Association of plasma fibrinogen, C-reactive protein and G-455>A polymorphism with early atherosclerosis in the VITA Project cohort

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Summary
While increased fibrinogen is associated with vascular events, only few data are available on its association with preclinical atherosclerosis. We aimed at evaluating the association between fibrinogen levels, fibrinogen polymorphism G-455>A and C-reactive protein and preclinical atherosclerosis in a population-based, cross-sectional study. A cohort of 2,580 subjects was enrolled. Fibrinogen was measured at time of original enrolment and at time of the second visit, when ultrasound examination of both left and right common carotid arteries was performed, together with evaluation of C-reactive protein (CRP) and of the fibrinogen G-455>A polymorphism. CRP and fibrinogen levels at baseline were the two variables mostly influencing fibrinogen levels at the follow-up visit (p<0.0001). Carriers of the H2H2 genotype of the G-455>A polymorphism had increased fibrinogen levels, particularly in association with increased CRP levels. Increased fibrinogen levels were independently associated with presence of carotid plaques, particularly in those subjects having a persistent increase of fibrinogen (odds ratio 1.98, 95% confidence interval 1.47–2.67). An association between the H2H2 genotype and presence of carotid plaques was observed only in a subgroup of subjects with CRP > 0.5 mg/dl. A persistent increase of plasma fibrinogen is associated with an increased risk of early atherosclerosis.

Keywords
Carotid atherosclerosis, fibrinogen, ultrasonography, epidemiology, C-reactive protein

Introduction
Fibrinogen is a plasma glycoprotein formed by the covalent union of three subunits (α, β and γ chains), the rate of synthesis of the β chains acting as the main determinant of fibrinogen secretion (1). Several prospective studies demonstrated that increased plasma fibrinogen correlate with risk of acute cerebral and myocardial vascular disease (2, 3). Both genetic and environmental factors contribute to increase plasma fibrinogen. In particular, a common polymorphism of the β chain gene (G-455>A) is associated with a mean increase of fibrinogen of 0.117 g/l per each A allele in normal subjects (4); plasma fibrinogen level also dramatically increases in acute-phase reactions, possibly due to the presence of interleukin-6 (IL-6)-sensitive elements in the β chain promoter (5).

A causal relation between plasma fibrinogen and arterial disease is still unproven. First, it is not known if fibrinogen is associated with early atherosclerotic changes or whether it contributes to overt clinical disease by promoting thrombus formation in complicated plaques. Second, the role of plasma fibrinogen as an independent causative factor has also been questioned, since other acute-phase reactants such as plasma C-reactive protein (CRP) correlated with incident arterial disease (6) and since fibrinogen is also unspecifically associated with all-causes mortality (3). Finally, in a recent meta-analysis, the G-455>A polymorphism was apparently not correlated with arterial events (4). Taken together, these observations suggest that fibrinogen could be a marker of inflammation associated with arterial disease rather than a causative factor by itself.

To further investigate the role of plasma fibrinogen in the development of arterial disease, we analysed the association between pre-clinical atherosclerosis, as determined by ultrasonography of the carotid arteries, with fibrinogen, CRP and the G-455>A polymorphism in a population-based cross-sectional investigation. The primary aim of this study was to evaluate the association between plasma fibrinogen and early atherosclerotic lesions; the secondary aim was to test whether CRP and the G-455>A polymorphism could modulate this possible association.

Patients and methods
Subjects
The VITA Project is a cross-sectional study on venous thrombophilia carried out in the township of Vicenza, Italy. From June 1993 to June 1997, 15109 subjects randomly selected from the census list of the Vicenza municipality and aged 18–65 were enrolled.
in this study, and underwent clinical examination and blood sampling (visit 1). Details of selection of the original VITA cohort have been already described (7). A sub-cohort of 2,580 subjects, randomly chosen from participants of the original cohort older than 40 years at January 2000, was reinvestigated from January 2000 to December 2002, and underwent a second examination and blood sampling (visit 2, see Fig. 1); the mean time span from the first to second visit 63.5 months. In all subjects, a standardised questionnaire investigating life habits, previous history of cardio- or cerebrovascular disease, and drug medications was administered. Anthropometric measurements, blood pressure and laboratory investigations were carried out as previously described in detail (8). Diabetes was defined as a fasting glycaemia above 140 mg/dl or use of blood glucose lowering drug. Low-density lipoprotein (LDL) cholesterol was estimated using the Friedewald’s formula. Hypertension was defined as a systolic pressure above 160 mmHg or use of antihypertensive drugs. Unless otherwise specified, clinical data collected during visit 2 were used for analysis.

Assessment of pre-clinical atherosclerosis

Bilateral ultrasound evaluation of common carotid artery (CCA), bifurcation, internal and external carotid arteries was carried out by two trained physician (C.B. e R.M) according to a standardised protocol, as previously described (9), during visit 2. Briefly, the carotid arteries were visualised with longitudinal (anterior, lateral and posterior) and transverse scans, and images of the CCA, frozen on the R wave of the electrocardiogram, were digitally stored. The intima media thickness (IMT) was therefore measured on a 10 mm segment of the far wall of the CCA with dedicated software (M’Ath, Metris, France). A random sample of all images was sent to the Central Reading office in Udine, where they were reviewed.

Definition of abnormal carotid findings

Based on a previous report, subjects were classified as having an abnormal carotid IMT if showing an IMT above the 97.5% age- and sex-adjusted percentile (1). Subjects were defined as having a carotid plaque when showing at least one localised focal protrusion into the examined vessels with a thickness greater than 1.0 mm.

Laboratory methods

In all subjects enrolled in the VITA Project visit 1 and in the 2,580 subjects enrolled in visit 2, a blood sample was obtained in fasting conditions and anticoagulated with sodium citrate (129 mM, 1:9 vol:vol). The plasma was obtained by centrifugation at 2,000 g for 20 minutes, then snap-frozen in liquid nitrogen and stored at –80°C within 2 hours. In both visit 1 and 2, fibrinogen was measured using the PT-derived method on an ACL 300 coagulometer (Instrumentation Laboratories, Milan, Italy), against an internal plasma standard calibrated with the clot-weighting method (10). Fibrinogen

Table 1: Characteristics of investigated subjects.

<table>
<thead>
<tr>
<th></th>
<th>Normal (n=1988)</th>
<th>Increased IMT (n=71)</th>
<th>P-value*</th>
<th>Plaque (n=454)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / female</td>
<td>832/1156</td>
<td>46 / 25</td>
<td>&lt;0.0001</td>
<td>253 /201</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>53.5 ± 4.4</td>
<td>54.1 ± 4.5</td>
<td>0.32</td>
<td>56.2 ± 4.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>58/1930 (2.9)</td>
<td>4/67 (5.6)</td>
<td>0.18</td>
<td>36/418 (7.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>276/1712 (13.8)</td>
<td>17/54 (23.9)</td>
<td>0.01</td>
<td>133/321 (29.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI &gt; 25 kg/m²</td>
<td>968/1020 (48.7)</td>
<td>42/29 (59.1)</td>
<td>0.08</td>
<td>275/179 (60.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Smoke use (%)</td>
<td>428/1560 (21.5)</td>
<td>16/55 (22.5)</td>
<td>0.83</td>
<td>155/299 (35.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td>62/1926 (3.1)</td>
<td>1/70 (1.4)</td>
<td>0.40</td>
<td>30/424 (6.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Menopause (%)</td>
<td>479/676 (41.4)</td>
<td>12/14 (46.1)</td>
<td>&lt;0.0001</td>
<td>119/82 (59.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL upper tertile (%)</td>
<td>703/1285 (35.4)</td>
<td>15/56 (20.8)</td>
<td>0.01</td>
<td>112/342 (24.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL upper tertile (%)</td>
<td>587/1399 (29.5)</td>
<td>28/43 (38.8)</td>
<td>0.08</td>
<td>217/237 (47.8)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* reports probabilities vs. normal subjects. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.
G–455>A polymorphism was determined after PCR amplification and digestion with HaeIII (11). CRP was measured using a sensitive test (Dade Behring N High Sensitivity CRP, Marburg, Germany). Unless otherwise specified, laboratory data derived from the blood samples collected during visit 2 were used for analysis.

Statistics

The relationship between plasma fibrinogen levels and other clinical or laboratory parameters obtained during visit 2 was tested using a multiple regression model (12), with independent variables entered as dummy variables to quantify the net effect of each variable on fibrinogen levels. Since CRP values were highly skewed, CRP was entered in all regression models after log transformation or categorised as a dichotomous variable (increased/not increased) using a cut-off of 0.5 mg/dl, corresponding to the 95th percentile of the distribution in our population. To this purpose, H1/H2 alleles, log transformed CRP levels, male gender, age above 60 years, smoking, presence of hypertension, diabetes, menopause, use of statins, LDL and HDL cholesterol above the upper third tertile were entered in the regression model. We subsequently tested the relation between fibrinogen levels and the presence of early carotid lesions (either increased IMT or plaque presence). For this analysis, subjects were classified as having no lesions, increased IMT or plaque and the effect of the same variables on different outcomes was evaluated in a polytomous logistic regression model (13), adjusting for the same variables used for the linear regression model. In this analysis, the effect of having increased fibrinogen levels (in the upper 3rd tertile) in both visit 1 and 2, one visit (either 1 or 2) or in no visit was also tested using a dummy variable coded as 0 (normal fibrinogen on both visits), 1 (increased fibrinogen on one visit) or 2 (increased fibrinogen on both visits).

To further test a possible relationship between fibrinogen genotype and presence of early atherosclerotic changes, we finally used

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**Table 2:** Mean (± 2 SD) fibrinogen level (mg/dl), by H1/H2 genotype and levels of CRP levels. Fibrinogen levels obtained at second evaluation (when CRP levels were also measured) are reported.

<table>
<thead>
<tr>
<th>CRP</th>
<th>H1/H1 (n=1524)</th>
<th>H1/H2 (n=871)</th>
<th>H2/H2 (n=118)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower tertile, n</td>
<td>516</td>
<td>307</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Mean fibrinogen</td>
<td>272 ± 54</td>
<td>284 ± 54</td>
<td>302 ± 52</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median tertile, n</td>
<td>511</td>
<td>273</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Mean fibrinogen</td>
<td>298 ± 61</td>
<td>313 ± 54</td>
<td>320 ± 48</td>
<td>0.001</td>
</tr>
<tr>
<td>Median tertile, n</td>
<td>497</td>
<td>291</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Mean fibrinogen</td>
<td>348 ± 70</td>
<td>368 ± 84</td>
<td>362 ± 83</td>
<td>0.002</td>
</tr>
<tr>
<td>Upper 95% percentile, n</td>
<td>151</td>
<td>99</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Mean fibrinogen</td>
<td>362 ± 81</td>
<td>389 ± 98</td>
<td>396 ± 89</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*reports univariate p for trend between alleles.
a Mendelian randomization approach, exploiting the fact that a genotype determining protein level is assumed to be randomly distributed in subjects with or without atherosclerotic changes. To this purpose, instrumental variable linear and probit regression was used to test the association between instrumental genetic factors (H1/H2 alleles), endogenous factor (fibrinogen level), exogenous factors (all previously considered covariates) and outcome (mean IMT for linear regression, presence of plaque for probit regression) (14). The ivreg and ivprobit procedures of the Stata package were used for the latter calculations (15).

Results

Evaluated subjects

Table 1 reports data of subjects enrolled in the study, stratified by carotid status. A total of 2,513 subjects had a valid ultrasonography and laboratory assessment and were included in the analysis.

Relationship between plasma fibrinogen, CRP and the G –455A polymorphism

In a multivariate regression model, CRP and the G–455A polymorphism were the most important determinants of the plasma fibrinogen level in the studied population (Fig. 2). Subjects having the H2H2 genotype at the –455 locus together with increased CRP resulted into a particularly high increase of fibrinogen levels, with an apparent additive effect between the H2 allele and CRP levels (Table 2). Furthermore, the H2 allele appears to be over-represented in those subjects with a persistently increased fibrinogen level (H2 allele frequency in those with fibrinogen in the upper tertile at both visits vs. those with fibrinogen in the lower two tertiles at both visits: 0.267 vs. 0.208, p=0.0003). However, even considering all the variables included in the regression model (CRP, H1H1, H1H2 or H2H2 genotype, age, gender, smoking and menopausal status), only 20% of the total fibrinogen variation could be explained in our population. Variation at the –455 locus accounted for about 1% of the total population variation of fibrinogen levels. In a separate model, baseline fibrinogen (visit 1) strongly correlated with levels measured at visit 2 (r²=0.27, p<0.0001); previous intra-individual levels of plasma fibrinogen remained the major determinant even after inclusion in the model of all previously considered the variables.

Relationship between plasma fibrinogen and CRP with preclinical atherosclerosis

In a multivariate polytomous logistic regression, an increased fibrinogen level (in the upper 3rd tertile) in at least one visit was clearly associated with presence of plaque but not with increased IMT (odds ratio [OR] 1.4, 95% confidence interval [CI] 1.08–1.84, Fig. 3). The association was stronger when subjects had consistently increased fibrinogen levels in both visits, and was independent from
the G-455>A polymorphism and from CRP levels, even after adjustment for other influencing factors (OR 1.98, 95% CI: 1.47–2.67).

**Interaction between the H2 allele and CRP with preclinical atherosclerosis**

Given i) the observed associations between fibrinogen, CRP and the H2 allele; ii) the association between persistent hyperfibrinogenaemia and plaque presence; and iii) the presence of a significant statistical interaction between the H2 allele and increased CRP in the logistic regression model (p=0.037 for interaction), we speculated that H2H2 subjects with increased CRP levels (above 0.5 mg/dl) could be at higher risk of carotid plaques. Since these subjects are a small fraction of the whole population (12/2,358, or 0.5%), interaction between the allele and CRP could be picked up only by a subgroup analysis. To avoid multiple comparisons, we again used a polytomous logistic regression to model the effect of G-455>A polymorphism on subjects with or without increased CRP levels (above 0.5 mg/dl). An increased risk of carotid plaques was present in H2H2 subjects having increased CRP, even after adjustment for other influencing factors (OR 2.9, 95% CI 1.14–5.39, ▶Fig. 4).

**Mendelian randomisation analysis**

The association between early atherosclerotic changes was finally evaluated using this approach, which evaluates the causative role of fibrinogen based on the presence of a locus regulating its plasma levels that is distributed in the population independently from other possible confounding factors. Instrumental variable linear regression did not provide evidence that fibrinogen level could be associated with increasing IMT (p=0.43), whereas probit regression showed an association between presence of plaque and fibrinogen level, even after correction for CRP, gender, age above 60 years, smoking, presence of hypertension, diabetes, menopause, statin use, LDL and HDL cholesterol above the third tertile (p=0.015).

**Discussion**

It is still debated whether increased plasma fibrinogen should be considered a cause or simply a marker of atherosclerosis (6, 16). Increased plasma fibrinogen has been demonstrated to be associated with cerebro- and cardiovascular events by several prospective studies since the late 1980s (17–19) and by a recent, individual-level meta-analysis (3). However, the observed reduction of the strength of the association after adjustment for confounders, the unspecific effect on all-causes mortality and the lack of association with fibrinogen genotypes known to increase basal fibrinogen level support the idea of a non-causal association between fibrinogen and arterial disease (3, 4).

Evaluation of preclinical atherosclerosis could be a particularly useful model to study those factors that are more specifically related with the inception of atherosclerosis. Increased carotid artery IMT (C-IMT) and presence of atherosclerotic plaques are markers...
of subclinical atherosclerosis that are associated with vascular risk factors (1, 20, 21), with occurrence of new carotid plaques (22, 23) and with the subsequent risk of new or recurrent stroke and myocardial infarction (24–27). Only few studies have addressed the relation between preclinical atherosclerosis and fibrinogen (21, 28–30), but without evaluating the mutual interaction between fibrinogen, fibrinogen polymorphisms and inflammatory markers.

This study confirms that only a minor fraction (about 20%) of the total fibrinogen variability is explained by personal clinical or laboratory characteristics, inflammatory markers such as C-reactive protein (CRP) being the most relevant explanatory variable, a finding in accordance with a recent meta-analysis (31). Although we were able to confirm a clear association between the beta fibrinogen gene G-455>A polymorphism and fibrinogen levels, the effect of the polymorphism was negligible at a population level since it resulted in a mean increase of only 23 mg/dl in carriers of the H2H2 genotype, that is present in less than 5% of our population. Notably, in a multiple regression model including also baseline fibrinogen, we found that the latter was the variable most associated with fibrinogen levels at the subsequent visit, even after inclusion of several covariates. This indicates that fibrinogen levels are possibly consistent thorough life, possibly with a genetic or behavioural background incompletely known (32, 33).

The main finding of this study is that increased fibrinogen was independently associated with the presence of early atherosclerosis, in particular carotid plaques. The risk of developing carotid plaques was indeed higher in those subjects with persistently high fibrinogen, as the risk given by a single finding (OR 1.38) fell tidal plaques was indeed higher in those subjects with persistently sclerosis, in particular carotid plaques. The risk of developing caro-

What is known about this topic?

- Increased levels of plasma fibrinogen are associated with a higher incidence of vascular events, although causality is still debated.
- An increased thickness of the intima-media (IMT) carotid layer and presence of carotid plaques are markers of preclinical atherosclerosis, predicting cerebro- and cardiovascular events also at a population level.

What does this paper add?

- Increased levels of plasma fibrinogen are associated with presence of asymptomatic plaques but not of increased IMT. The association was present after correction for vascular risk factors and C-reactive protein, and was stronger in those subjects having persistently increased fibrinogen levels.
- Carriers of the H2H2 genotype, predicting increased fibrinogen levels, were at risk of developing carotid plaques in the presence of increased C-reactive protein, suggesting a possible environment-genotype interaction.

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