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Validation of plasma fibrinogen as a marker of carotid atherosclerosis in subjects free of clinical cardiovascular disease

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A B S T R A C T

Background and Objectives. Fibrinogen has been found to be an independent risk factor for cardiovascular disease. The aim of this study was to validate the measurement of plasma fibrinogen as a marker of subclinical atherosclerosis in a series of asymptomatic subjects (n=519, median age 55.5 years, 80% men).

Design and Methods. All individuals had a complete clinical examination, lipid profile (cholesterol and its high and low density lipoprotein fractions and triglycerides), global vascular risk assessment (PROCAM), and B-mode ultrasonography of the carotid arteries to determine the intima-media thickness (IMT) and the presence of atheroma plaques. C-reactive protein (CRP), and von Willebrand factor (vWF) were also measured in all subjects as markers of inflammation/endothelial damage.

Results. In the univariate model, a positive relationship was found between plasma fibrinogen concentration and carotid IMT ($p<0.001$). Fibrinogen concentration also correlated positively with age ($p<0.001$), systolic blood pressure ($p<0.001$), smoking ($p<0.05$), diabetes ($p<0.05$), PROCAM ($p<0.001$), CRP and vWF ($p<0.001$). In the multivariate analysis, the association of fibrinogen with carotid IMT remained significant ($p=0.008$) after adjustment for all parameters analyzed.

Interpretation and Conclusions. In a population sample of adults without clinically overt atherosclerotic disease, elevated fibrinogen levels was related to carotid IMT independently of a wide range of important confounding variables. Plasma fibrinogen may represent a systemic marker of carotid atherosclerosis.

Key words: fibrinogen, inflammation, atherosclerosis, carotid intima-media thickness.

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I ncreasing evidence indicates that the hemostatic system plays an important role in the pathogenesis of atherosclerotic vascular disease.¹ Numerous epidemiological studies have documented the association of elevated plasma fibrinogen levels with increased incidence of cardiovascular disease.²⁻⁸ There is also evidence that fibrinogen may have a role in acute and inflammatory processes, being involved in the development of atherosclerosis and its complications.^{9,10} An important issue is whether fibrinogen is related to cardiovascular abnormalities in subjects in whom there is no evidence of overt atherosclerotic disease.¹¹ In a recent study,¹² we showed an independent association of fibrinogen concentration with carotid intima-media thickness (IMT), a non-invasive marker of subclinical atherosclerosis, known to be associated with prevalent heart disease and

with cardiovascular risk factors.^{13,14} However, controversy still exists as regards the relations of fibrinogen to markers of subclinical atherosclerosis.¹⁵

Therefore, we tried to validate the measurement of plasma fibrinogen both in relation to carotid IMT and atheroma plaques in a wider population of adults free of clinically overt cardiovascular disease.

Design and Methods

Subjects

A total of 519 consecutive apparently healthy subjects (420 males, 80%), median age 55.5±10.7 years (range 28 to 71 years) referred to the outpatient clinic for vascular risk assessment by the Internal Medicine Department at the University Hospital of Navarra were studied. All subjects were

interviewed to exclude individuals with a history of coronary artery disease (CAD) or previous vascular surgery. Additional exclusion criteria were the presence of severely impaired renal function, arteritis, collagen disease, and a history of alcohol abuse.

Assessment of cardiovascular risk factors

In addition to questions about symptoms of ischemic heart disease, peripheral vascular disease and stroke, information on the cardiovascular risk factors diabetes mellitus, arterial hypertension and smoking were obtained. The subject was defined as having hypertension if he or she was being treated with antihypertensive drugs at the time of examination or had a systolic blood pressure greater than 140 mmHg and/or diastolic pressure greater than 90 mmHg. Subjects with a positive history of diabetes mellitus or with fasting glucose levels higher than 7.0 mmol/L were considered diabetic. As far as concerns smoking, individuals were categorized as *ever smokers* or *non-smokers*. Obesity was estimated by the body mass index (BMI, kg/m²). Dyslipidemia was diagnosed in the presence of at least one of the following measurements: total cholesterol >200 mg/dL, low density lipoprotein (LDL)-cholesterol >135 mg/dL, and high density lipoprotein (HDL)-cholesterol <35 mg/dL.

Measurement of global cardiovascular risk

The PROCAM score for global CVD risk was obtained in every person as previously reported.¹⁶ Written informed consent was obtained from the subjects. Procedures followed were in accordance with the ethical standards approved by the institutional review board of the University Clinic of Navarra and with the Helsinki Declaration.

Laboratory data

Determination of plasma fibrinogen

Blood was collected by venipuncture between 9–11 a.m. after 12 hours of fasting. Venous blood was mixed (9:1) with sodium citrate solution 0.11 mol/L and immediately centrifuged at 2000g for 20 min at 4°C. The platelet-poor plasma was then frozen at -80°C until assayed. Plasma fibrinogen activity was measured on citrated plasma samples by the Clauss method¹⁷ on a Electra coagulometer 1600 (MLA, USA) using Hemoliance Q.F.A. thrombin (IL, USA). The intra- and inter-assay coefficients of variation for the fibrinogen assay were 4% and 5.5%, respectively.

Lipid measurements

Serum cholesterol, HDL and LDL-cholesterol, glucose and triglycerides were measured on fasting blood samples by standard enzymatic techniques.

Markers of inflammation and/or endothelial damage

von Willebrand factor antigen was quantified by an enzyme-linked immunosorbent assay (ELISA, Diagnostica Stago) using a STA compact analyzer (Diagnostica Stago, France). High-sensitivity C reactive protein (Immulyte hs-CRP) was measured by ELISA.¹⁸ Inter- and intra-assay coefficients of variation for all ELISAs were lower than 8%.

Measurement of carotid IMT and presence of atheroma plaques

All subjects underwent ultrasonography of the common carotid arteries. Ultrasonography was performed with a 5- to 12-MHz linear-array transducer (ATL 500 HDI). The IMT was measured 1 cm proximal to the carotid bulb of each common carotid artery at plaque-free sites. For each individual, the IMT was determined as the average of near-wall and far-wall measurements of each common carotid artery, which has been shown to be a reproducible strategy.^{1,12} Atheroma plaques were defined as echogenic structures encroaching the vessel lumen with a distinct area 50% greater than the intimal plus media thickness of neighboring sites.

Statistical analysis

The distribution of continuous variables in groups was expressed as mean±SD. Differences in the baseline characteristics were evaluated by the Student's t test and χ^2 tests when appropriate. Pearson's correlation tests were used to analyze the relationship between two continuous variables. Linear regression analysis and multivariate analysis were carried out to evaluate factors related to carotid IMT as well as the possibility of interactions. Statistical analysis was performed with SPSS for Windows software package version 11.0. For all these analyses the level of statistical significance was established as a *p* value < 0.05.

Results

Clinical and laboratory data

After exclusion criteria, 519 apparently healthy subjects (420 men, median age 55 years) were included. Baseline clinical characteristics, cardiovascular risk factors, PROCAM score and circulating levels of markers of inflammation and endothelial damage in the whole population are shown in Table 1. The prevalence of cardiovascular risk factors was as follows: dyslipidemia (90.2%), arterial hypertension (52.2%), smoking (33.8%), obesity (29.2%), and diabetes (11.4%). There were more smokers among males and their BMI

Table 1. Baseline clinical characteristics of the studied population.

	Total population (n=519)	Male (n=420)	Female (n=99)	p value
Age (years)	55.5±10.7	55.6±10.7	56.5±10.7	n.s.
Smokers (no/yes)	343/176	278/142	82/17	0.001
BMI (Kg/m ²)	28.0±4.1	28.4±3.7	26.2±4.4	<0.001
SBP (mm Hg)	132.1±19.9	131.8±19.9	133.4±20.4	n.s.
Arterial hypertension (no/yes)	248/271	198/222	47/52	n.s.
Diabetes mellitus (no/yes)	460/59	365/55	89/10	n.s.
Total cholesterol (mg/dL)	229.1±43.6	227.6±42.6	235.6±47.3	n.s.
HDL-cholesterol (mg/dL)	49.5±13.9	47.3±12.8	59.6±14.5	<0.001
LDL-cholesterol (mg/dL)	155.7±38.2	155.8±37.9	155.2±39.6	n.s.
Triglycerides (mg/dL) [†]	124.5±87.0	128.9±90.4	104.4±66.8	0.006
CRP (mg/L) [†]	4.7±9.5	4.7±9.4	4.9±10.4	n.s.
Fibrinogen (mg/dL)	306.6±77.4	304.7±77.9	315.2±74.6	n.s.
vWF (%)	120.4±58.5	120.3±59.8	121.1±52.8	n.s.
Risk score (PROCAM) [†]	1.3±1.5	1.3±1.5	–	–
Carotid IMT (mm)	0.74±0.16	0.75±0.16	0.68±0.14	<0.001
Carotid plaque (N/Y)	412/107	326/94	86/13	0.041

BMI: body mass index; SBP: systolic blood pressure; CRP: C-reactive protein; vWF: von Willebrand factor; (Mean±SD); [†]logarithmically transformed variables.

was significantly higher ($p<0.001$). In addition, compared with female patients, the male subjects had significantly higher plasma levels of triglycerides and lower HDL-cholesterol ($p<0.001$). Finally, carotid IMT was greater in males (0.75 mm vs 0.68 mm in females, $p<0.001$), and carotid plaques were more common in the males ($p=0.041$).

Fibrinogen levels in relation to ultrasound analysis, clinical and laboratory data

The median plasma fibrinogen level in the whole studied population was 306.6 mg/dL (range 83–508 mg/dL). A significant correlation was observed in the univariate analysis between plasma fibrinogen concentration and carotid IMT, as estimated by B-mode ultrasound ($r=0.18$, $p<0.001$). As shown in Table 2, fibrinogen was also positively associated with age ($r=0.18$, $p<0.001$), diabetes ($r=0.10$, $p<0.02$), smoking ($r=0.12$, $p<0.05$) and systolic blood pressure ($r=0.14$, $p<0.001$). The levels of fibrinogen also correlated significantly with CRP ($r=0.27$, $p<0.001$) and vWF ($r=0.25$, $p<0.001$). Finally, a significant correlation was found between baseline fibrinogen levels and the PROCAM score ($r=0.27$, $p<0.001$).

The carotid IMT (Table 3) also correlated positively with age ($r=0.32$, $p<0.001$), hypertension ($r=0.31$, $p<0.001$), gender ($r=0.16$, $p<0.001$), glucose ($r=0.17$, $p<0.001$), total cholesterol ($r=0.11$, $p<0.01$) and the PROCAM score ($r=0.32$ and $p<0.001$).

Finally, fibrinogen levels were significantly higher in patients with plaque ($n=107$) than in those with no plaque ($321.8±89.9$ vs $300.2±71.9$ mg/dL, $p=0.016$).

Table 2. Pearson's correlation coefficients between fibrinogen and cardiovascular risk factors, inflammatory and endothelial markers and carotid IMT.

Parameter	r	p value
Risk factors		
Age (years)	0.18	<0.001
BMI (kg/m ²)	0.07	n.s.
SBP (mm Hg)	0.14	0.001
Cholesterol (mg/dL)	0.05	n.s.
LDL-cholesterol (mg/dL)	0.02	n.s.
HDL-cholesterol (mg/dL)	-0.06	n.s.
Triglycerides (mg/dL)	0.01	n.s.
Diabetes mellitus (yes/no)	0.10	0.02
Smoking (yes/no)	0.12	0.036
Inflammatory/endothelial markers		
CRP (mg/L)	0.27	<0.001
vWF (%)	0.25	<0.001
PROCAM		
Risk score	0.27	<0.001
Ultrasound studies		
Carotid IMT (mm)	0.18	<0.001

Abbreviations as in Table 1.

Multivariate analysis

Because of the important associations of fibrinogen and carotid IMT with older age, male gender, hypertension and diabetes, further multivariate analysis was performed to assess the relationship between fibrinogen and carotid IMT after adjusting for these potential confounders (Table 4). Results showed that the relation between both parameters remained statisti-

Table 3. Correlation of the carotid IMT with all other variables investigated.

	<i>R</i>	<i>p</i> value
Age (years)	0.32	<0.001
Gender (M/F)	0.16	<0.001
BMI (Kg/m ²)	0.06	n.s.
SBP (mmHg)	0.31	<0.001
DBP (mmHg)	0.11	0.01
Total cholesterol (mg/dL)	0.11	0.02
HDL-cholesterol (mg/dL)	- 0.01	n.s.
LDL-cholesterol (mg/dL)	0.11	0.01
Triglycerides (mg/dL)	0.05	n.s.
Glucose (mg/dL)	0.17	<0.001
Smoking (yes/no)	0.06	n.s.

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.

Table 4. Correlation of the carotid IMT with fibrinogen in multiple linear regression analysis with carotid IMT (mm) as dependent variable.

	$\beta(\times 10^{-3})$	SE (β)	<i>p</i> *	Partial R ² (%)
Fibrinogen (mg/dL)	0.23	0.08	0.008	1.4
Sex (F/M)	73,2	10.0	<0.001	3.2
Age (years)	4.1	0.66	<0,001	6.9
SBP (mmHg)	1.7	0.34	<0.001	4.5
Glucose (mg/dL)	0.58	0.21	0.012	1.2
LDL-cholesterol	0.45	0.10	0.008	1.1
Smoking (no/yes)	38,9	14.3	0.006	1.4

*Adjusted for age, sex, BMI, SBP, LDL-cholesterol, glucose, smoking, CRP and vWF. The model R² for the total population was 21.3%

cally significant ($p=0.008$). Since there is evidence that fibrinogen plasma levels may reflect a systemic inflammatory state, further adjustment was made for markers of inflammation and endothelial damage. Results showed that the association between fibrinogen and carotid IMT was independently related to these parameters (adjusted R²= 0.21, $p<0.001$).

Discussion

In a population sample of adults free of clinically overt atherosclerotic disease, we found that an elevated plasma fibrinogen concentration was related to carotid IMT, a surrogate marker of atherosclerosis, independently of a wide range of important confounding variables, confirming previous data in a smaller population.¹² A significant relationship was also detected when fibrinogen was compared with the presence of carotid plaques. Our data underscore the

relevance of measuring the circulating levels of plasma fibrinogen as a marker of subclinical atherosclerosis in asymptomatic subjects. Elevated levels of fibrinogen are strongly associated with human vascular disease.^{19,20} The Northwick Park Heart Study found that elevated fibrinogen at recruitment was independently associated with subsequent cardiovascular risk, with an increase of approximately 70 mg/dL being associated with a 39% increase in cardiac death and a 60% increase in non-fatal myocardial infarction.² It is unknown, however, whether increased fibrinogen plays a causal role in vascular disease progression. Elevated plasma fibrinogen may promote vascular disease by increasing blood viscosity, promoting fibrin formation, enhancing platelet-platelet interactions, or by other mechanisms.¹⁹ On the other hand, elevated plasma fibrinogen could simply be a marker of vascular disease, but does not contribute to disease progression.²¹ Fibrinogen production in the liver is regulated by cytokines and is greatly enhanced in response to different inflammatory processes.^{9,10,22} It is, therefore, possible that moderately elevated fibrinogen levels simply reflect the chronic inflammation that characterizes atherosclerosis.²³

In the population analyzed, fibrinogen levels were significantly associated with the PROCAM score, and also with some traditional cardiovascular risk factors, such as hypertension and diabetes, suggesting a close relationship between fibrinogen and a variety of environmental factors, as previously reported.^{4,24} The levels of fibrinogen positively correlated with markers of inflammation and endothelial damage (CRP and vWF, respectively), also confirming previous findings in clinical and epidemiological studies.²⁵ The association between the plasma fibrinogen concentration and the levels of these inflammatory markers emphasizes the importance of inflammatory processes in the development of atherosclerosis, as already described for patients with coronary syndromes.^{25,26}

We, therefore, performed a multivariate analysis to determine whether the association between fibrinogen levels and carotid IMT still remained significant when the environmental factors showing significant associations in the univariate analysis were taken into consideration. Our results confirmed that fibrinogen concentration represents an independent marker of subclinical atherosclerosis in asymptomatic subjects. The association between fibrinogen and carotid IMT remained highly significant after adjusting for cardiovascular risk factors (age, BMI, diabetes, and arterial blood pressure) as well as for markers of inflammation (CRP) and endothelial damage (vWF). In contrast, CRP showed no association with carotid IMT, confirming that CRP levels do not correlate well with imaging measures of underlying atherosclerosis.²⁷ We also

showed that fibrinogen levels were significantly higher in patients with carotid plaques.

Higher fibrinogen levels could be one of the many mechanisms by which fibrinogen plays a role in the pathogenesis of atherosclerosis. It is interesting to note that hyperfibrinogenemia was associated with the histological composition of atherosclerotic carotid plaques in patients with transient ischemic attacks and coronary disease.^{28,29} Studies examining transgenic mice models of hyperfibrinogenemia also suggested that fibrinogen has a role in vascular disease progression.^{30,31} In a transgenic model of modest hyperfibrinogenemia, Kerlin *et al.* recently showed that fibrinogen alters vascular remodeling induced by ligation of the carotid artery, producing augmented intimal hyperplasia compared to that occurring in wild-type controls.³² Conversely, fibrinogen deficiency reduced vascular accumulation and development of atherosclerosis in apolipoprotein(a) transgenic mice.³³

Our study has some limitations. Although other chronic conditions that may influence fibrinogen synthesis were excluded, the possibility that the observed elevated fibrinogen was a result, rather than a cause of subclinical atherosclerosis, cannot be ruled out.

Since the majority of individuals enrolled were dyslipidemic, the results are mainly valid for this specific

vascular risk subgroup. In the multivariate analysis we tried to adjust for some but not all the possible confounders and it is known that other genetic, metabolic and environmental factors not included in the present analysis could influence the fibrinogen levels.^{34,35} However, in a previous pilot study we could not confirm a relationship between the -455 G/A β -fibrinogen polymorphism and carotid IMT.¹² Finally, the sample size means that the results cannot automatically be extrapolated to other populations at risk for CVD.

In conclusion, our results strongly suggest that plasma fibrinogen concentration can be an independent marker of subclinical atherosclerosis in asymptomatic subjects. Further studies are required to establish whether modest hyperfibrinogenemia contributes to the development of atherothrombotic diseases, particularly coronary heart disease.

JAP: conceived and supervised the study; OB: provided the material from patients and collected clinical data; CR: performed the biochemical analyses; JO: analyzed the data and performed the statistical analyses; AB: performed ultrasonography of carotid arteries. All authors participated in drafting the article and approved the final version. The authors reported no potential conflicts of interest.

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