ORIGINAL RESEARCH

Factor II Activity is Similarly Increased in Patients With Elevated Apolipoprotein CIII and in Carriers of the Factor II 20210A Allele

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***Background*—**Few studies have so far investigated the relationship between apolipoprotein CIII (Apo CIII) and coagulation pathway in subjects with or without coronary artery disease (CAD).

***Methods and Results*—**Serum Apo CIII concentrations and plasma coagulant activities of factor II (FII:c), factor V (FV:c), and factor VIII (FVIII:c), and activated factor VII (FVIIa) were analyzed in a total of 933 subjects, with (n=687) or without (n=246) angiographically demonstrated CAD and not taking anticoagulant drugs. Activated factor X (FXa) generation assay was performed on plasma from subgroups of subjects with low and high levels of Apo CIII. A statistical incremental concentration of FII:c, FV:c, and FVIIa levels was observed through the quartiles of Apo CIII distribution in the population considered as a whole. Significant results were confirmed for FII:c in CAD and CAD-free subgroup when separately considered. Subjects within the highest Apo CIII quartile (>12.6 mg/dL) had high FII:c levels not statistically different from those of carriers of 20210A allele (n=40; 4.28%). In a multiple linear model, Apo CIII was the best predictor of FII:c variability, after adjustment for age, gender, plasma lipids, CRP, creatinine, diagnosis, and carriership of 20210A allele. FXa generation was increased and its lag time shortened in plasmas with high Apo CIII levels. However, after thrombin inhibition by hirudin, differences between low and high Apo C-III samples disappeared.

***Conclusions*—**Elevated concentrations of Apo CIII are associated with an increase of thrombin activity to an extent comparable with the carriership of G20210A gene variant and mainly modulating the thrombin generation. **(*J Am Heart Assoc*. 2013;2: e000440 doi: 10.1161/JAHA.113.000440)**

Key Words: apolipoprotein • coagulation/thrombosis • thrombin

After its identification >40 years ago, apolipoprotein CIII (Apo CIII) has been intensively investigated, and evidence has accumulated showing its relevant role in mechanisms – whether related to the lipid metabolism or not – favoring the formation of atherosclerotic lesions.

Apo CIII is associated with high density cholesterol (HDL) and apoliporotein B-containing lipoproteins, but it is mainly an essential constituent of circulating particles rich in triacylglycerol, ie, chylomicrons and very low-density lipoproteins (VLDL).[1] The relative content in Apo CIII influences the catabolic rate of these triglycerides (TG)-rich particles by inhibiting their hydrolysis by lipoprotein lipase and apo E-mediated hepatic uptake, ultimately reducing their removal from blood and favoring fasting and postprandial hypertriglyceridemia.[2,3] Very recently, a prospective study regarding 2 US populations, initially free of coronary artery disease (CAD), has provided evidence that the relative risk for the top versus bottom quintile of basal Apo CIII-rich LDL were greater than those for LDL without Apo CIII, thus suggesting that the risk contribution by LDL actually largely results from the LDLs containing Apo CIII.[4]

Although the association of Apo CIII with atherogenesis has been commonly attributed to lipid mechanisms, in the last years other mechanisms have been also demonstrated, involving direct effects on endothelial cells or more generally on the "inflammatory" aspects of the arteriosclerotic process. Apo CIII has been shown to stimulate the adhesion of monocytes to the endothelial bed[5] by inducing expression of vascular adhesion molecule-1 in vascular cells.[6] HDL particles without Apo CIII are able to reduce monocyte adhesion but HDL particles rich in Apo CIII do not.[6] Moreover, antibodies against Apo CIII, but not antibodies against other Apo Cs or Apo E, impair these proadhesive properties, thus suggesting that the vascular protective role of HDL is specifically affected by Apo CIII.[6] Endothelial dysfunction, often anticipating the

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atherosclerotic damage, also seems to be facilitated. Exposure of vascular endothelial cells to Apo CIII indeed results in the inhibition of insulin-stimulated eNOS activity and NO production.[7]

This body of experimental findings has been confirmed by a number of clinical studies. Variation in the expression of Apo CIII has been associated with cardiovascular risk in several populations, eg, patients with hypertriglyceridaemia,[8,9] diabetes,[10] or metabolic syndrome,[11,12] and, more importantly, with cardiovascular disease both in cross-sectional[13–15] and prospective studies.[4,16–19]

For all these reasons, Apo CIII is currently considered as an important factor in the long-lasting atherosclerotic phase of the cardiovascular disease, whereas its role in late, more acute, thrombotic events has been so far poorly explored. In particular, few studies have examined Apo CIII prospectively in patients already presenting CAD[16–18] in order to unravel a possible unfavorable thrombogenic potential associated with the apolipoprotein.

In recent years, we have demonstrated not only that the serum concentrations of Apo CIII in CAD patients is an independent predictor of future total and cardiovascular mortality, but also that Apo CIII concentrations are associated with an enhanced plasma endogenous thrombin generation, suggesting a complex interplay between Apo CIII-rich particles and hemostatic balance.[19]

Based on these premises, we retrospectively analyzed the relationships between some key coagulation factors and the concentration of Apo CIII in a relatively large case-control study population of CAD and CAD-free patients of whose coagulant activities of factor II (FII:c), factor V (FV:c), factor VIII (FVIII:c), and activated factor VII (FVIIa) had been previously measured. In addition we set up a pilot fluorogenic assay of activated factor X (FXa) generation to provide further elements to interpret the observational results in our study population.

Materials and Methods

Verona Heart Study Cohort

We retrospectively analyzed the folders of 933 unrelated patients, previously recruited in the framework of the Verona Heart Study (VHS) project. Criteria for selection of the study population have been previously described in details.[19,20] Briefly, all subjects were classified as either being affected (n=687) or not affected (n=246, CAD-free) by CAD on the basis of the results of a coronary artery angiography, performed by 2 cardiologists unaware that the patients were participating in the study. Subjects with nonsignificant coronary stenosis (ie, <50%) were excluded from the study. CAD-free patients were examined with coronary angiography for reasons other than possible CAD (mostly valvular heart disease). They were required to have normal coronary arteries as documented by angiography and to have neither history of atherosclerosis nor clinical or laboratory evidence of atherosclerosis in other vascular beds. Because of the aim of the study, patients who were taking an anticoagulant drug at the moment of the recruitment were not considered for the statistical analysis.

At the time of blood sampling, a complete clinical history was collected, including the assessment of cardiovascular risk factors such as obesity, smoking, hypertension, and diabetes. The study was approved by the Ethic Committee of our Institution (Azienda Ospedaliera, Verona). After a full explanation of the study, all of the participants gave their written consent at the moment of enrollment.

Biochemical Analysis

Samples of venous blood were collected from each subject after an overnight fast. Serum lipids, apolipoproteins, and other biochemical routine parameters were determined as previously described.[19,20] In particular, Apo CIII concentration was measured using an automated turbidimetric immunoassay (Wako Pure Chemical Industries). Intraassay coefficient of variation (CV) was 1.84%, 2.02%, and 1.98% on 3 pools of control sera with low, medium, and high concentrations of Apo CIII, respectively; interassay CVs were 4.4%, 3.4%, and 2.29% for low, medium, and high concentration, respectively.[14]

For coagulation analysis, blood was drawn into vacuum tubes containing 0.1 part 0.129 mol/L buffered sodium citrate per 10 parts blood. FII:c, FV:c, and FVIII:c were measured on a Behring Coagulation Timer (BCT, Dade Behring) by modification of the one-stage clotting method with the use of relative deficient plasma (Dade Behring). Coagulation time by BCT was calibrated with standard human plasma (Dade-Behring). The intra-assay and interassay coefficients of variations were <5%. Results of factors activities were expressed in terms of IU/dL.

FVIIa was assayed with a kit utilizing a soluble recombinant truncated tissue factor that is selectively deficient in promoting factor VII activation but retains factor VIIa cofactor function, thus allowing direct quantification of factor VIIa 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 within-run and between-run coefficients of variation were 7.8% and 6.4%, respectively.

Genotype Analysis for FII G20210A Polymorphism

DNA was extracted using standard protocols and genotyping was performed as previously indicated (for details, see ref.21).

Because of the very limited number of homozygous subjects (only 1 subject presented the genotype 20210AA), the statistical calculations were performed categorizing the individuals as carrier or noncarrier of the variant.

Activated Factor X Generation

Activated factor X (FXa) generation in plasma was evaluated by the addition of a specific FXa fluorogenic substrate (Spectrafluor FXa, American Diagnostica). Plasma samples were diluted (1/10) in a HBS buffer (Hepes 20 mmol/L, NaCl 150 mmol/L, PEG-8000 0.1%, pH 7.4) and incubated for 3 minutes at 37°C. The generation of FXa was initiated by addition of a volume mixture of Innovin (Dade Behring) as a source of tissue factor, phospholipid surfaces, and calcium ions. Final concentrations of $CaCl_2$ and FXa fluorogenic substrate were 2.5 mmol/L and 150 μmol/L, respectively. The fluorescence was measured overtime in a fluorimeter (Fluoroskan Ascent BioMed) and the amount of the generated FXa was evaluated using a standard curve with serial dilutions (1/5 to 1/80) of normal pooled human plasma (Hyphen BioMed).

Specific parameters of FXa generation (lag time, peak, time to peak, and area under the curve) were obtained by a nonlinear regression analysis of the first derivative of relative fluorescence units (RFU) using the statistic software GraphPad Prism 5. In order to evaluate lag time, the cut-off threshold was arbitrarily set up to 2.0 RFU. All the experiments were performed in duplicate. The between-run coefficients of variation were 4.9% (lag time), 3.1% (peak), 3.8% (time to peak), and 1.4% (area under the curve).

In order to suppress the contribution of thrombin to FXa generation through the amplification pathway (Figure 1A and 1B), FXa generation was also performed by adding the thrombin-specific inhibitor hirudin[22] (Iketon Farmaceutici). Preliminary kinetic analysis was set up in order to determine the hirudin/thrombin ratio that is able to suppress the thrombin amplification reaction. In inhibitory conditions, the expected inverse correlation between FVIIa levels and lag time in FXa generation was clearly detectable in 10 plasma samples ($r=-0.666$, $P=0.025$). Taking into account the mean prothrombin plasma levels (0.07 to 0.1 mg/mL)[23] hirudin was added at a final concentration of 350 nmol/L, 5-fold higher than the mean prothrombin concentration (70 nmol/L) in the diluted samples (see also Figure 2) to assure a complete inhibition of prothrombinase activity. The substantially reduced FXa generation prevented the proper evaluation of peak, but allowed for the measurement of lag times that were prolonged. For this reason the cutoff threshold, after experimental evaluation of several first derivative values, was arbitrarily set up to 0.1 RFU.

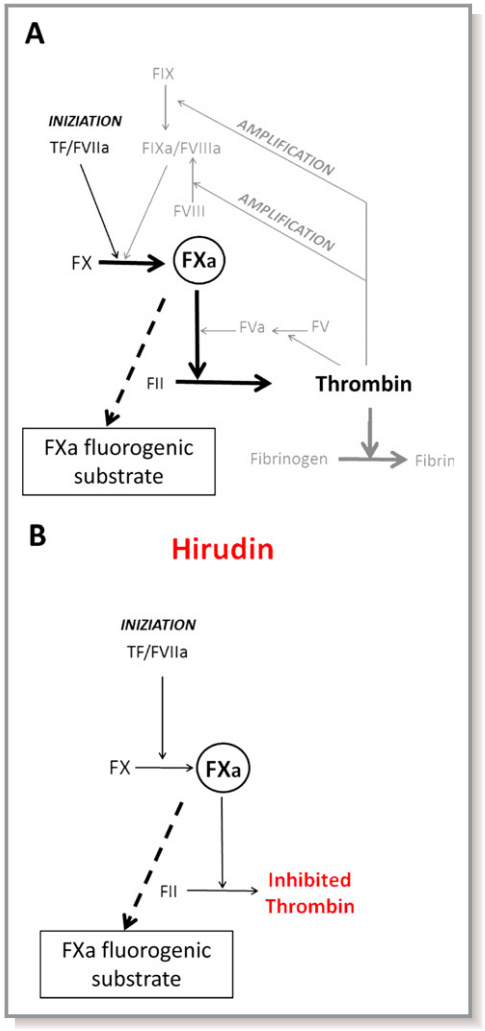

Figure 1. Activated factor X (FXa) generation assay. The generation of FXa in plasma was performed in the absence (A) or in the presence (B) of hirudin, a thrombin specific inhibitor. FXa generated was evaluated by using an FXa specific fluorogenic substrate. FII indicates factor II; FV, factor V; FVIIa, activated factor VII; FVIII, factor VIII; TF, tissue factor.

Statistical Analysis

Calculations were performed with IBM SPSS 20.0 statistical package (IBM Inc). Distributions of continuous variables were expressed as mean±standard deviation. Logarithmic transformation was performed on skewed variables, such as Apo CIII concentration or coagulation factors activities (with the only exception of FVIII, which showed a normal distribution). Thus, for these variables geometric means with 95% confidence intervals (CI) are given. Quantitative data were assessed using the Student *t* test or by ANOVA, with polynomial contrast for linear trend or Tukey post-hoc comparison when indicated. Correlations among quantitative variables were assessed using Pearson's correlation test. Qualitative data were analyzed with the χ^2-test. Linear regression models were performed to assess the independent

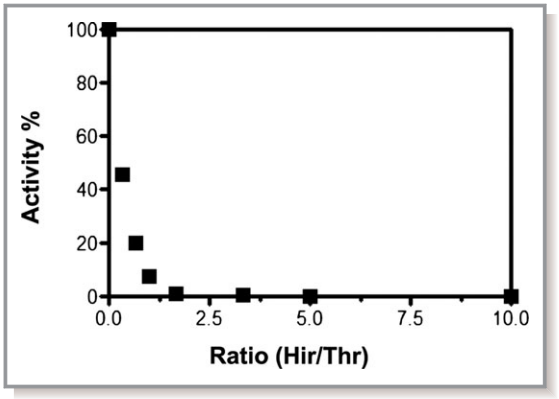

Figure 2. The amplification reactions of the coagulation cascade were suppressed by inhibition of thrombin activity by hirudin. Kinetic analysis was set up in order to determine the hirudin/thrombin ratio able to suppress the thrombin amplification reactions. Increasing concentrations of Hirudin (0 to 700 nmol/L) were mixed with purified thrombin (70 nmol/L) and the enzymatic activity was tested by using a specific thrombin fluorogenic substrate. The open square indicates the concentration used to investigate plasma from patients.

predictors of coagulation factor activity levels and to estimate the relative beta coefficients with 95% CI. Regression models with block entry were performed to provide a full adjustment for potential confounding factors. The results were then checked using regression models with backward stepwise selection of variables (removal if $P>0.10$). A value of $P<0.05$ was considered statistically significant.

Results

The clinical and laboratory characteristics of the study population, including lipid and coagulation parameters, subdivided in CAD (n=687) and CAD-free (n=246) subjects are summarized in Table 1. Patients affected by CAD presented obviously a greater number of risk factors and, importantly, more elevated levels of both Apo CIII concentrations and FII:c, FV:c, and FVIII:c levels as compared with CAD-free subjects (Table 1).

In the whole study population, the concentrations of Apo CIII were statistically correlated with the values of FII:c

Table 1. Clinical and Laboratory Characteristics of the Study Population Subdivided in CAD and CAD-Free Groups

	CAD-Free Patients, n=246	CAD Patients, n=687	*P* Value*
Age, y	57.5±13.4	60.6±9.4	<0.001
Male sex, %	71.0	81.0	0.001
History of smoke, %	43.3	68.9	<0.001
Hypertension, %	34.0	63.4	<0.001
Diabetes, %	6.5	17.1	<0.001
BMI, kg/m^2	25.5±3.5	26.7±3.3	<0.001
Creatinine, mmol/L†	89.2 (86.6 to 91.8)	94.7 (92.9 to 96.5)	0.001
hs-CRP, mg/L†	1.79 (1.53 to 2.09)	3.32 (3.04 to 3.63)	<0.001
Cholesterol, mmol/L	5.51±1.04	5.73±1.11	0.010
LDL-cholesterol, mmol/L	3.55±0.90	3.83±0.97	<0.001
HDL-cholesterol, mmol/L	1.44±0.42	1.21±0.30	<0.001
Triglycerides, mmol/L†	1.33 (1.27 to 1.40)	1.72 (1.66 to 1.78)	<0.001
Apo A, g/L	1.43±0.28	1.29±0.23	<0.001
Apo B, g/L	1.06±0.25	1.19±0.30	<0.001
Apo C-III, mg/dL†	10.2 (9.8 to 10.6)	11.4 (11.1 to 11.7)	<0.001
Apo E, g/L†	0.040 (0.038 to 0.042)	0.042 (0.041 to 0.043)	0.029
Statin therapy, %	3.2	28.4	<0.001
FII:c, IU/dL†	117 (113 to 122)	126 (124 to 128)	<0.001
FV:c, IU/dL†	126 (121 to 130)	134 (131 to 136)	0.002
FVIII:c, IU/dL	150±48	171±55	<0.001
FVIIa, mU/mL†	41.4 (38.0 to 45.1)	44.2 (42.3 to 46.3)	0.148

Apo indicates apolipoprotein; BMI, body mass index; CAD, coronary artery disease; FII:c, FV:c, and FVIII:c, coagulant activities of factor II, factor V, and factor VIII, respectively; FVIIa, activated factor VII; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein.

*By t test or χ^2-test.

†Variables with a skewed distribution (ie, creatinine, hs-CRP, triglycerides, Apo C-III, Apo E, FII:c, FV:c, and FVIIa) were log-transformed and then presented as geometric means with 95% confidence intervals.

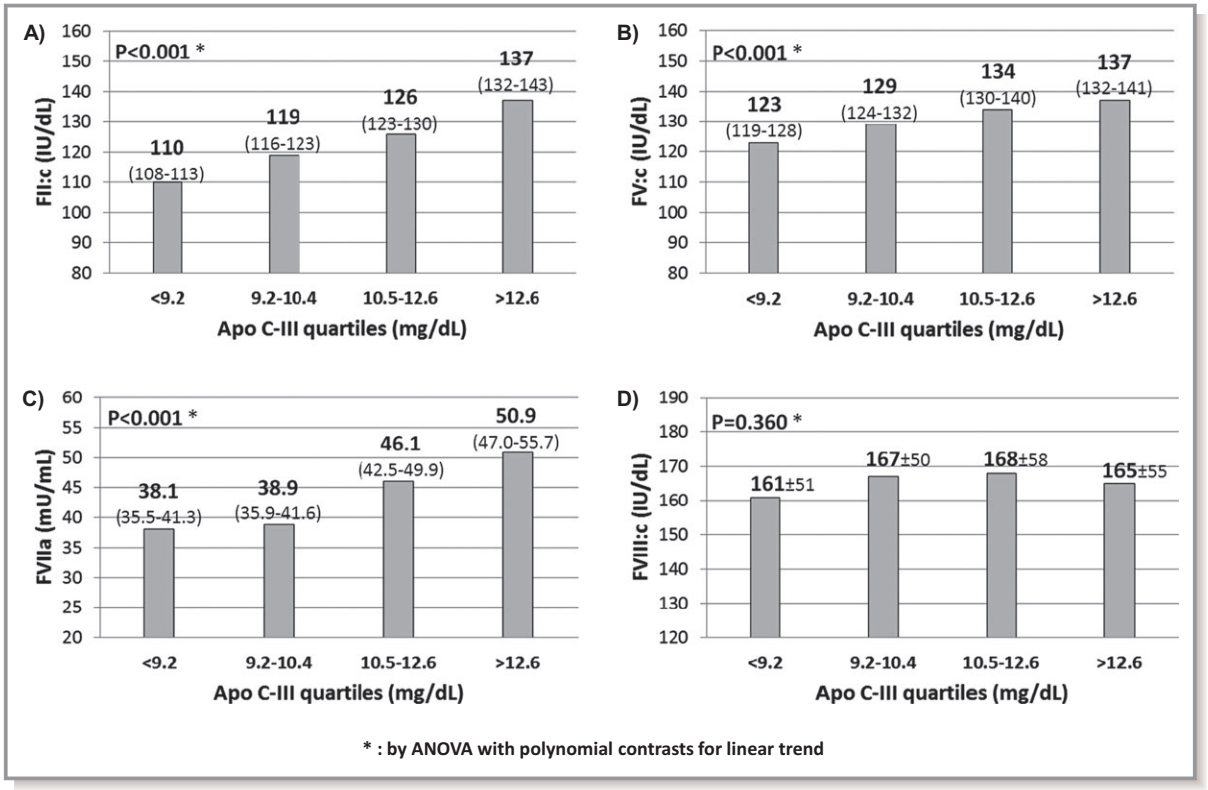

Figure 3. Distribution of coagulation factors activities (A) FII:c; (B) FV:c; (C) FVIIa; (D) FVIII:c according to apolipoprotein C-III (Apo C-III) quartiles. ANOVA indicates analysis of variance.

(R=0.303, P<0.001), FV:c (R=0.120, P<0.001), and FVIIa (R=0.203, P<0.001), but not with those of FVIII:c (R=0.017, NS). It is important to note that no statistical correlations were found between coagulation factors and Apo AI, Apo B, or plasma lipids (such as total, LDL and HDL cholesterol, or TG) (data not shown).

The correlation between Apo CIII concentrations and FII:c appeared particularly strong and the statistical significance was confirmed in either CAD or CAD-free subgroups, separately considered (R=0.327, P<0.001 for CAD group; R=0.213, P<0.001 for CAD-free group). Accordingly, as shown in Figure 3A, a linear statistical increase of FII:c was observed across Apo CIII quartiles with the highest levels in the subjects within the upper Apo CIII quartile (P<0.001 by ANOVA with polynomial contrast for linear trend). Similar findings were obtained within both CAD and CAD-free subgroups (data not shown). A statistically significant increase in the levels of both FV:c and FVIIa (P<0.001 for both), but not of FVIII:c (P=0.360), was also observed across the quartiles of Apo CIII in the whole study population (Figure 3B through D).

Finally, linear regression models were performed to assess the extent to which the different coagulation activities were associated with Apo CIII concentration. As shown in Table 2, Apo CIII was an independent predictor of FII:c variability in a multiple-adjusted model, with a β coefficient higher than most of those of the other variables in the model and similar to that of the carriership of FII 20210A allele. This result was

Table 2. Linear Regression Model for Factor II Coagulant Activity (FII:c)

Model for FII:c (n=933)	Beta-Coefficient With 95% CI	*P* Value
ln Apo C-III	0.206 (0.122 to 0.290)	<0.001
ln triglyceride	0.020 (−0.043 to 0.082)	0.542
LDL-cholesterol	0.021 (0.003 to 0.039)	0.022
HDL-cholesterol	0.035 (−0.025 to 0.094)	0.257
Sex (female)	0.036 (−0.008 to 0.081)	0.108
Age	−0.003 (−0.005 to −0.002)	<0.001
BMI	−0.003 (−0.008 to 0.002)	0.252
CAD diagnosis	0.134 (0.055 to 0.212)	0.001
CAD severity*	−0.030 (−0.057 to −0.003)	0.030
FII 20120G>A carriership	0.214 (0.140 to 0.288)	<0.001
ln CRP	0.020 (0.006 to 0.035)	0.006
ln creatinine	−0.039 (−0.113 to 0.035)	0.303

Apo indicates apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Number of affected coronary vessels.

Table 3. Linear Regression Model for Activated Factor VII (FVIIa)

Model for FVIIa (n=933)	Beta-Coefficient With 95% CI	*P* Value
ln Apo C-III	0.393 (0.185 to 0.600)	<0.001
ln triglyceride	−0.105 (−0.260 to 0.050)	0.185
LDL-cholesterol	0.040 (−0.004 to 0.084)	0.076
HDL-cholesterol	0.190 (0.041 to 0.339)	0.013
Sex, female	0.143 (0.034 to 0.251)	0.010
Age	0.001 (−0.003 to 0.005)	0.609
BMI	0.006 (−0.007 to 0.018)	0.373
CAD diagnosis	−0.053 (−0.249 to 0.144)	0.599
CAD severity*	0.043 (−0.024 to 0.110)	0.213
ln CRP	−0.018 (−0.054 to 0.018)	0.332
ln creatinine	0.096 (−0.089 to 0.282)	0.309

Apo indicates apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Number of affected coronary vessels.

confirmed by regression models with backward stepwise selection of variables, where Apo CIII levels remained an independent predictor of FII:c levels (β coefficient 0.216 with 95% CI 0.161 to 0.272; $P<0.001$) Apo CIII was also an independent predictor of FVIIa variability (Table 3, also confirmed by regression models with backward stepwise selection of variables: β coefficient 0.340 with 95% CI 0.204 to 0.475; $P<0.001$), while the statistical significance of the association with FV:c was lost after multiple adjustment in the regression model (Table 4). All these results were also

Table 4. Linear Regression Model for Factor V Coagulant Activity (FV:c)

Model for FV:c (n=933)	Beta-Coefficient With 95% CI	*P* Value
ln Apo C-III	0.067 (−0.027 to 0.161)	0.161
ln triglyceride	0.035 (−0.035 to 0.105)	0.324
LDL-cholesterol	0.007 (−0.013 to 0.027)	0.497
HDL-cholesterol	0.116 (0.048 to 0.183)	0.001
Sex, female	0.017 (−0.032 to 0.066)	0.497
Age	0.002 (−0.004 to 0.007)	0.075
BMI	0.002 (−0.004 to 0.007)	0.553
CAD diagnosis	0.104 (0.015 to 0.192)	0.022
CAD severity*	−0.011 (−0.041 to 0.019)	0.472
ln CRP	0.020 (0.004 to 0.036)	0.016
ln creatinine	−0.070 (−0.154 to 0.013)	0.100

Apo indicates apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Number of affected coronary vessels.

Table 5. FXa Generation in Subjects With Very Low and Very High Levels of Apo C-III

	Apo C-III group		
	Low (n=19) Mean Level 6.2 mg/dL (Min-Max 5.0 to 7.5 mg/dL)	High (n=17) Mean Level 20.8 mg/dL (Min-Max 16.9 to 24.1 mg/dL)	*P* Value
Lag time, s	132±6	113±6	0.038
Time to peak, s	275±41	248±56	0.103
Peak, RFU	25.6±3.8	33.1±10.2	0.005
AUC, RFU×s	3863±170	4140±247	<0.001

Apo indicates apolipoprotein; AUC, area under curve; FXa, activated factor X; RFU, relative fluorescence units.

confirmed after excluding subjects who were taking statin therapy (data not shown).

Because the concentrations of Apo CIII were statistically correlated not only with FII:c levels, but also with the values of FVIIa, we set up an appropriate assay to highlight the contribution of FVIIa in plasma through the FXa generation. This assay was performed in 2 well selected subgroups of individuals, representative of very low (6.2±0.7 mg/dL; n=19) and very high (20.8±2.2 mg/dL; n=17) Apo C-III plasma concentrations. The FXa generation assay was specifically tuned by using modest amounts of thromboplastin (lipids and tissue factor) to favor the detection of the potential contribution of Apo CIII components in triggering the coagulation cascade. The results showed a significantly higher FXa generation at the highest concentrations of Apo CIII (Table 5), with a definitely higher peak and area under curve. The lag time was also shorter in the highest Apo CIII level group, albeit the statistical evidence was less pronounced (lag time between low and high Apo CIII groups 132±6 s and 113±6 s, respectively, $P=0.038$). Because lag time is particularly associated with the levels of FVIIa[24] we performed additional assays to highlight its role by suppressing the contribution of thrombin by the positive feedback of the amplification pathway (Figure 1). Therefore, better evaluation of the activation of FX by the FVIIa/TF complex in the FXa generation in plasma was achieved by adding hirudin, a potent natural inhibitor of thrombin. It is worth noting that at the selected hirudin concentration we observed the expected inverse correlation between lag time in FXa generation and FVIIa levels. Noticeably, after thrombin inhibition by hirudin, the differences between low and high Apo C-III groups observed for lag time values completely disappeared (lag time 201±17 s and 197±25 s, respectively, $P=0.883$, Figure 4).

Overall, from a quantitative point of view, these results suggested FII:c as the coagulative parameter more strongly

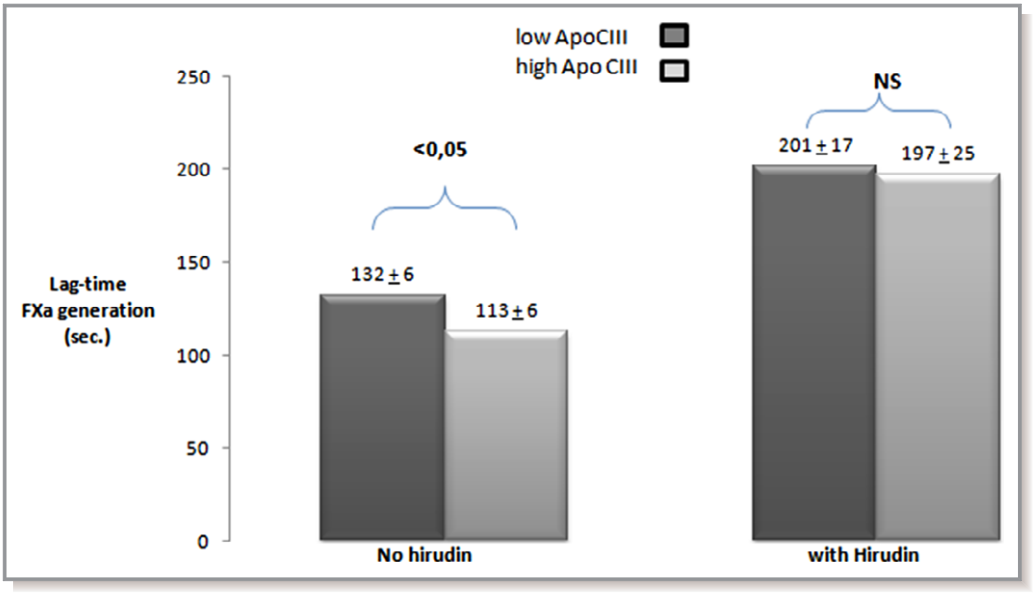

Figure 4. FXa generation lag time in low and high Apo CIII samples, with and without hirudin-mediated inhibition of thrombin activity (see also Figures 1 and 2). Apo C-III indicates apolipoprotein C-III; FXa, activated factor X; NS, not significant.

associated with Apo CIII. Moreover, FXa generation experiments (with and without hirudin) suggested that this association was poorly mediated by the upstream activation of FVIIa/TF complex. In accordance with this result, Apo CIII remained an independent predictor of FII:c variability in a further linear regression model also adjusted for FVIIa levels, as well as for the other assessed coagulant activities (Table 6, also confirmed by regression models with backward stepwise selection of variables: β coefficient 0.196 with 95% CI 0.142 to 0.250; $P<0.001$).

As shown in Table 2, the regression analyses indicated a similar (or noninferior) extent of association with FII:c of elevated concentrations of Apo CIII and FII 20210G>A polymorphism, ie, the most important genetic determinant of FII:c variability.[25,26] To further verify this hypothesis, we specifically analyzed the combined effects of FII 20210G>A polymorphism and Apo CIII concentration in determining FII:c levels. On the whole population, 40 individuals (4.28%) carried at least 1 FII 20210A allele (39 heterozygous carriers and 1 homozygous carrier). They did not differ from noncarriers for all lipid parameters and coagulation factors (data not shown) with the only remarkable difference of FII:c levels, that, as expected, were much more elevated in carriers than in noncarriers (153 with 95% CI 140 to 166 IU/dL versus 122 with 95% CI 120 to 124 IU/dL, respectively, $P<0.001$).

By evaluating FII:c levels according to both Apo CIII concentration and FII 20210A carriership, an additive effect was observed. The lowest levels were observed in noncarriers within the lowest Apo CIII quartile and the highest in carriers within the highest Apo CIII quartile (Figure 5A $P<0.001$ by ANOVA with polynomial contrast for linear trend). Interestingly, noncarriers within the highest Apo CIII quartile had yet lower FII:c levels than the carriers within the highest Apo CIII quartile ($P=0.020$ by ANOVA with Tukey post-hoc comparison;

Table 6. Linear Regression Models for Factor II Coagulant Activity, Including FVIIa, FV:c, and FVIII:c as Independent Variables

Model for FII:c (n=933)	Beta-Coefficient With 95% CI	*P* Value
ln Apo C-III	0.192 (0.109 to 0.274)	<0.001
ln triglyceride	0.012 (−0.049 to 0.073)	0.699
LDL-cholesterol	0.020 (0.003 to 0.038)	0.025
HDL-cholesterol	0.011 (−0.048 to 0.070)	0.724
ln FVIIa	0.001 (−0.028 to 0.030)	0.940
ln FV:c	0.205 (0.139 to 0.271)	<0.001
FVIII:c	0.0002 (−0.0001 to 0.001)	0.333
Sex (female)	0.031 (−0.013 to 0.075)	0.171
Age	−0.004 (−0.005 to −0.002)	<0.001
BMI	−0.003 (−0.008 to 0.002)	0.207
CAD diagnosis	0.107 (0.030 to 0.185)	0.007
CAD severity*	−0.026 (−0.053 to 0.000)	0.050
FII 20120G>A carriership	0.228 (0.155 to 0.301)	<0.001
ln CRP	0.015 (0.001 to 0.029)	0.045
ln creatinine	−0.026 (−0.099 to 0.047)	0.482

Apo indicates apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CI, confidence interval; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Number of affected coronary vessels.

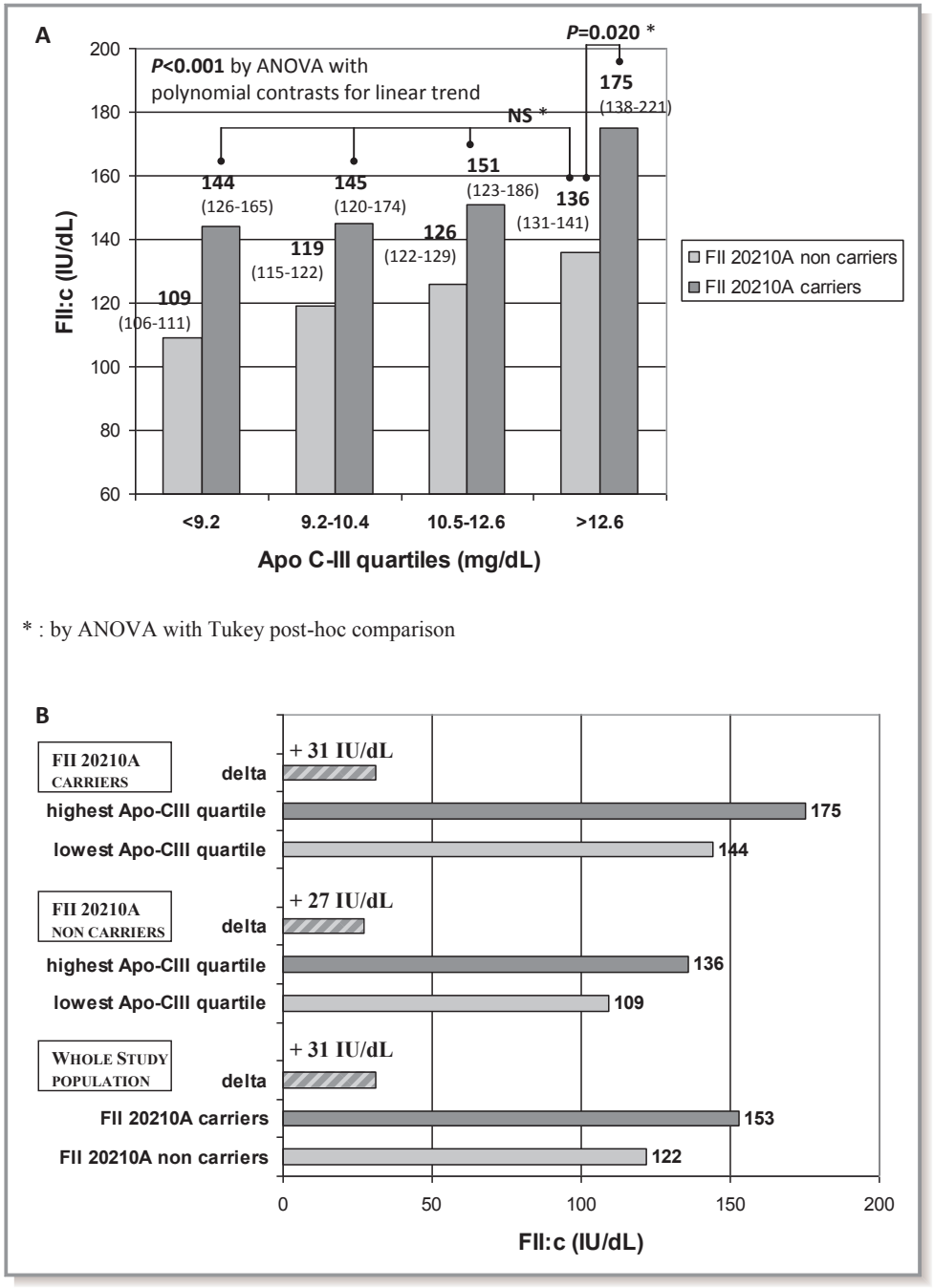

Figure 5. Coagulation factor II activity levels (FII:c) according to FII 20210G>A genotype and Apo C-III concentration quartiles (A). The increase of FII:c associated with FII 20210A carriership was quantitatively analogous to that observed comparing the highest to the lowest quartile of Apo C-III concentration in either carriers or noncarriers of the gene variant (B). Apo C-III indicates apolipoprotein C-III; ANOVA, analysis of variance.

Figure 5A), but no significant differences in FII:c levels as compared with the carriers within the 3 other quartiles ($P>0.5$ by ANOVA with Tukey post-hoc comparison; Figure 5A). More in detail, the increase of FII:c associated with the carriership of FII G20210A variant (ie, about +30 IU/dL) was very similar to that observed by comparing the highest to the lowest quartile of Apo C-III concentration in either carriers or noncarriers (Figure 5B).

Discussion

We have previously demonstrated that Apo CIII is an independent predictor of future cardiovascular mortality in patients already presenting CAD, and that Apo CIII concentration is associated with an enhanced plasma endogenous thrombin generation.[19] The results of the present work, (1) represent the accomplishment of the previous research by

providing evidence that increased Apo CIII concentrations significantly alter the hemostatic balance in a procoagulant way in both patients with and without angiographically demonstrated CAD; (2) in a noteworthy way indicate that this change is quantitatively comparable to that associated with the carriership of FII G20210A gene variant; and (3) report evidence in favor of the effect of Apo CIII concentration on the final part of the coagulation cascade.

In a relatively large population of CAD and CAD-free subjects, the coagulant activities of FVII, FV, and FII were indeed consistently increased in the presence of growing Apo CIII concentrations (Figure 3). Importantly, for FII:c this relationship was particularly strong, as well as in CAD-free patients. Taking into account that the atherosclerostic process is associated with a detectable activation of hemostatic system,[27–29] the results obtained in CAD-free subgroup are of relevance in supporting FII activation as an "atherosclerosis-independent" effect potentially induced by Apo CIII.

Apo CIII levels were previously demonstrated to be positively associated with endogenous thrombin potential,[19] suggesting a complex interplay between TG-rich particles and the coagulation cascade. Thrombin generation occurs in the terminal part of the coagulation cascade, and thus depends on a number of factors, including the levels of several coagulation factors, which in turn are determined by inherited and acquired components. Because the concentrations of Apo CIII were statistically correlated with the values of FVIIa, the Apo CIII-mediated effect on FII:c could be the result of the upstream formation of FXa by the activation of FVII/TF. For this reason, we set up an appropriate assay to highlight the contribution of FVIIa in plasma through the FXa generation. In our experimental condition, the FXa generation assay was specifically tuned by using modest amounts of thromboplastin (lipids and tissue factor) to favor the detection of the potential contribution of Apo CIII, fixed at low and high concentrations by an appropriate selection of the patients. The results showed a significantly higher FXa generation at the highest concentration of Apo CIII, confirming a procoagulant role of Apo CIII in terms of kinetics and concentration also on this essential intermediate step of the coagulation cascade. These data substantially strengthen the information previously obtained with a commercial thrombin generation chromogenic assay by using a well reproducible and sensitive fluorogenic assay.[19] Although the lag time of the FXa generation assay is strongly influenced by the FVIIa activity, this sensitive parameter also inversely reflects the combined effect on the production of FXa by the positive feedback of thrombin.[30] Thus, in the presence of a powerful thrombin inhibitor such as hirudin, the contribution of the FVIIa to the lag time should be highlighted. In our experiments with low or high Apo CIII patients, the previously observed difference for lag time between these 2 groups disappeared in the presence of hirudin, suggesting a direct role of the apolipoprotein on the thrombin-mediated, rather than FVIIa/TF-mediated, FXa generation. Interpretation of the present FXa generation experiments and previous data on endogenous thrombin potential[19] indicates Apo CIII as a potential modulator of the prothrombinase activity. Taking into account the pivotal role of thrombin in several biological pathways, beyond the sole blood coagulation, and its major role as a pathophysiological mediator bridging inflammation and clotting,[29] our experimental findings strongly support the hypothesis about thrombin-mediated effects of elevated Apo CIII levels.

The statistical association between Apo CIII and FII:c was also confirmed by linear regression analysis by which Apo CIII was the strongest determinant of FII:c among several variables including the carriership of prothrombin G20210A gene variant, a well recognized hereditary risk factor for venous thrombosis (VT) in turn associated with incremented FII:c values.[25,26] The effects of the G20210A carriership and elevated Apo CIII concentrations were additive on FII:c levels, with the greatest coagulant activity in carriers of the prothrombin mutation and Apo CIII concentrations in the highest quartile (Figure 5A). Interestingly, the separate effects on FII:c were statistically comparable for both these conditions. Individuals who were not carrying the gene variant but were within the highest Apo CIII quartile (>12.6 mg/dL) had no significantly different FII:c levels compared with carriers within the 3 other quartiles of apolipoprotein (Figure 5A). On the quantitative point of view, the increase of FII:c associated with the G20210A variant (about +30 IU/dL) was very similar to that observed by comparing the highest to the lowest quartile of Apo C-III concentration in either carriers or noncarriers (Figure 5B). In terms of procoagulant diathesis, these data suggest a functional equivalence for both FII G20210A gene mutation, genetically acting on the improved biosynthesis of prothrombin, and elevated concentrations of Apo CIII, improving thrombin generation activity. Moreover, these effects might well add each other as clearly inferred by comparison of quartiles and genotypes in Figure 5, potentially having pathophysiological implications albeit for small groups of subjects.

To the best of our knowledge this is the first description of a link between Apo CIII concentrations and FII:c levels. Moreover, it has never been reported that very high values of Apo CIII may be functionally equivalent to the FII G20210A gene variant as regards of FII activation. Of note, we did not observe any statistically significant relationships with other lipid compounds in particular with TG, despite the procoagulant role described for TG-rich lipoproteins in the past.[31–34] It is likely that only a selected fraction of TG-containing particles, ie, those rich in Apo CIII, are responsible for such procoagulant activity. Thus, the assessment of Apo CIII levels may represent a more direct tool to evaluate the plasma

lipid-related component of procoagulant diathesis. In this perspective, as a limitation of the study, the present data concerning total Apo CIII may underestimate the phenomenon as compared with the analysis of fractionated VLDL or LDL particles containing Apo CIII. This aspect may also explain the inconsistencies of the literature regarding TG-rich lipoproteins and procoagulant activity.

In any event, the main suggestion arising from our results is that the role of Apo CIII is probably multifaceted, and certainly more complex than previously expected. There is no doubt that until now the scientific interest has been exclusively focused on the lipid-related atherogenic and proinflammatory features of this apolipoprotein.[35] Indirect evidences from a few clinical studies regarding the thrombotic complications of CAD in the setting of secondary prevention[16–19] and, more directly, the results presented here extend the previous view and give support to an additional procoagulant role of Apo CIII. On such basis, Apo CIII may act potentially as a pleiotropically detrimental factor for cardiovascular disease leading not only to atherosclerotic damage but also to acute thrombotic complications.

The relationships between factors involved in atherogenetic and thrombogenetic risk has been widely debated in the past and several hemostatic factors have been associated with the development of cardiovascular disease including fibrinogen, von Willebrand factor, tissue plasminogen activator antigen, plasminogen activator inhibitor-1, and factor VII.[27–29] Although there is no clear clinical evidence of a role for the hemostatic system in the progression of atherosclerosis, experimental data indicate that platelets and the coagulation system are important determinants of atherothrombosis (for a recent review, see ref.[29]). Because of the cross-sectional design, our study is unable to establish whether the increase of coagulant factor activities precedes or is the result of either the abnormal lipoprotein milieu or the concomitant presence of atherosclerotic lesions. However, the relationship observed in CAD-free individuals between Apo CIII and FII:c is consistent with the latter rather than the former possibility.

On the other hand, the apparent dichotomy between the atherogenic and the thrombogenic role is likely to be an oversimplification. First impressively described in 2003 by Prandoni and colleagues[36] but also subsequently by others (revised in[37,38]), it has been observed that patients with venous thromboembolism are at higher risk of arterial events and vice versa, because they often share the same risk factors.[39] Several studies have indeed reported the association of factors common to both conditions such as waist circumference, diabetes, metabolic syndrome, low HDL, high LDL cholesterol, and high TG plasma concencentration.[37–39] It is quite impressive to note that many (if not all) of these conditions have been also documented to be associated with elevated levels of Apo CIII.[7–10,12,39]

Reasoning by similarity to FII G20210A gene polymorphism, the present data indicate Apo CIII as a potential culprit candidate for both arterial and venous thrombosis, according to the first hypothesis of Prandoni et al[36] of a common disease determinant. The clinical and functional impact of the carriership of G20210A gene variant, leading to the well known thrombophilic phenotype, is currently attributed to increased FII:c.[25,26] As clearly demonstrated in our population (Figure 5), levels of Apo CIII >12.6 mg/dL entail quantitatively similar FII activation so that an analogous condition of thrombophilia may be theoretically hypothesized. Thus, the current data may indicate elevated concentrations of Apo CIII as a potential candidate for both arterial and venous thrombosis. As a corollary, the recent notion that statins seem to be able to reduce VT rates[40] could be the indirect result of the simultaneous decrease of Apo CIII under the risk threshold, induced by these drugs.

Finally, it is important to underline that the value of the present findings, yet potentially relevant, is at the moment only circumstantial. The observations exposed here derive from a retrospective analysis and therefore are intrinsically flawed by the cross-sectional design. No insights on the causal mechanisms may be surely inferred from these results that however, for sample size and related statistical power, seem rather solid. Moreover, the population investigated was selected for arterial pathology so that all the considerations regarding the thrombotic risk in the venous district have to be considered merely speculative.

It is therefore necessary that further proof is collected by means of both clinical and biochemical studies specifically addressed to fully reveal the hemostatic implications of abnormal concentrations of Apo CIII-rich particles.

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Disclosures

None.

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