Functional Analysis of the Fibrinogen Aα Thr312Ala Polymorphism 
Effects on Fibrin Structure and Function

Kristina F. Standeven, MSc; Peter J. Grant, MD, FRCP; Angela M. Carter, PhD; Thomas Scheiner, MSc; John W. Weisel, PhD; Robert A.S. Ariëns, PhD

Background—The fibrinogen Aα Thr312Ala polymorphism occurs within the αC domain of fibrinogen, which is important for lateral aggregation and factor XIII–induced cross-linking of fibrin fibers. We have previously shown an association of Ala312 fibrinogen with poststroke mortality in subjects with atrial fibrillation and with pulmonary embolism in subjects with venous thrombosis.

Methods and Results—We studied the properties of clots formed from purified Ala312 and Thr312 fibrinogen and found that Ala312 fibrinogen produces stiffer clots, associated with increased α-chain cross-linking, as demonstrated by SDS-Page. On electron microscopy analysis, we found larger fiber diameters in the Ala312 clots and observed a lower number of fibers per square micrometer. The number of branch points per square micrometer was similar between genotypes.

Conclusions—Our study shows that Ala312 influences clot structure and properties by increased factor XIII cross-linking and formation of thicker fibrin fibers. These findings may provide a mechanism by which Ala312 fibrinogen could predispose to clot embolization. (Circulation. 2003;107:2326-2330.)

Key Words: fibrinogen ■ fibrin ■ thrombosis

The conversion of fibrinogen to fibrin and subsequent stabilization of the growing fibrin clot by factor XIII (FXIII) are the ultimate events in the activated coagulation cascade, leading to an effective seal on vascular injury. Abnormalities in this system can cause bleeding or thrombosis. Fibrinogen is a 340-kDa dimeric glycoprotein consisting of 6 chains (Aα, Bβ, γ). Factor XIIIa–dependent cross-linking of adjacent γ and α chains of polymerized fibrin gives rise to γ-chain dimers and α-chain multimers and is essential for the structural integrity of a fibrin clot.1

A couple of polymorphisms have been identified in the genes encoding the mature fibrinogen protein.2 A common polymorphism leading to a substitution from threonine to alanine at codon 312 occurs within the carboxy-terminal end of the fibrinogen Aα chain termed αC domain, which encompasses approximately two thirds of the Aα-chain.2 This region is important for FXIII-dependent processes, including fibrin α/α chain and fibrin α-chain/α2-antiplasmin cross-linking (amino acid residues Aα328 and Aα303, respectively).1,3 Residues Aα242 to 424, which surround the site of the polymorphism, promote the dissociation of the FXIII A and B subunits and thereby enhance activation of FXIII.4 Interactions between αC domains of adjacent fibrin molecules enhance lateral aggregation of fibers in the growing clot and therefore influence overall fiber thickness and the resulting clot architecture and stability.5 The molecular location of Thr312Ala suggests that the amino acid substitution has the potential to influence FXIII-dependent mechanisms that determine clot formation and stability, making it an interesting candidate for both clinical and functional studies.

We have previously reported an association of the Ala allele of the Thr312Ala polymorphism with poststroke mortality in subjects with atrial fibrillation and with pulmonary embolism in subjects with venous thrombosis.6,7 The aim of the present study was to investigate properties of clots formed by both fibrinogen variants in vitro to elucidate possible mechanisms that underpin the previously described clinical associations.

Methods

Genotyping
DNA was genotyped for fibrinogen Aα Thr312Ala and for a common polymorphism in the A-subunit of FXIII, Val34Leu, as described previously.7

Received November 27, 2002; revision received February 12, 2003; accepted February 18, 2003.

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000066690.89407.CE
Plasma Preparation and Purification of Fibrinogen
Subjects who were homozygous for either the Thr312 or the Ala312 allele and for the FXIII Val34 allele donated 50 mL venous blood, to which 15 IU/mL lithium heparin was added. Samples were processed at room temperature by centrifugation at 2540g for 20 minutes within 2 hours of collection. Platelet-poor plasma was separated and stored in 1.5-mL aliquots at −80°C until purification.

Fibrinogen was purified to homogeneity by affinity chromatography with a calcium-dependent monoclonal antibody (IF-1) coupled to Sepharose 4B, as described by Takebe et al. Purity of the preparations was examined with SDS polyacrylamide (8%, bis-acrylamide ratio of 1.37:5) gel electrophoresis. Concentration of the purified fibrinogen preparations was calculated from absorbency at 280 nm using an extinction coefficient of E (1 mg per mL, 280 nm)=1.61.

Purification of Factor XIII
Human FXIII was purified from outdated platelet poor plasma by ammonium sulfate precipitation and gel filtration, as described previously.

Cross-Linking of Fibrin by Factor XIII
The extent of cross-linking of α and γ chain for each variant was assessed by SDS-PAGE. Clots of both genotypes were formed by addition of thrombin (1 U/mL final concentration) to 1 mg/mL fibrinogen in 0.15 mol/L NaCl, 50 mmol/L Tris-HCl, and 10 mmol/L CaCl₂, pH 7.4, in the presence of commercial FXIII (0.022 mg/mL final concentration) (American Diagnostica, Inc). Clotting proceeded for 30 minutes in a moist environment. Clots were dissolved in reducing buffer consisting of 62.5 mmol/L Tris, 10% glycerol, 2% (wt/vol) SDS, 5% mercaptoethanol, and 0.05% (wt/vol) bromophenol blue, pH 6.8, and heated for 4 minutes at 95°C. SDS-PAGE was carried out on a 7.5% separation gel with a 4% stacking gel using a Miniprotean 3 (Biorad) electrophoresis unit. Gels were dried and digitized. Densitometry of bands was carried out using ImageJ 1.23y software (National Institutes of Health). Six samples per genotype on 6 separate gels were analyzed.

Viscoelastic Properties
A Plazek torsion pendulum was used to determine viscoelastic properties of clots, including clot stiffness (as described earlier) formed from both variants. Clots were formed by addition of 1 U/mL α-human thrombin and 10 mmol calcium chloride to 1 mg/mL fibrinogen and 22 μg/mL FXIII in Buffer A (100 mmol/L NaCl, 50 mmol/L Tris, pH 7.4). Data recorded were used to calculate G’ (the dynamic storage modulus), G” (the loss modulus), and the tan delta, which is a function of G” over G’ and gives information about irreversible deformation of the clot. Ten samples per genotype were prepared.

Scanning Electron Microscopy and Measurement of Fiber Diameter
Scanning electron microscopy was used to determine the fibrin structure of clots formed from each variant through direct examination and by assessing fiber thickness and the numbers of branch points and fibers.

Human α-thrombin (final concentration, 1/U mL) and calcium chloride (final concentration, 10 mmol/L) in Buffer A were added to purified fibrinogen (final concentration, 1 mg/mL, 8 clots per genotype, 4 of which were formed in the presence of 22 μg/mL purified FXIII). Samples were prepared as described previously. Clots were observed and photographed digitally in at least 2 different areas per clot, using a scanning electron microscope (XL 20, Philips Electron Optics). Fiber thickness was measured using ImageJ 1.23y software (National Institutes of Health). To exclude bias, digital photographs were analyzed for fiber thickness and the number of branch points and fibers by an operator blind to genotype.

Turbidity Curves
To obtain information regarding rates of polymerization, including protofibril formation and overall fiber thickness in a clot, turbidity experiments were carried out. To compare the data generated from turbidity experiments, 2 parameters were determined: the time point of each curve where the optical density at 350 nm had reached 0.01 (lag phase) and the optical density at 350 nm of each curve at 20 minutes.

Polimerization of purified fibrinogen of both genotypes in Buffer A at a concentration of 1 mg/mL was initiated with 0.5 U/mL human thrombin and 10 mmol/L calcium chloride. Immediately on addition of the thrombin/calcium chloride mixture, absorbency was read every 10 seconds at 350 nm for 20 minutes on a MRX Microplate Reader (Dynex Technologies). Twenty-two replicate measurements for both variants were performed.

Permeation Properties
Permeation properties of clots were investigated according to a protocol as previously described Human α-thrombin (final concentration, 0.5 U/mL) and calcium chloride (final concentration, 10 mmol/L) were added to start polymerization of purified Ala312 and Thr312 fibrinogen (1 mg/mL in Buffer A). The Darcy constant K, was calculated from the flow rate of buffer through the clot, which is directly related to the pore size of the fibrin meshwork.

Results
SDS-PAGE Analysis of Fibrin Cross-Linking by Factor XIIIa
Cross-linking by FXIII caused the formation of multiple α-chain polymers. Because it was difficult to obtain density measurements for these multiple bands, we determined the densitometric difference in disappearing α-monomers instead, which is inversely correlated with the extent of α-chain cross-linking. Figure 1 shows a representative gel (of 6),
indicating that in Ala312 clots formed in the presence of FXIII, approximately 54% of α-chain monomer was cross-linked compared with 45% in Thr312 clots (mean difference, 9±6%, \(P=0.013\)). There was no difference in the extent of γ dimer formation between the 2 variants.

**Viscoelastic Properties of Clots Formed From Fibrinogen Variants Determined by Torsion Pendulum**

To determine whether the difference in α-chain cross-linking described above resulted in a difference in clot stiffness, we evaluated clot stiffness and deformability with a torsion pendulum. Parameters calculated were the storage modulus or \(G'\), a measure of the stiffness of the clot, the loss modulus or \(G''\), which assesses the energy dispersed by nonelastic, viscous processes, and the tan delta, a function of \(G''/G'\), which quantifies irreversible deformation of the clot as a fraction of the reversible deformation. There were significant differences in \(G'\) and tan delta between Thr312 clots (\(G'=259\pm51\), tan delta=0.057±0.016) and Ala312 clots (\(G'=329\pm64\), tan delta=0.050±0.009, \(P<0.05\) for both) but no significant difference in \(G''\) (Thr312=15.1±7.7, Ala312=16.4±4.1). Thus, Ala312 clots were stiffer (higher \(G'\)), with a lower proportion of irreversible deformation (lower tan delta), than Thr312 fibrin clots.

**Scanning Electron Microscopy**

We additionally characterized the influence of the Aα Thr312Ala polymorphism on fibrin structure by examining clots of each genotype with scanning electron microscopy. Analysis of the micrographs revealed a significant difference in fiber thickness between the variants. As shown in Figure 2, Ala312 clots formed thicker fibers compared with Thr312 clots. The difference between the variants remained consistent on the addition of FXIII (Table 1). The numbers of fibers and branch points per unit volume were counted for each clot, revealing a significantly lower number of fibers per square micrometer in the Ala312 clots compared with Thr312, but there was no significant difference in the number of branch points per square micrometer between the variants (Table 2).

**Fibrin Polymerization Measured by Turbidity**

Fibrin polymerization was investigated by turbidity experiments, which give information about various stages of clotting.

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**TABLE 1. Fibrin Fiber Diameters Measured From Electron Micrographs**

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Mean, nm</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr312</td>
<td>300</td>
<td>117</td>
<td>44</td>
</tr>
<tr>
<td>Ala312</td>
<td>300</td>
<td>142*</td>
<td>45</td>
</tr>
<tr>
<td>Thr312+FXIII</td>
<td>300</td>
<td>126</td>
<td>36</td>
</tr>
<tr>
<td>Ala312+FXIII</td>
<td>330</td>
<td>138†</td>
<td>12</td>
</tr>
</tbody>
</table>

*\(P<0.001\) vs Thr312; †\(P=0.002\) vs Thr312+FXIII (ANOVA with Scheffe post hoc analysis).

**TABLE 2. Numbers of Fibers and Branch Points Measured From Electron Micrographs**

<table>
<thead>
<tr>
<th></th>
<th>No. of Squares Counted</th>
<th>No. of Fibers per (\mu m^2)</th>
<th>No. of Branch Points per (\mu m^2)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr312</td>
<td>4</td>
<td>0.48</td>
<td>0.03</td>
<td>0.23</td>
</tr>
<tr>
<td>Ala312</td>
<td>5</td>
<td>0.41*</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Thr312+FXIII</td>
<td>4</td>
<td>0.45</td>
<td>0.01</td>
<td>0.18</td>
</tr>
<tr>
<td>Ala312+FXIII</td>
<td>5</td>
<td>0.37†</td>
<td>0.04</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*\(P=0.005\) vs Thr312; †\(P=0.02\) vs Thr312+FXIII (ANOVA with Scheffe post hoc analysis).

1 square = 375.7 \(\mu m^2\).
ting, including the rate of initial oligomer formation and lateral aggregation of protofibrils. The lag phase of the turbidity curve corresponds to the time required for initial protofibril formation, and measurements of turbidity at 20 minutes give an indication of the overall fiber thickness as well as number of branch points in the clot. Mean lag time to OD=0.01 was 36.4 seconds in clots formed by both Thr312 and Ala312, showing that there was no difference in the rate of initial protofibril formation between the variants. There was also no significant difference in the OD at 20 minutes (Ala 312=0.402±0.057, Thr312=0.384±0.047, P=NS).

**Clot Permeability**

The Darcy constant (Kd), which is calculated from measurements of permeability or flow of fluid through the clots, gives information about pore size in the fibrin clot. There was no significant difference in the permeability of Thr312 and Ala312 clots (Thr312: Kd=0.234 [SD=0.097]; Ala312: Kd=0.263 [SD=0.093], P=NS).

**Discussion**

Elevated plasma fibrinogen is an established risk factor for thrombotic vascular diseases, although the mechanisms by which it contributes to disease pathogenesis have not been clearly characterized. Cleavage of fibrinogen by thrombin to form fibrin is the central event in clot formation, and fibrinogen level has been shown to be a major predictor of the architecture of clots. To date, there are only a limited number of studies that have examined indicators of fibrin clot structure; some of these have shown differences in clot permeability and fibrin fiber thickness in those with coronary artery disease and relatives of those with coronary artery disease.

Previous clinical studies have found that the Ala allele of the fibrinogen AαThr312Ala polymorphism influences clot stability and predisposes clots to embolization in both the arterial and venous vascular trees. We have investigated fibrin clot formation and structure of both Thr312 and Ala312 variants in an in vitro system using purified fibrinogen. From our present in vitro studies we found that Ala312 fibrin clots had thicker fibers and more extensive α-chain cross-linking. It is possible that the substitution of threonine to alanine at position 312 of the C-terminal α-chain enhances lateral aggregation of the fibers, leading to thicker fibers, consistent with the role of the αC domains in lateral aggregation. The increased α-chain cross-linking is in agreement with earlier studies that have shown that thicker fibers tend to have more α-chain cross-links. The presence of thicker fibers in Ala312 clots compared with Thr312 clots, both in the presence and absence of FXIII, suggests that the presence of Alanine at residue 312 directly affects lateral aggregation via the αC domains.

Turbidity experiments showed no difference in early polymerization stages between the variants. There was also no significant difference in maximum absorbency of clots, as observed by turbidity. There is the possibility that the turbidity and permeation measures are relatively insensitive to the alterations in fibrin clot structure induced by the Aα Thr312Ala polymorphism compared with the other methods. However, differences between the techniques used to analyze fibrin structure and function are more likely a consequence of the complex relationships that exist between fiber thickness, number of branch points, and pore size. Although maximum absorbency has been used as an indicator of fiber size, in the case reported in our present study, it is more likely to reflect the similar number of branch points, and consequently pore size, between the variants. Equally, and in support of this, there was no difference in permeability between the variants, suggesting again a similar measure of pore size. These results are in contrast to those reported by Curran et al, who found a decrease in plasma clot permeability associated with Ala312. Because the amount of fibrinogen in clots is a major determinant of fibrin structure, it is difficult to determine accurately the influence of genotypes in plasma samples. Differing fibrinogen concentrations as well as concentrations of other proteins may affect clot structure. In our study, we used a purified system rather than plasma clots to eliminate the known confounding influence of differences in plasma fibrinogen levels and of other proteins that can alter the architecture of clots. For instance, we have previously shown that the FXIII Val34Leu polymorphism has a significant influence on clot structure. We evaluated the influence of Thr312Ala on clot structure/function in a purified system from subjects only possessing the FXIII Val34 genotype to exclude the influence of this variable, which could represent one of the reasons for the difference in results between our study and that by Curran et al.

We have previously hypothesized that the Ala312 allele predisposes clots to embolization by influencing α-fibrin monomer cross-linking. In this study, we have shown that the fibrinogen AαThr312Ala polymorphism indeed affects clot structure and stiffness and changes FXIII-induced cross-linking of α-chains. How an increase in clot stiffness leads to a tendency of the clot to embolize is at present unclear. It is possible that stiffer clots are more brittle, leading to an increased tendency to fragmentation under conditions of blood flow. Studies to determine the effect of clot stiffness on the fate of the clots under flow conditions will be required to confirm the relationship between our clinical and functional studies on Thr312Ala.

Our data indicate that this common genetic variant of fibrinogen influences fibrin clot structure in an in vitro system. However, the contribution of an individual polymorphic variant to overall risk of thrombotic disease is likely to be small, and in complex polygenic disorders, it is the accumulation of genetic risk factors and their interaction with the environment that will determine overall susceptibility to disease and disease outcome.

In the present study, we have characterized the effect of fibrinogen Aα Thr312Ala on fibrin clot structure. Previous studies from our laboratory also reported the effects of a common coding polymorphism in the FXIII A subunit (FXIII Val34Leu) on fibrin structure/function. It will be important to functionally characterize all of the common coding region polymorphisms in the genes encoding proteins involved in the terminal events of clot formation and their interactions to more clearly define their influence on thrombotic risk.
understanding of the genetic and environmental factors that influence clot structure will provide important information regarding the etiology of thrombotic vascular diseases and may identify potential targets for therapeutic intervention.

Acknowledgments
This study was supported by the British Heart Foundation, Medical Research Council, and National Institutes of Health.

References
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Circulation. 2003;107:2326-2330; originally published online April 21, 2003; doi: 10.1161/01.CIR.0000066690.89407.CE
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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