Subunit Antigen and Activity Levels of Blood Coagulation Factor XIII in Healthy Individuals
Relation to Sex, Age, Smoking, and Hypertension

R.A.S. Ariëns, H.P. Kohler, M.W. Mansfield, P.J. Grant

Abstract—Factor (F) XIII covalently cross-links and stabilizes the fibrin clot. Recent evidence suggests a role for FXIII in atherothrombotic diseases, but no information is available regarding the association of FXIII with common risk factors. The aim of this study was to investigate the relationship of FXIII with age, sex, smoking, and hypertension. Plasma levels of FXIII A-subunit antigen, FXIII B-subunit antigen, and FXIII cross-linking activity were measured in 612 healthy individuals (250 men and 362 women). FXIII A- and B-subunit levels were correlated significantly with age in both men \( (r=0.21, P=0.001, \text{ and } r=0.17, P=0.008, \text{ respectively}) \) and women \( (r=0.20, P<0.0005, \text{ and } r=0.13, P=0.011, \text{ respectively}) \). FXIII B-subunit levels and activity were correlated significantly with FXIII A-subunit levels \( (r=0.60, P<0.0005, \text{ and } r=0.14, P<0.0005, \text{ respectively}) \) and fibrinogen \( (r=0.26, P<0.0005, \text{ and } r=0.14, P=0.001, \text{ respectively}) \). Women had higher levels of FXIII A-subunit \( (111.8\% \text{ versus } 105.2\%, P<0.01) \) and B-subunit \( (109.5\% \text{ versus } 103.8\%, P<0.01) \) than did men. FXIII A-subunit was significantly increased in smokers \( (117.0\% \text{ versus } 104.6\%, P<0.0005) \) and in subjects with hypertension \( (114.9\% \text{ versus } 107.8\%, P<0.05) \). In a multiple regression model, FXIII A-subunit was significantly increased by female sex \( (+6.4\%, P<0.007) \), smoking \( (+12.3\%, P<0.0005) \), and increasing age \( (+3.7\% \text{ per 10 years}, P<0.0005) \). FXIII B-subunit was significantly related to female sex and fibrinogen, and FXIII activity was significantly related to fibrinogen levels. In conclusion, the FXIII A-subunit level increases significantly with female sex, age, and smoking, whereas FXIII B-subunit and FXIII activity are associated with FXIII A-subunit level and fibrinogen. Although evidence for a causal relationship between FXIII A-subunit and vascular disease is not available, these results might suggest a role for elevated FXIII A-subunit levels in the pathogenesis of vascular disease. (Arterioscler Thromb Vasc Biol. 1999;19:2012-2016.)

Key Words: factor XIII  ■  sex  ■  age  ■  smoking  ■  hypertension

The transglutaminase blood coagulation factor XIII (FXIII) covalently cross-links and therefore stabilizes the fibrin clot, which initially is held together solely by electrostatic interactions.1,2 The mature proenzyme circulates as a tetramer consisting of 2 A-subunits containing the active site and 2 B-subunits serving as carriers of the A-subunits in plasma. In addition to introducing peptide bonds between the transglutaminase blood coagulation factor XIII (FXIII) covalently cross-links and therefore stabilizes the fibrin clot, which initially is held together solely by electrostatic interactions.1,2 The mature proenzyme circulates as a tetramer consisting of 2 A-subunits containing the active site and 2 B-subunits serving as carriers of the A-subunits in plasma. In addition to introducing peptide bonds between the aligned fibrin α- and γ-chains, FXIII incorporates αvβ3 von Willebrand factor,3 thrombospondin,5 and fibronectin7,8 into the fibrin network. Through these cross-linking reactions, the mechanical, chemical, and proteolytic vulnerability of the fibrin clot is decreased by FXIII. Because a deficiency of FXIII leads to severe bleeding, the critical role of FXIII in blood clotting has long been acknowledged. The involvement of FXIII in thrombotic disease is instead starting to emerge only now. It has been shown that FXIII levels were increased in non-insulin-dependent diabetic patients with microangiopathy and macroangiopathy9 and in patients with obliterator atherosclerosis of the lower limbs.10 Increased cross-linking of fibrin γ-chains has been found in patients with acute myocardial infarction.11 In addition, plasma samples from men with myocardial infarction at a young age formed fibrin gel structures that were more tight and rigid than did plasma samples from normal subjects.12 Recently, we have shown that a common genetic polymorphism in the FXIII A-subunit, coding for a substitution of valine with leucine at residue 34, is protective against myocardial infarction.13 These studies suggest that FXIII may be a risk factor for the development of atherothrombotic disorders. To date, there is no information available regarding the relation of FXIII with other known risk factors of cardiovascular disease, such as age and smoking. The aim of the present study was to investigate the influence of sex, age, smoking, and hypertension on FXIII activity and subunit antigen levels in a population of healthy individuals.

Methods
Plasma samples were obtained from 612 healthy, white northern Europeans, 250 men and 362 women, aged between 22 and 93 years.
The subjects were recruited from large employers in Leeds, randomly selected from the registers of Leeds Health care, and invited by letter to participate in the study. A clinical history was recorded for each subject, and all were apparently free from vascular disorders, thrombosis, and other diseases. Subjects were classified as smokers if they were current, regular smokers. All other individuals were classified as nonsmokers. The presence of hypertension was defined as a blood pressure of 160/95 mm Hg or greater as recorded before blood sample withdrawal, or as previously diagnosed and currently treated hypertension. Characteristics of the study population are depicted in Table 1. All subjects gave informed consent according to a protocol approved by the Research Ethics Committee of the United Leeds Teaching Hospitals NHS Trust.

Blood Sampling and Processing
Venous blood was collected in 0.1 mol/L trisodium citrate, 9 parts blood to 1 part trisodium citrate. Within 1 hour after collection, the samples were centrifuged at 2560g for 20 minutes at room temperature to obtain platelet-poor plasma, frozen in aliquots in LN₂, and stored at −80°C until analysis. Pooled normal plasma was obtained from 47 healthy donors (different from the subjects of the study population) at the local blood transfusion center and was used as reference plasma throughout the study.

Fibrinogen Assay
Fibrinogen levels were measured with a clotting assay according to Clauss. The intra-assay CV was 3.9% (n=20) and the interassay CV 10.0% (n=10).

FXIII Activity Assay
FXIII activity was determined with a microtiter assay using fibrinogen and 5-(biotinamido)pentylamine as substrates, based on a method described by Song et al. Nunc Immuno Maxisorp microtiter plates (Nunc A/S) were coated with 100 μL fibrinogen at 40 μg/mL in 40 μL Tris-HCl, 140 mmol/L NaCl, and 0.02% (wt/vol) NaN₃, pH 8.3 (TBS1), for 45 minutes at room temperature. The microtiter plate was emptied and blocked with 150 μL of 0.5% (wt/vol) nonfat dried milk in TBS1 for 20 minutes at 37°C and washed twice with 200 μL of TBS1. Ten microliters of citrated plasma samples, diluted 1/10 in TBS1, were added in 2 duplicates to the wells, and the cross-linking reaction was started by addition of 90 μL of reaction mixture: 1.11 IU/mL α-thrombin (Sigma Chemical Co), 1.11 mmol/L 5-(biotiamido)pentylamine (Pierce Chemical Co), 0.56 mmol/L DTT (Sigma), and 0.11 mol/L CaCl₂ in TBS1. After incubation at room temperature, the reaction was stopped at 3 and 10 minutes by addition of 200 μL of 0.2 mol/L EDTA. Wells were washed twice with 200 μL TBS1 and incubated with 100 μL of 10 μg/mL streptavidin–alkaline phosphatase (Sigma) in 0.5% (wt/vol) nonfat dried milk/TBS1 for 1 hour at 37°C. After 2 washes with 200 μL of TBS1 containing 0.01% (vol/vol) Triton X-100 and 2 washes with 200 μL of TBS1, color was developed by incubation with 100 μL of 1 mg/mL p-nitrophenyl phosphate (Sigma) in 1 mol/L diethanolamine, pH 9.8, for 10 to 30 minutes at room temperature. The development reaction was stopped by the addition of 100 μL of 4 mol/L NaOH to the wells. Absorbance was measured at 405 nm, and at 550 nm as a reference, on a Titertek absorbance reader (Flow Laboratories). FXIII activities were determined from the change in absorbance after 3 and 10 minutes and expressed as a percentage of pooled normal plasma. The intra-assay CV was 3.9% (n=20) and the interassay CV 10.0% (n=10).

FXIII A- and B-Subunit Antigen Assays
FXIII A- and B-subunit antigen levels were determined by sandwich-ELISA. Microtiter plates (Nunc Immuno Maxisorp) were coated overnight at 4°C with 100 μL of polyclonal sheep anti-human FXIII per well (Binding Site Ltd) at a concentration of 5 μg/mL in 50 mmol/L sodium carbonate, pH 9.6, followed by washing and overcoating with 150 μL of 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% BSA for 1 hour at room temperature. The polyclonal sheep anti-human FXIII antibody binds both purified FXIII A-subunit and purified FXIII B-subunit.

After the plates were washed, 100 μL of plasma samples, diluted 1/4000 for the A-subunit and 1/16000 for the B-subunit assays in 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20, were loaded into the wells in duplicate and incubated for 1 hour at room temperature. Plates were next washed and incubated with 100 μL of polyclonal rabbit anti-human FXIII A- or FXIII B-subunit (both from Diagnostic Stago) at a dilution of 1/1000 in TBS2 for 1 hour at room temperature, followed by washing and incubation with 100 μL of polyclonal alkaline phosphatase–labeled goat anti-rabbit IgG (Sigma) at a dilution of 1/1000 in TBS2 for 1 hour at room temperature. Every washing step was performed by 5 cycles of 150 μL per well of 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20. After a final washing step, color was developed by incubation of the plates at room temperature with 100 μL of 1 mg/mL p-nitrophenyl phosphate (Sigma) in 1 mol/L diethanolamine, pH 9.8, containing 0.5 mmol/L MgCl₂. The reaction was stopped by the addition of 100 μL of 4 mol/L NaOH, and absorbance was read at 405 nm, and at 550 nm as a reference, on a Titertek absorbance reader (Flow Laboratories). A standard curve was obtained with pooled normal plasma, diluted from 1/1000 to 1/16000 for the A-subunit and from 1/4000 to 1/64000 for the B-subunit, and sample levels were interpolated from this standard curve and expressed as percentages. Testing a sample from a patient with homozygous FXIII A-subunit deficiency showed that there was no cross-reactivity with other proteins for the A-subunit antigen assay. Incubation with purified A-subunit and/or purified B-subunit showed that the A-subunit ELISA reacts with purified A-subunit only and that the B-subunit ELISA reacts with purified B-subunit only. Incubation with solutions of purified B-subunit with or without purified A-subunit showed that the FXIII B-subunit ELISA equally measures both the free form of the B-subunit as well as the B-subunit in complex with the A-subunit. The intra-assay CVs (n=20) were 5.4% for the A-subunit antigen assay and 6.2% for the B-subunit antigen assay.

The interassay CVs (n=12) were 9.3% for the A-subunit antigen assay and 9.8% for the B-subunit antigen assay.

Statistical Analysis
Results were analyzed using the SPSS for Windows software package (version 6.0, SPSS Inc). Levels of FXIII activity, FXIII A-subunit antigen, and FXIII B-subunit antigen were not normally distributed and were positively skewed toward higher values. Logarithmic transformation resulted in a sufficient fit of the data to the normal distribution (Kolmogorov-Smirnov test) to validate parametric statistical analysis. Accordingly, between-group differences were analyzed with Student’s t test for unpaired data by using logarithmically transformed values and expressed as geometric means and 95% CIs. Bivariate correlations between variables were analyzed with Pearson’s correlation coefficient, and trendlines were calculated with linear regression analysis on logarithmically transformed data. Multiple regression modelling was used to test multivariate relationships of variables with logarithmically transformed FXIII levels. Regression coefficients were reverse-transformed to geometric values and expressed as percentage changes to allow interpretation and assessment of effect. Significance was taken as P<0.05.

Results
Correlation of FXIII With Age
Plasma levels of FXIII A-subunit (log₁₀FXIIIA=1.66×10⁻³·age+1.93, r=0.21, P<0.0005) and FXIII B-subunit
antigen (log \( \beta \)) = 0.95 \times 10^{-7} \times \text{age} + 1.97, \quad r = 0.16, \quad P < 0.0005) but not FXIII activity showed a significant correlation with age. When the regression coefficients were transformed to geometric values, a 10-year increase in age was associated with a 3.9% increase in A-subunit levels and a 2.2% increase in B-subunit levels. The correlation of FXIII A-subunit and FXIII B-subunit antigen levels with age was found in both male and female parts of the population (the Figure).

FXIII B-subunit antigen and FXIII activity levels were correlated significantly with fibrinogen levels (6.8% per 1-g/L fibrinogen increase, \( r = 0.26, \quad P = 0.0005 \), and 5.7% per 1-g/L fibrinogen increase, \( r = 0.14, \quad P = 0.001 \), respectively). There was a strong correlation between FXIII A-subunit and FXIII B-subunit antigen levels (\( r = 0.60, \quad P < 0.0005 \)), and both FXIII A- and B-subunit antigen levels showed a relatively weak but significant correlation with FXIII activity (\( r = 0.14, \quad P < 0.0005 \), and \( r = 0.18, \quad P < 0.0005 \), respectively).

### Association of FXIII With Sex, Smoking, and Hypertension

FXIII A-subunit antigen levels and FXIII B-subunit antigen levels were higher in women than in men (Table 2). There were no significant differences for FXIII activity levels between women and men. Smokers had markedly increased FXIII A-subunit antigen levels when compared with non-smokers, and subjects with hypertension had significantly higher FXIII A-subunit antigen than did subjects without hypertension. No significant differences were found for the FXIII B-subunit antigen and FXIII activity levels when

### TABLE 2. Levels of FXIII A-Subunit Antigen, FXIII B-Subunit Antigen, and FXIII Activity in Relation to Sex, Smoking, and Hypertension in 612 Healthy Individuals

<table>
<thead>
<tr>
<th></th>
<th>FXIII A-Subunit</th>
<th>FXIII B-Subunit</th>
<th>FXIII Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women (n=362)</td>
<td>111.8 (108.6–115.1)*</td>
<td>109.5 (107.0–112.2)*</td>
<td>96.0 (92.5–99.7)</td>
</tr>
<tr>
<td>Men (n=250)</td>
<td>105.2 (101.6–109.0)</td>
<td>103.8 (101.1–106.5)</td>
<td>92.0 (88.0–96.1)</td>
</tr>
<tr>
<td>Smokers (n=229)</td>
<td>117.0 (112.6–121.6)†</td>
<td>109.3 (106.2–112.4)</td>
<td>95.9 (91.7–100.3)</td>
</tr>
<tr>
<td>Nonsmokers (n=383)</td>
<td>104.6 (101.9–107.5)</td>
<td>105.9 (103.5–108.3)</td>
<td>93.5 (90.1–97.0)</td>
</tr>
<tr>
<td>Hypertension (n=116)</td>
<td>114.9 (109.5–120.6)‡</td>
<td>109.6 (105.8–113.6)</td>
<td>93.7 (88.3–99.5)</td>
</tr>
<tr>
<td>No hypertension (n=496)</td>
<td>107.8 (105.1–110.5)</td>
<td>106.6 (104.4–108.7)</td>
<td>94.5 (91.5–97.6)</td>
</tr>
</tbody>
</table>

All values are expressed as percentages of pooled, normal plasma. Values shown are antilogs of geometric means and (95% CIs).

*P < 0.01 for women vs men.
†P < 0.0005 for smokers vs nonsmokers.
‡P < 0.05 for hypertension vs no hypertension.
TABLE 3. Multiple Regression Models of the Relation Between FXIII Measurements and Age, Sex, Smoking, Hypertension, and Fibrinogen

<table>
<thead>
<tr>
<th></th>
<th>FXIII A-Subunit</th>
<th>FXIII B-Subunit</th>
<th>FXIII Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficient*</td>
<td>P</td>
<td>Coefficient</td>
<td>P</td>
</tr>
<tr>
<td>Smoker</td>
<td>+12.3</td>
<td>&lt; 0.0005</td>
<td>+3.1</td>
</tr>
<tr>
<td>Age (10-year increase)</td>
<td>+3.7</td>
<td>&lt; 0.0005</td>
<td>+0.8</td>
</tr>
<tr>
<td>Female sex</td>
<td>+6.4</td>
<td>0.007</td>
<td>+3.8</td>
</tr>
<tr>
<td>Hypertension</td>
<td>+3.8</td>
<td>NS</td>
<td>+0.14</td>
</tr>
<tr>
<td>Fibrinogen (1-g/L increase)</td>
<td>-2.1</td>
<td>NS</td>
<td>+5.5</td>
</tr>
<tr>
<td></td>
<td>(-5.1 to +0.6)</td>
<td></td>
<td>(+3.2 to +7.9)</td>
</tr>
<tr>
<td>Model</td>
<td>$r^2$ = 0.11</td>
<td>&lt; 0.0005</td>
<td>$r^2$ = 0.08</td>
</tr>
</tbody>
</table>

*Values shown are antilogs of regression coefficients expressed as percentage change and (95% CIs).

comparing smokers with nonsmokers or subjects with hypertension versus those without.

Multiple Regression Analysis

Multiple regression models were constructed with FXIII A-subunit antigen, FXIII B-subunit antigen, or FXIII activity as the dependent variable and age, sex, smoking, hypertension, and fibrinogen as independent predictor variables. In this model, smoking, age, and female sex were significantly related to FXIII A-subunit antigen levels (Table 3). The regression coefficient between A-subunit levels and age in this model (3.7% increase per 10 years) was very similar to that found with bivariate analysis (3.9%). Smoking had the most marked relationship with FXIII A-subunit antigen levels, increasing them by 12.3%. The univariate association of elevated FXIII A-subunit levels in subjects with hypertension was not confirmed in this model.

On a similar multivariate analysis, female sex and fibrinogen levels were significantly related to levels of FXIII B-subunit. The correlation of FXIII B-subunit antigen levels with age was no longer significant after adjustment for confounding effects in this model. FXIII activity levels were related significantly to fibrinogen levels only in the multivariate model. The regression coefficients between fibrinogen and B-subunit levels or activity levels (5.5% and 6.7% increase per 1-g/L fibrinogen) were similar to those found with bivariate analysis (6.8% and 5.7%, respectively).

Discussion

We found that age, female sex, and smoking were significantly related to increased circulating levels of the A-subunit, which contains the activation peptide and the active site of FXIII. Individuals with hypertension also had higher FXIII A-subunit levels in univariate analysis, but this increase was not confirmed in a multiple regression model. It is known that fibrinogen levels increase with age and smoking, and increased fibrinogen levels are thought to be a risk factor for vascular disease. In the present study, we found that FXIII A-subunit levels also increase with age and smoking, which, together with previous studies that have reported an increase of FXIII in patients with atherosclerotic disorders, might suggest a role of the fibrin cross-linking enzyme FXIII in the pathogenesis of vascular disease. However, this cross-sectional study offers no evidence to support a causal relationship between FXIII levels and vascular disease. An elevation of FXIII measurements in subjects with a higher risk for vascular disease, such as smokers and the elderly, may therefore also be an epiphenomenon of systemic effects associated with the disease state.

One previous study has reported that FXIII is correlated significantly with age in healthy individuals who were enrolled as control group for a study of patients with diabetic angiopathy. The authors found that FXIII A-subunit, B-subunit (both measured with rocket immunoelectrophoresis), and FXIII activity (measured with a dansylcadaverine-into-casein incorporation assay) were all correlated significantly with age in their control group of 35 subjects. The regression lines between the measurements and age were much steeper than in our study. However, the number of subjects studied by Kloczko et al was too small to provide conclusive evidence regarding the regression lines and significance of the correlation between FXIII and age. Another study has reported on the relation of FXIII to smoking in pregnant women. van Wersch et al found that FXIII activity levels, measured with a commercially available photometric assay, were higher in women in the second half of pregnancy who were smoking than in those who were not. Also in that study, the number of subjects studied was rather small: 35 smoking, pregnant women were compared with 27 nonsmoking, pregnant women.

Both FXIII A- and B-subunit antigen levels were significantly higher in women than in men, even after adjusting for the confounding effects of smoking, hypertension, fibrinogen levels, and age. Because of the lower incidence of vascular disease in women, this finding appears contrary to the speculation that increased FXIII A-subunit levels may be a risk factor for vascular disease. However, levels of fibrinogen, FVII, and total cholesterol are also higher in women than in men, particularly after the menopause. The cause of these higher FXIII antigen levels in women is not clear,
although it has been suggested that changes in female hormone metabolism are related to changes in other clotting factors. Further studies on the effect of menopausal status and estrogen replacement therapy on FXIII levels are needed to investigate whether or not FXIII levels are related to female hormone metabolism.

A correlation between FXIII B-subunit levels and age was found, but this association was not confirmed by multivariate regression analysis. It has been proposed that levels of circulatory A-subunit control the total concentration of the B-subunit,21 which mainly serves as a carrier protein of the A-subunit in plasma. This idea is in agreement with the highly significant correlation between the A-subunit and B-subunit levels we found in the present study. It therefore appears that levels of the FXIII B-subunit show a significant correlation with age through its association with the A-subunit, which significantly increases with age in both sexes. Besides being correlated with the A-subunit, FXIII B-subunit levels were significantly associated with fibrinogen levels. This result is in agreement with previous studies that have shown a strong affinity between the FXIII B-subunit and fibrinogen.22 One might expect that FXIII activity plasma levels would increase significantly with age, smoking, and female sex, in a manner similar to the A-subunit, which contains the active site of the enzyme. However, we found that FXIII activity was associated significantly with fibrinogen levels only. We have previously shown that FXIII activity levels were markedly affected by a polymorphism in the FXIII A-subunit with an allele frequency of ≈25% that codes for a substitution of valine with leucine only 3 amino acids away from the thrombin activation site.23 It appears that FXIII activity, as measured with a pentylamine-into-fibrin incorporation assay, is particularly sensitive to the thrombin activation step and hence, to the common polymorphism FXIII Val34Leu. Owing to this sensitivity of the FXIII activity assay to the Val34Leu polymorphism, a high variability of FXIII activity levels is present in the normal population.24 This variability in activity may lead to the relatively weak correlation between FXIII activity levels and FXIII A-subunit antigen levels as found in the present study and may explain why, in contrast to FXIII A-subunit, FXIII activity levels were not associated significantly with age, smoking, and female sex.

In conclusion, FXIII A-subunit antigen levels are increased significantly by age, female sex, and smoking. FXIII B-subunit antigen levels and FXIII activity levels are correlated significantly with FXIII A-subunit and fibrinogen levels. Although there is no evidence for a causal relationship between increased FXIII A-subunit and vascular disease or thrombosis, the association of FXIII A-subunit with the cardiovascular risk factors of age and smoking might suggest that increased FXIII A-subunit levels are involved in the pathogenesis of vascular disease.

Acknowledgments

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References

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