

Apolipoprotein C-III strongly correlates with activated factor VII-antithrombin complex: an additional link between plasma lipids and coagulation

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Running head: Apolipoprotein C-III and activated factor VII-antithrombin

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SUMMARY TABLE

WHAT IS KNOWN ABOUT THIS TOPIC?
<ul style="list-style-type: none">• Plasma lipoproteins can stimulate coagulation cascade, with triglyceride-rich lipoproteins (TRLs) being apparently the most efficient.• Tissue factor (TF) and activated factor VII (FVIIa) are involved in phospholipid interactions which enhance their coagulant activity.• Activated factor VII–antithrombin complex (FVIIa-AT) in plasma may reflect tissue factor (TF) exposure and TF-FVIIa interaction.
WHAT DOES THIS PAPER ADD?
<ul style="list-style-type: none">• Apolipoprotein C-III (Apo C-III), a crucial player of TRLs metabolism, showed the strongest correlation with FVIIa-AT among all plasma lipid parameters• The <i>APOC3</i>-tagging polymorphism rs964184, linked by GWAS with cardiovascular diseases, was consistently associated with both Apo C-III and FVIIa-AT plasma levels.• An increased high Apo C-III concentration may identify subjects with a prothrombotic diathesis characterized by an enhanced TF-FVIIa interaction

SUMMARY

Activated factor VII-antithrombin (FVIIa-AT) complex is a potential biomarker of prothrombotic diathesis reflecting FVIIa-tissue factor (TF) interaction and has been associated with mortality in patients with coronary artery disease (CAD). Previous data indicated plasma lipids as predictors of FVIIa-AT variability, and plasma lipoproteins as potential stimulators of the coagulation cascade. Our aim was to evaluate the relationships between FVIIa-AT plasma concentration and a broad apolipoprotein profile (including Apo A-I, Apo B, Apo C-III, and Apo E). Within the framework of the observational Verona Heart Study we selected 666 subjects (131 CAD-free and 535 CAD, 75.4% males, mean age 61.1 ± 10.9 years) not taking anticoagulant drugs and for whom plasma samples were available for both FVIIa-AT assay and a complete lipid profile. Plasma concentration of FVIIa-AT levels significantly and directly correlated with total and HDL cholesterol, triglycerides, Apo A-I, Apo C-III, and Apo E levels. Apo C-III showed the strongest correlation ($R=0.235$, $P=7.7 \times 10^{-10}$), confirmed in all the subgroup analyses (males/females and CAD/CAD-free). Only Apo C-III remained associated with FVIIa-AT plasma concentration, even after adjustment for sex, age, CAD diagnosis, BMI, renal function, **smoke**, lipid-lowering therapies, and FVIIa levels. The *APOC3* gene locus-tagging polymorphism rs964184, previously linked with cardiovascular risk and plasma lipids by genome-wide association studies, was associated with both Apo C-III and FVIIa-AT plasma concentration. Our results indicate a strong association between Apo C-III and FVIIa-AT levels, thereby suggesting that an increased Apo C-III concentration may identify subjects with a prothrombotic diathesis characterized by an enhanced TF-FVIIa interaction and activity.

KEYWORDS

Activated factor VII-antithrombin complex, *APOC3* gene polymorphism, apolipoprotein C-III, coagulation, plasma lipids

INTRODUCTION

Plasma lipids play a crucial role in the pathogenesis of cardiovascular disease (CVD) [1].

Lipoproteins are the main actors of a finely tuned system to transport lipids through the whole body according to its metabolic needs. Lipoproteins have a mono-layer phospholipid and cholesterol outer shell and heterogeneous lipid core. Apolipoproteins are embedded in the outer shell, both stabilizing the structure and determining their functional characteristics.

Alterations in levels of plasma lipids are well recognized among the most important risk factors of CVD and lipid-lowering drugs are among the most widely used drugs in Western world [2,3]. The increase of cardiovascular risk due to alterations in plasma lipids concentration is usually attributed to atherosclerosis-related processes. However, lipoproteins are endowed with various biological properties and have been also demonstrated to influence the coagulation pathway.

Coagulation needs lipids and the cascade is greatly accelerated by lipid binding [4]. Several coagulation factors are equipped with specialized membrane phospholipid-binding domains, which are essential for proper clot formation [4]. Tissue Factor (TF), whose interaction with activated factor VII (FVIIa) starts the coagulation cascade, is not only an integral membrane protein but its ectodomain is also involved in phospholipid interactions which enhance FVIIa activity [5]. Thrombin generation is dependent on assembly of the prothrombinase complex on a phospholipid surface [6]. In clinical studies plasma levels of both cholesterol and triglycerides have been directly correlated with plasma levels of coagulation factors, especially with the vitamin K-dependent ones, [7] as well as with thrombin generation [8]. Experimental data suggest that all plasma lipoproteins stimulate coagulation cascade, with triglyceride-rich lipoproteins (TRLs) being apparently the most efficient [9,10].

Activated factor VII-antithrombin (FVIIa-AT) complex is an indirect marker of intravascular exposure of TF and, consequently, of activation of the extrinsic coagulation pathway. While still incompletely understood, the transition from inactive (encrypted) to active (decrypted) form of TF and its binding and activation of FVII is the key initiator of coagulation cascade [11,12,13]. Both antithrombin (AT) and tissue factor pathway inhibitor (TFPI) acts as inhibitors of the TF-FVIIa pathway. TFPI forms with TF, FVIIa and activated coagulation factor X (FXa) a stable quaternary complex which remains bound to the cellular surface. On the other hand, once the TF-FVIIa-AT complex is formed, FVIIa loses affinity for TF and FVIIa-AT is released and accumulates in the plasma where it can be measured [14]. Therefore, FVIIa-AT plasma levels indirectly reflect TF-FVIIa interaction and have been suggested as a biomarker of prothrombotic diathesis [15-17]. A slight increase in FVIIa-AT plasma concentration was observed in subjects with previous arterial and/or venous thrombosis [15], as well as in survivors after myocardial infarction [16]. In the setting of primary prevention of coronary artery disease (CAD) within the Stockholm study of 60-year-old individuals FVIIa-AT had no predictive value [16], while in the secondary prevention setting within the Verona Heart Study (VHS) population high FVIIa-AT levels predicted total and cardiovascular mortality in patients with clinically stable CAD [17]. This suggests that FVIIa-AT levels may be more related with later thrombotic complications of CAD, rather than with the early development of atherosclerotic plaques. Accordingly, a recent analysis in participants to the large Cardiovascular Health Study has shown that high FVIIa-AT levels may reflect an increased risk of mortality [18].

In our previous study, high-density lipoprotein (HDL) cholesterol and triglycerides, were independent predictors of FVIIa-AT variability, even after adjustment for FVIIa levels [17], thus addressing the interest on the complex and still controversial link between plasma lipids and coagulation.

On the basis of such premises, in order to investigate more in depth the relationship between FVIIa-AT and plasma lipids, we performed a new series of analyses evaluating a broad apolipoprotein profile, including apolipoprotein A-I (Apo A-I), B (Apo B), C-III (Apo C-III), and E (Apo E), in the original VHS population cohort previously assessed for FVIIa-AT.

MATERIALS AND METHODS

Study population

This study was performed within the framework of the Verona Heart Study (VHS), a regional survey aimed to look for new CAD risk factors in subjects with angiographic documentation of their coronary vessels [19]. Briefly, 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 syndrome were excluded from this study. Subjects with severe renal failure (estimated glomerular filtration rate (eGFR) < 30 ml/min) and those with severe hepatic impairment (clinically defined diagnosis of liver cirrhosis) were also excluded from this study. The study design is summarized in Supplemental Figure 1. Within the original study population not taking any anticoagulant drugs who have been assessed for FVIIa-AT levels [17], we selected the 666 subjects for whom plasma samples for apolipoprotein assays were available (75.4 % males, mean age 61.1±10.9 years). One hundred and thirty-one subjects having 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 also no history, or clinical or instrumental evidence of atherosclerosis outside the coronary bed. Five hundred and thirty-five subjects had angiographically-proven CAD (CAD group), with at least one of the major epicardial coronary arteries (left anterior descending, circumflex, and right) affected with ≥ 1 significant stenosis ($\geq 50\%$ lumen reduction). All the CAD patients were newly diagnosed at time of enrolment, i.e. at time of coronary angiography. The angiograms were assessed blind 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 Ethic Committee of our Institution (Azienda Ospedaliera Universitaria Integrata, Verona). A written informed consent was obtained from all the participants.

Biochemical analysis

Samples of venous blood were drawn from each subject, after an overnight fast, at the time of enrolment before coronary angiography. Serum lipids, as well as other CAD risk factors, including high-sensitivity C Reactive Protein (hs-CRP), were determined as previously described [17,19]. **Very low-density lipoprotein (VLDL) cholesterol was calculated by subtracting from total cholesterol the concentration of HDL and low-density lipoprotein (LDL) cholesterol.** The four variables version of the Modification of Diet in Renal Disease (MDRD) equation was used to estimate the glomerular filtration rate (GFR) from serum creatinine levels [20].

Apolipoprotein assays

ApoA-I, ApoB and ApoE were measured by commercially available nephelometric immunoassays, as previously described [21]. ApoC-III concentration was measured using an automated turbidimetric immunoassay (Wako Pure Chemical Industries). Intra-assay coefficients of variation (CVs) were 1.84%, 2.02%, and 1.98% on 3 pools of control sera with low, medium, and high concentrations of Apo CIII, respectively; inter-assay CVs were 4.4%, 3.4%, and 2.29% for low, medium, and high concentration, respectively [22,23].

FVIIa-AT and FVIIa assays

The concentration of FVIIa-AT was measured by ELISA (Asserachrom VIIa-AT, Diagnostica Stago, Asnieres, France) on frozen citrate plasma samples, never 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 one hour after sample collection. Plasma samples were thawed in a water bath at

37°C for 5 minutes before FVIIa-AT assay. All testing was performed in duplicate. The intra-assay and inter-assay CVs were <5% [17].

FVIIa was assessed with a kit utilizing a soluble recombinant truncated TF that is selectively deficient in promoting FVII activation but retains r FVIIa cofactor function, thus allowing direct quantification of FVIIa in plasma (Staclot VIIa-rTF, Diagnostica Stago). Values were expressed in milliunits per milliliter, 30 such units being equivalent to 1 ng of FVIIa. The standard was a recombinant FVIIa supplied with the kit. The intra-assay and inter-assay CVs were 7.8% and 6.4%, respectively [21]. Plasma samples for FVIIa assay were available for 291 subjects (43.7%).

Single nucleotide polymorphisms and genotyping

Genomic DNA was prepared from whole blood samples by phenol-chloroform extraction.

The rs964184 C/G polymorphism is located on chromosome 11q23.3, tagging *ZNF259*, *APOA5*-*APOA4*-*APOC3*-*APOA1* gene region. Genome-wide association studies (GWAS) proved that the G allele is associated with high levels of triglycerides and LDL-cholesterol, low levels of HDL-cholesterol, and an increased risk of CAD [24-26]. rs964184 SNP was genotyped using Taqman platform. DNA samples for rs964184 analysis were available for 540 subjects (81.0%).

Polymorphisms in the *APOE* gene, rs429358 (Cys112Arg) and rs7412 (Arg158Cys), encode three common alleles, $\epsilon 2$ (Cys122 and Cys158), $\epsilon 3$ (Cys112 and Arg158) and $\epsilon 4$ (Arg112 and Arg158), which combine to form 6 genotypes, $\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 3$, $\epsilon 2/\epsilon 4$, $\epsilon 3/\epsilon 3$, $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$. *APOE* $\epsilon 2/\epsilon 3/\epsilon 4$ polymorphisms are among the most investigated gene variants being a key regulator of lipoprotein metabolism [27,28]. *APOE* polymorphisms were genotyped according to a previously described multilocus assay [29] and data were available for 495 subjects (74.3%).

Statistics

All calculations were performed using the IBM SPSS 20.0 (IBM Inc., Armonk, NY) statistical package.

Distributions of continuous variables in groups were expressed as means \pm standard deviations.

Skewed variables, including FVIIa-AT, FVIIa, hs-CRP, VLDL cholesterol, triglyceride, Apo C-III, and Apo E, were logarithmically transformed and then geometric means with 95% confidence interval (CI) were reported. Quantitative data were assessed using the Student's t-test or by ANOVA, with polynomial contrast for linear trend when indicated. Qualitative data were analyzed with the χ^2 -test and with χ^2 for linear trend analysis when indicated. The frequencies of the genotypes associated with each of the assessed polymorphisms were compared by using the chi-square test with the values predicted on the basis of the Hardy-Weinberg equilibrium.

Significant associations between FVIIa-AT plasma concentration and lipid parameters (i.e. traditional plasma lipids and apolipoproteins) were evaluated at first by Pearson's correlation coefficient (R) in the whole study population, as well as in either males or females and in either CAD or CAD-free subgroups. 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. Further adjustments were performed including in the regression models sex, age, body mass index (BMI), eGFR, smoke, CAD diagnosis, lipid lowering therapy, and FVIIa. Taking into account the low number of subjects for whom FVIIa levels were available (n=291), data of FVIIa were added in a separate adjusted regression model.

A value of $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and laboratory characteristics of the study population are reported in Table 1. As previously observed [17], no difference in FVIIa-AT levels was found between CAD and CAD-free subjects, ~~while—as expected—plasma lipids and apolipoproteins showed significantly different distributions~~ (Table 1). As regards plasma lipids and apolipoproteins, HDL cholesterol and Apo A-I were lower while triglyceride and Apo C-III were higher in patients with CAD than in CAD-free subjects, although with a substantial overlap in plasma levels between cases and controls (Table 1). In the whole study population, several significant direct correlations were found between FVIIa-AT and lipid variables. Total and HDL cholesterol, triglycerides, Apo A-I, Apo C-III, and Apo E correlated directly with FVIIa-AT levels (Table 2). The association with Apo C-III was the strongest with high statistical significance ($R=0.235$, $P=7.7\times 10^{-10}$ – Figure 1) and was the only confirmed in all the subgroup analyses (i.e. males and females, CAD and CAD-free – Table 2). Including total and HDL cholesterol, triglycerides, Apo A-I, Apo C-III, and Apo E (i.e. the variables showing an association with the FVIIa-AT complex at univariate analysis) in a regression model for FVIIa-AT variability, only Apo C-III remained associated with FVIIa-AT (Table 3). Such association was confirmed after adjustment for sex, age, CAD diagnosis, BMI, eGFR, *smoke*, and lipid-lowering therapies (standardized β -coefficient=0.158, $P=0.011$ – Table 3). Taking into account that Apo C-III levels correlated with all plasma lipid parameters (Supplemental Table 1), a further regression model including all plasma lipids and apolipoproteins was performed and Apo C-III remained associated with FVIIa-AT also by this analysis (standardized β -coefficient=0.209, $P=0.004$). The strong correlation between Apo C-III and FVIIa-AT was confirmed in the subgroup of subjects not taking lipid-lowering therapies at enrolment ($n=497$; $R=0.251$, $P=1.3\times 10^{-8}$), even in full-adjusted regression model (standardized β -coefficient=0.183, $P=0.007$). Considering the potential influence of smoke on both TRLs metabolism and TF-FVIIa pathway, analyses according to smoking status

were also performed. The strong direct correlation between Apo C-III and FVIIa-AT plasma levels was confirmed in both smokers ($R=0.221$, $P=7.0\times 10^{-6}$) and non-smokers ($R=0.269$, $P=3.6\times 10^{-5}$).

Within the subgroup of subjects with available data of FVIIa ($n=291$), Apo C-III plasma concentration correlated directly also with FVIIa levels ($R=0.291$, $P=4.4\times 10^{-7}$). In an adjusted regression model both Apo C-III (standardized β -coefficient= 0.178 , $P=0.001$) and FVIIa (standardized β -coefficient= 0.479 , $P<0.001$) remained significant predictors of FVIIa-AT variability (Supplemental Table 2).

Stratifying the study population on the basis of Apo C-III plasma concentration, FVIIa-AT levels increased progressively from the lowest to the highest quartile (Figure 2 and Supplemental Table 3) and such trend was confirmed in all the main subgroup analyses (Figure 2), including those in smoker and non-smoker subpopulations (Supplemental Figure 2). As expected, subjects with high Apo C-III levels had an unfavorable lipid profile (Supplemental Table 3).

Considering the strong direct correlation of Apo C-III with triglycerides ($R=0.559$, $P=2.6\times 10^{-54}$) and Apo E ($R=0.290$, $P=2.2\times 10^{-14}$), which are all parameters characterizing TRLs, we performed a further analysis stratifying the study population on the basis of triglycerides, Apo C-III, and Apo E median values. All the subgroups having Apo C-III above the median plasma concentration had increased levels of FVII-AT rather than those with low Apo C-III, independent of both triglycerides and Apo E plasma concentration (Figure 3).

We investigated also genetic components supporting the association between Apo C-III and FVII-AT. Within the study population 540 subjects were genotyped for rs964184 polymorphism, which tags *APOA5-APOA4-APOC3-APOA1* locus and has been linked by GWAS with both dyslipidemia and CAD. The genotype distribution was consistent with Hardy-Weinberg equilibrium. As expected, the carriers of the minor allele (G) were more represented among CAD and had an unfavorable lipid

profile (Table 4). While no significant difference was found for Apo A-I, Apo B, and Apo E, the carriers of the G allele had higher Apo C-III plasma concentration and also higher FVIIa-AT levels (Figure 4). Within the genotyped population data on FVIIa data were available for 253 subjects and G allele carriers had increased levels of FVIIa (Table 4). On the other hand, *APOE* $\epsilon 2/\epsilon 3/\epsilon 4$ genotypes were not related with FVIIa-AT levels, while – as expected – the associations with Apo E, triglycerides, and VLDL cholesterol were confirmed (Supplemental Table 4).

DISCUSSION

In the present study we show that i) Apo C-III has the strongest association with FVIIa-AT among plasma lipid parameters, and ii) carriers of the minor allele of rs964184 gene polymorphism, tagging *APOC3* locus and known to influence Apo C-III plasma concentration and cardiovascular risk [24-26], had FVIIa-AT levels higher than non-carriers. Thereby, these results suggest a link between Apo C-III and the TF-related initiation phase of the blood clotting cascade.

Apo C-III is a crucial player of TRLs metabolism and is recognized as a key determinant of cardiovascular risk by correlating with an unfavorable lipoprotein metabolism [30-31]. Apo C-III, synthesized mainly in the liver, resides mostly on the surface of VLDLs, but also on LDLs and HDLs, and can reduce the clearance of Apo B lipoproteins from the circulation by interfering with their binding to hepatic Apo B/Apo E receptors [32]. Moreover, Apo C-III inhibits the action of lipoprotein lipase to hydrolyze lipoprotein triglycerides. Finally, Apo C-III contributes to the formation of TRLs in liver cells, stimulating the secretion of VLDL [32]. GWAS have identified polymorphisms tagging *APOC3* that strongly associated with both dyslipidemia and CVD, according with the hypothesis of an increased cardiovascular risk mediated by TRLs [24-26]. Loss-of-function mutations of *APOC3* gene have been associated with a decreased risk of CVD paving the way to Apo C-III-lowering therapies as innovative approaches to reduce cardiovascular risk [33].

Volanesorsen – an anti-Apo C-III antisense oligonucleotide – has been demonstrated in phase II clinical trials to be potentially a new powerful tool for the therapy of hypertriglyceridemia [34].

Noteworthy, the mechanisms linking TRLs (including Apo C-III rich lipoproteins) and CVD go beyond the traditional view of atherosclerotic plaque development due to lipid accumulation and may include excessive free fatty acid release and production of proinflammatory cytokines, which in turn could increase TF expression, as well as stimulation of coagulation factors and impairment of fibrinolysis [3].

In this paper we add a further tile to the mosaic of pleiotropic effects of Apo C-III, emphasizing its potential procoagulant capability, which has been previously been addressed by our group [22,23]. We originally showed that increased Apo C-III concentrations correlated with an amplified plasma endogenous thrombin generation [22]. Then, we found that high Apo C-III levels were associated with an increase of factor II (FII) coagulant activity to an extent comparable with the carriership of the A allele of *F2 20210G>A* gene polymorphism [23]. We also observed a direct correlation of Apo C-III plasma concentration with both FVIIa and factor V (FV) coagulant activities. Finally, high levels of Apo C-III were related with an enhanced activated factor X (FXa) generation [23].

The results of the present study, showing a strong direct correlation between Apo C-III and FVIIa-AT, appear consistent with earlier findings (Supplemental Figure 3). The correlation of Apo C-III with FVIIa-AT suggests increased intravascular amounts of TF-FVIIa complex in subjects with high Apo C-III plasma concentrations. An increased exposure of decripted TF could be hypothesized, leading to an enhanced FVII activation and consequently to a greater FXa generation [23]. The extrinsic pathway potentiation would be reflected in an amplified common pathway, resulting into increased FV and FII coagulant activities [23] and thrombin generation [22].

In our previous work both triglycerides and HDL cholesterol correlated directly with FVIIa-AT [17], but in the current analysis such correlations were no longer evident after adjustment for Apo C-III. Apo C-III is expressed on both TRLs and HDLs and recent data support that it may not only characterize the harmful effects of TRLs but also may adversely affect the antiatherogenic properties of HDL [35,36]. This result prompts that Apo C-III may be a key player of procoagulant effects associated to both TRLs and HDLs. Considering all the parameters characterizing TRLs which were available in our study (i.e. triglyceride, Apo C-III, and Apo E), only an elevated Apo C-III concentration was unvaryingly associated with high FVIIa-AT levels. Our results are consistent with previous works showing the procoagulant properties of VLDL, which typically express Apo C-III

[9,10]. Thrombin generation sustained by VLDLs was found to be 19.4-fold greater than that sustained by HDLs and 11.7-fold greater than that sustained by LDLs [9,10]. VLDLs support all the components of the extrinsic coagulation pathway [10] and can both stimulate TF expression and enhance TF-independent FVII activation [37]. VLDLs are functionally characterized by Apo C-III expression. However, it is not known if Apo C-III might participate in VLDL microdomains with distinct local lipid compositions able to support/enhance TF-FVIIa interaction. It may be also speculated that the rise of FVIIa-AT is secondary to the TRLs-related increase of FVIIa levels, whose concentration has been found to be the main determinant of FVIIa-AT in plasma [16]. However, in our analysis the association between Apo C-III and FVIIa-AT was independent of FVIIa, thus suggesting that Apo C-III may influence expression and/or activity of TF rather than merely FVIIa levels. It is worthy to note that in a recent study high FVIIa-AT levels were related with postprandial lipemia [38]. Since postprandial lipemia is characterized by high concentrations of Apo C-III [39], such observation appears consistent with our findings. Moreover, considering the rapid change in levels observed within short time intervals (< 6h) [38], it could suggest involvement of release/activation processes rather than biosynthesis of new TF and FVII molecules.

Our hypothesis of a procoagulant role of Apo C-III is strengthened by the analysis of rs964184 polymorphism, which tags the gene cluster containing the *APOC3* locus and has been linked by GWAS with both dyslipidemia and CAD [24-26]. This variant exerts multiple functional effects on the expression of the gene cluster [40]. The higher levels of FVIIa-AT found in carriers of the risk allele G further supports a link between Apo C-III and extrinsic coagulation pathway. Noteworthy, another key gene variant in TRLs metabolism, i.e. the *APOE* $\epsilon 2/\epsilon 3/\epsilon 4$ polymorphism, did not associate with FVIIa-AT levels, consistently with the analysis of FVIIa-AT on the basis of triglyceride, Apo C-III, and Apo E levels (Figure 3). Up to now GWAS have linked approximately 60

genetic loci to CAD [41]. Although coagulation plays a crucial role in CAD pathophysiology, very few of these GWAS-identified loci have been related with hemostatic processes so far [42,43]. The here reported association suggests that one of the SNPs with the strongest association with CAD identified by GWAS [25], beyond the effects on lipid metabolism, may influence also blood coagulation.

Our results, supporting a connection between Apo C-III/TRLs and prothrombotic diathesis marked by FVIIa-AT, may have implications for both arterial and venous thrombosis. As a matter of fact, high levels of FVIIa-AT have been observed in patients with venous thromboembolism (VTE) [15], hypertriglyceridemia has been also related with VTE [44] and lipid-lowering therapies may reduce VTE risk [45].

It is important to underline some limitations of this work. First, our results may be intrinsically flawed by the cross-sectional design. Statistical association does not mean biological causality and no insights on the possible causal mechanisms linking Apo C-III and coagulation can be inferred. It could be argued that apolipoproteins could remodel the phospholipid distribution in cellular membranes [46,47], which in turn influences TF-FVIIa activity at the cell surface [48-50]. However, our work does not provide any proof about the molecular mechanisms potentially linking Apo C-III and TF-FVIIa interaction. **It could be not excluded that Apo C-III plasma levels may merely alter either directly or indirectly the clearance of FVIIa-AT complex (e.g. by interfering with hepatocyte-related clearance), without any significant effects on TF-FVIIa interaction. Nonetheless, the present result is consistent with previous works [22,23] demonstrating that high Apo C-III concentrations are associated with an increased activation of extrinsic and common pathways of coagulation cascade, as shown in Supplemental Figure 3. Therefore, the whole of these observations may suggest a link between Apo C-III and procoagulant diathesis, more than an isolated influence on the mechanisms of FVIIa-AT complex clearance.** As further limitations, the

population sample was limited and the polymorphisms for genetic analysis were selected on the basis of *a priori* hypothesis (i.e. gene variants known to influence the levels of apolipoproteins and plasma lipids). It should be noted that a recent GWAS identifies only F7 promoter region and PROC1R gene as loci associated with circulating levels of FVIIa-AT [18]. Finally, we recognize the lack of some laboratory parameters, like other apolipoproteins which are known to be involved in TRLs metabolism (e.g. Apo C-II and Apo A-V).

In summary, our results indicate a strong association between Apo C-III and FVIIa-AT, thereby suggesting that an increased high Apo C-III concentration may identify subjects with a prothrombotic diathesis characterized by an enhanced TF-FVIIa interaction. Our results should be confirmed by further studies, both investigating the underlying molecular mechanisms and validating our findings in larger populations. Nonetheless, they support the fascinating hypothesis of an additional and clinically relevant link between plasma lipids and coagulation which may pave the way to new approaches in the management of CVD.

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Disclosure of Conflicts of Interests

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

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Table 1: Clinical and laboratory characteristics of the study population, considered as a whole or divided in subgroups with or without coronary artery disease (CAD).

	Total population (n=666)	CAD-free (n=131)	CAD (n=535)	P *
Age (years)	61.1±10.9	60.4±11.9	61.3±10.7	NS
Males (%)	75.4	69.5	76.8	NS
BMI (kg/m²)	26.6±3.8	26.0±3.6	26.8±3.8	0.042
Diabetes (%)	19.0	8.1	21.6	0.001
Hypertension (%)	64.1	45.3	68.7	<0.001
Smoke (%)	63.8	43.7	68.5	<0.001
eGFR (mL/min) #	73.7±19.9	74.2±21.0	73.5±19.7	NS
hs-CRP (mg/L)	4.23 (3.78-4.73)	2.33 (1.84-2.94)	4.98 (4.40-5.64)	<0.001
Lipid-lowering therapy (%)	25.4	8.4	29.5	<0.001
Total cholesterol (mmol/L)	5.18±1.10	5.21±1.04	5.17±1.11	NS
LDL cholesterol (mmol/L)	3.41±0.94	3.34±0.95	3.42±0.93	NS
HDL cholesterol (mmol/L)	1.20±0.33	1.38±0.41	1.16±0.29	<0.001
VLDL cholesterol (mmol/L)	0.67 (0.65-0.69)	0.54 (0.50-0.58)	0.71 (0.68-0.73)	<0.001
Triglyceride (mmol/L)	1.58 (1.53-1.64)	1.28 (1.19-1.36)	1.67 (1.61-1.73)	<0.001
Apo A-I (g/L)	1.28±0.26	1.36±0.30	1.26±0.25	<0.001
Apo B (g/L)	1.04±0.28	1.01±0.25	1.05±0.28	NS
Apo C-III (mg/dL)	10.5 (10.2-10.8)	9.9 (9.3-10.5)	10.6 (10.3-11.0)	0.038
Apo E (g/L)	0.037 (0.035-0.038)	0.038 (0.035-0.039)	0.036 (0.035-0.037)	NS
FVIIa-AT (pM)	84.7 (81.5-88.0)	85.2 (77.4-93.9)	84.5 (81.1-88.1)	NS
FVIIa (mU/mL) §	46.9 (43.8-50.1)	46.1 (40.0-53.0)	47.2 (43.7-50.9)	NS

* by t-test or χ^2 -test, when appropriate

by MDRD formula

§ data were available for 291 subjects (76 CAD-free and 215 CAD)

Table 2: Correlations between activated factor VII-antithrombin complex (FVIIa-AT) and plasma lipids and apolipoproteins, in the whole study population and in the different subgroups on the basis of coronary artery disease (CAD) diagnosis and gender. Significant correlations are reported in bold type.

	Whole Study Population (n=666)		CAD-free (n=131)		CAD (n=535)		Males (n=502)		Females (n=164)	
	R	P	R	P	R	P	R	P	R	P
Total cholesterol	0.081	0.041	0.234	0.008	0.040	NS	0.098	0.030	-0.006	NS
LDL cholesterol	0.040	NS	0.161	NS	0.008	NS	0.050	NS	0.028	NS
HDL cholesterol	0.117	0.005	0.170	NS	0.091	0.049	0.101	0.034	0.035	NS
VLDL cholesterol	0.058	NS	0.145	NS	0.042	NS	0.045	NS	0.122	NS
Triglyceride	0.110	0.005	0.218	0.014	0.091	0.038	0.120	0.008	0.121	NS
Apo A-I	0.158	<0.001	0.132	NS	0.168	<0.001	0.183	<0.001	0.035	NS
Apo B	-0.020	NS	0.107	NS	-0.023	NS	-0.021	NS	0.009	NS
Apo C-III	0.235	<0.001	0.336	<0.001	0.211	<0.001	0.244	<0.001	0.187	0.016
Apo E	0.125	0.001	0.090	NS	0.135	0.002	0.161	<0.001	-0.024	NS

Table 3: Linear regression analysis for variability of activated factor VII-antithrombin complex (FVIIa-AT) plasma levels. FVIIa-AT was considered as dependent variable, while all plasma lipids and apolipoproteins showing a significant correlation at univariate analysis were included as independent variables (Model 1). The regression model was then adjusted for sex, age, CAD diagnosis, BMI, renal function, and lipid-lowering therapies at enrollment (Model 2). Significant associations are reported in bold type.

	Model 1		Model 2	
	Standardized β coefficient	P	Standardized β coefficient	P
Total cholesterol	-0.087	0.076	-0.060	0.264
HDL cholesterol	0.108	0.113	0.065	0.395
Triglyceride	0.048	0.426	0.047	0.465
Apo A-I	0.053	0.422	0.114	0.108
Apo C-III	0.200	0.001	0.158	0.011
Apo E	0.066	0.135	0.028	0.550

Model 1: total cholesterol, HDL cholesterol, triglyceride, Apo A-I, Apo C-III, and Apo E as independent variables

Model 2: Model 1 plus sex, age, CAD diagnosis, BMI, eGFR, **smoke**, lipid-lowering therapies at enrollment as independent variables

Table 4: Clinical and laboratory characteristics, including plasma lipids and activated factor VII-antithrombin complex (FVIIa-AT) levels, according rs964184 genotype (data available for 540 subjects).

rs964184 polymorphism				
	CC (n=400)	CG (n=127)	GG (n=13)	P *
Age (years)	60.5±11.2	62.1±9.5	65.0±10.0	NS
Males (%)	76.3	76.4	76.9	NS
CAD diagnosis (%)	80.3	86.6	92.3	0.058
BMI (kg/m²)	26.5±3.7	26.9±3.9	26.5±5.3	NS
Lipid-lowering therapy (%)	24.8	28.3	23.1	NS
Total cholesterol (mmol/L)	5.19±0.98	5.24±1.35	5.25±1.02	NS
LDL cholesterol (mmol/L)	3.34±0.85	3.69±1.10	3.54±0.79	0.007
HDL cholesterol (mmol/L)	1.20±0.33	1.17±0.27	1.15±0.20	NS
VLDL cholesterol (mmol/L)	0.66 (0.63-0.69)	0.71 (0.66-0.76)	0.89 (0.72-1.11)	0.011
Triglyceride (mmol/L)	1.57 (1.50-1.64)	1.69 (1.57-1.82)	2.18 (1.85-2.57)	0.005
Apo A-I (g/L)	1.26±0.26	1.26±0.26	1.27±0.22	NS
Apo B (g/L)	1.05±0.26	1.05±0.32	1.02±0.35	NS
Apo C-III (mg/dL)	10.5 (10.1-10.8)	10.8 (10.1-11.5)	13.9 (11.6-16.7)	0.033
Apo E (g/L)	0.036 (0.035-0.038)	0.036 (0.034-0.038)	0.038 (0.028-0.052)	NS
FVIIa-AT (pM)	81.4 (77.7-85.4)	89.9 (83.0-97.4)	97.7 (75.7-126.0)	0.018
FVIIa (mU/mL) #	43.8 (39.9-48.0)	53.4 (45.9-62.1)	51.7 (42.0-63.7)	0.042

* : by ANOVA with polynomial contrasts for linear trend or χ^2 for linear trend, when appropriate

: data were available for 253 subjects (CC n=188, CG n=56, GG n=7)

FIGURE LEGENDS

Figure 1: Correlation between plasma concentrations of activated factor VII-antithrombin complex (FVIIa-AT) and apolipoprotein C-III (Apo C-III) in the whole study population.

* : by Pearson's correlation test

Figure 2: Activated factor VII-antithrombin complex (FVIIa-AT) levels according to apolipoprotein C-III (Apo C-III) plasma concentration quartiles in the whole study population, in subjects with or without coronary artery disease (CAD) and in male and female subgroups.

* : by ANOVA with polynomial contrasts for linear trend

Figure 3: Activated factor VII-antithrombin complex (FVIIa-AT) levels according to triglyceride (TG), apolipoprotein C-III (Apo C-III), and apolipoprotein E (Apo E) plasma concentrations.*

* : the whole study population has been stratified according the median levels of TG (1.56 mmol/L), Apo C-III (10.4 mg/dL), and Apo E (0.037 g/L). Therefore, eight subgroups were obtained, each characterized by a combination of high or low plasma concentration of TG, Apo C-III, and Apo E. The horizontal dotted line indicates the median level of FVIIa-AT in the whole study population (78.5 pM).

† : by ANOVA with Tukey's post-hoc comparison

Figure 4: Plasma concentrations of Apolipoprotein C-III (Apo C-III) and activated factor VII-antithrombin complex (FVIIa-AT) according to rs964184 genotypes.