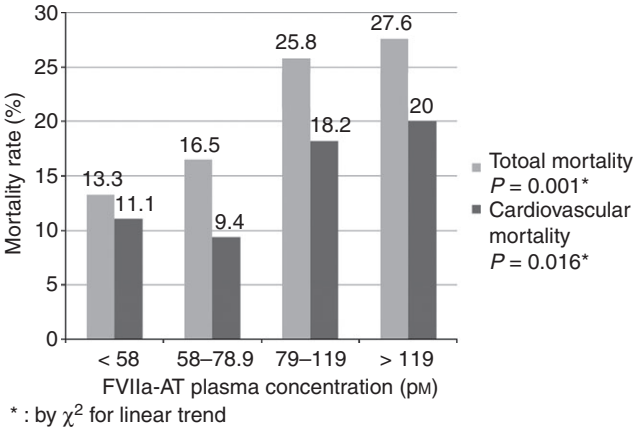


Fig. 2. Total and cardiovascular mortality rate according to quartiles of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration. The survival analysis was performed on 502 patients with coronary artery disease after a median follow-up of 64 months with 105 deaths (20.9%), 68 (13.5%) of whom had cardiovascular causes. Subjects who died perioperatively ($n = 8$) were excluded from this analysis.

Kaplan–Meier survival curves show total and cardiovascular mortality according to FVIIa-AT quartiles (Fig. S2). This finding is particularly clear if the median value (79 pmol L^{-1}) was considered as the threshold level (Fig. 3A,B). The subjects with FVIIa-AT levels higher than the median value (≥ 79 pmol L^{-1}) had a two-fold greater risk of both total and cardiovascular mortality. This was confirmed in a Cox regression model adjusted for sex and age (Table 4, Model 1), as well as for the other predictors of mortality at univariate analysis (i.e. BMI, history of MI, degree of CAD, type of therapeutic approach, hypertension, diabetes, renal function [MDRD], fibrinogen, hsCRP, and drug therapy at discharge) (Table 4, Models 2 and 3). Stratifying the study population on the basis of FVIIa-AT plasma concentration and MI history, high levels of FVIIa-AT were predictive of total and cardiovascular mortality in CAD patients both with and without MI (Fig. 4A,B). Subjects with MI and high levels of FVIIa-AT had the worst prognosis, while subjects with high levels of FVIIa-AT without a history of MI showed similar survival curves to those with MI and low levels of FVIIa-AT (Fig. 4A,B). Moreover, high levels of FVIIa-AT were predictors of total mortality in CAD patients who were taking an 'optimal' medical therapy (i.e. with both β-blockers and statins, in addition to antiplatelet agents—176 subjects, with 23 deaths during follow-up, 10 of which had cardiovascular causes (HR 2.41, 95% CI 1.02–5.73, in a sex- and age-adjusted model).

Data on left ventricular ejection fraction (LVEF) were available for a subgroup of 412 subjects (with 79 deaths during follow-up, 48 of which had cardiovascular causes). Even including LVEF in a regression model, high levels of FVIIa-AT were a predictor of total and cardiovascular

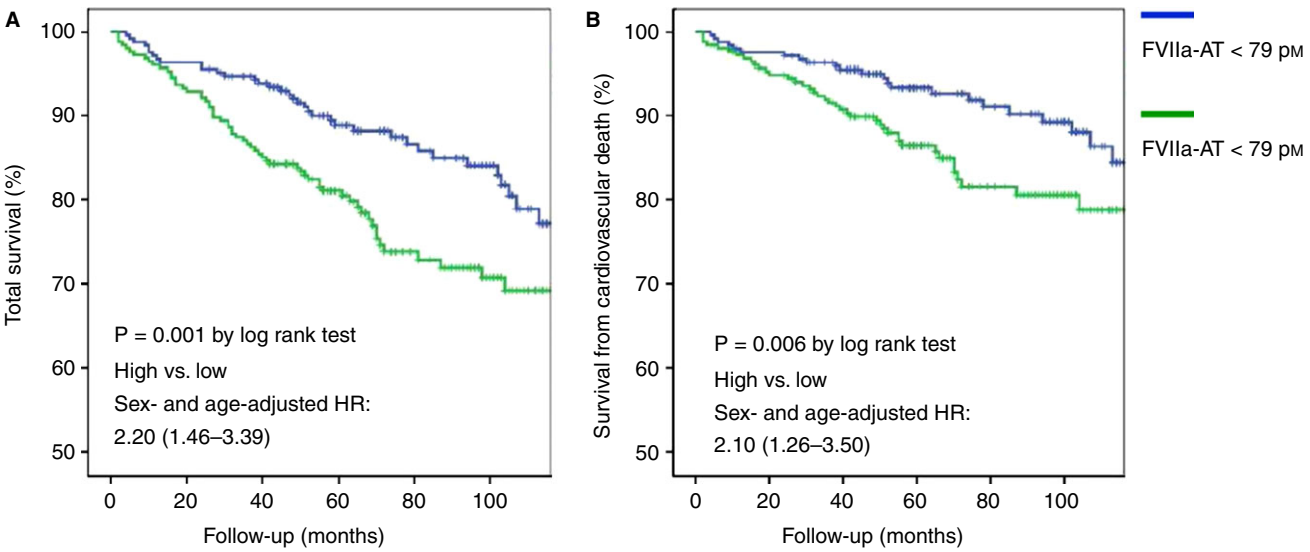


Fig. 3. Total (A) and cardiovascular (B) mortality according to median value of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration (79 pmol L^{-1}).

Table 4 Plasma concentration of activated factor VII–antithrombin complex (FVIIa-AT) above the median level (≥ 79 pmol L^{-1}) as a predictor of total and cardiovascular mortality in different Cox regression and multiple imputation analysis models

	Total mortality						Cardiovascular mortality					
	Cox regression				Multiple imputation*		Cox regression				Multiple imputation*	
Models	B	SE	HR	*P*	HR	*P*	B	SE	HR	*P*	HR	*P*
1: Sex-and age adjusted	0.778	0.209	2.20 (1.46–3.31)	< 0.001	–†	–†	0.743	0.260	2.10 (1.26–3.50)	0.004	–†	–†
2: Risk predictors adjusted	0.716	0.266	2.05 (1.22–3.45)	0.007	1.90 (1.24–2.91)	0.003	0.664	0.333	1.94 (1.01–3.73)	0.046	1.71 (1.00–2.96)	0.050
3: Drug therapy adjusted	0.593	0.207	1.81 (1.21–2.71)	0.004	–†	–†	0.577	0.256	1.78 (1.08–2.94)	0.024	–†	–†
4: Plasma lipids adjusted	0.558	0.218	1.75 (1.14–2.68)	0.011	1.99 (1.33–2.99)	0.001	0.587	0.272	1.80 (1.06–3.07)	0.031	2.05 (1.24–3.38)	0.005
5: Coagulation times adjusted	0.616	0.208	1.85 (1.23–2.78)	0.003	2.00 (1.34–3.00)	0.001	0.642	0.257	1.90 (1.15–3.15)	0.012	2.02 (1.22–3.35)	0.006
6: LVEF adjusted	0.648	0.235	1.91 (1.21–3.03)	0.006	1.92 (1.28–2.87)	0.002	0.618	0.300	1.86 (1.03–3.34)	0.039	1.94 (1.17–3.20)	0.010

Subjects with FVIIa-AT levels below the median value are considered as reference group. Model 1: sex and age adjusted. Model 2: adjusted for sex, age, body mass index, history of myocardial infarction, degree of coronary artery disease, type of therapeutic approach, hypertension, diabetes, renal function (estimated glomerular filtration rate (GFR) by the abbreviated Modification of Diet in Renal Disease (MDRD) equation), fibrinogen, and high-sensitivity C-reactive protein. Model 3: adjusted for antiplatelet, β-blocker, and statin drug therapy at discharge. Model 4: adjusted for high-density lipoprotein cholesterol and triglyceride plasma concentration. Model 5: adjusted for classical coagulation times, prothrombin time, and activated partial thromboplastin time. Model 6: adjusted for left ventricular ejection fraction (LVEF; data were available for 412 on 502 subjects). *Missing data were imputed using multiple imputation by chained equations with 100 imputations; estimated hazard ratios from multiply imputed data sets were combined into an overall estimate using Rubin's rules. †No missing values were present in this dataset. Therefore, multiple imputation analysis was not necessary.

mortality (Table 4, Model 6). Taking into account the previously demonstrated correlations of FVIIa-AT with either plasma lipids or classic coagulation times (Table S1), further regression models were set up. High levels of FVIIa-AT remained a significant predictor of both total and cardiovascular mortality after adjustment for HDL-C and triglycerides, as well as for prothrombin time and activated partial thromboplastin time (Table 4, Models 4 and 5). All these associations were confirmed by multiple imputation analysis if indicated (Table 4), as well as by censoring follow-up times for subjects lost in follow-up ($n = 36$) at date of change of domicile (Table S5, Fig. S3).

In the subgroup analysis of subjects for whom FVIIa data were available (212 subjects, with 30 deaths during follow-up, 21 of whom had cardiovascular causes), FVIIa-AT plasma concentration but not FVIIa level was a predictor of total mortality in a sex- and age-adjusted Cox regression model (Table S6).

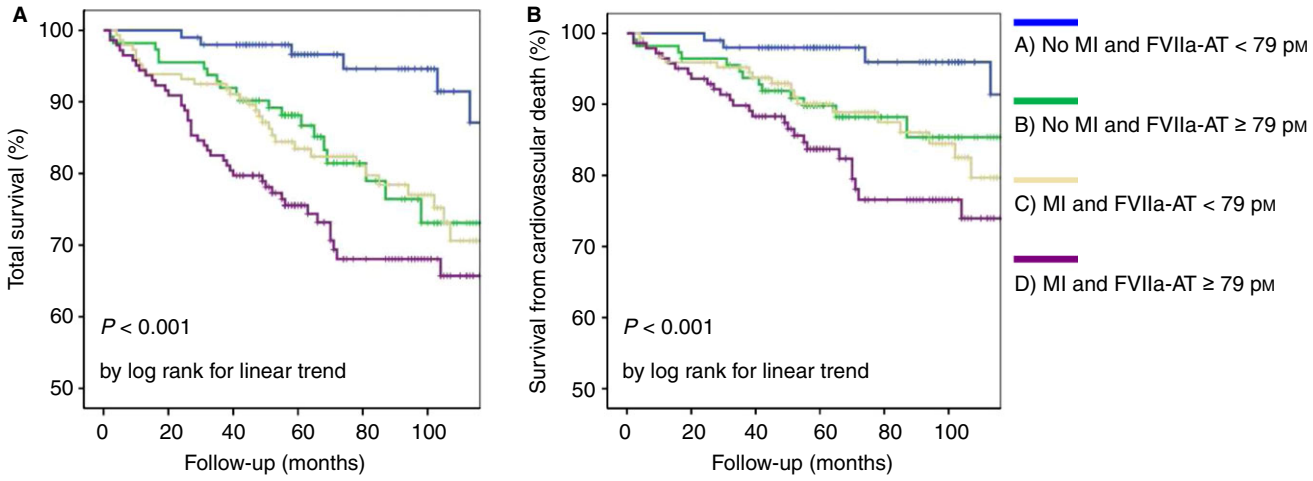
Finally, discrimination and reclassification analyses were performed. The addition of FVIIa-AT levels to a model that included the other predictors of mortality improved the discrimination (at 48, 72, and 96 months), although the magnitude of the improvement of C-statistic was modest (Table S7). In cNRI analysis, the risk classification of a model including the other predictors was slightly improved by FVIIa-AT for total mortality (at 72 and 96 months) but not for cardiovascular mortality (Table S8).

Discussion

In this study, high FVIIa-AT levels were consistently associated with thrombin generation, in particular with an increased ETP, supporting the hypothesis of FVIIa-AT as a marker of hypercoagulability. High FVIIa-AT levels were shown to be a predictor of total and cardiovascular mortality in patients with established and clinically stable CAD, which prevented potential biases due to 'acute phase' activation of the coagulation cascade.

Our results confirm previous observations showing that FVIIa level is the strongest predictor of FVIIa-AT levels [22,23]. In our study, population plasma concentrations of HDL-C and triglyceride, although with much lower coefficients, were also weak predictors of FVIIa-AT levels even after adjustment for FVIIa levels, emphasizing the extensive connection between plasma lipids and the coagulation pathway [40,41].

In our study population, no difference in FVIIa-AT levels was observed between CAD and CAD-free subjects, suggesting the concept that FVIIa-AT plasma concentration could be more related with the prognosis rather than the occurrence of CAD. It is worth noting that baseline levels of FVIIa-AT in CAD patients who died during follow-up were higher not only than those in survived CAD patients but consistently also than those in CAD-free subjects ($P = 0.029$ by sex- and age-adjusted analysis).



	Sex- and age-adjusted HR
B vs. A	3.73 (1.58–8.77)
C vs. A	3.66 (1.60–8.35)
D vs. A	6.80 (3.05–15.14)
C vs. B	0.98 (0.56–1.71)
D vs. B	1.76 (1.10–2.80)

	Sex- and age-adjusted HR
B vs. A	3.07 (1.10–8.58)
C vs. A	3.27 (1.22–8.77)
D vs. A	6.18 (2.38–16.03)
C vs. B	1.07 (0.53–2.13)
D vs. B	1.85 (1.04–3.31)

Fig. 4. Total (A) and cardiovascular mortality (B) stratifying the study population on the basis of myocardial infarction (MI) history and the median value of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration (79 pmol L^{-1}). Hazard ratios (HRs) between the different pair of subgroups were calculated by sex- and age-adjusted Cox regression.

Only a few studies have investigated the clinical significance of FVIIa-AT plasma levels in thrombotic disease [42]. Spiezia and colleagues [22] found a difference in FVIIa-AT levels between acute and previous arterial or venous thrombosis, attributing the decrease during acute thrombotic events to the presence of TFPI–FXa complexes that could create the TF–FVIIa–TFPI–FXa quaternary complexes which in turn may inhibit the formation of the FVIIa–AT complex. In the Stockholm Coronary Atherosclerosis Risk Factor (SCARF) study, patients with previous MI had slightly increased FVIIa-AT levels compared with healthy controls, with a large overlap of intervals [23]. However, the SCARF study cannot be appropriately compared with our study, because the case-control design was not angiography based.

In our study population, high plasma concentrations of FVIIa-AT were a strong predictor of mortality in patients with clinically stable CAD. Notably, the prognostic impact of high FVIIa-AT levels on mortality appeared similar to that related to a history of previous MI (Fig. 3). In the Stockholm study of 60-year-old individuals, no predictive value of FVIIa-AT plasma concentration was found for cardiovascular events [23]. However, the Stockholm study aimed at using the FVIIa-AT assay in the setting of primary prevention of CAD: the outcome variables were first cardiovascular events (i.e. angina, MI, and ischemic stroke) and only subjects without a previous history of cardiovascular disease were recruited [23]. By contrast, in our prospective study, we only considered lethal events in patients with angiographically proven severe CAD [24]. Starting from the hypothesis that FVIIa-AT is a marker of a prothrombotic state, we consider it biologically plausible that its prognostic role may be most relevant when there are preexisting atherosclerotic plaques.

If confirmed in other similar populations, our results may point toward high FVIIa-AT levels as predictors of clinical 'vulnerability' due to prothrombotic diathesis in CAD patients [43]. High levels of FVIIa-AT remained a significant predictor of both total and cardiovascular mortality also after adjustment for prothrombin time and activated partial thromboplastin time. Such a result underlines the potential ability of FVIIa-AT to disclose components of the prothrombotic diathesis complementary to and more advanced than the classic clotting times, as well as more specifically associated with clinical outcomes in CAD.

There is great interest in prognostic biomarkers in CAD patients already receiving a full cardiovascular therapy, in whom the traditional risk factors for atherosclerosis or markers of cardiovascular risk, like low-density lipoprotein cholesterol [44] or hsCRP [45], may be not

related with clinical outcomes. It is worth noting that in our study population FVIIa-AT plasma concentration was a predictor of mortality even in patients using all the usual medications for CAD treatment [1]. This leaves open the hypothesis of possible benefits in using more aggressive therapeutic approaches in this high-risk group. We can speculate on the possible beneficial use of oral direct FXa inhibitors in patients with high FVIIa-AT levels, by blocking the excess of FX activation mediated by FVIIa–TF pathway [46,47].

Clearly, our findings have to be confirmed by further larger prospective studies. At the moment, caution should be advised in disentangling the potential prognostic role of FVIIa-AT. Adding FVIIa-AT levels to established CAD risk factors resulted in only modest improvement of discrimination (C-statistic), while reclassification analysis by cNRI revealed minimal improvement for total mortality only.

We recognize that the present study has some limitations, such as the unbalanced distribution of subjects with or without CAD in the case-control study and the low number of female participants enrolled. Nonetheless, considering the distribution and the difference of FVIIa-AT levels detected in CAD patients who survived or died during the follow-up period, the statistical power of this analysis in our study population was about 85% by Altman's nomogram with a significance level of 0.05. There was a lack of data about the effect of variations of AT concentration on the FVIIa-AT complex, but a previous study did not support a strong relationship between AT and FVIIa-AT levels [22]. There was no consideration of TFPI, but it should be noted that as any TFPI–FVIIa (along with TF and FXa) complex formed is not released into the plasma, it is not possible to measure it [48].

The lack of assessment of non-fatal cardiovascular events in the follow-up represents a limitation of our findings. Therefore, we emphasize that the implications of the present results are restricted to the setting of mortality in CAD population. Nonetheless, the robustness of the endpoints considered (i.e. total and cardiovascular mortality) is a strength of our study. Another strength is the angiographic evaluation of coronary arteries in both cases and controls, which allowed for a clear-cut definition of the clinical phenotype.

In summary, in our study a single measurement of FVIIa-AT at the time of coronary angiography, usually before intervention of coronary revascularization, was a predictor of both total and cardiovascular mortality in clinically stable CAD patients. Our data also point toward FVIIa-AT as a marker of hypercoagulability.

Addendum

N. Martinelli designed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; D. Girelli, B. Woodhams, F. Bernardi, and O. Olivieri designed research, interpreted data, and wrote the manuscript; M. Baroni, B. Lunghi, and A. Branchini performed thrombin generation analysis, analyzed data, and contributed to subsequent manuscript discussion; P. Guarini, F. Tosi, and F. Sartori collected data, analyzed data, and contributed to subsequent manuscript discussion; M. Sandri analyzed data, performed statistical analysis, and contributed to subsequent manuscript discussion.

Acknowledgments

We thank Maria Zoppi for her invaluable secretarial help and Diego Minguzzi and Patrizia Pattini for their excellent technical help.

This work was supported by the Cariverona Foundation, Verona, Italy, the Italian Medicines Agency, Agenzia Italiana del Farmaco (AIFA RF-null-2008-1235892), and the Emilia Romagna Region-University Strategic Programme, Italy. We are grateful to Diagnostica Stago, Asnieres, France, for financial support and for providing the reagents for the FVIIa-AT assay.

Disclosure of Conflicts of Interests

N. Martinelli reports receiving a payment from Diagnostica Stago, Asnieres, France, for management and conduct of the study. B. Woodhams reports being a consultant for Diagnostica Stago via Haemacon Ltd. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Plasma concentration of activated factor VII–antithrombin complex (FVIIa-AT) as an affordable marker of intravascular exposure to tissue factor.
Fig. S2. Total (A) and cardiovascular (B) mortality according to quartiles of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration.
Fig. S3. Total and cardiovascular mortality rate according to quartiles of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration in the whole coronary artery disease population, censoring follow-up times for subjects lost in follow-up ($n = 36$) at date of change of domicile (i.e. their last documented certification of being alive). Subjects who died perioperatively ($n = 8$) were excluded from this analysis.
Table S1. Distribution of subjects with or without coronary artery disease (CAD) according to deciles of activated factor VII–antithrombin complex (FVIIa-AT) plasma concentration.
Table S2. Associations of activated factor VII–antithrombin complex (FVIIa-AT) plasma levels with clinical and

laboratory characteristics by linear regression models in the study population.

Table S3. Predictors of activated factor VII–antithrombin complex (FVIIa-AT) variability by adjusted linear regression models in the whole study population (A, $n = 686$) and in subgroup of subjects for whom data of FVIIa levels were available (B, $n = 305$).

Table S4. Correlation between activated factor VII–antithrombin complex (FVIIa-AT) and thrombin generation parameters.

Table S5. Plasma concentration of activated factor VII–antithrombin complex (FVIIa-AT) above the median level (≥ 79 pmol L^{-1}) as a predictor of total and cardiovascular mortality in different Cox regression models, censoring follow-up times for subjects lost in follow-up ($n = 36$) at date of change of domicile (i.e. their last documented certification of being alive). Subjects with FVIIa-AT levels below the median value are considered as reference group.

Table S6. Activated factor VII–antithrombin complex (FVIIa-AT) and FVIIa levels as predictor of total mortality in a sex- and age-adjusted Cox regression model (212 subjects with 30 deaths).

Table S7. Assessment of the improvement in prediction performance gained by adding activated factor VII–antithrombin complex (FVIIa-AT) levels to a set of baseline predictors in Cox models for total and cardiovascular mortality.

Table S8. Continuous net reclassification improvement (cNRI) analysis for total (A) and cardiovascular mortality (B) by adding activated factor VII–antithrombin complex (FVIIa-AT) levels to a reference model that included the other predictors of mortality.

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