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Serum phospholipids are the main environmental determinants of activated factor VII in the most common FVII genotype

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ABSTRACT

Background and Objective. **Numerous studies have emphasized the role of triglyceride-rich lipoproteins and of Factor VII (FVII) polymorphisms in determining levels of FVII activity.**

Design and Methods. **This study was undertaken to evaluate the role of other lipid fractions and the interaction between lipids and FVII in subjects with recognised genotypes. Volunteer subjects (n=459) from 5 European countries were studied. Blood samples were drawn irrespective of the time of day or fasting status. Levels of FVII activity (FVIIc), activated FVII (FVIIa) and FVII antigen (FVIIAg) were evaluated with reference to a number of lipid parameters (HDL-, LDL- and total cholesterol, triglycerides, phospholipids, lipoprotein(a), and apolipoptein A1). The two most common FVII polymorphisms were analyzed in combination (353R/Q and 5'F7; alleles M1/M2 and A1/A2, respectively).**

Results. **Homozygotes for the A1 and M1 alleles (M11/A11) had significantly higher FVII levels. At multiple regression analysis the strongest predictor of FVIIa and FVIIc was the concentration of phospholipids. This interaction was confined to the A11M11 genotype subjects.**

Interpretation and Conclusions. **These data indicate that lipids contribute mainly to FVIIa levels through their phospholipid content, and that the degree of this contribution is strictly dependent on FVII genotypes.**

Key words: serum phospholipids, factor VII

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It is now widely accepted that both atheroma and thrombosis play a key role in ischemic heart disease (IHD).

Abnormal lipid levels are considered of great importance in the pathophysiology of atheroma and are believed to be important risk factors for IHD.[1] A number of metabolic variables affecting these levels and the distribution of lipids among lipoprotein subfractions should also be taken into account in determining IHD risk. Total cholesterol (CHL), high- and low-density lipoproteins (HDL-C and LDL-C) and triglycerides (TriG)[1-3] have been extensively studied and CHL, LDL-C and TriG are now widely accepted as risk factors for IHD.

Among the hemostatic IHD risk variables, factor VII (FVII) has attracted attention because of its association with CHL and TriG.[4-14] Recent technical developments allow direct assay of the active form of FVII (FVIIa) in human plasma.[15,16] The study of FVII polymorphisms[14,17-23] has also led to the identification of genotypes associated with different FVII levels and has thrown light on the relation between FVII and TriG. Furthermore, in a very recent work by our group, a strong contribution of FVII genotype to FVIIa was illustrated.[24]

Our aim was to evaluate the interactions between lipids and FVII in cohorts of apparently healthy subjects with a wide age range focusing on FVII genotypes and FVIIa in such interactions.

Design and Methods

Study population

Four hundred and fifty-nine volunteer subjects from 5 European countries (France, Italy, The Netherlands, Norway and Spain) were examined. Blood samples were taken irrespective of the time of day or subjects' fasting status. The study population was divided into three age groups: 19-35, 36-50, 51-75. All participants declared themselves to be in

good health and free from cardiovascular disease, diabetes and cancer. Exclusion criteria were pregnancy and treatment with anticoagulant drugs.

Blood sampling

Blood for coagulation studies was taken in 5 mL Vacutainer tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France) containing 0.5 mL of 0.129 M buffered sodium citrate. Tubes without anticoagulant were used for the lipid assays. Serum was prepared by incubating blood for ≥ 2 hours at 37°C. All samples were centrifuged at 2,000 g for 15'. Sera and plasmas were harvested and aliquoted in plastic tubes (Sorenson BioScience, Salt Lake City, USA). Samples were frozen to –80°C in cryo-tubes and -boxes (CryoStore Systems, Nunc Inc. Naperville, IL, USA) and subsequently sent in dry-ice to the central repository in the co-ordinating institution (Thrombosis Center, University of Rome) for redistribution. For the genetic evaluations, pellets from the citrated blood samples were harvested in plastic tubes and frozen to –10°C.

Assay procedures

FVIIc and FVIIAg assays were carried out as previously reported.[36] FVIIa was assayed with a commercial kit (Staclot VIIa-rTF, Diagnostica Stago, Asnières, France). Values were expressed in mU/mL, 30 mU being equivalent to 1 ng of FVIIa.

For FVIIa the standard was a recombinant protein and for FVIIc and FVIIAg assays, the standard was a home-made, pooled plasma (20 males and 20 females in a fasting state).

FVII genetic markers were evaluated as previously reported.[23] Comparisons were made between the most frequent FVII genotypes. The alleles of the polymorphism in the promoter region (5'F7) were denominated A2 (single decamer insertion) and A1 (absence of decamer) and the alleles of the 353R/Q polymorphism, characterized by a mutation in the second position of the 353 codon, were denominated M1 (codon for arginine) and M2 (codon for glutamine). Tight linkage disequilibrium between the A1 and M1 alleles as well as between the A2 and M2 alleles was found (Δ values ranging from 0.85 to 0.93), whatever the population. Genotype distributions were in Hardy-Weinberg equilibrium.[24]

Total CHL was determined with a commercial kit (Cholesterol, Du Pont, Wilmington, USA) based on the production of stoichiometric amounts of hydrogen peroxide generated by cholesterol-esterase and -oxidase.[25] HDL-C was determined by the same procedure after the precipitation of the other CHL-containing lipoprotein fractions by a phosphotungstate solution buffered to pH 5.7[26] (HDL-CHOL Du Pont, Wilmington, USA). LDL-C was evaluated by the indirect procedure as proposed by Friedewald *et al.*[27] TriG were assayed by a kinetic NAD-coupled procedure[28] (Triglycerides, Du Pont, Wilmington, USA). Choline-containing PhL were evaluated by a choline oxidase determination of the amount of choline liberated by phospholipase D (Phospholipids, SGM Italia, Rome, Italy).[29] ApoA1 was determined by a turbidimetric end-point measurement using a specific polyclonal antibody and 10 mM polyethylene glycol[30] APO A1, Du Pont, Wilmington, USA). Lp(a) was determined by an enzyme immunoassay using a monoclonal antibody anti-kringle IV, and a polyclonal anti-Lp(a) antibody conjugated with horseradish peroxidase[31] (Macra Lp(a), Strategic Diagnostic, Newark, USA).

Statistical analysis

The procedures used were from the BMDP software. The distribution of variables was assessed for deviation from normality, and the appropriate normalizing (logarithmic) transformation was used in order to analyze data using parametric methods. Tables were computed on untransformed data. Parametric analyses of variance (one-way, two-way) and of co-variance (using age as co-variate) were used, including main effects and interactions in the models. Pearson's linear correlation coefficients were used to detect any association between variables. A fixed multiple linear regression model was fitted to the data, to estimate the effect of high concentrations of each independent lipid variable (after adjustment for age, sex and country effects) on the dependent one, in the overall population and in the most frequent FVII genotypes. Problems due to colinearity were checked and ruled out during the analysis. The appropriate Student's t-tests were performed to assess the significance of correlation and regression coefficients, and of differences in coefficients between subgroups.

Results

Basic characteristics of the study group

Of the 459 subjects, 219 (47.7%) were females and 240 males. Subjects were evenly distributed over the three age groups: 19-35 y (n=155, 33.8%), 36-50 y (n=137, 29.8%) and 51-75 y (n=167, 36.4%) and among the populations studied. Mean values and SD for the study population are set out in Table 1, whilst Table 2 shows the age- and sex-specific mean values (SD), and the percentage change in the variables for a 10-year increase in age.

Age and sex effects

Age exerted an important effect on most variables: highly significant (p <0.0001) increases in the levels of FVII (all assays), CHL, LDL-C, TriG, PhL, ApoA1 were found whereas no significant changes were detected for HDL-C and Lp(a). Sex-related differences were highly significant (p <0.0001) for HDL-C, TriG, PhL and ApoA1, and were also found, to a lesser degree (p <0.02) for FVIIc levels. Females had higher levels of FVIIc, FVIIAg, HDL-C and ApoA1 as well as, but only in the third age group, FVIIa and PhL levels. There were no sex significant differences among the remaining

Table 1. Phenotypic characteristics of the subjects (age-adjusted data, when necessary).

Variable	U. of measure	Mean	SD	Centiles 2.5	Centiles 97.5
FVIIc	(%PNP)	122.8	29.7	72	186
FVIIAg	(%PNP)	102.7	18.0	70	144
FVIIa	(mU/mL)	77.1	35.7	23	163
CHL	(mmol/L)	5.5	1.1	3.6	8.0
LDL-C	(mmol/L)	3.6	1.0	2.0	5.9
HDL-C	(mmol/L)	1.3	0.4	0.8	2.2
TriG	(mmol/L)	1.1	0.8	0.4	3.5
PhL	(mmol/L)	3.0	0.5	2.1	4.1
ApoA-I	(mmol/L)	47.0	9.1	33.6	66.1
Lp(a)	(mmol/L)	0.7	0.7	0.1	2.5

Table 3. Associations (as partial correlation coefficients) between hemostatic and lipid variables in the general population, after adjustment for age, sex and country effects.

	FVIIAg	FVIIa	CHL	HDL–C	LDL–C	TriG	ApoA1	PhL
FVIIc	.66	.72	.27	.18	.14	.21	.25	.32
FVIIAg	–	.50	.19			.17	.15	.20
FVIIa		–		.15			.16	.13
CHL			–	.16	.92	.35	.25	.82
HDL –C				–		–.41	.90	.38
LDL –C					–	.21		.62
TriG						–	–.23	.39
ApoA1							–	.49

p<0.01 for correlation coefficients $|r| \geq .12 - .18$;
p<0.001 for correlation coefficients $|r| \geq .19$.

Table 2. Age- and sex-specific mean values and (SD) of the variables studied and analysis of the percentage variation corresponding to a 10-year increase in age (same units of measure as in Table 1).

	age 19-35		age 36-50		age 51-75		% var./10y.	
	men (n= 89)	women (n=66)	men (n=72)	women (n=65)	men (n=79)	women (n=88)	men	women
FVIIc	107.4 (22.3)	115.2 (27.4)	120.6 (26.8)	121.5 (26.9)	128.2 (31.9)	141.9 (31.9)	+5.1	+5.3
FVIIAg	97.3 (15.5)	98.5 (14.7)	103.1 (16.0)	106.1 (20.6)	102.9 (18.1)	108.3 (20.0)	+2.0	+2.2
FVIIa	65.5 (27.3)	63.1 (23.0)	74.4 (33.8)	75.9 (30.0)	84.5 (36.0)	94.9 (45.7)	+8.5	+9.6
CHL	4.9 (1.0)	4.9 (0.9)	5.7 (1.1)	5.2 (0.8)	5.7 (1.0)	6.4 (1.1)	+4.6	+8.0
LDL-C	3.3 (0.9)	3.1 (0.8)	3.9 (1.0)	3.3 (0.7)	3.8 (0.9)	4.3 (1.0)	+4.9	+9.3
HDL-C	1.2 (0.3)	1.4 (0.4)	1.2 (0.3)	1.5 (0.4)	1.3 (0.3)	1.5 (0.4)	+2.4	+0.2
TriG	1.0 (0.5)	0.7 (0.3)	1.3 (0.7)	0.8 (0.6)	1.4 (0.9)	1.5 (0.9)	+8.9	+20.8
PhL	2.7 (0.4)	2.7 (0.4)	3.0 (0.4)	3.0 (0.4)	3.0 (0.5)	3.4 (0.5)	+3.1	+5.9
ApoA1	42.0 (6.1)	46.7 (8.7)	43.4 (6.5)	50.5 (9.3)	46.4 (8.8)	52.3 (9.7)	+2.6	+2.4
Lp(a)	0.7 (0.7)	0.6 (0.6)	0.5 (0.6)	0.5 (0.5)	0.7 (0.8)	0.8 (0.8)	+6.2	+6.7

variables. A significant (p <0.0001), positive interaction between age and sex was found only for LDL-C, TriG and PhL.

Correlation coefficients for the associations between FVII and lipid variables were calculated and Table 3 shows those with p <0.01. Partial correlation coefficients adjusted for sex and age did not differ between the five ethnic groups (data not shown).

Associations

Between FVII variables. The strongest association found was that between FVIIc and FVIIa (0.72), followed by that between FVIIc and FVIIAg (0.66).

Between FVII and lipids. The strongest association between FVIIc and lipids was that with PhL (0.32) and the lowest with HDL-C (0.14). These associations were more evident in the fasting subjects (n=88) where the correlation coefficients with PhL were 0.43 for FVIIc, 0.44 for FVIIa, and 0.30 for FVIIAg.

Between lipids. The lipid parameter having the widest spectrum of strong associations with the other ones was PhL, which correlated most strongly with CHL and LDL-C, and somewhat less with ApoA1, TriG and HDL-C (Table 3). When assessed individually, the highest correlation coefficients were those between CHL and LDL-C and between HDL-C and ApoA1 (both ≥ 0.90).

Influence of FVII genotypes on FVIIc, FVIIAg and FVIIa levels

There was a clear difference in FVIIc, FVIIAg and FVIIa levels in the genotypes studied: homozygotes for the A1 and M1 alleles displayed significantly higher mean values than those of the heterozygotes or the homozygotes for the rarer A2 or M2 alleles, and even more so for FVIIa and FVIIc than for FVIIAg (Table 4).

Multiple regression analyses of the effect of high lipid concentrations on FVII levels; influence of the genotypes

High PhL concentrations were associated with high FVIIa and FVIIc levels (Table 5). High concentrations of TriG or CHL were found not to be independent predictors of high FVII levels. When genotypes were evaluated separately, very high FVIIa and FVIIc levels were found to be associated with high PhL concentrations, but only in the A11M11 genotype (Table 6). The difference between genotypes was not significant when TriG were considered as the independent variable (Table 6).

Table 4. Influence of the most common genotypes on FVII-related parameters (mean[SD]) (same units of measure as in Table 1).

Genotypes	*n.*	*FVIIa*	*FVIIc*	*FVIIAg*
A11M11	330	84.4 (3.2)	128.7 (25.3)	104.9 (17.7)
A12M12 + A22M22	88	51.0 (2.4)	103.2 (25.6)	95.8 (18.2)
Difference between genotypes				
		F 122.0	F 84.0	F 21.9
		p <.00001	*p* <.00001	*p* <.00001

Table 5. Multiple regression analysis concerning the effect of PhL, TriG and CHL concentrations on FVII levels as dependent variable (age, sex and center included in the regression model). The values of the standardized regression coefficients (SRC) are shown, together with the effect caused by a hypothetical 50% and 100% increase of the lipid variables.

	Increase	*FVIIc*	*FVIIa*	*FVIIAg*
	SRC	*+0.25°*	*+0.20**	*+0.10*
PhL	+50%	+16.9%	+26.1%	+4.2%
	+100%	+30.7%	+48.7%	+7.3%
	SRC	*+0.12**	*+0.07*	*+0.10*
TriG	+50%	+2.4%	+2.8%	+1.3%
	+100%	+4.2%	+4.8%	+2.3%
	SRC	*+0.04*	*–0.14*	*+0.09*
CHL	+50%	+2.2%	–12.8%	+3.2%
	+100%	+3.7%	–20.9%	+5.5%

*Slope significantly different from 0: *p<.05; °p<.01.*

Discussion

There is increasing evidence of the important role played by the tissue Factor-FVII pathway in the initiation of blood coagulation. Population-based studies have provided evidence that elevated FVIIc levels may be involved in the pathogenesis of IHD.[32-39] The clinical impact of elevated FVII levels has given rise to controversy because of the different forms of FVII that exist in plasma[15,34,40,41] and the different methods employed for measuring FVIIc.[23,32,34,41] Further complexity stems from the genetic and environmental determinants of FVII levels. Very recently, the importance of FVII genotype was stressed in the analysis of a large cohort of subjects with a personal and family history of IHD.[42] On the other hand, numerous studies have analyzed the impact of lipids and, in particular, of TriG.[5-16,19,20]

The most important features of this study are the wide array of lipid and genetic assays carried out, and the fact that the subjects were enrolled irrespective of time of day and fasting status. The fact that subjects were analyzed irrespective of their fasting status, has certainly meant that overall correlations are weaker, but description of the variables and their interactions is closer to reality, since it is made up of diverse elements which may be present during the course of acute ischemic events.

Table 6. Multiple regression analysis concerning the effect of PhL on FVII levels (dependent variable) in a population selected by genotype. Age, sex, center, TriG, and CHL were included in the regression model. The values of the standardized regression coefficients (SRC) are shown, together with the effect caused by a hypothetical 50% and 100% increase of the lipid variables.

Genotype	*increase*	*FVIIa*	*FVIIc*	*FVIIAg*
SRC	+0.34**	+0.47**	+0.27**	
A11M11	+50%	+41.0%	+27.6%	+11.4%
	+100%	+79.9%	+51.6%	+20.4%
	SRC	–0.17	–0. 11	–0.14
A12M12+	+50%	–16.2%	–6.8%	–5.7%
A22M22	+100%	–26.1%	–11.4%	–9.5%

Slope significantly different from 0: p<.05 ;** p<.01.*

Differences between genotypes:

a) PhL as independent variable	FVIIc	t=5.82, *p* <0.001
	FVIIa	t=5.09, *p* <0.001
	FVIIAg	t=4.50, *p* <0.001
b) TriG as independent variable (data not shown in the table)		
	FVIIc	t=0.47, n.s.
	FVIIa	t=0.12, n.s.
	FVIIAg	t=0.58, n.s.

Effect of age and sex

FVIIc and FVIIAg levels were, as previously reported,[23,33,43-45] influenced by age and this influence was more pronounced in females than in males. This was even more evident as regards the increase of FVIIa (9%/10 years). The overall evaluation of the changes in lipid levels with respect to sex and age, can be ascribed to menopausal hormonal changes.[46,47]

FVII genotypes

Polymorphic markers within the FVII gene (the 5'F7 and the 353R/Q polymorphisms) contribute to determining FVII levels.[17,23] Members of this group[23] have calculated that these markers are associated with about 1/3 of the total FVIIc variation, a finding which has been extended, more recently, also to FVIIa.[24] In this study, two groups were characterized: one including the homozygotes for the A1 and the M1 alleles, and the other one made up of the heterozygotes and the homozygotes for the rarer A2 and M2 alleles. Our data not only provide new and strong evidence that FVIIa levels are significantly different in the genotypes examined, but also that a significant part of the variation is due to particular lipid constituents.

Lipids and FVII

The multiple regression analysis demonstrated that the major determinants of FVII are the PhL, whose high concentrations were associated with significant increases of FVIIa, FVIIc and FVIIAg. Equally high concentrations of other lipid fractions were associated with insignificant changes in FVII levels (Table 5). When focused on the effect of high PhL concentrations on FVII levels in subjects characterized by genotype, the analysis confirmed that the highest FVII levels were indeed associated with increased PhL concentrations, but only in the A11-M11 genotype (Table 6). In subjects with the A2 and/or M2 alleles FVII levels were lower, regardless of the PhL concentrations. The genetic analysis highlighted an important association that would have been missed if the phenotype, alone, had been considered. It is important to note that in previous studies the association between FVIIa and TriG was reported to be weak or absent.[14,40,48,49]

For methodological reasons, namely to use a reproducible and standardized procedure, our investigation was limited to choline-containing PhL. The non-choline-containing PhL were previously reported to make up a small part (<30%) of these serum lipid constituents.[50] We have checked this aspect in an appropriate number of subjects (n=91) and found that non-choline-containing PhL average less than 10% of the whole PhL concentration (ranging from 3.6 to 13). This does not exclude that the rare PhL compounds (in particular the acidic ones) could play a role in the interaction with FVII.

It is important to note that Berliner *et al.*[51] have indicated substantial roles for oxidized PhL in atherogenesis, namely the stimulation of the monocyte-endothelial interaction and the production of platelet-derived growth factor by smooth muscle cells. These interactions may have some connections with the presence of increased levels of activated FVII.

Our findings may revive interest in the so-called *phospholipase C (PLC)-sensitive FVII complex*.[41,52-55] The most accredited hypothesis concerning the nature of this form of FVII is that of a complex made up of activated FVII and phospholipids.[54] Our data supports this hypothesis, which, nonetheless, is still in need of further experimental corroboration.

The nature of the association of FVII with PhL may be different from that with TriG. Under physiological conditions, TriG in chylomicrons increase sharply 3-5 hours after meals and then rapidly decrease (half-life of 5 min.);[56] the same rapid change holds for FVII. In sustained hypertriglyceridemia, on the other hand, FVIIc remains elevated.[9,57,58] It is this chronic increase of FVII levels that may be associated with high PhL levels.

One may wonder whether the weak association we found between FVII and TriG is related to methodological issues (the non fasting status of the majority of the subjects). We ruled out this possibility by analyzing the fasting subjects separately and found that the association between FVII and PhL was much stronger than that with TriG (data not shown).

The most important findings of this study indicate that PhL are the main environmental determinants of FVII and that the interaction between these ubiquitous lipid components and FVII is confined to the subjects homozygous for the A1 and M1 alleles of the 5'F7 and ^{353}R/Q polymorphisms. Further studies are needed to understand the molecular basis of the interaction between the FVII molecules expressed under the control of the A11/M11 genotype and PhL.

In conclusion, the interaction between FVII and lipids is complex, since many lipid fractions are involved. Clarification of these interactions will allow us to understand better the mechanisms underlying thrombosis in the atherosclerotic vessel.

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Disclosures

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